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Genomic patterns of DNA methylation: targets and function of an epigenetic mark

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Methylation of cytosines can mediate epigenetic gene silencing and is the only known DNA modification in eukaryotes. Recent efforts to map DNA methylation across mammalian genomes revealed limited DNA methylation at regulatory regions but widespread methylation in intergenic regions and repeats. This is consistent with the idea that hypermethylation is the default epigenetic state and serves in maintaining genome integrity. DNA methylation patterns at regulatory regions are generally stable, but a minor subset of regulatory regions show variable DNA methylation between cell types, suggesting an additional dynamic component. Such promoter *de novo* methylation might be involved in the maintenance rather than the initiation of silencing of defined genes during development. How frequently such dynamic methylation occurs, its biological relevance and the pathways involved deserve investigation.

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Introduction

In prokaryotes, DNA can be methylated on both cytosines and adenines and this methylation is involved in various processes, including DNA repair and defense against foreign DNA [1]. Eukaryotes show methylation almost exclusively at cytosines: in mammals it occurs only in the context of CpG dinucleotides (CpGs), while in fungi (e.g. *Neurospora crassa*) and plants (e.g. *Arabidopsis thaliana*) methylation is seen in various symmetrical and asymmetrical sequence contexts (reviewed in [2]). However, cytosine methylation does not occur in all eukaryotes, as it is absent in Saccharomyces cerevisiae and in many invertebrates, like the nematode *Caenorhabditis elegans*. Regarding insects, low levels of cytosine methylation have been reported in *Drosophila melanogaster* and substantial methylation has been found in the honey bee *Apis mellifera* [3].

Four DNA methyltransferases (DNMTs) sharing a conserved DNMT domain have been identified in mammals. The founding member, DNMT1, maintains DNA methylation during replication by copying the DNA methylation of the old DNA strand onto the newly synthesized strand [4]. DNMT3a and DNMT3b are responsible for *de novo* methylation, as they are able to target unmethylated CpG sites [5]. They also cooperate with DNMT1 to propagate methylation patterns during cell division [6]. DNMT2 has only weak DNA methyltransferase activity *in vitro* and has recently been shown to efficiently methylate a tRNA [7•].

DNA methylation is generally associated with a repressed chromatin state and inhibition of promoter activity. Two models of repression have been proposed: first, cytosine methylation can prevent the binding of some transcription factors, and second, DNA methylation can affect chromatin states indirectly through the recruitment of methyl-CpG-binding proteins (MBPs) [8]. DNA methylation is essential for mammalian development, as shown by the lethality of various DNMT deficiencies in mice [5,9]. DNA-methylation-mediated repression has been directly implicated in X-chromosome inactivation and genomic imprinting (see review by Edwards and Ferguson-Smith in this issue); however, other functions of DNA methylation in developmentally regulated gene expression remain less definite.

Mammalian genomes are globally depleted for CpGs, except at short DNA stretches called CpG islands, which are frequently associated with gene promoters. This unequal distribution of CpGs needs to be considered when interpreting global maps of DNA methylation, because the amount of methylated cytosines at a given region depends both on the degree of methylation and on the density of CpGs.

Here we review how recent advances in determining the sites of DNA methylation on a genome-wide scale have given new insights into the biological function of DNA methylation in maintaining genome integrity and cell identity. Due to space limitations we will not focus on aberrant DNA methylation in cancer, for which we refer the reader to recent summary articles [10,11].

Going global: technologies for genome-wide mapping of DNA methylation

Until recently the distribution of DNA methylation in eukaryotic genomes (the 'methylome') remained poorly characterized, despite its utility for defining global rules

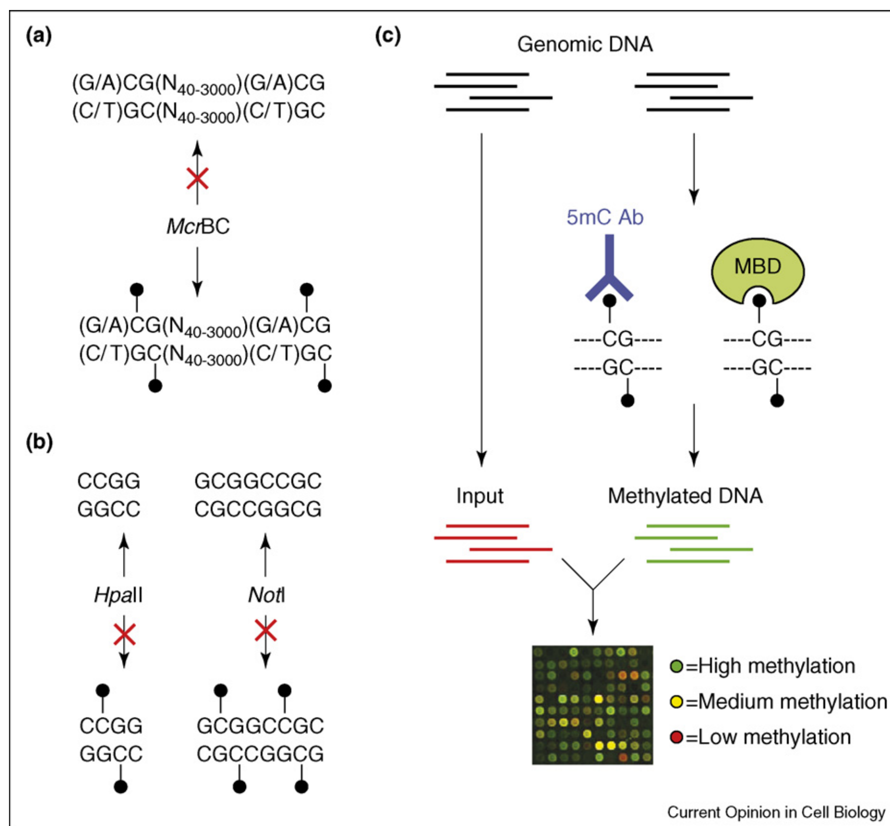
that govern the distribution of DNA methylation and identifying potential exceptions. Within recent years, however, approaches have been developed to map DNA methylation genome-wide. Some of these are variations of classical approaches using methylation-sensitive restriction enzymes [12–14] or digestion with the methylation-specific enzyme *Mcr*BC [15,16] (Figure 1a,b). One constraint imposed by the use of restriction enzymes is that only particular sequence motifs are analyzed (see [17] for details). To circumvent such limitations, approaches have been developed that rely on affinity purification of methylated DNA that has been fragmented by random shearing (Figure 1c). In the methylated DNA immunoprecipitation assay (MeDIP), a monoclonal antibody against 5-methylcytidine is utilized to purify methylated DNA, which can be used for genomic profiling with DNA miroarrays [18•,19•]. Other strategies isolate methylated DNA with an MBD domain fused to a human IgG [20], or with MBD proteins bound to a sepharose matrix [21], in a variation of a previous approach using a column [22]. One caveat with affinity approaches is that methylated CpG-rich sequences give higher enrichments than methylated CpG-poor sequences. In addition, microarray-based detection has limited ability to measure allelic DNA methylation or DNA methylation of individual repetitive elements. Bisulfite genomic sequencing to determine DNA methylation is less limited in that regard, but requires extensive resources when applied genome-wide [23••].

In addition to novel experimental strategies, computational approaches have been developed to predict DNA methylation from DNA sequence [24,25]. These appear to have a high success rate in predicting steady state methylation, and it will be interesting to see if these can also predict changes in methylation in development and disease.

Genomic distribution of DNA methylation

It has long been speculated that most coding regions in mammalian genomes show a high degree of DNA methylation, and this has now been confirmed across the genome by independent studies. Hybridization of methylated DNA to a BAC microarray representing the entire human genome showed that DNA methylation of unique sequences is abundant in genic regions [18•]. This is in agreement with earlier studies of selected genes showing

Figure 1



Technologies to map DNA methylation genome-wide. Classical approaches to study DNA methylation use restriction enzymes that cut only (**a**) methylated (*Mcr*BC) or (**b**) unmethylated (*Hpa*II, *Not*I) DNA; however, these methods limit the analysis to particular sequence motifs. (**c**) Alternative methods use isolation of methylated DNA with antibodies or MBD proteins. The methylated DNA (labeled in green) can be used for cohybridization with input DNA (labeled in red) on any existing microarray. Lollipop shapes denote methyl groups.

that exons are methylated in both human and mouse [26]. Recent bisulfite sequencing data on 2524 amplicons from three human chromosomes confirmed that sequences outside of promoters have a high degree of DNA methylation [23••]. Thus in mammals most DNA outside regulatory regions (intergenic DNA, coding DNA and repeat elements) appears to be methylated (Figure 2a).

In contrast to mammals, DNA methylation is restricted to specific genomic regions in plants and fungi. In *Arabidopsis thaliana*, most of the methylated fraction of the genome is composed of local tandem or inverted repeats, transposons and other dispersed repeats that are found around centromeres and in euchromatin. RNAi pathways have been directly involved in guiding DNA methylation to tandem repeats, leading to the model that siRNA produced from repeats selectively guides DNA methylation to homologous sequences [27] (Figure 2b). The same pathways are involved in guiding DNA methylation to transposons [28], although this does not appear to be the case in the fungus *Neurospora crassa* [29]. Recent genome-wide mapping in *Arabidopsis thaliana* using high resolution tiling arrays also revealed that DNA methylation is found in the transcribed regions of a significant fraction (>20%) of expressed genes [30••,31••], pointing to a transcription-coupled targeting of DNA methylation in genes. One proposed model is that cryptic initiation in gene bodies leads to aberrant double-stranded RNA transcripts that are processed by the RNAi pathway and in turn recruit the DNA methylation machinery [31••] (Figure 2b).

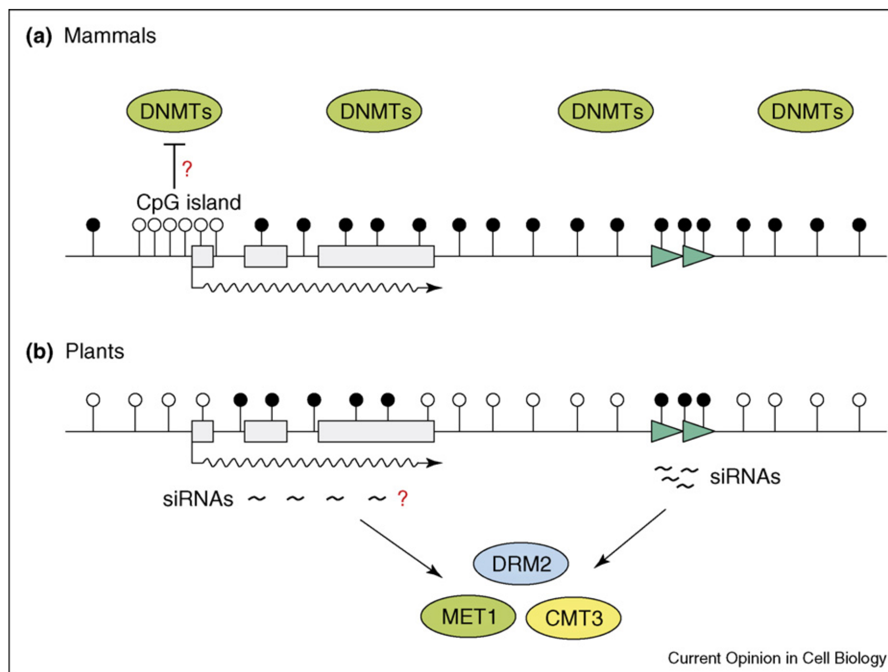
DNA methylation and genome integrity

The fact that most DNA methylation in mammals is found outside regulatory regions suggests a role for DNA methylation in the global maintenance of the genome, and several functional models have been proposed.

Inhibition of cryptic initiation

Methylation of coding regions is common among eukaryotes, as it has been described not only in mammals and plants (see above), but also in a chordate (*Ciona intestinalis*) [32] and more recently in the honey bee [3]. However its function remains enigmatic, especially because it carries a cost: DNA methylation has been shown to inhibit transcription elongation in *Neurospora crassa* [33], and to reduce elongation rates in plants [31••] and mammals [34]. One potential role for intragenic DNA methylation could be to inhibit cryptic transcriptional initiation outside gene promoters. The process of

Figure 2



Distribution of DNA methylation in the genome. (**a**) In mammals, all sequences may be accessible to DNA methyltransferases (DNMTs), except CpG islands, which often colocalize with gene promoters. How CpG islands are protected from DNA methylation is currently unknown. (**b**) The *A. thaliana* genome contains DNA methylation in repeats (both CG and non-CG) and in gene bodies (mostly CG). CG methylation is established by DRM2 and maintained by MET1, while non-CG methylation is established by DRM2 and maintained by DMR2 and CMT3. RNAi pathways producing siRNAs have been shown to guide methylation to repetitive DNA, although it is still unclear how double-stranded RNA substrates are specifically produced from local tandem repeats as opposed to unique sequences [27]. Similarly, cryptic antisense transcription within genes could generate double-stranded RNA substrates processed into siRNAs that guide CG methylation to gene bodies [31••]. Boxes denote exons; triangles denote repeated sequence; lollipop shapes denote methylated (black) or unmethylated (white) cytosines.

transcription itself disrupts chromatin structure and leads to nucleosomal displacement, therefore exposing potential cryptic initiation sites. Recent evidence in yeast showed that methylation of lysine 36 of histone H3 occurs at active genes, where it recruits histone deacetylase (HDAC) activity to prevent transcription initiation at cryptic sites that are exposed as a consequence of transcription-coupled nucleosomal disruption [35]. Since recruitment of HDAC activity by DNA methylation is well established, it might play a role in compacting chromatin in coding sequences to inhibit cryptic initiation. However, experimental data to support this model are still lacking.

Protection against mobile elements

Transcriptional inactivation and immobilization of mobile elements that are densely interspersed in eukaryotic genomes is presumably important to ensure genomic integrity, and DNA methylation plays a role in this process. In plants, loss of CG and non-CG DNA methylation leads to the reactivation of mobile elements and increases the frequency of transposition, consistent with a primary role for DNA methylation in genome defense [36]. In mammalian genomes, most repeats are found to be methylated [37] and there is evidence that DNA methylation is directly involved in their silencing. IAP retrotransposons are transcriptionally reactivated in *Dnmt1*$^{-/-}$ mouse embryos [38], whereas IAP and L-INE-1 elements are reactivated in germ cells lacking Dnmt3L or Lsh, two cofactors required for the establishment of DNA methylation patterns in germ cells [39,40•]. However, in contrast to plants, there is as yet no conclusive evidence for a mechanism that targets DNA methylation directly to repeats in mammals and fungi.

Maintenance of genome stability

Several genetic studies indicate that global hypomethylation is associated with increased genome instability in mammalian cells. Inactivation of Dnmt3b in mouse embryonic fibroblasts leads to partial loss of DNA methylation, changes in ploidy and chromosomal abnormalities [41]. Absence of Lsh, a chromatin remodeler required for global DNA methylation patterns, also leads to chromosomal abnormalities in embryonic fibroblasts and germ cells [40•,42]. In humans, deletion of *Dnmt1* and *Dnmt3b* induces chromosomal abnormalities in cell lines [43,44•], and partial loss of function of DNMT3b is linked to ICF syndrome, characterized by chromosomal rearrangements in hypomethylated centromeric regions [45]. The connection between DNA hypomethylation and genome instability is also well documented in the context of cancer. Many cancer cells display global hypomethylation of their genome, which has been causally linked to increased chromosomal instability and tumor progression [46]. Recent studies in gastrointestinal cancers showed that global hypomethylation precedes copy number changes [47], and that the extent of hypomethylation correlates with the extent of genomic damage [47,48]. In that context, genome-wide mapping of DNA methylation will be valuable to test if sites of hypomethylation coincide with sites of genome instability in cancer cells [49]. It is currently unclear how DNA methylation counteracts genomic instability, but one possibility is that hypomethylation leads to increased frequency of illegitimate recombination between homologous repeats [39,40•]. In support of this hypothesis, hypomethylation of telomeric regions in *DNMT1*$^{-/-}$ and *DNMT3a,3b*$^{-/-}$ mouse ES cells is associated with an increased frequency of telomere recombination [50•].

DNA methylation and maintenance of cell identity

Developmental restriction by repression of genes represents a key paradigm in epigenetics. On the basis of its potential to silence promoters, DNA methylation has been hypothesized to play an important role in cell-type-specific gene expression. Rare examples of tissue-specific promoter DNA methylation exist [12,51], while other studies on individual genes failed to establish a strong connection between changes in expression and dynamic methylation [52]. Indeed, ~60% of genes in mammalian genomes contain promoter-proximal CpG islands, which are believed to be always unmethylated [2]. Recent comprehensive datasets confirmed that most CpG island promoters are unmethylated in different types of human primary cells, but revealed that a subset (2–5%) of CpG island promoters show high DNA methylation in primary tissues [12,18•,23••,37,53,54••]. Moreover, CpG island methylation has also been reported to regulate lineage-specific expression in the *Rhox* gene cluster [55••]. Thus, protection of CpG islands from *de novo* methylation can be overcome in primary cells on specific target genes. Notably, a recent comprehensive study of human promoters identified many novel targets and suggests that de novo methylation of CpG islands in somatic cells preferentially occurs at germline-specific genes as well as promoters with an intermediate CpG frequency [54••]. Furthermore, comparison of methylation profiles between tissues suggests that a large proportion of differentially methylated regions is located outside promoters [23••,53], suggesting that DNA methylation could also modulate the activity of distal enhancers.

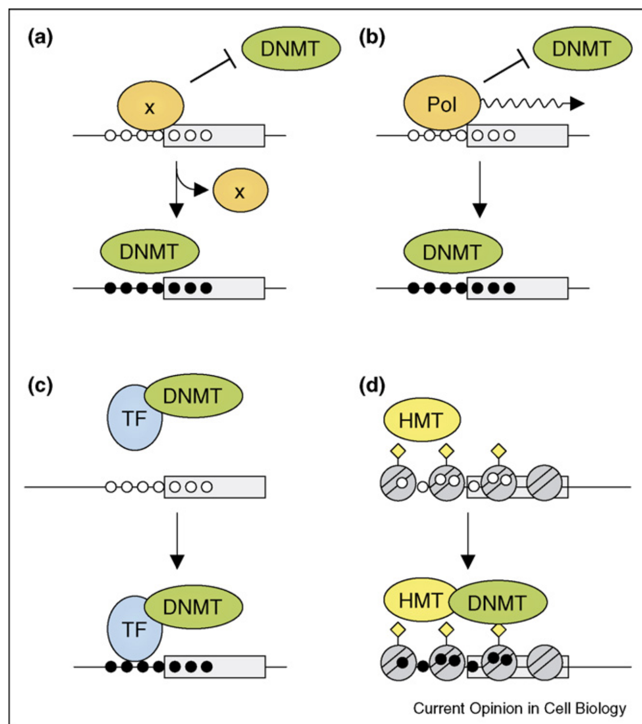
Several studies indicate that DNA methylation is involved in the maintenance rather than the initiation of gene silencing. In the case of epigenetic reprogramming of the OCT4 promoter during stem cell differentiation, DNA methylation is a late event; it is not required to silence the gene but is required to stably prevent its re-expression [56••]. Similarly a CpG-free transgene undergoes transcriptional silencing to the same extent as a transgene containing CpGs and becoming DNA-methylated, but the transgene is resistant to reactivation only if methylated [57•].

Together these data are in line with a function of DNA methylation in epigenetic repression of a subset of genes during development, which might function in maintaining lineage restriction.

Reprogramming of DNA methylation

How is DNA methylation specifically targeted to a subset of promoters? As most of the genome is methylated, one could envision that *de novo* methylation of selected CpG island promoters may entail loss of protection against a default program yielding DNA methylation (Figure 3a). Currently we know little about what protects CpG islands from DNA methylation, and understanding this phenomenon is likely to provide a key to understanding the mechanisms of dynamic promoter methylation. In most studied examples, *de novo* methylation coincides with or follows transcriptional shutdown, making it possible that transcription or the associated chromatin state could provide protection from DNA methylation (Figure 3b). However, as most CpG island promoters remain methylation-free even when inactive [54••], this is unlikely to be a general mechanism. Alternative models suggest active recruitment of DNMT activity to targeted promoters. This could involve sequence-specific transcription factors, as suggested for Myc [58] or PU.1 [59] (Figure 3c). Several studies are also compatible with the recruitment of DNMT activity by histone modifications (Figure 3d). For example, H3K9 methylation is required to target DNA methylation to pericentric repeats [60] or to the promoter of the OCT4 gene during differentiation of stem cells [56••]. A connection between DNA methylation and Ezh2-mediated H3K27 methylation has also been proposed in the context of cancer [61•,62–64] and it remains to be seen if similar mechanisms could function during normal development. Global analysis should help to discriminate between these models and reveal if reprogrammed promoters share common features in regards to chromatin state and sequence motifs. Ultimately however these mechanistic models need to be tested genetically.

Figure 3

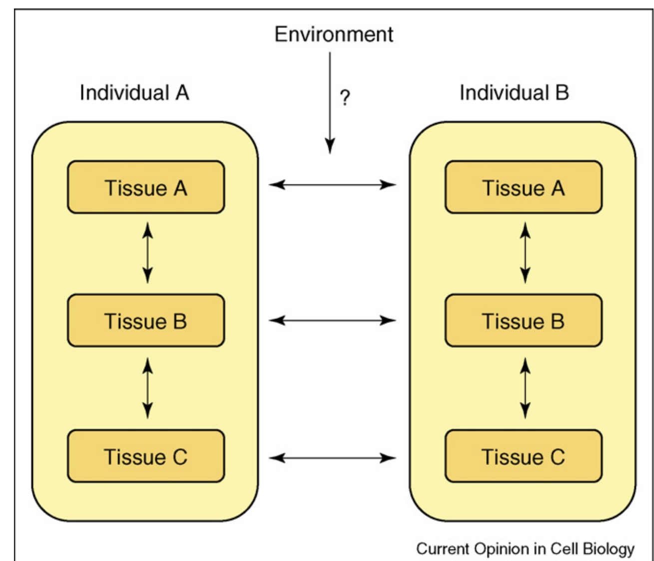


Models for targeting DNA methylation to gene promoters in mammalian cells. (**a**) The specificity of promoter DNA methylation could be conferred by the selective loss of an as-yet-unidentified protecting factor, X. (**b**) Absence of a transcribing polymerase complex could initiate DNA methylation on some promoters. (**c**) Some transcription factors (TFs) have been proposed to interact with DNMTs and recruit them to their target sites. (**d**) DNMTs could be targeted by histone methylation through an interaction with the histone methyltransferase (HMT) or the histone mark itself. Box denotes first exon; circles denote methylated (black) or unmethylated (white) CpGs.

Variation and heritability of DNA methylation patterns

The possibility that DNA methylation patterns are variable between individuals and that epigenotypes could contribute to phenotypic diversity and disease susceptibility has drawn considerable attention in recent years (Figure 4). Pilot studies aimed at estimating variability in DNA methylation patterns between individuals gave quite different results. Bisulfite sequencing of 2524

Figure 4



Intra- and inter-individual variation in DNA methylation patterns. There is evidence for variation in DNA methylation patterns between tissues and individuals; however, the phenotypic consequences and heritability of these variations are unclear. More work is needed to study how DNA methylation is involved in establishing and maintaining cell identity, and how the environment might influence these patterns of methylation.

amplicons revealed very little variation with age and sex [23••], whereas another study reported a high frequency of epigenetic differences between aging monozygotic twins [65]. It remains to be seen if this discrepancy originates from the tested samples or the analytical methods applied. Nevertheless there is little doubt that epimutations can occur in mammals. A classic example is the agouti locus in the mouse, where gene expression depends on variable and inheritable methylation of a promoter proximal repeat [66]. Recently, examples of potential heritable epimutations in the promoters of the *MLH1* and *MSH2* genes that lead to increased susceptibility to cancer have been described in humans [67,68••]. Another pioneering work showed heritable altered DNA methylation in rats exposed to endocrine disruptors [69••], suggesting that DNA methylation patterns can be influenced by the environment. However, more comprehensive analyses are needed to estimate the frequency of these phenomena and, more importantly, to link observed epigenetic differences to phenotypes.

Conclusions

Recent advances in epigenomic approaches allow mapping of the methylation state of the genome with high accuracy, enabling the testing of models for the function of this DNA modification. Emerging evidence suggests that hypermethylation is the default state of mammalian genomes, but that dynamic DNA methylation of regulatory regions can occur during development. Moreover, epigenetic differences might also exist between individuals. Studying the extent of both these phenomena and their biological relevance represents major challenges for future research.

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This study from the Human Epigenome Project (HEP) reports bisulfite genomic sequencing of 2524 amplicons from three chromosomes analyzed in 12 human tissues. Interestingly, the cell type, rather than the age or sex of the individual, appears to account for most of the differences in DNA methylation patterns. Tissue-specific differentially methylated regions (T-DMRs) are found not only in promoters but also in conserved intergenic elements, leading the authors to propose that DNA methylation regulates the activity of distant enhancers.

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