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Tissue-specific 5-hydroxymethylcytosine landscape of the human genome

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ABSTRACT

5-Hydroxymethylcytosine (5hmC) is an important epigenetic mark that regulates gene expression. Charting the landscape of 5hmC in human tissues is fundamental to understand its regulatory functions. Here, we systematically profiled the whole-genome 5hmC landscape at single-base resolution for 19 types of human tissues. We found that 5hmC preferentially decorates gene bodies and outperforms gene body 5mC in reflecting gene expression. Approximately one-third of 5hmC peaks are tissue-specific differentially hydroxymethylated regions (tsDhMRs), which are deposited in regulatory elements that regulate the expression of nearby tissue-specific functional genes. In addition, tsDhMRs are enriched with tissue-specific transcription-factor-binding sites and may rewire tissue-specific gene expression networks. Moreover, tsDhMRs are associated with SNPs identified by genome-wide association study (GWAS), linked to tissue-specific phenotypes and diseases. Collectively, our results show the tissue-specific 5hmC landscape of the human genome and demonstrate that 5hmC serves as a fundamental regulatory element affecting tissue-specific development and diseases.

Introduction

DNA methylation at the fifth position of cytosine (5mC), which is established and maintained by DNA methyltransferases (DNMTs), is a predominant epigenetic modification that is critical for various biological and pathological processes, including silencing of transposable elements, regulation of gene expression, genomic imprinting and X-chromosome inactivation^{1,2}. 5-Hydroxymethylcytosine (5hmC), also known as the “sixth base” of DNA, was discovered as another relatively abundant form of cytosine modification in Purkinje neurons and mouse embryonic stem cells (mESCs)^{3,4}. Further studies found that the ten-eleven translocation (TET) family of Fe(II)- and α -ketoglutarate (α -KG)-dependent DNA dioxygenases (including TET1, TET2, and TET3) catalyzes the sequential oxidation of 5mC to 5hmC, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)⁴⁻⁶. Subsequently, the DNA repair enzyme thymine-DNA glycosylase (TDG) can excise 5fC and 5caC to generate abasic sites, which eventually results in the regeneration of unmodified cytosines by base excision repair (BER) pathway⁷⁻⁹. This TET-TDG pathway is known as the active DNA demethylation pathway.

In addition to being an intermediate of 5mC oxidation, evidence shows that 5hmC is a stable epigenetic mark with regulatory functions¹⁰. The specific genomic distribution pattern of 5hmC, such as its high enrichment in the gene bodies of transcriptionally active genes, promoters and enhancers, hints at specific biological roles of 5hmC¹¹⁻¹³. In addition, the 5hmC level undergoes highly dynamic changes during development, differentiation and cancer^{4,14,15}. For instance, the global 5hmC

content is dramatically reduced in multiple human cancers compared with that in the normal tissues adjacent to the cancer¹⁶⁻¹⁸, suggesting that dysregulation of genomic 5hmC may be involved in tumorigenesis. Mechanistically, abnormal hydroxymethylation status impacts chromatin structure by interrupting the interaction of 5hmC-specific binding proteins with 5hmC¹⁹. Together, existing data have suggested a critical role of 5hmC in developmental processes and an association of dysregulation of 5hmC with human diseases.

Different methods have been developed to map the genomic distribution of 5hmC, including affinity-based methods (5hmC-DIP-Seq, CMS-Seq, GLIB and hMe-Seal) and high-resolution methods (oxBS-Seq, TAB-Seq, hmC-CATCH, TAPS and ACE-Seq)^{20,21}. For instance, we previously developed hmC-CATCH²², which is a bisulfite-free method for genome-wide detection of 5hmC. hmC-CATCH couples selective chemical labeling and biotin pulldown²²⁻²⁵, thus allowing 5hmC enrichment and detection at single-base resolution. While hydroxymethylome maps have been obtained for mammalian cell lines and mouse tissues, 5hmC profiles in human tissues are poorly characterized thus far.

In this study, we generated genome-wide, base-resolution 5hmC data via hmC-CATCH across 19 human tissue types, represented by 60 tissue samples derived from 6 Chinese donors. We found that 5hmC is enriched in gene bodies and that gene body 5hmC exhibits a better positive correlation with gene expression than gene body 5mC. Tissue-specific differentially hydroxymethylated regions (tsDhMRs) may serve as regulatory elements and colocalize with transcription-factor-binding sites to regulate

the tissue-specific gene expression program. Furthermore, tsDhMRs were shown to enrich disease-related GWAS SNPs, linking 5hmC to human phenotypes and pathologies. Collectively, our results provide a high-resolution, high-quality atlas of DNA hydroxymethylation across diverse human tissues and provide a resource for exploring the role of this modification in development and human diseases.

Results

5-Hydroxymethylcytosine landscape of diverse human tissues

To systematically investigate the dynamics of DNA hydroxymethylation across different human tissues, we collected samples of 19 tissue types from 6 Chinese donors, including 3 males and 3 females (Fig. 1a, Supplementary Table 1). We profiled the 5hmC signals of various human tissues via hmC-CATCH (60 hmC-CATCH samples; 83-183 million paired-end reads per sample; average, 128 million paired-end reads) (Supplementary Table 2). We found that the 5hmC-containing spike-in sequence was specifically and efficiently enriched (Extended Data Fig. 1a) and displayed a high detection rate for 5hmC (~91% and ~98% before and after pull down) (Extended Data Fig. 1b, c, Supplementary Table 3), both of which showed the high quality of the hydroxymethylome generated by hmC-CATCH. In addition, tissues derived from the same organ system from different donors were clearly clustered together (Fig 1b, Extended Data Fig. 1d), further demonstrating the confidence of the 5hmC data.

We next analyzed the genomic features of 5hmC. We identified 721,404 reproducible 5hmC peaks, most of which were located in intron and intergenic regions (Fig. 1c). The peak number of each tissue type ranged from 272,391 to 525,080 (Extended Data Fig. 1e). We found that 5hmC is highly enriched in gene bodies, especially in exon regions, while it is depleted in intergenic regions (Extended Data Fig. 1f). Taking the HOX gene clusters as examples, we observed 5hmC signals in distal regulatory elements, promoters and gene bodies; such signals can also be dynamic among tissues (Fig. 1d, Extended Data Fig. 2). Hence, via hmC-CATCH, we were able to generate the whole-genome 5hmC landscape of different human tissues.

Hydroxymethylome at single-base resolution

To obtain a more detailed and clearer picture of the hydroxymethylome, we analyzed 5hmC sites at single-base resolution. We identified 9,416,937 reproducible 5hmC sites, with the site number of each tissue type ranging from 1,217,850 to 2,429,878 (approximately 3%-10% CpG sites were hydroxymethylated) (Extended Data Fig. 3a). More than half of the 5hmC sites were found in at least two tissues, suggesting that a substantial number of 5hmC sites could be conserved (Extended Data Fig. 3b). Most of the 5hmC sites were located in intron and intergenic regions (Fig. 2a) and were highly enriched in gene bodies (Extended Data Fig. 3c). The number of 5hmC sites in the brain was significantly higher than that in other tissues (p value: 6.57×10^{-7}), consistent with the higher 5hmC content in the brain²¹.

5mC in the CG context (5mCG) is almost symmetric due to the maintenance of DNA methyltransferase 1 (DNMT1). A total of 98.46% of the 5hmC sites identified are located in the CG context (Fig. 2b), but only approximately 13% of the 5hmCG sites are symmetric (Extended Data Fig. 3d). In addition, the proportion of 5hmC in the non-CG context (5hmCH) is variable in different tissues, ranging from 0.96% to 2.79% (Fig. 2c, Extended Data Fig. 3e). Previous research suggests that DNA methylation is rapidly accumulated in 5mCH sites during synaptogenesis²⁶, and we also found a higher 5hmCH ratio in the brain than in other tissues (Fig. 2c, Extended Data Fig. 3e), indicating that 5hmCH may play a role during brain development.

We next analyzed the 5hmC context in tissues and found a “CA^{hm}CGT” motif for 5hmCG (Fig. 2d, Extended Data Fig. 3f, g), which coincides with the binding site of ARNT (Fig. 2e), a housekeeping gene that participates in important metabolic processes. Within ARNT ChIP-seq signals, cytosines are hydroxymethylated in most tissues (Fig. 2f), further indicating that 5hmC may positively impact the genomic occupancy of ARNT. Consistent with this, it has been reported that the ARNT binding motif loses 5hmC signals in esophageal cancer patients compared to healthy individuals²⁷. With regard to the 5hmCH modification, we found that the most frequent base following 5hmCH is adenine (Fig. 2g, h, Extended Data Fig. 3h, i), consistent with the 5mCH sequence preference²⁸.

Taken together, the base-resolution hydroxymethylome analysis results presented above revealed the varied 5hmCH ratio among tissues, the asymmetric distribution of

5hmCG and the sequence preference of 5hmC, providing a potential mechanism for how 5hmC can be modified, recognized and dynamically regulated.

Gene body 5hmC excels 5mC in reflecting gene expression

Because the 5hmC level in the gene body shows a positive correlation with gene expression in mECSs and the mouse brain¹⁹, we analyzed whether the gene body 5hmC level in human tissues also corresponds to gene expression. Indeed, we found that stronger gene body 5hmC signals were correlated with higher gene expression levels (Fig. 3a, Extended Data Fig. 4a). For instance, the genes that escape X chromosome inactivation display higher gene body 5hmC levels than the inactive genes (Extended Data Fig. 4b, c). Because the gene body 5mC level is reported to be positively correlated with transcription (Fig. 3b, c)¹, we also compared the gene body 5mC and 5hmC levels. Although 5mC colocalizes with 5hmC in all gene bodies (Fig. 3d), we found that the gene body 5hmC level exhibits a stronger quantitative correlation with gene expression levels than the gene body 5mC level.

To further explore the role of the 5hmC gene body in controlling the expression of tissue-specific genes, we defined 4,031 such genes using RNA expression data generated by the Genotype-Tissue Expression project (GTEx)²⁹ (Extended Data Fig. 4d). The expression of tissue-specific genes and gene body 5hmC levels also demonstrated a positive correlation, which was absent for 5mC (Extended Data Fig.

4d-g). Correlations of representative tissue-specific marker genes are shown in Fig. 3e-g. For instance, *PGA4* is specifically expressed in the stomach and encodes the precursor of pepsin; we found the highest level of gene body 5hmC signals in the stomach among all tissues (Fig. 3e). In contrast, gene body 5mC failed to display a positive correlation with tissue-specific gene expression (Fig. 3f). Using *CYP4A11* as another example showed the following: this gene is specifically expressed in the liver and is involved in drug metabolism and the synthesis of cholesterol; consistent with this function, we also found higher gene body 5hmC levels in the liver than in other tissues (Fig. 3h). Again, this pattern was not observed for 5mC (Extended Data Fig. 4h). Collectively, our data show that the gene body 5hmC level correlates well with gene expression and could play an important role in maintaining tissue-specific functions.

tsDhMRs function as tissue-specific regulatory elements

5hmC is dynamic during development and differentiation^{4,14,15,30}; nevertheless, the dynamics of 5hmC in different tissues have not been reported. Based on our hydroxymethylome of human tissues, we identified 33.31% (240,269 out of 721,404 peaks tested) of the 5hmC peaks as being differentially hyperhydroxymethylated in different tissues, which we term tissue-specific differentially hydroxymethylated regions (tsDhMRs) (Fig. 4a, See Methods). A majority of the tsDhMRs were located in intron and intergenic regions (56.0% and 31.5%, respectively, Fig. 4b, Extended Data Fig. 5a). We further analyzed the histone marks using data from the ENCODE project³¹

and found that tsDhMRs are showed high enrichment of histone modification signals, including H3K4me1 and H3K27ac, in the corresponding tissues (Fig. 4c). Thus, tsDhMRs may predominantly function as regulatory elements. In addition, tsDhMRs show higher evolutionary conservation than random regions (Extended Data Fig. 5b), suggesting the importance of such functional elements.

We then investigated how these tsDhMRs may regulate the gene expression programs of specific tissues. We adopted an existing method to identify putative regulatory element-gene linkages occurring within a 500-kb window³². In total, we identified 4,154 genes whose expression showed significant correlations with 5hmC signals in tsDhMRs (Fig. 4d). These are genes with tissue-specific expression, such as *CYP11B2* in adrenal glands and *NPPB* in heart. Gene ontology (GO) analysis demonstrated that these genes are enriched in tissue-specific functions, such as learning or memory (brain), kidney epithelium development (kidney), female gonad development (ovary) and sex differentiation (uterus) (Fig. 4e). Similarly, KEGG pathway analysis revealed that tsDhMR-associated genes participate in tissue-specific functional pathways (Extended Data Fig. 5c). *CYP2C8*, which is involved in the metabolism of xenobiotics, is highly expressed in liver, with sharp and specific 5hmC peaks present upstream of *CYP2C8* in liver (Fig. 4f). Meanwhile, *PTF1A* plays a vital role in mammalian pancreatic development and displays increased 5hmC levels around the gene (Extended Data Fig. 5d). In summary, tsDhMRs act as regulatory elements specifically affecting the expression of nearby functional genes.

Tissue-specific transcription factors are enriched in tsDhMRs

We next analyzed, within tsDhMRs, potential transcription-factor-binding site (TFBS) clusters profiled by the ENCODE project. We found that more than half of the tsDhMRs overlapped with at least one TFBS, and 20.3% of the tsDhMRs even overlapped with no less than four TFBSs (Fig. 5a). We further investigated different transcription factors (TFs) enriched in tsDhMRs derived from each tissue type and found that tissue-specific TFs were significantly enriched in the corresponding tsDhMRs (Fig. 5b, Extended Data Fig. 6a). Taking NEUROD1 and HNF4A as examples, NEUROD1 plays an important role in the regulation of the differentiation process of various nervous system cells during development, while HNF4A regulates the expression of several hepatic genes. By comparing to ENCODE ChIP-seq data, we found that NEUROD1 specifically binds to brain-specific DhMRs, while HNF4A specifically binds to liver-specific DhMRs (Fig. 5c, Extended Data Fig. 6b). We further used *LINGO1* and *CYP2C18*, which are two downstream targets of NEUROD1 and HNF4A in the brain and liver, respectively, to demonstrate the tissue-specific coexistence of 5hmC signals (Fig. 5d). Thus, key tissue-specific TFs may rewire their tissue-specific network through binding to tsDhMRs.

To further understand the regulatory functions, we constructed regulatory networks mediated by HNF4A and NEUROD1 with their corresponding tsDhMRs. We found that HNF4A can regulate genes with tissue-specific expression, such as *CYP4F2*,

CYP8B1, and *UGT1A9*, via a process potentially mediated by liver-specific DhMRs (Fig. 5e). Within the NEUROD1 network, brain-specific DhMRs regulate *GRIK3*, *CSMD2* and other genes to perform their brain-specific functions (Fig. 5f). Through network analysis, we illustrated that the key TFs interact with tsDhMRs, which may further affect the expression of tissue-specific functional genes.

GWAS SNPs prefer to locate within tsDhMRs

We next analyzed the potential relationship of tsDhMRs with functional single-nucleotide polymorphisms (SNPs). We found that the tsDhMRs derived from each tissue highly overlapped with the GTEx single-tissue eQTL SNPs (Fig. 6a). In fact, tsDhMRs contain SNPs that are functional in the corresponding tissues. Moreover, tsDhMRs are also enriched for GWAS SNPs³³ with phenotypes related to the corresponding tissue functions (Extended Data Fig. 6c), indicating that tsDhMRs contribute to tissue-related diseases (Fig. 6b). We used several examples to elaborate the findings: (1) SNPs related to electrocardiographic traits and QRS duration are highly enriched in heart-specific DhMRs; (2) SNPs related to metabolite levels and LDL cholesterol are enriched in liver-specific DhMRs; and (3) SNPs related to type 2 diabetes are enriched in pancreas- and adrenal gland-specific DhMRs.

More specifically, we used an example to illustrate the potential mechanism by which distal GWAS SNPs may impact diseases. *HCN4*, which is necessary for the

cardiac pacemaking process, is specifically expressed in the heart (Fig. 6c). We found that several heart-related GWAS SNPs were localized in the heart-specific DhMR (Fig. 6c), indicating that the DhMR is associated with heart diseases. Moreover, several enhancers of the heart identified by ENCODE candidate cis-regulatory elements (cCREs) were located in this DhMR (Fig. 6c). We further integrated the VISTA enhancer data³⁴, which were validated by transgenic mouse assays, and found an enhancer (enhancer element 2161 from VISTA) within the DhMR. This enhancer is specifically expressed in the mouse heart (images from VISTA database) (Fig. 6d). These data confirm that DhMR, as an enhancer, is functional in the heart and is related to heart diseases. These results indicate that dysregulation of tsDhMRs may be involved in human disease pathologies. Collectively, our data show that tsDhMRs could help us understand the function of distal GWAS SNPs in the corresponding tissues.

Discussion

In this study, we present a base-resolution atlas of 5hmC in human tissues. Hundreds of thousands of 5hmC peaks and millions of 5hmC sites were identified in this dataset, expanding the epigenomic landscape determined by previous large-scale efforts, for example, the ENCODE project.

Gene body 5hmC levels are positively correlated with gene expression, especially of genes with tissue-specific expression. Thus, the gene body 5hmC levels may be used to infer gene expression in tissues. This can be particularly useful in some precious clinical samples, where RNA degradation is severe (frozen samples, formalin-fixed

paraffin-embedded samples, body fluids and so on). For instance, 5hmC levels in cell-free DNA (cfDNA) have been utilized as biomarkers for cancer diagnosis^{22,35-37}. While it is anticipated that healthy and cancerous tissues may have different hydroxymethylomes, cancer-specific 5hmC signatures, which may reflect the gene expression program in different cancers, could be identified for noninvasive cancer diagnosis. Thus, the relative stability of epigenetic modifications makes 5hmC a promising candidate for prediction of gene expression in clinical samples.

Using the hydroxymethylome of multiple tissues, we discovered that approximately one-third of all 5hmC peaks are tissue-specifically hyperhydroxymethylated. We found that tsDhMRs, as cis-regulatory elements, are positively correlated with gene expression, which is in contrast to the fact that differentially methylated regions (DMRs) show a negative correlation³⁸. Moreover, tissue-specific TFs are enriched in tsDhMRs, providing a mechanism by which key TFs may regulate tissue-specific gene expression via tsDhMRs. Based on our identified tsDhMRs, future studies could be designed to illustrate the specific mechanisms by which 5hmC can regulate tissue development and differentiation.

Through integration of GWAS SNP and 5hmC data, we discovered that tsDhMRs were significantly enriched with GWAS SNPs. Our data indicate that tsDhMRs, as cis-regulatory elements, contribute to tissue-related diseases. Although GWAS SNPs are consistent among somatic cells, they may affect tsDhMR 5hmC levels and ultimately lead to dysfunctions of the corresponding tissues. Our analysis provides new insights into the understanding of GWAS data, where distal GWAS SNPs interact with tsDhMRs

to regulate target genes. Disruption of tsDhMR 5hmC levels may result in tissue-related disease phenotypes.

Collectively, our data provide a rich resource for understanding the 5hmC landscape in human tissues. The reported human tissue hydroxymethylome adds to the knowledge of how this epigenetic mark may affect tissue-specific differentiation and diseases.

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Author Contributions

C.Y., W.T. and Q.Z. designed the study. 5fC labeling chemical compounds are synthesized by B.D. X.Z., B.H., Y.F. and H.Z. performed experiments. C.Z. and B.H. analyze the high-throughput sequencing data with help from H.M. B.H., C.Z., X.Z., W.T. and C.Y. wrote the manuscript.

Competing Interests Statement

B.X., A.Z., C.Z. and C.Y. are co-inventors on filed patents (PCT/CN2014/087479 and 201710111600.9) for the labeling strategies of 5fC and 5hmC.

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Methods

Biospecimen Collection

We collected a total of 60 tissue samples at Zhongshan Hospital (Shanghai, China) after obtaining research consent from the families; we targeted 19 distinct tissues from 6 postmortem Chinese individuals, including 3 males and 3 females. The collected samples were transferred to cryovials for long-term storage at -80°C.

Genomic DNA library construction and sequencing

Genomic DNA was isolated from tissues using the Blood/Cell/Tissue Genomic DNA Extraction Kit (TIANGEN, DP304) following the manufacturer's specifications. The 5hmC sequencing libraries were constructed with 200 ng of genomic DNA through the hmC-CATCH method reported previously²², which is a bisulfite-free, base-resolution method for genome-wide detection of 5hmC. Briefly, the DNA was fragmented with a Covaris device (Gene Company Limited, ME220) to 300-500 bp, followed by end repair and addition of the 3'dA nucleotide. Endogenous 5fC was blocked by hydroxylamine, and endogenous 5hmC was oxidized to 5fC using K₂RuO₄. The newly generated 5fC was labelled with 5-(2-azidoethyl)-1,3-indandione (AI) (J&K, 2793948), and the AI-labeled ssDNA was used directly for library preparation with the TELP protocol³⁹. It is worth mentioning that we designed a homemade adapter with a unique molecular identifier (UMI) for deduplication⁴⁰. Subsequently, click chemistry was performed by adding DBCO-S-S-PEG3-Biotin (Click Chemistry Tools, A112-10), followed by pull-down for enrichment of 5hmC-containing DNA. Finally, we obtained

libraries by performing PCRs on subsets of 5hmC-containing DNA.

Data processing

Illumina sequencing adapters and low-quality reads were removed from raw sequencing data to obtain clean data. We added a sequencing barcode through the hmC-CATCH protocol to mark PCR duplicated reads. Then, the PCR-duplicated reads were filtered out, and only one read was retained using an in-house script. The final cleaned reads were mapped to hg38 by Bismark (Version: v0.15.0). To enhance the signal-to-noise ratio, we used only read pairs with more than one C-to-T conversion for further analysis.

Assessing the C-to-T conversion rate of 5hmC sites

We added a model sequence as a spike-in before constructing the hmC-CATCH library, of which one C site was 100% hydroxymethylated. After treatment through the hmC-CATCH protocol, we observed a C-to-T conversion signal in this 5hmC site. We used the $T/(C+T)$ of the sequencing data at this site to estimate the C-to-T conversion rate.

Identification of 5hmC sites

We used Bismark (Version: v0.15.0) to extract single-base-resolution information. Sites with less than five total bases (NT+NC) or three NTs were discarded for 5hmC calling. Then, we used the binomial distribution with N as the sequencing depth (NC+NT) and p as the normal cytosine conversion rate to assess the probability of observing NT by

chance. We considered 5hmC sites with the Holm-Bonferroni method-adjusted $P < 0.001$ and located within a type of tissue 5hmC-enriched region as high-confidence 5hmC sites.

Identification of 5hmC peaks

Our hmC-CATCH approach could enrich DNA fragments with 5hmC. Here, we used a peak calling method to identify these regions with 5hmC. MASC2 was applied to call peaks in each sample with the following command:

```
“macs2 callpeak -t <5hmC bam> -c <input bam> -g hs -f BAMPE --keep-dup  
all --outdir <outdir> -n <sample name>”
```

Annotation of 5hmC sites and peaks

The 5hmC sites or peaks were annotated by `annotatePeaks.pl` (Homer, Version: v4.5), and the “-annStats” parameter was added to quantify the enrichment of genomic elements compared to the background. Then, the $\log_2$ ratios of observation to expectation in all tissues were plotted as a bar chart by `ggplot2` (R package).

tSNE cluster of the global 5hmC signals

The whole hg38 genome was first cut into 10-kb nonoverlapping bins. Then, the read counts in each 10-kb bin of all samples were calculated by “`Bedtools multicov`” (Version: v2.27.1). After normalizing the sequencing depth (`DESeq2`, R package) and batch effect (`limma`, R package), we performed tSNE clustering (`Rtsne`, R package) to reduce the high-dimensional data to two dimensions. `ggplot2` (R package) was used to

visualize the data.

Analysis of the correlation of gene body 5hmC and 5mC

The 5mC data of human tissues were downloaded from the ENCODE project. Then, the RPKM values of all protein-coding genes were calculated and normalized as mentioned above. Spearman's correlation coefficients of the RPKM values between 5hmC and 5mC in matched tissues were calculated by R (`cor, method="spearman"`).

Identification of genes with tissue-specific expression

We used GTEx gene expression data (RNASeQCv1.1.9_gene_median_tpm) to identify the genes with tissue-specific expression that were defined as being highly expressed in one tissue. We first filtered out the genes with a mean TPM less than 1, which was regarded as low expression in all tissues. Then, we calculated the fold changes in the expression of protein-coding genes in one tissue over the mean values for other tissues. We ordered the genes according to fold change levels, and the top 300 in each tissue were regarded as genes with tissue-specific expression. To ensure that the top 300 genes with tissue-specific expression in each tissue were significantly more highly expressed than others, we filtered out the genes with fold changes lower than 2.

Identification of tissue-specific differential 5hmC regions

We first merged all 5hmC peaks from the 60 samples to obtain the total peaks using "Bedtools merge". Then, the read counts in each merged peak of all samples were

calculated by “Bedtools multicov” and normalized as mentioned above. We merged all biological replicates from the same tissue to enhance the signals. We used the Poisson distribution to estimate the p value of each peak in a tissue. The probability of read counts in each peak of one tissue was estimated by the one-tail Poisson distribution with the parameter λ as the mean for other tissues. The p values were further adjusted by the Bonferroni method. The peaks with adjusted p values less than 0.05 and fold changes more than 2 were regarded as significant tissue-specific differential 5hmC regions.

Identification of tsDhMR-associated genes

We adapted a method to link tsDhMRs to putative genes³². We first identified all possible genes linked to tsDhMRs by searching any TSS of GENCODE protein-coding genes within 500 kb of the tsDhMRs. Then, we calculated the Pearson correlation coefficients between normalized 5hmC signals and gene expression (TPM). To avoid spurious associations, we used cor.test (R) to assess the stochastic links. Finally, we required that the confident links had a Pearson correlation over 0.8 and p value lower than 0.05.

Motif enrichment analysis

For each tissue, we used findMotifsGenome.pl (Homer) to find the motifs enriched in tsDhMRs with the following command:

```
“findMotifsGenome.pl <tsDhMRs bed> hg38 <output> -p 5”
```

Analysis of tsDhMRs associated with GWAS SNPs

We downloaded previously published GWAS datasets from the GWAS catalog. Then, we calculated the associations of GWAS phenotypes and tsDhMRs. A GWAS phenotype always has multiple associated SNPs. For each phenotype, we used Fisher's test to compute the significance and odds ratios between the phenotype-associated SNPs and tsDhMRs.

Data availability

Sequencing data have been deposited into the Gene Expression Omnibus (GEO) under the accession number GSE134078.

(<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE134078>).

Figure legends

Figure 1. Hydroxymethylation landscape of human tissues. **a.** Human tissues analyzed in this study. Samples are denoted by the two-letter code in parentheses. Colors indicate each tissue type. **b.** tSNE cluster of all tissues using the global 5hmC signals under 10-kb bins. **c.** Pie chart showing the percentages of 5hmC peaks in each class of genomic features. The promoter regions are defined as 2000 bp around the TSS. **d.** IGV visualization of the 5hmC signals surrounding the HOXD gene cluster on chromosome 2. 5hmC signals in promoter, gene body or intergenic region are highlighted.

Figure 2. Single-base resolution profiles of 5hmC. **a.** Pie chart showing the percentages of 5hmC sites in each class of genomic features. **b.** Distribution of 5hmCG, 5hmCHH and 5hmCHG sites. **c.** Proportions of 5hmCH in different tissues. **d.** Sequence context ± 10 bp around 5hmCG sites. **e.** The most significant 5hmC motif (the upper panel) demonstrates partial sequence overlap with the known transcription factor motif of ARNT (the lower panel). **f.** IGV visualization of the 5hmC site signals. 5hmC is asymmetric at the 5hmCG site. **g.** Sequence context ± 10 bp around 5hmCHG sites. **h.** Sequence context ± 10 bp around 5hmCHH sites.

Figure 3. Gene body 5hmC correlates well with gene expression in human tissues.

a. 5hmC profiles of genes expressed at high (yellow), low (cyan) and silenced (blue) levels in heart tissue. **b.** Spearman's correlation of gene body 5hmC (5mC) signals and

gene expression levels. Random regions were selected as controls. *** represents P values < 0.0001 (Wilcox test was performed to compare the difference). **c.** 5mC profiles of genes expressed at high (yellow), low (cyan) and silenced (blue) levels in heart tissue. **d.** Scatter plot showing the correlation of 5mC and 5hmC signals at gene bodies. **e.** Heatmap displaying the expression levels of tissue-specific marker genes. Gene expression of matched tissue samples was downloaded from the GTEx project. **f.** Heatmap displaying the normalized gene body 5hmC signals of tissue-specific marker genes. The order of the row is identical to (e). **g.** Heatmap displaying the normalized gene body 5mC signals of tissue-specific marker genes. The order of the row is identical to (e). The 5mC data were downloaded from ENCODE. **h.** IGV visualization of the 5hmC signals at the gene body of *CYP4A11* and surrounding regions. Gene expression levels of the gene in different tissues are shown on the right.

Figure 4. tsDhMRs are tissue-specific regulatory elements. **a.** Heatmap showing the normalized 5hmC signals in tsDhMRs. **b.** Pie chart showing the percentages of tsDhMRs in each class of genomic features. **c.** Profiles of H3K27ac and H3K4me1 modifications around tsDhMRs in the liver (LI) and spleen (SX). **d.** Heatmap showing the expression of genes associated with tsDhMRs within 500 kb. **e.** GO enrichment and representative genes of tsDhMR-associated genes. **f.** IGV visualization of the 5hmC signals near *CYP2C8* on chromosome 10. The highlighted regions are liver-specific DhMRs.

Figure 5. tsDhMRs are enriched for tissue-specific TFBSs. **a.** Overlap of tsDhMRs with ENCODE TFBSs. **b.** The TF motifs enriched in tsDhMRs of each tissue. The color scale represents the $-\log_{10}(p \text{ value})$. **c.** ChIP-seq signals of NEUROD1 and HNF4A around BR-specific DhMRs and LI-specific DhMRs. **d.** IGV visualization of 5hmC signals and ChIP-seq signals of NEUROD1 and HNF4A in the brain and liver. **e.** Key TF regulatory networks in the liver and brain. Hexagon represents Key TF; rhombus represents tsDhMRs; circle represents regulated genes. The line linking the hexagon and rhombus indicate that the TF binds to the tsDhMRs, which is supported by TF ChIP-seq data.

Figure 6. tsDhMRs enrich tissue-specific, phenotypic GWAS SNPs. **a.** Overlap of tsDhMRs with single-tissue eQTL SNPs. eQTL SNP data are from GTEx. **b.** Representative GWAS phenotypes enriched in tsDhMRs. **c.** IGV visualization of the 5hmC signals around *HCN4* on chromosome 15. The locations of GWAS SNPs and VISTA enhancers are also shown. The highlighted region shows the LI-specific DhMRs. **d.** In vivo reporter assay of enhancer activity for VISTA enhancer element 2162, as obtained from the VISTA enhancer browser³⁴.

Figure 1

a

b

c

d

Figure 2

Figure 3

Figure 4

Figure 5

a Heatmap showing the enrichment of tsDhMRs across 19 tissues (AG, BR, ES, HR, LI, LN, OV, PA, SE, SK, ST, SX, TR) for single-tissue eQTLs. The color scale represents the Enrichment p value from -4 to 4.

b Heatmap showing the enrichment of tsDhMRs across 20 GWAS phenotypes (AG, AO, BD, BR, ES, HR, KD, LE, LI, LN, OV, PA, SE, SK, ST, SX, TC, TR, UT). The color scale represents the Enrichment p value from 0 to 8.

c Genomic tracks for the HCN4 locus (Chr15:73,315,277-73,387,668). Tracks include GWAS SNPs, VISTA Enhancer hg38, ENCODE heart cCREs, and HCN4 gene structure. A yellow box highlights the VISTA Enhancer Element 2161. A dashed line separates the NA (Non-Active) and A (Active) regions.

d VISTA Enhancer Element 2161 visualization. The image shows a 3D model of the enhancer element and a corresponding 2D heatmap of the enhancer activity across 19 tissues.

Figures

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