Charting a dynamic DNA methylation landscape of the human genome

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DNA methylation is a defining feature of mammalian cellular identity and is essential for normal development1–2. Most cell types, except germ cells and pre-implantation embryos3–5, display relatively stable DNA methylation patterns, with 70–80% of all CpGs being methylated5. Despite recent advances, we still have a limited understanding of when, where and how many CpGs participate in genomic regulation. Here we report the in-depth analysis of 42 whole-genome bisulphite sequencing data sets across 30 diverse human cell and tissue types. We observe dynamic regulation for only 21.8% of autosomal CpGs within a normal developmental context, most of which are distal to transcription start sites. These dynamic CpGs co-localize with gene regulatory elements, particularly enhancers and transcription-factor-binding sites, which allow identification of key lineage-specific regulators. In addition, differentially methylated regions (DMRs) often contain single nucleotide polymorphisms associated with cell-type-related diseases as determined by genome-wide association studies. The results also highlight the general inefficiency of whole-genome bisulphite sequencing, as 70–80% of the sequencing reads across these data sets provided little or no relevant information about CpG methylation. To demonstrate further the utility of our DMR set, we use it to classify unknown samples and identify representative signature regions that recapitulate major DNA methylation dynamics. In summary, although in theory every CpG can change its methylation state, our results suggest that only a fraction does so as part of coordinated regulatory programs. Therefore, our selected DMRs can serve as a starting point to guide new, more effective reduced representation approaches to capture the most informative fraction of CpGs, as well as further pinpoint regulatory elements.

Changes in DNA methylation patterns and the resulting DMRs have been the focus of numerous studies in the context of normal development and disease6. These studies have characterized many different DMR classes including partially methylated domains7, condition-specific8, cell-type-specific9,10 and tissue-specific DMRs11,12, as well as DMRs arising in diseases such as cancer13,14. Owing to the relatively small fraction of genomic CpGs assayed or small sample cohorts, the question of what fraction of genomic CpGs changes its methylation state in the context of normal development as well as their regulatory context remains underexplored.

In this study, we systematically investigated the DNA methylation state of most human autosomal CpGs to determine those that show dynamic changes and hence may participate in genome regulation in a developmental context (dynamic CpGs). In total, we included 42 whole-genome bisulphite sequencing (WGBS) data sets, comprising a range of human cell and tissue types (n = 30). The combined 40.4 billion reads enabled us to assay 25.71 million autosomal CpGs (≥5× coverage in at least 50% of all samples; 96% of all hg19 autosomal CpGs) (Supplementary Table 1). We organized the samples into four classes: human embryonic stem (ES) cells and human ES-cell-derived cell populations, primary cells, disease conditions, and long-term cultured cell lines (Fig. 1a and Supplementary Table 1). On a global scale, human ES cells and their derivatives exhibit the highest DNA methylation levels, followed by primary cells (~5% less), which is in sharp contrast to the global hypomethylation observed in colon cancer (~10–15% less) and long-term cultured cell lines (10–30% less).

Focusing initially on our developmental sample set (n = 24 total, ES cells, in-vitro-derived cell types and primary cells; Supplementary Table 1) we identified ~5.6 million dynamic CpGs (minimum methylation difference ≥ 0.3, false discovery rate (FDR) = 10.4%, 21.8% of captured autosomal CpGs; Fig. 1b, Supplementary Fig. 1e and Supplementary Information) distributed across 716,087 discrete DMRs (19.2% of the mappable human genome; Supplementary Table 2). In addition to this moderately stringent cut-off, we also tested thresholds as low as 10% methylation difference that may account for DNA methylation changes arising from relevant small subpopulations in heterogeneous tissue samples or noise, but still only find 10.4 million CpGs to be dynamic (Supplementary Fig. 1a–d).

Focusing on the more stringent set (≥0.3 difference), we find approximately 70% are on average highly methylated (>75% methylation ratio), whereas less than 2% are on average unmethylated (<10% methylation ratio) (Supplementary Fig. 1h). In line with this observation, we find that hypomethylation of DMRs shows greater sample specificity than hypermethylation (Fig. 1c). Interestingly, most of the DMRs are small (>75% are smaller than 1 kilobase (kb); Supplementary Fig. 1i) and located distal to transcription start sites (Supplementary Fig. 1j). However, the average variation in DNA methylation levels across all ReSeq promoters (n = 30,990) does still exhibit a clear increase specifically at the transcription start sites, with most of this variation occurring at intermediate and low CpG density promoters (Fig. 1d). For CpG islands in general, we observe distinct dynamic regimes, highlighting that different classes of CpG islands are probably subject to different modes of regulation15,16. Consistent with previous reports15, we find CpG island shores (regions within 2 kb of an island)15 to be among the most variable genomic regions (Supplementary Fig. 1o).

These observations are exemplified at the OCT4 (also known as POU5F1) locus, in which the promoter and large parts of the gene body exhibit high DNA methylation dynamics, whereas the strong downstream CpG island as well as the surrounding CTCF-binding sites remain static (Fig. 1e). Only 12.2% of our DMR set overlap with at least one of four annotated classic gene-centric genomic features (promoter, exon, CpG island (CGI), or CGI-shore; n = 568,430) (Fig. 1f). To gain insights into the role of the remaining set, we first investigated their co-localization with DNase I hypersensitive sites across 92 distinct cell types as well as a catalogue of putative enhancer elements for 31 cell and tissue types. Notably, we found that 42.3% of our DMRs overlap with at least one

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Figure 1 | Identification and characteristics of DMRs in the human genome. a, Principal component (PC) analysis based on CpG methylation levels for 1-kb tiles across 30 diverse human cell and tissue samples. Colouring indicates classification of samples into subgroups and group-wise mean DNA methylation. Detailed sample annotations are listed in Supplementary Table 1. Grey area indicates Alzheimer’s disease (AD) samples. b, Density scatterplot of CpG-wise DNA methylation level differences (x axis, \( P \leq 0.01 \)) and CpG median methylation (y axis) across the 24 developmental samples (excluding cancer and long-term culture). Colouring indicates CpG density from low (blue) to high (red). The red box highlights dynamic CpGs (\( \alpha \geq 0.3 \)). c, Cumulative distribution of DMR specificity. High hypo/hypermethylation specificity indicates that a particular region is methylated/unmethylated in most tissues and deviates from this default pattern across various genomic features. Each region is assigned to only one of these genomic categories according to the ranking promoter, CGI, CGI shore, 5’ upstream, 3’ downstream, DNase I hypersensitive (DHS), TFBS cluster track, Enhancer-ChIP (EtoH), and RefSeq (Fig. 2a). Grey areas mark twenty-fifth, fifty-fifth, and seventy-fifth percentiles. The bottom distribution of mean DNA methylation difference for each genomic feature. Black bars indicate twenty-fifth and seventy-fifth percentiles; white dots mark the median. For CGI islands, a smaller, experimentally determined set (eCGI; \( n = 25,490 \)) is also shown. Promoters are broken down into high CpG content (HCP; \( n = 24,899 \)), intermediate CpG content (ICP; \( n = 10,920 \)) and low CpG content (LCP; \( n = 7,946 \)) regions \( (n = 43,765 \text{ total}) \). Shore denotes regions within 2 kb of an island; eShore denotes experimentally determined shore. pEnhancer, putative enhancer. e, Methylation level variation across the OCT4 locus (chr6: 31,119,000–31,162,000) (top). Blue bars indicate significant DMRs at \( P \leq 0.01 \), and exhibit a minimum difference \( \alpha \geq 0.3 \) across the 24 developmental samples. Grey boxes (1–3) are examples of regions that are specific to one promoter (1), both pEnhancers (2), or that do not meet the threshold of dynamic (3). f, Distribution of DMRs across various genomic features. Each region is assigned to only one of these genome features according to the ranking promoter, CGI, CGI shore, 5’ exon, exon, intron, putative enhancers, DNase I hypersensitive site (DHS) or other.

DNase I hypersensitive site (Fig. 1f), and 26.1% co-localize with enhancer-like regions, which cover more than 50% of all H3K27ac regions in our catalogue \( (n = 285,344) \) and represents one of the most differentially methylated features (Fig. 1d). Next, we examined DMR overlap with transcription-factor-binding site (TFBS) clusters compiled from 165 transcription factors profiled by the ENCODE project\(^2\) and uncovered a highly significant overlap of the two feature classes \( (\text{odds ratio} \text{ 1.14, } P < 0.01 \text{ empirical test, Supplementary Information}) \). Interestingly, we find that more than 50% of all DMRs overlap with at least one and 25% with more than three TFBSs, accounting for an additional 13.0% of DMRs. Consistent with this, we find markedly increased variation in DNA methylation levels specifically across TFBS (Supplementary Fig. 2a). In summary, we were able to attribute 64.2% of all DMRs to at least one putative gene regulatory element or coding sequence...
We determined all cell-type-specific hypomethylated regions \((n = 430,250; \text{see Supplementary Information})\) and investigated the enrichment for 161 ENCODE factors (excluding MBD4, SETDB1, POL2P and HDAC2 from the previous set). Notably, we observe significant enrichment of cell-type-specific transcription factors that are known to be involved in the regulation of the respective cellular states (Fig. 2b). For instance, the top three factors enriched in HUES64-specific DMRs are OCT4, SOX2 and NANOG (Fig. 2b). Similarly, PU.1 and TAL1 are highly enriched in CD34 cells and hepatocyte nuclear factors in adult liver (Fig. 2b).

In further support of this, motif enrichment analysis revealed many more interesting cell-type-specific transcription factor associations, such as enrichment of distinct NKX factors in fetal heart and brain, and ESRRG in fetal adrenal cells (Supplementary Fig. 2b and Supplementary Table 3). Moreover, we tested whether the DMR set can be used to gain insights into the combinatorial control of cellular states by transcription factors. To that end, we determined all unmethylated \((\leq 10\% \text{ methylation})\) PAX5 motif instances \(\geq 100\) base pairs (bp)) across the human genome in CD34 or fetal brain cells (Fig. 2c). Although both footprint sets show a large overlap \((11,031\) sites), regions exclusively unmethylated in CD34 or fetal brain are enriched for distinct sets of other known lineage-specific transcription factor motifs; such as PU.1 in CD34 and LMX1A or EN1 in fetal brain (Fig. 2c). Taken together, these findings highlight that cell-type-specific DNA methylation patterns can be used to detect footprints and infer potential co-regulation by transcription factors. In fact, more than 60% of all ENCODE TFBSs are hypermethylated in most samples, but become hypomethylated in other known lineage-specific transcription factors. To that end, we determined all unmethylated \((\leq 10\% \text{ methylation})\) PAX5 motif instances \(\geq 100\) base pairs (bp)) across the human genome in CD34 or fetal brain cells (Fig. 2c). Although both footprint sets show a large overlap \((11,031\) sites), regions exclusively unmethylated in CD34 or fetal brain are enriched for distinct sets of other known lineage-specific transcription factor motifs; such as PU.1 in CD34 and LMX1A or EN1 in fetal brain (Fig. 2c). Taken together, these findings highlight that cell-type-specific DNA methylation patterns can be used to detect footprints and infer potential co-regulation by transcription factors. In fact, more than 60% of all ENCODE TFBSs are hypermethylated in most samples, but become hypomethylated very specifically in only one or two cell types (Fig. 2d), whereas 25% are constitutively unmethylated and never change (Fig. 2d).

Breaking down this distribution of TFBSs reveals distinct patterns of variation for different types of transcription factor (Supplementary Fig. 2e). More generally, we find that DNA methylation variation across TFBSs is strongly correlated with its median methylation level and therefore the hypo- methylation specificity (Supplementary Fig. 2c), as well as the tissue specificity of transcription factor expression \(22\) (Supplementary Fig. 2d). These observations support the notion \(23\) that selective transcription factor binding creates and/or maintains spatially highly constrained hypomethylated regions and confers cell type specificity.

On the basis of these findings and previous reports \(24\), we asked whether DMRs are more susceptible to point mutations that are functionally consequential. Even with strict filtering criteria, we found a significant enrichment of single nucleotide polymorphisms (SNPs) in DMRs compared to genomic background as well as different sets of random control regions \(\text{odds ratio } 1.06, P < 10^{-16}, \text{binomial test; Supplementary Information})\). We then determined the overlap of DMRs specifically enriched human-specific CpGs, termed CpG beacons \(25\), which shows a marked enrichment \(\text{odds ratio } 1.37–1.6 \text{ compared to genomic background and random control regions, } P < 10^{-16}\). This suggests overall higher genetic intra-species variability specifically at regions that change their DNA methylation state. In concordance with the increased SNP frequency, DMRs are also significantly enriched for genome-wide association study (GWAS) SNPs from the GWAS catalogue \(26\) \(\text{odds ratio } 1.16, P = 3.27 \times 10^{-10}, \text{binomial test})\).

It is well known that many cancers exhibit considerable DNA methylation changes \(27\), we therefore compared a colon cancer to a matched control and found 532,665 differentially methylated CpGs. Forty per cent of these overlapped with the previously identified developmental dynamic set (Fig. 4a). Similarly, 37% of differentially methylated CpGs found in Alzheimer’s disease samples compared to normal controls

**Figure 2** | Dynamic CpG methylation regions frequently co-localize with TFBSs. **a**, Overlap of DMRs with ENCODE TFBSs. **b**, Enrichment of the top four TFBSs significantly overrepresented \((P < 0.01, \text{empirical test})\) in DMRs specific to the cell type indicated (specificity > 0.15). Colour code quantifies median enrichment odds ratio compared to size-matched random control regions. Cmucosa, colon mucosa; F denotes fetal. **c**, Overlap of PAX5 motifs \(\geq 100\text{-bp}\) unmethylated in CD34 cells or fetal brain across the entire human genome. Regions specifically unmethylated in CD34 or fetal brain were subjected to motif analysis, and top differentially co-occurring motifs are highlighted on the left for CD34 and on the right for fetal brain. **d**, Density scatterplot of maximum DNA methylation difference across 24 developmental samples for TFBS cluster track \((n = 2.7\) million) and median methylation level across all samples. Colour code indicates density of TFBSs from low (blue) to high (red).

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Our study highlights and defines a relatively small subset of all genomic CpGs that change their DNA methylation state across a large number of representative cell types. Although we expect that number to remain unchanged over the entire sample set (n = 30). CpGs. Class name indicates sample group in which a CpG was observed dynamic development (developmental (n = 24), cell culture (n = 3), cancer (n = 2)) or remained unchanged over the entire sample set (n = 30). c, Repeat content distribution of DMRs (sets as in b). d, Hierarchical clustering using Pearson correlation coefficient (PCC) of the DMR values across the entire sample set (n = 30). e, Distance of the fetal brain sample to different sets of signature regions defined for sample classes or individual samples, but excluding regions identified by means of the fetal brain sample. f, Contribution of individual sample signature region sets to an in-silico-generated hybrid sample (HUES64 and hippocampus).

**METHODS SUMMARY**

**Biological materials and sequencing libraries.** Genomic DNA was fragmented to 100–500 bp using a Covaris S2 sonicator. DNA fragments were cleaned-up, end-repaired, A-tailed and ligated with methylated paired-end adapters (purchased from ATDBio). See Supplementary Information for details.

**Data processing and analysis.** In-house-generated WGBS libraries were aligned using MAQ<sup>26</sup> in bisulphite mode to the hg19/GRCh37 reference assembly. Subsequently, CpG methylation calls were made using custom software, excluding duplicate, regulatory CpGs will drop rapidly once all major cell and tissue types have been mapped, mostly owed to the fact that between tissue variability exceeds within tissue variability by one order of magnitude (Supplementary Fig. 3a, b). Future studies are likely to fine map dynamics occurring in more specific subpopulations, giving rise to smaller changes in DNA methylation that we were unable to detect or include because of power constraints. Extreme conditions in vitro or in vivo such as loss or misregulation of the maintenance methylation machinery will affect a larger subset including many intergenic CpGs that are generally static, but most of these additional CpGs are unlikely to overlap with functional elements such as TFBSs or enhancers. In combination with the fact that sequencing of WGBS libraries is very inefficient, as only 80% of sequencing depth on non-informative reads and static regions. Furthermore, once defined, it will probably be sufficient in most cases to profile only a representative subset of CpGs across a comprehensive set of DMRs using an array-based<sup>27</sup> or hybrid-capture-based<sup>28</sup> technology to recover representative dynamics and measure regulatory events. Using these results as a guiding principle, we expect further improved efficiencies in mapping DNA methylation and enhance its applicability as a marker for various regulatory dynamics in normal and disease phenotypes.
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Author Contributions M.J.Z. and A.M. conceived the study and interpreted the results. M.J.Z. designed the statistical framework, analysis strategy and analysed the data. H.G. performed in-house WGBS library production, F.M. contributed bioinformatics tools and J.D. performed cell culture experiments. L.T.-Y.T. and E.D.R. provided adipocyte cell culture experiments. H.G., A.G. and A.M. established the WGBS at the Broad Institute. A.M. supervised the project. H.G., A.G. and A.M. conceived the study and interpreted the results. M.J.Z. designed the statistical framework, analysis strategy and analysed the data. H.G. performed in-house WGBS library production, F.M. contributed bioinformatics tools and J.D. performed cell culture experiments. L.T.-Y.T. and E.D.R. provided adipocyte cell culture experiments. H.G., A.G. and A.M. established the WGBS at the Broad Institute. A.M. is supported by the National Institutes of Health (NIH) Roadmap Epigenomics Project (ES017690). D.A.B. is supported by NIH grants P01AG10161, R01AG17917, R01AG15819 and R01AG36042. A.M. is supported by the Pew Charitable Trusts and is a New York Stem Cell Foundation, Robertson Investigator. This work was funded by NIH grants (U01ES017155 and P01GM099117) and The New York Stem Cell Foundation.

Author Information WGBS data are deposited at the Gene Expression Omnibus (see Supplementary Table 1 for the specific accession numbers). Supplementary Table 2 is available under GEO accession number GSE46644. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.M. (alexander_meissner@harvard.edu).