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Loss of Tet1 associated 5-hydroxymethylcytosine is concomitant with aberrant promoter hypermethylation in liver cancer.

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Abstract

Aberrant hypermethylation of CpG islands (CGI) in human tumors occurs predominantly at repressed genes in the host tissue, but the preceding events driving this phenomenon are poorly understood. In this study, we temporally tracked epigenetic and transcriptomic perturbations which occur in a mouse model of liver carcinogenesis. Hypermethylated CGI events in the model were predicted by enrichment of the DNA modification 5-hydroxymethylcytosine (5hmC) and the histone H3 modification H3K27me3 at silenced promoters in the host tissue. During cancer progression, CGI underwent hypo-hydroxymethylation prior to hypermethylation, whilst retaining H3K27me3. In livers from mice deficient in Tet1, a tumor suppressor involved in cytosine demethylation, we observed a similar loss of promoter core 5hmC, suggesting that reduced Tet1 activity at CGI may contribute to epigenetic dysregulation observed during hepatocarcinogenesis. Consistent with this possibility, mouse liver tumors exhibited reduced Tet1 protein levels. Similar to humans, DNA methylation changes at CGI in mice did not appear to be direct drivers of hepatocellular carcinoma progression, rather, dynamic changes in H3K27me3 promoter deposition correlated strongly with tumor-specific activation and repression of transcription. Overall, our results suggest that loss of promoter-associated 5hmC in liver tumors licenses reprogramming of DNA methylation at silent CGI during progression.

Introduction

Significant advances have been made in the study of epigenetic reprogramming associated with specific cancer and tumour subtypes(1). Much of the focus has been on DNA methylation reprogramming or alterations in the post translation modifications (PTM) of DNA bound histone proteins such as tri-methylation at lysine 27 on Histone H3 (H3K27me3)(2,3). The identification of several new forms of modified cytosine in vertebrate DNA has added to this narrative by providing new patterns for interpretation, hypothesis building and functional outcomes (4). In particular, 5-hydroxymethylcytosine (5hmC), generated from a 5-methylcytosine (5mC) precursor by the Ten-Eleven-Translocation (TET) enzymes, is hypothesised to be part of a predicted demethylation pathway and that differs significantly in its genomic distributions from that of 5mC (5-8). Whilst 5mC tends to be found over heterochromatic and repetitive portions of the genome and has known roles in

the maintenance of transcriptional silencing, 5hmC is more restricted to the bodies of expressed genes, enhancer elements and a cohort of promoter regions in many tissues(8-10). Aside from a predicted role as a demethylation intermediate, the functional significance of the 5hmC modification is still largely unknown. However there is evidence that the genic levels of 5hmC can correlate with the transcriptional activity of the associated genes in tissues whilst low-level direct enrichment of 5hmC over the core of promoters surrounding the transcription start site (TSS) appears to be related to transcriptional silencing events and maintenance of CpG hypomethylation (11-13).

Normal 5hmC patterns are dramatically altered in several human cancer types such as melanoma and hepatocellular carcinoma; as well as in cultured cancer cell lines and colon cancer (9,11,14-16). In previous work, we have identified hypermethylation prone promoters in various human cancer types which overlap with Polycomb regulated gene sets (17). We speculated that these genes are prone to methylation due to their inactivity in cancer-host tissue and loss of a demethylase activity (2). A possibility is that 5hmC/Tet enzymes are involved in this process, as loss of Tet activity from such promoters may licence the occurrence of aberrantly hypermethylated CpG islands (CGI) in cancer.

Previously we carried out a number of studies investigating the epigenetic perturbations that occur in liver following exposure to the well-studied rodent non-genotoxic carcinogen (NGC), Phenobarbital (PB), to elucidate potential drug induced epigenetic changes that anticipate cellular transformation (13,18,19). PB is an antiepileptic drug which promotes hepatocarcinogenesis in rodents when administered subsequent to an initiating carcinogen such as N-nitrosodiethylamine (DEN) (20). This results in selective clonal outgrowth of cells harbouring activating mutations in the proto-oncogene *Ctnnb1*, encoding β -Catenin, a key mediator in the canonical Wnt signaling pathway (21). In this study we attempt to delineate the progression of hepatocarcinogenesis through combined epigenetic (5mC, 5hmC & H3K27me3 patterns) and transcriptomic analysis... Our study tracks the initial epigenetic and transcriptomic perturbations throughout the early stages of toxicological insult and follows them to an 'end-state' that is characteristic of liver tumour formation in three models (21,22). As a result we link initial loss of 5hmC at CGI promoters with the occurrence of aberrant hypermethylated CGIs in tumours. This occurs at genes that are repressed in normal liver and remain silent in the resulting tumours, possibly due to the continuous presence of the inactivating mark H3K27me3.

Materials and Methods

Study material and animal treatment

For full description of mouse strains and treatment regimes, please see supplemental materials and methods.

Methyl and hydroxymethyl DNA immunoprecipitation (MeDIP & hMeDIP)

Genomic DNA was extracted from frozen ground-up samples and fragmented to a range between 300 bp to 1,000 bp in size (Bioruptor, Diagenode) prior to immunoprecipitation with 5hmC (active motif #39769) or 5mC (Eurogentec # BI-MECY-1000) antibodies. For HmeDIP and MeDIP protocols see;(13,18). DNA was purified using DNA Clean & Concentrator™ (Zymo Research). 5hmC and 5mC patterns were then generated following dye labelling and hybridisation to promoter specific microarrays – either on 2.1M Deluxe mouse promoter tiling microarrays (Roche Nimblegen) for the control, 12 week PB, Ctnnb1 and Ha-ras tumours or on 1M mouse promoter microarrays (Agilent) for control and NASH hepatocellular carcinoma (HCC) tumour samples.

Genome wide ChIP-sequencing of H3K27me3

25 µg of chromatin was taken for the control liver, a 12 week PB treated liver and a PB exposed liver tumour and H3K27me3 chromatin immunoprecipitation carried out using 4 µg of antibody (Millipore, cat # 07-449) as described previously (23). Following this, Illumina libraries were prepared by Active Motif (Active Motif) and samples sequenced by HiSeq and mapped against the mouse reference genome (mm9 build).

Preparation of RNA for strand specific RNA sequencing

Total RNA was extracted from the 2 control livers, 2 livers of mice treated with PB for 12 weeks and 3 tumours which have arisen through sequential DEN/PB treatment. Strand-specific mRNA-seq libraries for the Illumina

platform were generated through poly-A enrichment and sequenced at BaseClear BV (BaseClear, Leiden, the Netherlands). For full methods see supplemental information.

Data Access

All data for this study can be found at the Gene Expression Omnibus (GEO) in the super series GSE77731. Published mouse liver 5hmC datasets were analyzed from GEO series GSE40540 (13,19). Published fetal liver RNAseq data analyzed from GEO samples GSM850909. Mouse embryonic stem cell TET1 genome wide datasets were analyzed from GEO sample GSM611192 .

Results

5-methylcytosine and 5-hydroxymethylcytosine patterns are decoupled during hepatocarcinogenesis progression

As PB is thought to promote carcinogenesis through non-genotoxic mechanisms, epigenetic reprogramming through long term exposure are likely to be an important indicator of liver tumour formation. Our work has shown that mice exposed to tumour promoting doses of PB exhibit changes to both their 5hmC and 5mC liver patterns at the promoter proximal regions of many genes (13,18,19). Here we expand upon these findings to investigate perturbations arising in hepatocellular tumours generated by a DEN/PB regimen (Supplementary Fig. S1A).

Immunohistological staining of sections of mouse liver, following DEN/PB exposure, with antibodies against glutamine synthetase (GS) identify *Ctnnb1*-mutated hepatocellular tumour-lesions which are dependent on an activated Wnt/ β -catenin signalling pathway (Fig. 1A) (24). Staining with antibodies against 5mC or 5hmC reveals that these *Ctnnb1*-mutated tumours have reduced signals for both DNA modifications (Fig. 1A), an observation also reported in several types of human cancer including HCC (16). DNA modification patterns were directly investigated by immunoprecipitation with specific antibodies towards either 5hmC or 5mC prior to hybridization on high density promoter microarrays with a set of control mouse liver DNAs (n=2)

and liver DNAs from mice that had been exposed to phenobarbital in their drinking water for 12 weeks (n=2) or in the excised hepatic tumours from mice which had received PB for 35 weeks (n=3). Analysis of overall Pearson's correlation scores between the samples reveals that the 5hmC pattern is strongly perturbed following drug dosing (and in the tumours), whilst 5mC patterns are largely similar following drug dosing but differ greatly in the tumour samples (Fig. 1B). This result suggests that a change in 5hmC profiles might precede that of 5mC.

Next we defined differentially hydroxymethylated regions (dHMRs) or differentially methylated regions (dMRs) between the control and PB exposed livers or between the control and tumour samples (Supplementary Fig. S1B). Mapping of the resulting dHMRs and dMRs to one of five genomic compartments (inter-genic, promoter distal, promoter proximal, promoter core or intra-genic; Supplementary Fig. S1B) reveals that genomic regions of epigenetic perturbation are largely distinct for the two modifications. Following PB exposure both at 12 weeks and in the resulting liver tumours, 5hmC was strongly lost over the promoter proximal and core regions and both reduced and elevated in the bodies of genes (Supplementary Fig. S1B). In contrast, an increased number of 5mC peaks were observed in the tumour samples compared to those exposed to short term PB. Additionally, although 5mC was seen to be both acquired and lost at intergenic and intragenic loci following 12 week PB exposure, hypo-methylation was typically observed at such sites in the resulting tumours.

Tumour specific promoter hyper-methylation occurs after phenobarbital mediated loss of 5hmC

As the 5hmC modified DNA is itself derived from TET oxidised hydroxylation of a 5mC precursor, we set out to test the relative changes in both marks across all of the promoters. Firstly, we identified a subset of promoter core regions are marked by 5hmC in the normal livers in this study (Fig. 1C). Independent validation of select loci by glucosylation-mediated restriction enzyme sensitive qPCR (gRES-qPCR; see supplementary experimental procedures) reveals that these sites contain ~15% to 20% 5hmCpG, whilst a control region of low 5hmC and 5mC at *Gapdh* was only found to contain less than 5% 5hmCpG (Supplementary Figs. S2 & S3).

Analysis of the DNA modification patterns reveal that there is a dramatic loss in promoter core 5hmC levels in the livers of mice exposed to PB for 12 weeks as well as in the resulting tumours (Supplementary Fig.

S4). In contrast, 5mC levels were unaffected following chronic PB dosing. However we observed a strong acquisition of 5mC at a subset of CpG islands in the tumour samples - the majority of which were initially marked by 5hmC in the healthy liver (Figs. 1C, 1D, Supplementary S4 & S5 and Supplementary Table S1). Extension of this analysis with published liver datasets from mice receiving different lengths of PB dosing reveals that loss of promoter core 5hmC does not occur following acute drug exposure (i.e. 1/7 days) but instead requires longer chronic exposure (i.e. 28days dosing; Supplementary Fig. S6)(19). A reciprocal 5hmC loss/5mC accumulation was also observed at a common set of promoter elements in the PB exposed tumour samples (n=2037), indicating that such a “switch” may be a hallmark of hepatocarcinogenesis progression (Fig. 1E and 1F).

Promoter hypo 5hmC/hyper 5mC is a common feature of mouse liver tumour types with differing activating mutations

To test if the promoter core loss of 5hmC and gain of 5mC is a feature of chemical exposure or is instead a more general hallmark of hepatocarcinogenesis, we also profiled 5hmC and 5mC patterns in two mouse liver tumours of differing pathology (Supplementary Fig. S7). One was a mouse liver tumour that had arisen following DEN induction only, resulting in a Ha-ras mutated tumour (22). The second set of liver tumours resulted from an obesity based mouse model in which neonatal male mice develop multiple HCC's following non-alcoholic steatohepatitis (NASH) onset (21). In both cases we observed a gain of promoter core 5mC at loci normally marked by 5hmC in the healthy tissue (Fig 1G & Supplementary S7). The reciprocal nature of the changes in 5hmC/5mC persist to some degree in all of the three tumour types with a general loss of 5hmC accompanied by a gain in 5mC (Supplementary Figs. S7 & S8). While there was a large degree of commonality in the promoter core spanning probes which exhibited a loss of 5hmC or gain of 5mC between the three tumour types, there were also a number of regions which were unique to the particular tumour type which may reflect stratification of particular cancer subtypes (Supplementary Fig. S9).

Promoter epigenetic dysregulation events are related to only a handful of transcriptional perturbations

In order to test the relationship between promoter core epigenetic dysregulation and transcriptional perturbation during hepatocarcinogenesis progression, we carried out RNA-sequencing on matched control livers (n=2), PB treated livers (n=2) and DEN/PB induced *Ctnnb1* mutated liver tumour samples (n=3). Pearson correlation analysis and principal component analysis (PCA) reveals that the global transcriptomic patterns of the *Ctnnb1* mutated tumours were distinct from both control and PB treated liver (Supplementary Fig. S10). No clear relationship was evident between changes in the epigenetic state at promoter core elements with expression alterations of the associated genes (Fig. 2A). This was most notable at the promoters of genes exhibiting no significant change in gene expression following PB exposure or in the resulting tumours (grey plots Fig. 2A). However there is a significant retention of 5hmC levels over the promoter cores of the genes that are repressed in the tumour samples (Fishers P-value 1.99E-0.7) and a reduction of 5mC levels over tumour induced genes (Fishers P-value 2.52E-0.15); implying that there may be a functional relationship between epigenetic and transcriptomic perturbations in the progression of hepatocarcinogenesis (Fig. 2B). A handful of genes exhibit a strong epigenetic remodelling associated with transcriptional activation, in particular the Cytochrome P450 genes *Cyp2b10* and *Cyp2c55*, which in addition to being two of the most strongly induced genes following PB exposure undergo a strong loss of both promoter core 5hmC and 5mC following PB exposure (Fig. 2C). In agreement with recent research, the genes which were identified as containing promoter core elements which lost 5hmC and gained 5mC upon tumorigenesis correspond to low expressed/transcriptionally silent genes in the healthy control liver (Fig. 2D) – highlighting that aberrant methylation at these loci is not directly linked to a change in their transcriptional status in the tumour (17).

Key signalling pathways are perturbed during hepatocarcinogenesis progression

We next identified differentially expressed genes that arise following PB exposure (control to PB) or upon tumour formation (control to tumour) ($>\log_2$ 2 fold change with associated unadjusted P-values <0.05). Genes exhibiting a change in their gene expression patterns were grouped into one of six classes (“i” to “vi”, Fig. 3A and 3B, & Supplementary Table S2) based on their profile in control, PB treated and the tumours. Functional analysis of the genes in each class reveals that distinct pathways are de-regulated following PB exposure as well as in the PB exposed liver tumours (Fig 3. B). For example genes de-regulated exclusively in the *Ctnnb1*-

mutated tumours are linked to pathways involved in production of cell adhesion molecules, diabetes, PPAR signalling and TGF-beta signalling pathway. As well as genes frequently mutated or transcriptionally perturbed in the progression of many cancers (such as the Wnt signalling protein *axin1* and the cell cycle regulator *ccnd1*) (25,26). Close inspection of the data reveals strong changes in the expression of genes related to control of proliferation (*Cdkn1a*), apoptosis and DNA damage response (*Trp53* & *Gadd45b*), as well as genes associated with known cancer related signalling pathways (the *Tfg- α* gene, the *Tgf- β* pathway related tumour suppressor, *Chd1* and the Wnt signalling component, *Ctnnb1*). In addition we observe strong PB specific responses at select genes previously reported to exhibit transcriptional changes following toxicological challenge (*Gtl2*, *Prom1* & Cytochrome P450 genes) (Fig. 3C). Together this data highlights progressive transcriptomic misregulation that is indicative of physiological changes and a detoxification response.

We also observed specific expression of a series of genes in the tumour samples normally associated with fetal liver (Fig. 3D). For example α -fetoprotein 1 (*Afp1*) and Glypican-3 (*Gpc3*), as well as the hepatocyte nuclear factors 1a, 1b and 4a (*Hnf1a*, *1b* & *4a*), are highly expressed in the developing fetal liver compared to the mature adult tissue, whilst there is reduced expression of albumin (*Alb*) and CCAAT/enhancer-binding protein alpha (*C/EBPa*), which are normally found in the adult liver (Fig. 3D). This may represent either a dedifferentiation of mature hepatocytes into a more fetal like state or positive selection of liver cancer stem cells during tumorigenesis –which would have a potential growth advantage due to their elevated proliferative abilities (27). Similar changes were also observed in published expression array datasets for both PB exposed (*Ctnnb1* mutated) and non-PB exposed (Ha-ras mutated) liver tumours (Fig. 3E) (28).

A unique Polycomb mediated repression signature at CGIs is established in PB exposed mouse liver tumours

It has been widely reported that the global levels and genome wide patterns of the repressive histone modification, H3K27me3, as well as the enzymes responsible for its deposition, are altered in many cancer types including HCC (29-31). We carried out genome wide ChIP sequencing for the H3K27me3 modification to determine if these Polycomb mediated silencing mechanisms are also perturbed in the PB derived liver tumours. Peak finding analysis indicates a general elevation in the levels of H3K27me3 signals in the PB liver

tumours compared to control liver (94,341 200bp window regions gain H3K27me3, 31,995 windows lose H3K27me3 (Fig. 4A and Supplementary Fig. S11).

Focussing on promoter core regions we find that these are relatively unchanged in response to PB but elevated in the resulting tumour (Fig. 4B). Studies suggest that promoter regions marked by H3K27me3 in mouse embryonic stem cells become hyper-methylated upon mammary cancer formation (32). To test this possibility in our mouse liver tumour we directly compared our H3K27me3 and 5hmC/5mC datasets. Plots of promoter core 5hmC and 5mC change against changes in the H3K27me3 signal following PB exposure reveal little change in the histone modification, even though 5hmC levels are lost at a number of loci (Fig. 4C). However, compared to the normal genomic profile there is a strong increase in the number of hyper-H3K27me3 peaks at promoter spanning loci upon tumour progression. Interestingly a significant proportion of promoters acquire 5mC without a change in H3K27me3 occupancy (Fig. 4C). Interestingly we observe only a very modest elevation of the H3K27me3 mark at promoters following PB exposure but a strong elevation in signal is observed at many promoters in the resulting tumours (Fig. 4D & Supplementary Fig. S12). Promoters that become hyper-methylated and hypo-hydroxymethylated in the tumours do not exhibit this elevation as they are already strongly enriched for H3K27me3 in normal healthy livers (Figs. 4D & 4E). In agreement with the notion that the H3K27me3 mark is inhibitory towards transcription, analysis of the promoter regions of genes induced in the tumour indicates full loss of H3K27me3 is coincident with gene activation in the resulting tumours. Conversely the genes that become repressed in the tumour have dramatic gains of H3K27me3 over their promoters in the resulting tumours (Figs. 4D, 4E & Supplementary Fig. S12).

To further test the chromatin landscape of this promoter set in normal control livers we analysed the profiles of several other commonly studied histone tail modifications using publically available mouse liver epigenomics datasets produced by the ENCODE project (<http://www.genome.gov/encode/>) (Fig. 4F). Although there was no observable difference, compared to the total promoter set, in the levels of Histone H3 lysine 4 mono-methylation (H3K4me1), H3 lysine 27 acetylation (H3K27ac) or H3 lysine 36 tri-methylation (H3K36me3), there was an enrichment at these promoters for the typically euchromatic mark H3 lysine 4 tri-methylation

(H3K4me3). This suggest that a proportion of these 5hmC marked promoters are bivalent (simultaneously enriched for H3K4me3 and H3K27me3). Combined analysis of the promoters which lose 5hmC and gain 5mC in the tumours (n=2035) reveals that a strong epigenetic transition occurs during hepatocarcinogenesis at promoter core elements originally bivalent/5hmC marked and transcriptionally silent genes in the normal healthy liver, without leading to expression changes (Fig. 4G & Supplementary Figs. S13 & S14).

Tet1 protein levels are reduced in mouse HCC

In order to better understand the epigenetic dysregulation occurring during hepatocarcinogenesis we investigated the transcriptomic changes in a selection of key epigenetic modifying enzymes (Fig 5A). In general, the majority did not exhibit a strong change in gene expression (>2 fold change vs control tissue). We did, however, observe a strong induction of the histone H3 lysine 9 demethylase *Jmjd1c* and the histone deacetylase *Hdac11* alongside a low to moderate elevation (1.5-2 fold) of several HDACs, methyl-binding proteins (*Mbd1*), Trithorax (*Mll3*) and polycomb group proteins (*Suz12*). The levels of the methyltransferases Dnmt-1 (1.64 FC), -3a (1.51 FC) and -3b (0.98 FC) were not significantly altered. We did not observe changes in levels of expression of the Tet genes (Tet1, 2 and 3) responsible for conversion of 5hmC from 5mC. This result was validated both by microarray and RT-qPCR; thus loss of 5hmC in mouse HCC cannot be explained by the mis-expression of the Tet genes (supplementary Fig. S15) (14,19,33). As levels of the Tet1 protein were previously shown to be reduced in human HCC, we carried out immunohistochemistry for Tet1 in mouse liver tumours (16)(Fig. 5B). In agreement with the results of the human study, we did observe a strong loss of Tet1 staining in MAPK positive tumour cells. Although levels of Tet1 expression in the mouse liver are low (supplementary Fig. S15), this result indicates that protein levels are readily detectable in the normal healthy liver tissue. Published *in vitro* studies have shown that loss of Tet1 is coincident with loss of 5hmC from promoter regions (9,34,35). Moreover in human embryonic carcinoma NCCIT cells Tet1 loss was seen to correlates with hypermethylation of CGIs and CpG island shores (9). Interestingly analysis of Tet1 binding sites in published mESC data reveals an enrichment over the promoters that we identify as becoming aberrantly hypermethylated in mouse liver tumours (Supplementary Fig. S16). To test for the consequences resulting from loss of the Tet1 protein observed in the liver tumours, we profiled 5hmC levels across the promoter

regions in control and Tet1 KO mouse livers by hmeDIP-seq (36). This revealed that loss of Tet1 in the mouse liver results in a dramatic reduction of 5hmC over a large number of promoters (Fig. 5C). This was also observed over the promoter regions identified as becoming aberrantly methylated following a loss of 5hmC during tumorigenesis (Supplementary Fig. S17) – suggesting that direct reduction in Tet1 protein levels or inhibition of TET activity during the progression of tumorigenesis could account for a decrease of promoter associated 5hmC and enable subsequent gain of 5mC.

Discussion

DNA methylation reprogramming has been previously noted for a host of tumour types including HCC (2,14,16,37). In addition, changes to the normal 5hmC patterns have also been reported in both mouse and rat livers following exposure to either non-genotoxic or genotoxic agents (13,19,38). In this present study we build on these early observations to show for the first time in mouse models of liver cancer that early perturbations in the epigenetic landscape over transcriptionally silent CGIs that are marked by low levels of 5hmC and H3K27me3 predicate hyper-methylation that occurs in tumours. We utilised several models of rodent liver tumour models (PB exposed Ctnnb1 mutant tumours, DEN treated Ha-Ras mutant tumours and NAFLD driven tumours) to demonstrate the same observation for each. The reciprocal nature of these changes fits with the notion that these two modifications exist within a programmed DNA methylation/demethylation pathway that is mediated by Tet enzyme activity (39). A reduction of Tet1 protein levels in mouse HCC may account for the observed epigenetic disruption, however this occurs without obvious changes in Tet transcript levels (Figs. 1 & 5). We propose that reduced Tet1 binding and/or activity at target CGIs may be responsible for aberrant epigenetic events in many cancers (Fig. 5D). Direct loss of Tet1 function in liver results in a similar dramatic reduction in promoter 5hmC levels (Fig. 5C). In PB exposed mice reduction of Tet1 and 5hmC levels may also be linked to an elevated rate of proliferation in tumour cells and altered cellular intermediary metabolism (28,40). Indeed it was recently shown that both Tet1 expression and global 5hmC levels are reduced in proliferating cells in culture as well as in rapidly proliferating hepatocytes following partial hepatectomy (41,42). Finally the function of the Tet enzymes may also be affected by altered metabolic programs in these tumours, such as changes in the levels and utilization of the Tet enzyme cofactor α -ketoglutarate (28).

Emerging data suggest that the deregulation of normal Polycomb group proteins (PcG), critical mediators of the “silencing” chromatin modification H3K27me₃, plays causative roles in oncogenesis (31,43). EZH2 is often highly expressed in HCC and can have important roles in tumour progression and fetal liver development (44). H3K27me₃ profiling during mouse PB exposure implies that an “epigenetic switch” takes place at the promoters of genes which are linked to changes in their expression levels during hepatocarcinogenesis; gains in H3K27me₃ are associated with repression of genes in the tumours, while loss is accompanied with their activation (Fig. 4D & 4E). In contrast at the promoters which exhibit aberrant hypermethylation/hypo-hydroxymethylation in the liver tumours, we observe a strong enrichment of H3K27me₃ levels in the healthy tissue, which is not significantly altered in the tumours (Figs. 4C, 4D & 4G). Fittingly these promoters tend to be associated with transcriptionally silent genes - a result which matches with observations in human cancers (17). These findings are also in line with recent reports in which H3K27me₃ modified histone tails were found to be associated with 5mC marked DNA in cultured cancer cells (45,46). Our data builds on previous work which indicated that promoters that are marked with H3K27me₃ in embryonic stem cells are more likely to gain DNA methylation during differentiation and carcinogenesis than those lacking H3K27me₃ (47,48). We suggest that Tet1 binding along with PRC2 components mark promoters that are destined to become hypermethylated in cancer, this can be inferred by the presence of 5hmC at these regions in control liver samples. Similar findings have been published in human cancer cell lines where promoters marked by H3K27me₃ and H3K4me₃ were initially enriched for 5hmC but lose this modification upon depletion of Tet1 by siRNA (9). The finding that 5hmC and H3K27me₃ marked promoters mark sites which later become hypermethylated in the tumours supports a model in which general aberrant methylation at CGIs is not linked with silencing of tumour-suppressor-genes, but instead indicates a de-coupling of TET function from PRC2 complexes at these genes. These relationships between DNA and H3K27 methylation clearly warrant more careful molecular examination *in vivo*, especially as culturing of somatic cells *in vitro* leads to a reduction in Tet levels and subsequent de novo methylation at H3K27me₃ marked promoters (42).

A recent study in human colon cancer reports that promoter proximal regions ~1kb upstream of the TSS marked by 5hmC in the normal colon appear to correlate to sites which is resistant to hypermethylation in colon cancer progression. These results contrast with both our findings and recent observations in mouse mammary tumours and human embryonic carcinoma cells (9,32), in which Polycomb marked promoters in the

normal state may become hypermethylated in the resulting cancer. This indicates that epigenetic disruption during cancer progression is linked to the type of cancer in question and is likely dependant on a combination of both TET protein miss-regulation, tissue background and genetic mutation. Further combined epigenetic and transcriptomic studies, particularly in cancers arising in different tissues, will be essential in order to better understand the precise contribution of epigenetic pathways to hepatocarcinogenesis in particular and to carcinogenesis in general.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Gu L, Frommel SC, Oakes CC, Simon R, Grupp K, Gerig CY, et al. BAZ2A (TIP5) is involved in epigenetic alterations in prostate cancer and its overexpression predicts disease recurrence. *Nat Genet* 2015;47(1):22-30.
2. Sproul D, Meehan RR. Genomic insights into cancer-associated aberrant CpG island hypermethylation. *Brief Funct Genomics* 2013;12(3):174-90.
3. Reddington JP, Perricone SM, Nestor CE, Reichmann J, Youngson NA, Suzuki M, et al. Redistribution of H3K27me3 upon DNA hypomethylation results in de-repression of Polycomb target genes. *Genome Biol* 2013;14(3):R25.
4. Laird A, Thomson JP, Harrison DJ, Meehan RR. 5-hydroxymethylcytosine profiling as an indicator of cellular state. *Epigenomics* 2013;5(6):655-69.
5. Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 2009;324(5929):929-30.
6. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009;324(5929):930-5.
7. Shen L, Zhang Y. 5-Hydroxymethylcytosine: generation, fate, and genomic distribution. *Curr Opin Cell Biol* 2013.
8. Song CX, Yi C, He C. Mapping recently identified nucleotide variants in the genome and transcriptome. *Nat Biotechnol* 2012;30(11):1107-16.
9. Putiri EL, Tiedemann RL, Thompson JJ, Liu C, Ho T, Choi JH, et al. Distinct and overlapping control of 5-methylcytosine and 5-hydroxymethylcytosine by the TET proteins in human cancer cells. *Genome Biol* 2014;15(6):R81.
10. Neri F, Incarnato D, Krepelova A, Rapelli S, Pagnani A, Zecchina R, et al. Genome-wide analysis identifies a functional association of Tet1 and Polycomb repressive complex 2 in mouse embryonic stem cells. *Genome Biol* 2013;14(8):R91.
11. Nestor CE, Ottaviano R, Reddington J, Sproul D, Reinhardt D, Dunican D, et al. Tissue type is a major modifier of the 5-hydroxymethylcytosine content of human genes. *Genome Res* 2012;22(3):467-77.
12. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, et al. Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nat Biotechnol* 2011;29(1):68-72.
13. Thomson JP, Lempiainen H, Hackett JA, Nestor CE, Muller A, Bolognani F, et al. Non-genotoxic carcinogen exposure induces defined changes in the 5-hydroxymethylome. *Genome Biol* 2012;13(10):R93.
14. Lian CG, Xu Y, Ceol C, Wu F, Larson A, Dresser K, et al. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell* 2012;150(6):1135-46.
15. Uribe-Lewis S, Stark R, Carroll T, Dunning MJ, Bachman M, Ito Y, et al. 5-hydroxymethylcytosine marks promoters in colon that resist DNA hypermethylation in cancer. *Genome Biol* 2015;16(1):69.
16. Liu C, Liu L, Chen X, Shen J, Shan J, Xu Y, et al. Decrease of 5-hydroxymethylcytosine is associated with progression of hepatocellular carcinoma through downregulation of TET1. *PLoS One* 2013;8(5):e62828.
17. Sproul D, Kitchen RR, Nestor CE, Dixon JM, Sims AH, Harrison DJ, et al. Tissue of origin determines cancer-associated CpG island promoter hypermethylation patterns. *Genome Biol* 2012;13(10):R84.
18. Lempiainen H, Muller A, Brasa S, Teo SS, Roloff TC, Morawiec L, et al. Phenobarbital mediates an epigenetic switch at the constitutive androstane receptor (CAR) target gene *Cyp2b10* in the liver of B6C3F1 mice. *PLoS One* 2011;6(3):e18216.

19. Thomson JP, Hunter JM, Lempiainen H, Muller A, Terranova R, Moggs JG, et al. Dynamic changes in 5-hydroxymethylation signatures underpin early and late events in drug exposed liver. *Nucleic Acids Res* 2013;41(11):5639-54.
20. Aydinlik H, Nguyen TD, Moennikes O, Buchmann A, Schwarz M. Selective pressure during tumor promotion by phenobarbital leads to clonal outgrowth of beta-catenin-mutated mouse liver tumors. *Oncogene* 2001;20(53):7812-6.
21. Fujii M, Shibazaki Y, Wakamatsu K, Honda Y, Kawauchi Y, Suzuki K, et al. A murine model for non-alcoholic steatohepatitis showing evidence of association between diabetes and hepatocellular carcinoma. *Med Mol Morphol* 2013;46(3):141-52.
22. Rignall B, Braeuning A, Buchmann A, Schwarz M. Tumor formation in liver of conditional beta-catenin-deficient mice exposed to a diethylnitrosamine/phenobarbital tumor promotion regimen. *Carcinogenesis* 2011;32(1):52-7.
23. Dunican DS, Cruickshanks HA, Suzuki M, Semple CA, Davey T, Arceci RJ, et al. Lsh regulates LTR retrotransposon repression independently of Dnmt3b function. *Genome Biol* 2013;14(12):R146.
24. Giera S, Braeuning A, Kohle C, Bursch W, Metzger U, Buchmann A, et al. Wnt/beta-catenin signaling activates and determines hepatic zonal expression of glutathione S-transferases in mouse liver. *Toxicol Sci* 2010;115(1):22-33.
25. Okegawa T, Pong RC, Li Y, Hsieh JT. The role of cell adhesion molecule in cancer progression and its application in cancer therapy. *Acta Biochim Pol* 2004;51(2):445-57.
26. Gorin A, Gabitova L, Astsaturov I. Regulation of cholesterol biosynthesis and cancer signaling. *Curr Opin Pharmacol* 2012;12(6):710-6.
27. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 2006;7(1):21-33.
28. Unterberger EB, Eichner J, Wrzodek C, Lempiainen H, Luisier R, Terranova R, et al. Ha-ras and beta-catenin oncoproteins orchestrate metabolic programs in mouse liver tumors. *Int J Cancer* 2014;135(7):1574-85.
29. Hosogane M, Funayama R, Nishida Y, Nagashima T, Nakayama K. Ras-induced changes in H3K27me3 occur after those in transcriptional activity. *PLoS Genet* 2013;9(8):e1003698.
30. Sasaki M, Ikeda H, Itatsu K, Yamaguchi J, Sawada S, Minato H, et al. The overexpression of polycomb group proteins Bmi1 and EZH2 is associated with the progression and aggressive biological behavior of hepatocellular carcinoma. *Lab Invest* 2008;88(8):873-82.
31. Huether R, Dong L, Chen X, Wu G, Parker M, Wei L, et al. The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. *Nat Commun* 2014;5:3630.
32. Pathania R, Ramachandran S, Elangovan S, Padia R, Yang P, Cinghu S, et al. DNMT1 is essential for mammary and cancer stem cell maintenance and tumorigenesis. *Nat Commun* 2015;6:6910.
33. Neri F, Dettori D, Incarnato D, Krepelova A, Rapelli S, Maldotti M, et al. TET1 is a tumour suppressor that inhibits colon cancer growth by derepressing inhibitors of the WNT pathway. *Oncogene* 2014.
34. Ficiz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, et al. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 2011;473(7347):398-402.
35. Xu Y, Wu F, Tan L, Kong L, Xiong L, Deng J, et al. Genome-wide Regulation of 5hmC, 5mC, and Gene Expression by Tet1 Hydroxylase in Mouse Embryonic Stem Cells. *Mol Cell* 2011;42(4):451-64.
36. Thomson JP, Fawkes A, Ottaviano R, Hunter JM, Shukla R, Mjoseng HKC, R., et al. DNA immunoprecipitation semiconductor sequencing (DIP-SC-seq) as a rapid method to generate genome wide epigenetic signatures. *Sci Rep* 2015;5:9778.
37. Timp W, Feinberg AP. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. *Nat Rev Cancer* 2013;13(7):497-510.

38. Lian CG, Xu S, Guo W, Yan J, Frank MY, Liu R, et al. Decrease of 5-hydroxymethylcytosine in rat liver with subchronic exposure to genotoxic carcinogens riddelliine and aristolochic acid. *Mol Carcinog* 2014.
39. Ficiz G, Gribben JG. Loss of 5-hydroxymethylcytosine in cancer: cause or consequence? *Genomics* 2014.
40. Luisier R, Lempiainen H, Scherbichler N, Braeuning A, Geissler M, Dubost V, et al. Phenobarbital induces cell cycle transcriptional responses in mouse liver humanized for constitutive androstane and pregnane x receptors. *Toxicol Sci* 2014;139(2):501-11.
41. Neri F, Incarnato D, Krepelova A, Dettori D, Rapelli S, Maldotti M, et al. TET1 is controlled by pluripotency-associated factors in ESCs and downmodulated by PRC2 in differentiated cells and tissues. *Nucleic Acids Res* 2015.
42. Nestor CE, Ottaviano R, Reinhardt D, Cruickshanks HA, Mjoseng HK, McPherson RC, et al. Rapid reprogramming of epigenetic and transcriptional profiles in mammalian culture systems. *Genome Biol* 2015;16(1):11.
43. Au SL, Ng IO, Wong CM. Epigenetic dysregulation in hepatocellular carcinoma: focus on polycomb group proteins. *Front Med* 2013;7(2):231-41.
44. Koike H, Ouchi R, Ueno Y, Nakata S, Obana Y, Sekine K, et al. Polycomb group protein ezh2 regulates hepatic progenitor cell proliferation and differentiation in murine embryonic liver. *PLoS One* 2014;9(8):e104776.
45. Statham AL, Robinson MD, Song JZ, Coolen MW, Stirzaker C, Clark SJ. Bisulfite sequencing of chromatin immunoprecipitated DNA (BisChIP-seq) directly informs methylation status of histone-modified DNA. *Genome Res* 2012;22(6):1120-7.
46. Takeshima H, Wakabayashi M, Hattori N, Yamashita S, Ushijima T. Identification of co-existence of DNA methylation and H3K27me3 specifically in cancer cells as a promising target for epigenetic therapy. *Carcinogenesis* 2014.
47. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet* 2007;39(2):232-6.
48. Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet* 2007;39(2):237-42.

Fig. Legends

Fig. 1. Levels and distributions of DNA modifications are altered following PB exposure and in resulting liver tumours. **A.** Immunostaining of liver sections with glutamine synthetase (GS) reveals *Ctnnb1* mutated nodules. 5hmC and 5mC levels are reduced in the tumour relative to the surrounding regions. **B.** Pearson's correlation clustering of datasets for 5hmC (i) and 5mC (ii) profiles. **C.** Heat map analysis of log₂ 5hmC (blue) and 5mC (red) signals over promoter regions (TSS +/- 2kb) ranked by tumour 5mC level (Tum 1-3, right panel). Normal control livers: "Cntl", PB exposed for 12 weeks: "PB", *Ctnnb1* mutated liver tumours: "Tum". CGIs are denoted by black bars in the far right plot. Average 5hmC and 5mC patterns are plotted below (solid black lines) against the mean signal from the control samples (dashed green line). Dashed box outlines promoters which lose 5hmC/gain 5mC in the tumours. **D.** Changes in promoter core 5hmC vs 5mC are plotted for each gene promoter between replicate control livers (i), between average control : 12 week PB treated livers (ii) and between average control and average tumour samples (iii). **E.** Venn diagram displaying the level of overlap in promoters which both lose 5hmC and gain 5mC in three *Ctnnb1* tumour samples relative to the control livers. The total number of hypo 5hmC/hyper 5mC promoter cores in each tumour is shown between brackets. **F.** Examples of 2 promoters which exhibit PB loss of 5hmC/ tumour gain of 5mC. All data is plotted between log₂ -1.5 to log₂ +1.5. **G.** Heat map analysis of log₂ 5hmC (blue) and 5mC (red) signals over the promoter regions (TSS +/- 2kb) present on the array ranked by Ha-ras tumour 5mC level for average normal control livers (n=2), average *Ctnnb1* tumours (n=3) and a Ha-ras mutated liver tumour. CGIs are denoted by black bars in the far right plot. Average 5hmC and 5mC patterns across all promoters are plotted below

Fig. 2. Epigenetic perturbations weakly associate with changes in transcriptional state during hepatocarcinogenesis. **A.** Scatter plot analysis of change in promoter core 5hmC (i) and 5mC (ii) signal versus log₂ fold change in expression following PB exposure or resulting tumours. Green: induced (>log₂ 2 fold elevated), red: repressed (>log₂ 2 fold repressed), grey: unchanged expression. Select genes are highlighted. **B.** Boxplot of change in promoter core 5hmC (i) and 5mC (ii) over the total gene set (grey), induced (green and repressed (pink) genes between control and tumour samples. Although typically following the global trend (i.e. loss of 5hmC/gain in 5mC in all three gene sets) there is significantly more 5hmC over the promoter cores of repressed genes and less 5mC over induced gene sets. Fisher's P-values are shown between plots. **C.** Example

of epigenetic perturbation and transcriptional changes at the *Cyp2b10* gene. 5mC and 5hmC microarray data plotted from log₂ -1.5 to log₂ +1.5. RNA-seq data plotted from RPKM of 0 to 2000. **D.** Average RPKM value across total gene set for control (C1, C2: greens), PB (PB1, PB2: blues) and tumour (T1, T2 & T3: reds).

Fig. 3. The normal liver transcriptome is perturbed following PB exposure and in resulting tumour samples.

A. Sets of genes which change their transcriptional activity following either PB treatment, in the PB tumours or in both sets of samples were stratified into one of 6 groups (i-vi) plotted as change in expression relative to the control liver group. Examples of select induced genes (blue) and repressed genes (red) are shown under each group in a dashed box. **B.** Plots of P-values associated with significantly enriched pathways based analysis of groups of genes induced (i-iii) or repressed (iv-vi) following PB treatment or in the tumour samples relative to control livers. No pathways were found enriched in the gene set from group vi (down PB only). **C.** Plots of RPKM values (reads per kilobase per million) over candidate genes of interest which have potential roles in cell signalling, cancer progression and xenobiotic response. Roman numeral above each gene is related to the groups identified in Fig 3A. **D.** Plots of change in the levels of established fetal and adult liver specific transcripts between control livers to those exposed to PB for 12 weeks (blue bars), to PB exposed liver tumours (red bars) or to fetal livers (yellow bar). Change in expression is plotted as log₂ fold change vs average signal from the control livers. **E.** Fold change in expression signals for fetal and adult liver transcripts taken from published microarrays relative to control livers for PB exposed *Cttnb1* mutated liver tumours (red), non-PB driven Ha-Ras mutated tumours (pink) and long term PB exposed livers without tumours (grey).

Fig. 4. Polycomb signatures are perturbed in the PB exposed & *Cttnb1* mutated liver tumours.

A. Distribution of hyper- and hypo-H3K27me₃ peak windows normalised to the number of each feature. Hyper-H3K27me₃ peaks: blue bars, hypo-H3K27me₃ peaks: yellow bars. Promoter distal : TSS -2kb to -1kb, promoter proximal: TSS -1kb to -250bp, promoter core TSS+/-250bp, Intra-genic: TSS+250bp to TES, Inter-genic: all remaining features **B.** Box plot of promoter core H3K27me₃ signals for control (grey), 12 week PB (yellow) and tumour (maroon) samples. **C.** Density scatter plot of change in promoter-core DNA modification state versus change in H3K27me₃ signal for average 5hmC and 5mC upon PB exposure (i & iii) or in the tumour samples (ii & iv). **D.** Plots of average control and tumour H3K27me₃ patterns across the total promoter cohort, promoters

identified as hypo-5hmC/ hyper-5mC in the tumours, promoters of tumour induced genes and promoters of tumour repressed genes. Control liver patterns: Black line, Tumour patterns: brown line, Control liver H3K27me3 pattern from total promoter set: dashed green line. Plots cover promoter regions TSS +/- 2kb. **E.** Examples of H3K27me3 ChIP-seq patterns, 5hmC & 5mC microarray patterns and RNAseq expression states at loci identified in Fig. 4D. **F.** Average patterns of several histone modifications in the control livers at the tumour hypo-5hmC/ hyper-5mC promoter regions. black line: patterns across the total promoter set. Plots shown for TSS +/-2kb regions. **G.** Heat map analysis of the bivalency modifications H3K27me3 and H3K4me3, DNA modifications and expression status over the tumour hypo-5hmC/hyper-5mC promoter-core regions. A random selection of 1000 promoters were tested as a comparison. H3K27me3: green, H3K4me3: purple, 5hmC and 5mC (blue: high, red: low), RNAseq RPKM (grey).

Fig. 5. The role of Tet1 in the maintenance of epigenetic state during hepatocarcinogenesis. **A.** Heat-map of expression change (fold change in RPKM) for key epigenetic proteins following PB exposure and in tumour samples. Green: induced, red: repressed. **B.** Immunostaining of control healthy liver and two liver tumours for glutamine synthase (GS: hepatocytes at central vein), Mitogen-activated protein kinase (MAPK: tumour positive cells) and Tet1. **C.** Heatmap analysis of 5hmC landscape at promoters (TSS +/- 2kb) in control healthy liver versus those in a Tet1 deficient mouse (*Tet1*^{-/-}). 5hmC levels ranked by control promoter core level. Blue = high, Red= low). **D.** Model illustrating how Tet1 may regulate the CGI epigenetic landscape in liver. Examples of epigenetic landscapes are shown for the promoter regions of genes transcriptionally induced or repressed during hepatocarcinogenesis or at the promoters of genes which exhibit aberrant promoter hypo hydroxymethylation/hypermethylation in the liver tumours. H3K27me3: Orange triangle, H3K4me3: green oval, 5hmCpG: blue lollypop, 5mCpG: black lollypop, unmodified CpG: white circle. Possible mechanisms to explain the role of Tet1 at CGIs are outlined (i-iv). Transcription factor (TF) binding events are also shown. For reference the global changes in the three epigenetic marks are shown alongside with a gain in a mark represented by a green arrow, a loss in the mark by a red arrow and no change by a black bar.

Figure 1

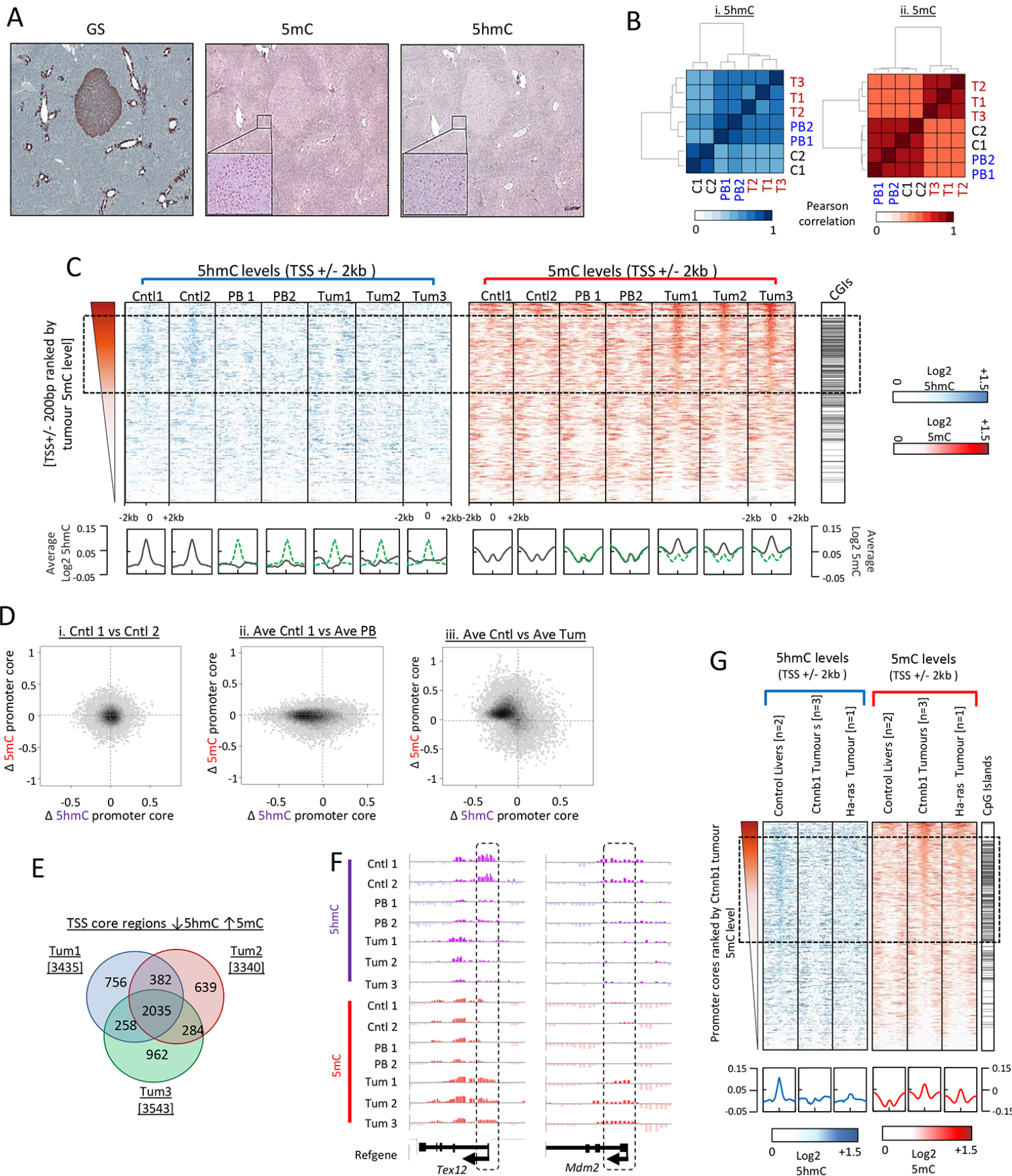


Figure 2

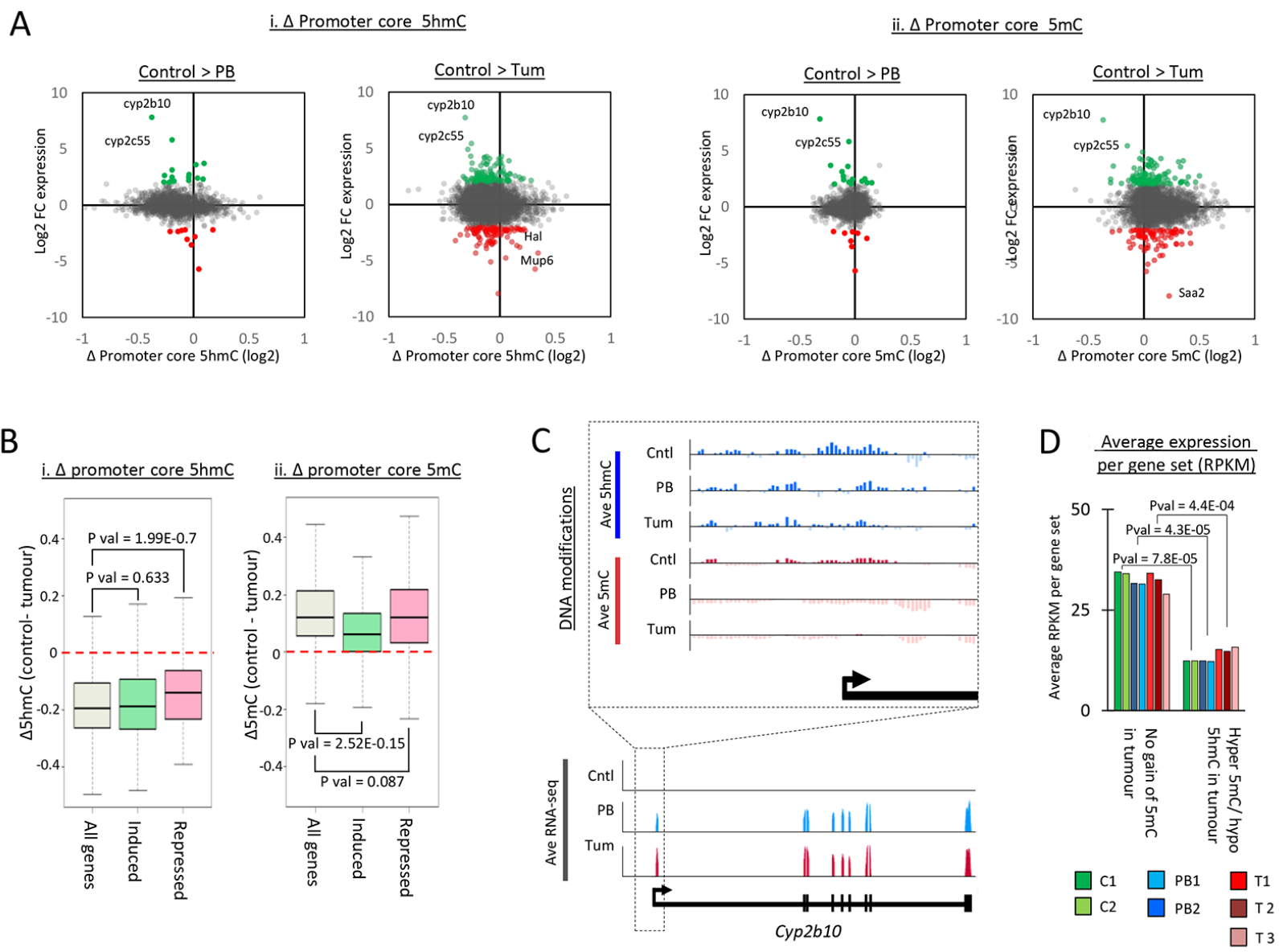


Figure 3

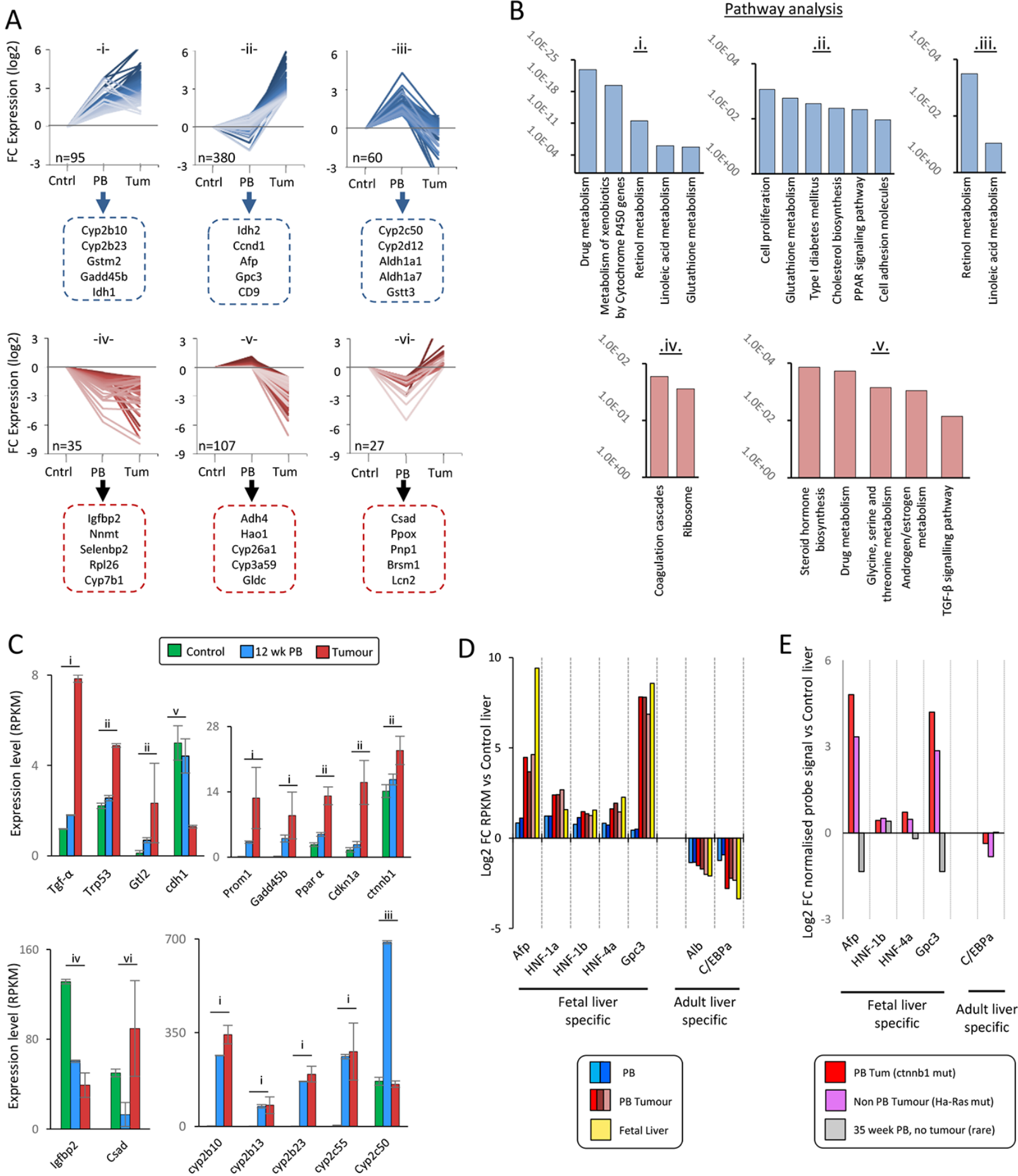


Figure 4

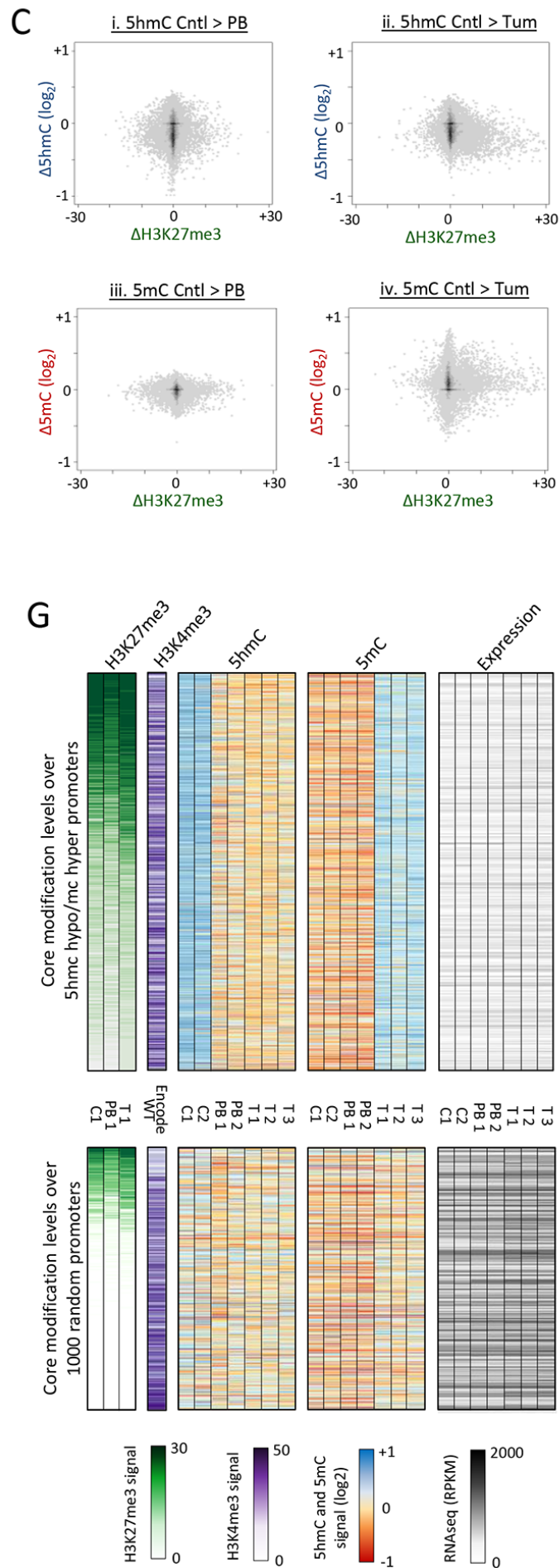
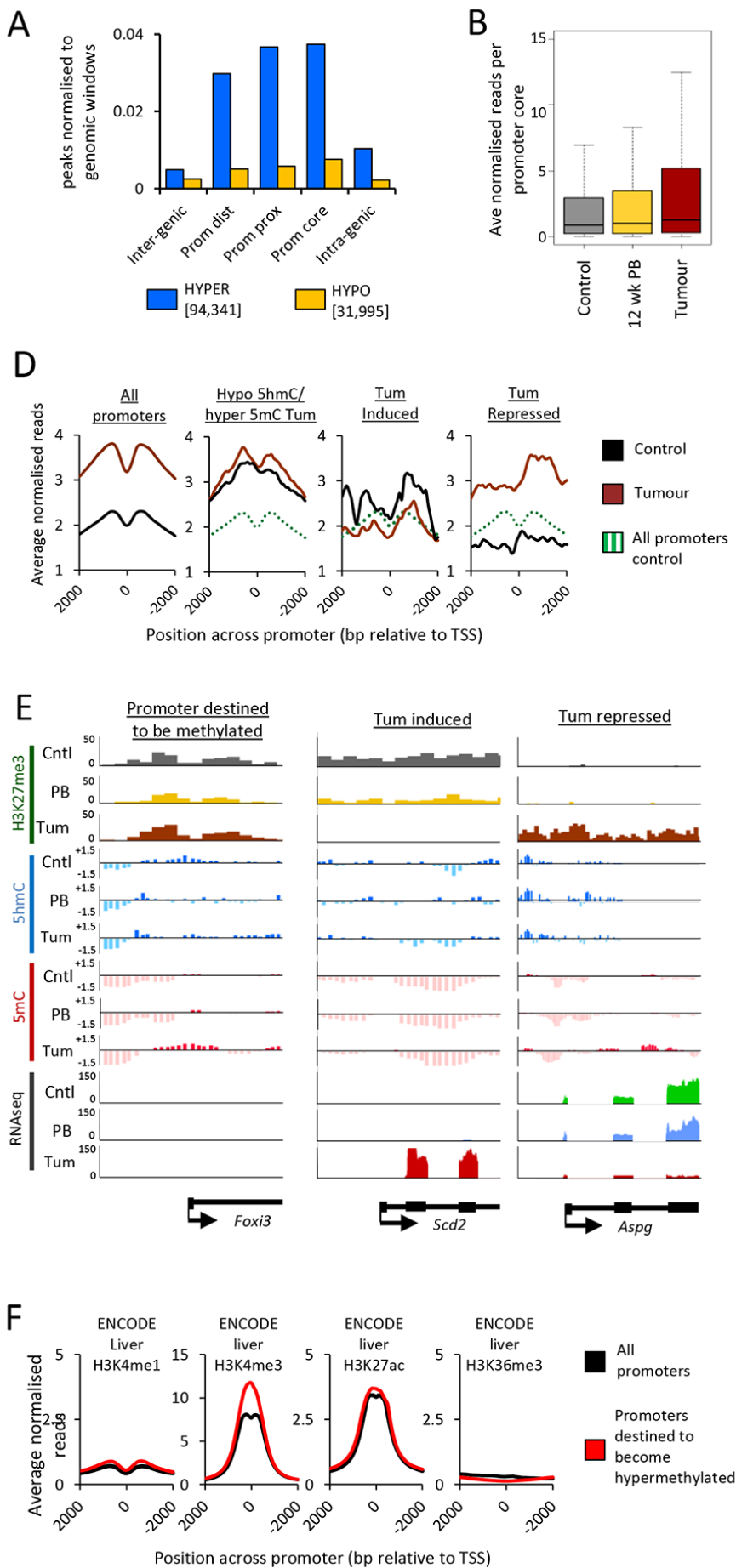
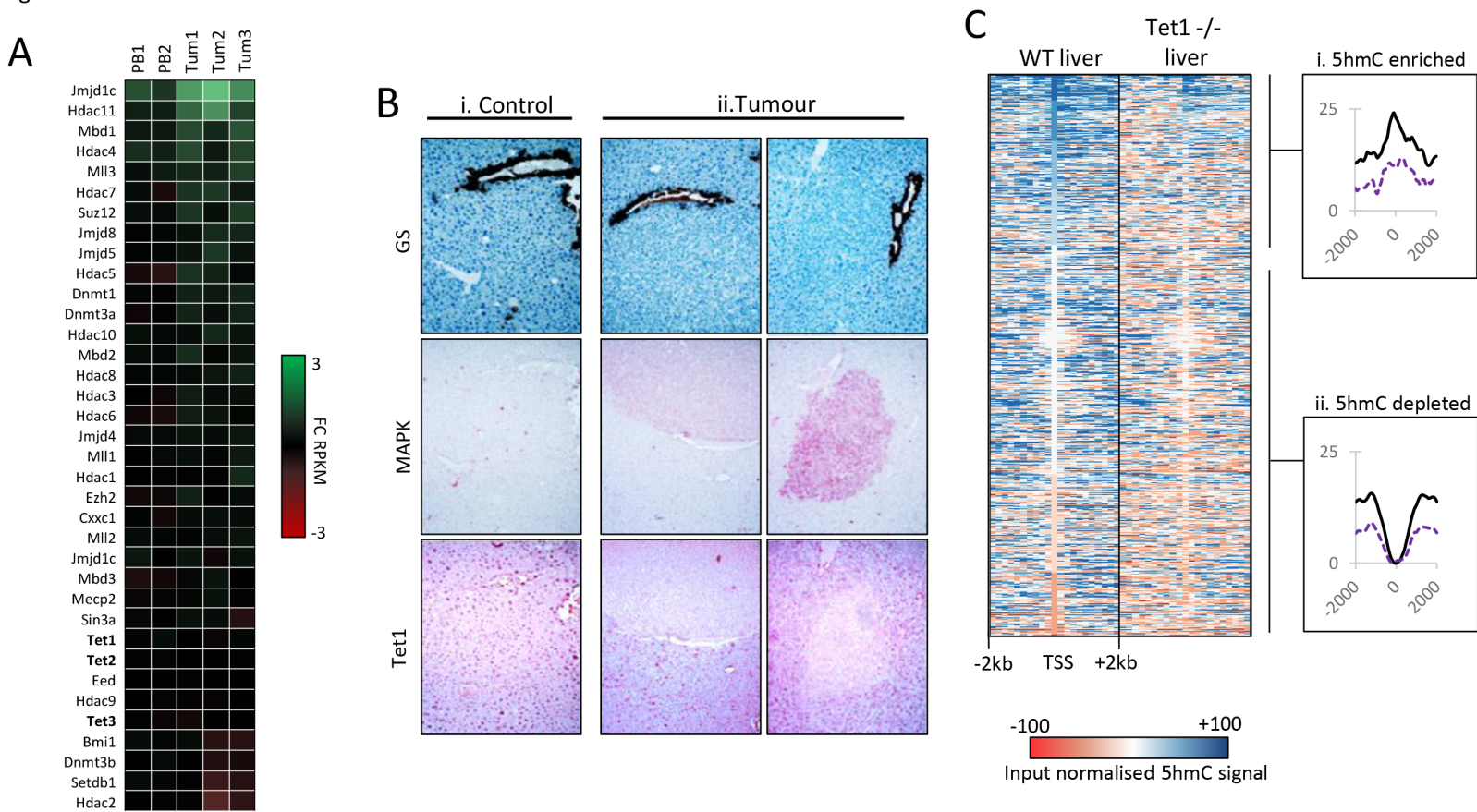


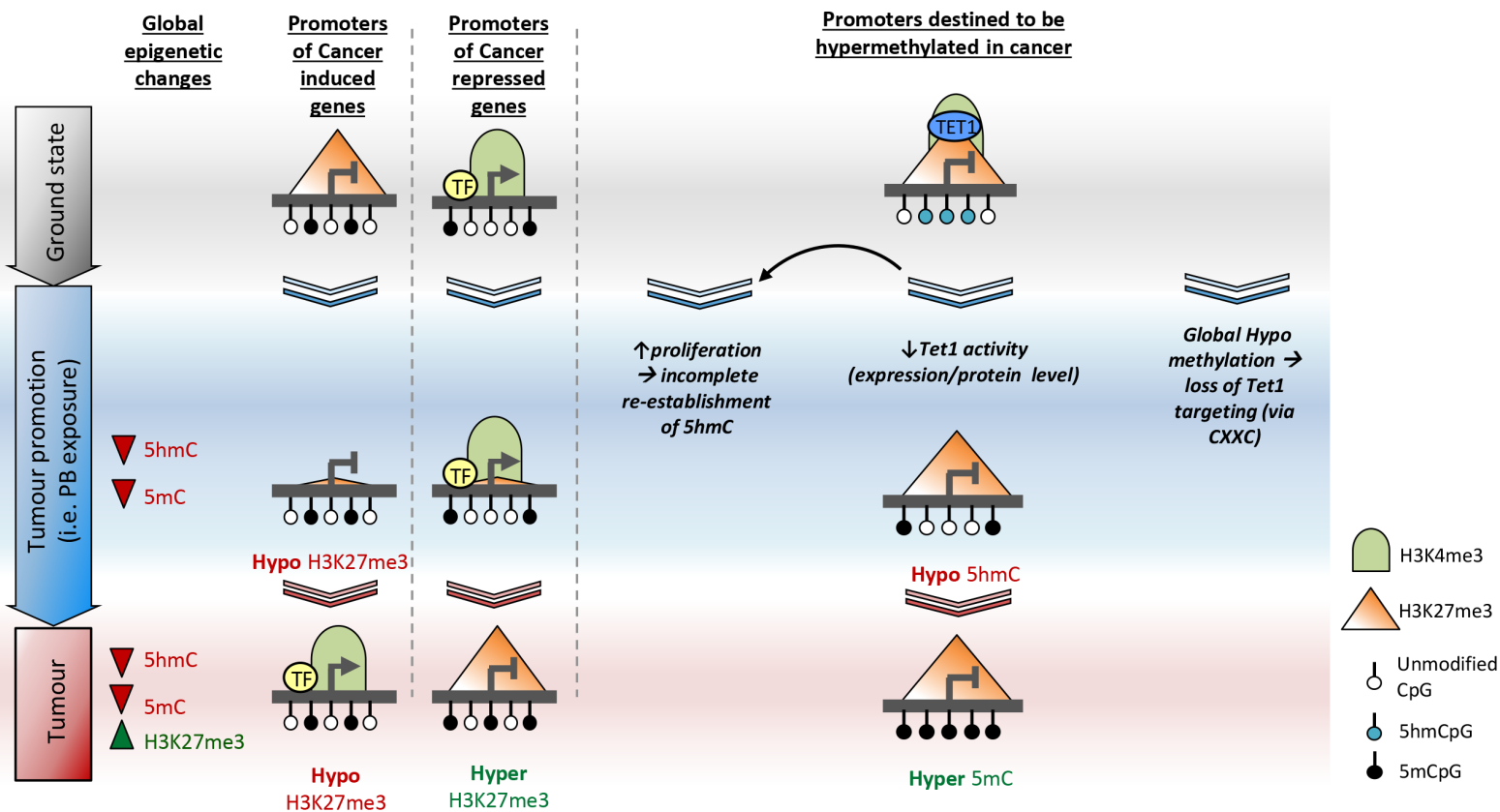
Figure 5



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Global

Gene Promoters



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Loss of Tet1 associated 5-hydroxymethylcytosine is concomitant with aberrant promoter hypermethylation in liver cancer

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