Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals

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Cells of a multicellular organism are genetically homogeneous but structurally and functionally heterogeneous owing to the differential expression of genes. Many of these differences in gene expression arise during development and are subsequently retained through mitosis. Stable alterations of this kind are said to be 'epigenetic', because they are heritable in the short term but do not involve mutations of the DNA itself. Research over the past few years has focused on two molecular mechanisms that mediate epigenetic phenomena: DNA methylation and histone modifications. Here, we review advances in the understanding of the mechanism and role of DNA methylation in biological processes. Epigenetic effects by means of DNA methylation have an important role in development but can also arise stochastically as animals age. Identification of proteins that mediate these effects has provided insight into this complex process and diseases that occur when it is perturbed. External influences on epigenetic processes are seen in the effects of diet on long-term diseases such as cancer. Thus, epigenetic mechanisms seem to allow an organism to respond to the environment through changes in gene expression. The extent to which environmental effects can provoke epigenetic responses represents an exciting area of future research.

The term 'epigenetics', which literally means 'outside conventional genetics', is now used to describe the study of stable alterations in gene expression potential that arise during development and cell proliferation. This has effectively replaced an earlier usage, which referred to the study of 'epigenesis', meaning interpretation of the genotype during development to give the phenotype[1]. Epigenetic processes are essential for development and differentiation, but they can also arise in mature humans and mice, either by random change or under the influence of the environment[2]. Epigenetic mechanisms also guard against viral genomes that would otherwise hijack cellular functions for their own ends[3]. How does the genome adapt in this way to developmental or environmental cues? Our rapidly growing understanding of epigenetic processes identifies postsynthetic modification of either the DNA itself or of proteins that intimately associate with DNA as the key mediators. These modifications seem to be interpreted by proteins that recognize a particular modification and facilitate the appropriate downstream biological effects.

It was proposed in 1975 that DNA methylation might be responsible for the stable maintenance of a particular gene expression pattern through mitotic cell division[4,5]. Since then, ample evidence has been obtained to support this concept, and DNA methylation is now recognized to be a chief contributor to the stability of gene expression states. Specifically, DNA methylation establishes a silent chromatin state by collaborating with proteins that modify nucleosomes[6,7].

In this review, we focus on advances in understanding DNA methylation in mammals and on some of the key components that translate this DNA modification into specific chromatin states. Many reviews on epigenetic regulation have been published[8–12], and here we have not attempted a comprehensive coverage of this topic but rather have made a personal selection of epigenetic phenomena that are exciting at present or promise to be so in the future.

Establishing and maintaining patterns of DNA methylation

DNA methylation in mammals is a post-replication modification that is predominantly found in cytosines of the dinucleotide sequence CpG. The extent of DNA methylation changes in an orchestrated way during mammalian development, starting with a wave of demethylation during cleavage, followed by genome-wide *de novo* methylation after implantation[13]. Demethylation is an active process that strips the male genome of methylation within hours of fertilization[14,15]; by contrast, the maternal genome is only passively demethylated during subsequent cleavage divisions[12]. The extent of methylation in the genome of the gastrulating embryo is high owing to *de novo* methylation, but it tends to decrease in specific tissues during differentiation[16]. *De novo* methylation occurs rarely during normal postgastrulation development but is seen frequently during the establishment of cell lines *in vitro*[17,18] and in cancer (see below).

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Table 1 • Phenotypes of mouse mutants of epigenetic regulatory factors

Protein	Function	Mutant phenotype	References
methyltransferases			
Dnmt1	maintenance of methylation	embryonic lethal, loss of imprinting and X-linked gene expression, ES cells viable	20,21
Dnmt1o	oocyte-specific isoform	loss of maternal imprints	23
Dnmt2	non-CpG methylation in *Drosophila*	no phenotype	28
Dnmt3a, Dnmt3b	*de novo* methyltransferases, establishment of methylation	embryonic lethal, ICF syndrome	24,134
Dnmt3L	no catalytic activity, colocalizes with Dnmt3a and Dnmt3b	abnormal maternal imprinting	26,27
methyl binding proteins			
MeCP2	methyl binding proteins, recruit HDACs	RTT	80,81,84
MBD1	methyl binding proteins, recruit HDACs		
MBD2	methyl binding proteins, recruit HDACs	behavior abnormalities	76
MBD3	methyl binding proteins, recruit HDACs	lethal	76
MBD4	repair enzyme	increased mutation frequency	180
histone-modifying proteins			
HDAC1	histone deacetylase	embryonic lethal	181
Suvar39	Lys9 methylation in histone H3	embryonic lethal, chromosomal instability, increased tumor risk	182

Mechanistic insights into the role of DNA methylation and the establishment of methylation patterns during development have come from phenotypic analyses of mice with mutations in the various DNA methyltransferase (DNMT) genes (Table 1)[19]. Salient observations can be summarized as follows. First, deletion of *Dnmt1* in mice[20,21] and antisense RNA–mediated inhibition of *xDnmt1* expression in frogs[22] results in global demethylation and embryonic lethality. All evidence indicates that the Dnmt1 enzyme acts as a maintenance methyltransferase. Likewise, the oocyte-specific isoform Dnmt1o[23] is responsible for maintaining but not establishing maternal imprints.

Second, Dnmt3a and Dnmt3b are expressed highly in the developing mouse embryo and are responsible for global *de novo* methylation after implantation[24], consistent with the finding that Dnmt3a can carry out *de novo* methylation in transgenic flies[25]. Dnmt3L, a protein that by itself has no DNMT activity, colocalizes with Dnmt3a and Dnmt3b and is essential for establishing methylation imprints in the female germ line[26,27]. Last, the deletion of *Dnmt2*, a member of the DNMT family that lacks biochemically detectable methyltransferase activity, has no obvious phenotype in mice[28]; however, this gene is conserved in *Drosophila*, highly expressed during oogenesis and may be responsible for the small amount of non-CpG methylation seen in the fly embryo[29,30].

Genetic analysis of the various DNMTs has established that DNA methylation is essential for vertebrate development. Loss of methylation causes apoptosis in embryos[31,32] and fibroblasts[33], but not in embryonic stem (ES) cells[20,21] or human cancer cells[34], and leads to both widespread derepression of ectopic gene expression[33] and transcriptional activation of transposable elements[35]. Deletion of *Dnmt1* during brain development results in perinatal respiratory distress and causes the elimination of mutant neurons within 3 weeks of postnatal life[36]. Potential explanations for the evolution of DNA methylation invoke its ability to silence transposable elements[37] or its function as a mediator of developmental gene regulation or transcriptional noise reduction[11]. This issue has yet to be resolved, but all may be partially correct.

In non-embryonic cells, methylated sites are distributed globally on about 80% of CpGs. Interrupting this relatively featureless sea of genomic methylation are the CpG islands—short sequence domains that generally remain unmethylated at all times, regardless of gene expression[38–41]. Most CpG islands are associated with genes and all may contain promoters[42,43], the control of which may involve the transcription factor Sp1 and perhaps the recently identified CpG-binding proteins[44,45]. In a few specific cases, CpG islands do become methylated during development, leading to long-term shutdown of the associated gene[46–48]. How CpG islands remain methylation-free in a globally methylated genome is an open question[49,50].

In cancer cells, methylation of CpG islands is known to contribute to gene silencing . For example, studies of unscheduled *de novo* methylation at the CpG island of the retinoic acid receptor-β (*RARβ*)[51] have implicated a protein fusion that is known to induce leukemia in humans. The promyelocytic leukemia (PML)-RAR fusion protein binds to the *RARβ* promoter, and a transcriptional repression domain of PML directs repression of *RARβ*, causing the recruitment of DNMTs. These enzymes seem to methylate the *RARβ* CpG island and to shut down the gene permanently. A related scheme has been proposed for the silencing and methylation of a fraction of ribosomal RNA genes in mammalian cells[52]. In this way the reported interactions[53,54] between DNMTs and histone deacetylases (HDACs) may allow genes that are already silenced by co-repressors to invite the *de novo* methylation that can make silencing permanent.

Histone modifications other than deacetylation are also strongly implicated in triggering the *de novo* methylation of DNA. Most markedly, methylation of histone H3 at Lys9, which is clearly linked to gene silencing, has been shown to be essential for DNA methylation in the fungus *Neurospora crassa*[55] and in the plant *Arabidopsis thaliana*[56]. Evidence for an equivalent dependence in mammalian systems is eagerly sought. In the case of methylation of Lys9 on histone H3, and in the PML-RAR example described above, the silent chromatin state provokes DNA methylation, supporting the view that DNA methylation is acting as a system of cellular memory that is not directly involved in initiating gene silencing but senses and propagates the silent state[11].

Biochemical evidence indicates that DNA methylation is just one component of a wider epigenetic program that includes other postsynthesis modifications of chromatin[7,57,58]. Several chromatin modification states are either mutually reinforcing or mutually inhibitory. The resulting feedback loops may function to ensure functional polarization of chromatin domains, stably committing them to either transcriptional activity or transcriptional silence. Under such a scheme (Fig. 1*a*), uncommitted

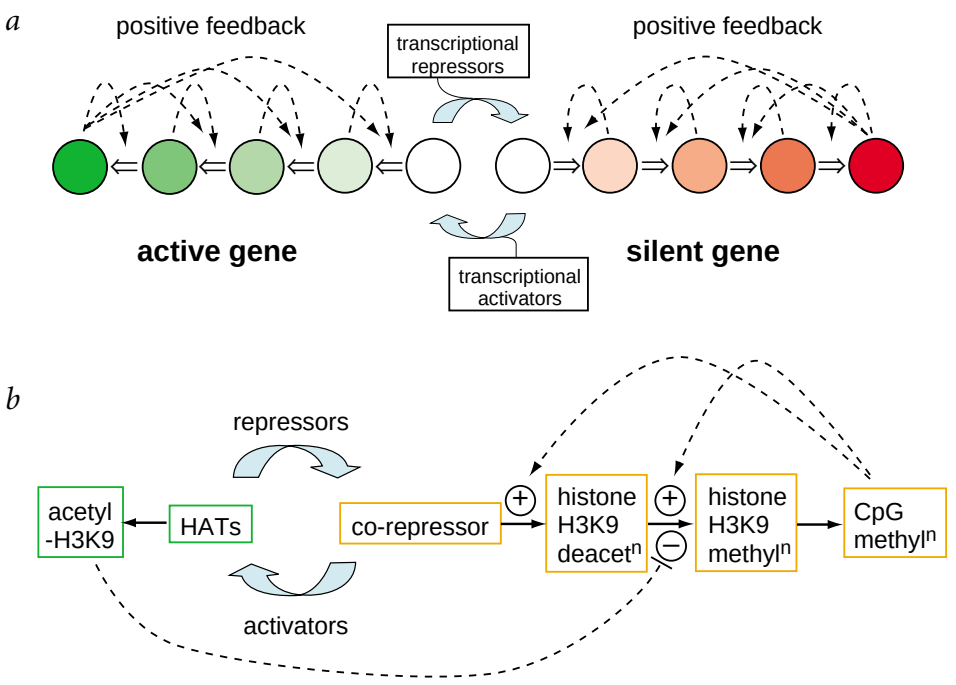

Fig. 1 Polarization of active and silent chromatin states owing to feedback loops between intermediate states. ***a***, Different chromatin states. Each circle represents a particular DNA or histone modification status (examples are shown in *b*). The effect of positive feedback is to polarize domains at either the active or the inactive extremity. Broken lines indicate positive feedback loops between products and reactions that interconvert intermediate states. Negative interactions between the silent and active states of the gene occur (see *b*) but are not shown. The likelihood that a chromatin domain will occupy a particular state is indicated by the color intensity, with dark shades representing more probable states. ***b***, Potential examples of the polarization scheme shown in ***a***. Both positive feedback of CpG methylation on Lys9 of histone H3 (H3K9) mediated by MeCP2 (ref. 185) and stimulation of histone deacetylation by CpG methylation through methyl-CpG binding proteins have both been reported. HATs, histone acetyltransferases.

chromatin states would be unstable and therefore rare. Switching between active and inactive states would be unlikely to occur by chance, because several self-reinforcing steps would work against the transition, pulling a domain that is 'drifting' towards an alternative activity state back to the 'poles'. Current data offer support for such a scheme, because methylation of lysine 9 on histone H3 can promote DNA methylation, and acetylation and methylation of lysine 9 are mutually exclusive (Fig. 1*b*). Moreover, CpG methylation stimulates both histone deacetylation and methylation of lysine 9 on histone H3.

Interpreting the DNA methylation signal

CTCF and chromatin boundaries. There seem to be several ways in which DNA methylation can repress transcription. A general route is through the exclusion of proteins that affect transcription through their DNA binding sites[59]. An example of such exclusion applies to the chromatin boundary element binding protein, CTCF, which can block interactions between an enhancer and its promoter when placed between the two elements[60,61]. CpG methylation blocks the binding of CTCF to DNA and thus allows an enhancer to stimulate promoter activity across the inert boundary site. This binary switching of CTCF binding through DNA methylation is clearly important in imprinting of the *Igf2* gene, which is expressed exclusively from the paternal allele during development[61,62]. In addition to the binding of CTCF, several other transcription factors are known to be blocked by CpG methylation[63], but the biological consequences of this process are not known.

The methyl-CpG binding proteins. Although DNA methylation is clearly repulsive to some DNA binding proteins, it is singularly attractive to others. Early studies detected methyl-CpG binding activities and implicated these as mediators of transcriptional repression. Progress in understanding the mechanism of repression came with the characterization of MeCP2 (ref. 64) and its relatives, the methyl-CpG binding domain proteins MBD1–MBD4 (ref. 65), as well as the unrelated protein Kaiso[66]. Biochemical and transient transfection studies identified a domain in each protein (except mammalian MBD3) that could target it specifically to methylated CpG sites *in vitro* and *in vivo*[65,67].

Chromatin immunoprecipitation has shown at higher resolution that MeCP2 and MBD2 associate with loci in a manner that is strictly dependent on DNA methylation[68–70]. Studies with crude nuclear extracts have supported the view that methyl-CpG binding proteins such as MeCP2, MBD2 and MBD1 function as transcriptional repressors, because in their absence methylated reporter genes are expressed[71]. A breakthrough in understanding how methylation-mediated repression worked was the finding that MeCP2 interacts with a co-repressor complex containing HDACs[72,73]. MBD2 also associates with HDACs[74,75], extending a relationship between histone modification and DNA methylation that is likely to be better understood in the future.

Loss of the methyl-CpG binding protein MBD2 significantly affects gene expression. Mice without MBD2 (ref. 76) are viable and fertile but lack the methylated DNA binding complex MeCP1, which comprises MBD2 plus the NuRD chromatin remodeling complex[74,76,77]. *Mbd2*$^{-/-}$ cells are defective in the methylation-mediated repression of transiently transfected genes, and significant gene derepression of endogenous genes has been detected in cells from mutant mice. In particular, *Mbd2*$^{-/-}$ mice express the gene (*Il4*) encoding interleukin-4 in a subset of naive T helper cells in which it is normally silent. They also express *Il4* in abnormally high amounts in differentiating, type 2 T helper cells[78]. MBD2 is bound at the silenced *Il4* gene in wild-type cells, suggesting that it may act directly on the methylated gene.

In contrast to the profound effects of DNMT loss[20], the consequences of losing a methyl-CpG binding protein seem to be much less severe. A possible explanation is the apparent redundancy of methyl-CpG binding proteins. Mutations in DNMTs greatly reduce the extent of genomic DNA methylation and therefore interfere with all proteins that interpret the DNA methylation signal. Loss of one methyl-CpG binding protein, by contrast, leaves intact the other proteins that recognize the DNA methylation signal. Recent studies[79] support the view that repression is multilayered and involves many collaborating processes (see Table 1). If so, a combination of mutations in several methyl-CpG binding proteins should produce a more severe phenotype. So far, however, a *Mbd2*$^{-/-}$;*Mecp2*$^{-/y}$ double-mutant

mouse has not shown any phenotypic enhancement[80], but further analysis of this kind is required to test whether methyl-CpG binding proteins have redundant or non-overlapping functions.

MeCP2 and Rett syndrome. Mutations in the X-linked *MECP2* gene are responsible for at least 80% of all cases of Rett syndrome (RTT), an extreme neurological disorder that affects female heterozygotes[81]. The condition is almost always due to *de novo* mutations that are distributed widely in the protein coding sequence. Notably, most missense mutations are tightly concentrated in the methyl-CpG binding domain and lead to reduced binding to methylated DNA[82,83]. This suggests that, as expected, binding to methyl-CpG is an essential attribute of functional MeCP2.

Mice with null mutations in *Mecp2* show phenotypic similarities to the human condition, including delayed onset of symptoms, small brain size, reduced dendritic arborization, tremors, inertia, abnormal gait and arrhythmic breathing[80,84,85]. The mouse model therefore promises to shed light on the etiology of RTT—the molecular causes and detailed pathology of which are poorly understood. Deleting *Mecp2* in the early mouse brain only leads to the same phenotype as deleting it in all cells of the mouse[80,84], emphasizing the neurological basis of RTT.

Heterozygous female mice, which constitute the genetic model for RTT, acquire symptoms between 4 and 12 months of age. Given the vast difference in developmental timing between mice and humans, this is unexpectedly close to the human age of onset (6–18 months). On the basis of the known biochemical properties of MeCP2, an obvious hypothesis is that the symptoms of RTT are due to a failure of effective silencing of methylated genes. But microarray analysis of brain RNA from wildtype and mutant mice has detected few, if any, gene expression differences[86]. It is possible that these findings indicate that MeCP2 has a function in the brain that is not related to the repression of methylated genes. Alternatively, the absence of one methyl-CpG binding protein may be so well compensated by other related proteins that only subtle effects on gene expression occur, which are below detectable levels using current technology.

Gene activation during development

In general, the known developmental effects of DNA methylation on gene expression involve long-term silencing of gene expression. The attractive idea that genes are transcriptionally activated by removing DNA methylation has lacked strong experimental support until recently. Many correlations between expression and loss of DNA methylation have been reported, and the methylation of some reporters has been shown to inhibit expression. A notable recent example is the human maspin gene, the promoter of which is unmethylated in expressing cells but methylated when silent[87].

Compelling evidence for the induction of premature gene activation by demethylation has been reported for the frog *Xenopus laevis*[22,88]. Widespread gene expression normally occurs at the 'mid-blastula transition' and is correlated in time with promoter demethylation; however, induced hypomethylation of the genome through the inhibition of *Dnmt1* leads to the premature activation of many genes. Similarly, widespread activation of tissue-specific genes has been seen in fibroblasts after conditional inactivation of *Dnmt1* (ref. 33). Thus, DNA methylation ensures that gene activation is delayed until the mid-blastula stage in the frog and assures the silencing of genes in somatic cells of mammals.

Epigenetic regulation and dysregulation

Genomic imprinting and X-chromosome inactivation. The hallmark of both X-chromosome inactivation (X inactivation) and genomic imprinting is monoallelic gene expression. X inactivation is random in somatic cells, whereas the expression of imprinted genes and X inactivation in the extraembyonic lineages are dictated by parental origin. Indeed, the epigenetic mechanisms that are involved in X inactivation and genomic imprinting share some marked similarities.

First, choice of the inactive X chromosome and initiation of the inactivation process are dependent on the expression of *Xist* RNA—a noncoding transcript that originates at the X inactivation center (Xic) and coats the inactive X chromosome[89]. Similarly, expression of the noncoding *H19* RNA correlates reciprocally with monoallelic expression of *Igf2* (refs. 9,10), although the *H19* transcript itself is not involved in silencing[90]. Second, *Tsix*, a noncoding antisense transcript that is initiated downstream of *Xist*, acts as a negative regulator of *Xist* expression[91]. Similarly, an antisense transcript originating from the unmethylated form of the differentially methylated region (DMR) in an intron of the paternal *Igf2r* allele has been shown to interfere with its expression[92]. CTCF binds to both the *Tsix* imprinting center[93] and the DMR upstream of the *H19* promoter[94] and may mediate the action of epigenetic switches that regulate X inactivation and imprinting.

The 'maintenance' of imprinting and X inactivation depends strictly on the continuous activity of Dnmt1 (refs. 31,95,96) and an oocyte-specific isoform of Dnmt1 that maintains the methylation marks of imprinted genes during cleavage[23]. To reset imprinting, methylation imprints are removed actively during primordial germ-cell development[97,98]. Once lost, methylation imprints cannot be re-established in the postzygotic embryo or adult, indicating that the establishment of allele-specific methylation marks is dependent on activities that are specific for oogenesis and spermatogenesis[99].

Evidence[26,27] suggests that the 'establishment' of methylation imprints is dependent on the expression of Dnmt3L, which by itself has no detectable methyltransferase activity but cooperates with the *de novo* methyltransferase Dnmt3a to establish maternal imprints (Table 1). By contrast, DNA methylation seems not to be involved in establishing X inactivation because *Xist* activation and late replication precedes *de novo* methylation of CpG islands by a few days[100–102]. DNA methylation is, however, involved in maintaining the inactive state, which requires the cooperation of several mechanisms including methylation of CpG islands, continuous expression of *Xist* and deacetylation of histones[102].

Nuclear cloning and epigenetic reprogramming. The cloning of mammals by nuclear transfer is inherently inefficient. Few embryos survive to birth, and many of the survivors die in the postnatal period or succumb prematurely to a variety of abnormalities[103–105]. The etiology of these pathologies is probably due to epigenetic rather than genetic abnormalities because cloned animals can reproduce sexually, yielding normal offspring[106]. Epigenetic reprogramming of the genome normally occurs during germ-cell development and ensures that the gamete genome provides an appropriate platform for the genetic program that drives embryonic development[103,107].

For cloned embryos to develop successfully, this epigenetic program must be reset and activated in the brief period between nuclear transfer and the initiation of cleavage. It is a failure to reprogram faithfully the 'embryonic' genes that seems to cause early death[103,107]. Faulty epigenetic reprogramming is reflected, for example, in the aberrant methylation of cloned cleavage-stage embryos[108–110]. Indeed, most clones derived from cumulus donor nuclei do not activate *Oct4* (ref. 111) or other '*Oct4*-like' genes correctly[112]. *Oct4* is known to be important for early mouse development[113]. The few cloned mice that do survive show dysregulation of many imprinted and non-imprinted genes regardless of the type of donor nucleus[114–116]. By contrast, X inactivation[117] and adjustment of telomere

Table 2 • Possible consequences of changes in genomic methylation on selective advantages leading to tumor outgrowth

Change in methylation status	Possible consequences	Mechanism	References
Hypermethylation	A. Epigenetic: silencing of tumor suppressor genes, biallelic expression of imprinted growth factors	*De novo* methylation of CpG islands, silencing of imprinted genes that inhibit growth factor activation (*H19*)	129,135,183
	B. Genetic: increase in point mutations	C→T transitions caused by spontaneous or by DNMT-mediated deamination	134
Hypomethylation	A. Epigenetic: protection against intestinal tumors	Demethylation may cause activation, inhibit silencing of suppressor genes	184
	B. Genetic: genomic instability	Altered mitotic recombination (LOH)	138,139

length[118–120]—reprogramming events that normally occur postzygotically—are accomplished faithfully and do not seem to impair the survival of cloned animals.

The efficiency of nuclear reprogramming depends on the differentiation state of the donor nucleus: the survival of clones derived from ES cell donor nuclei is 10–20 times higher than that of clones derived from somatic donors[121–123]. In fact, cloning from terminally differentiated B and T cells is so inefficient that monoclonal mice have been obtained only through a modified, two-step procedure and not through direct transfer of the cloned embryo into the uterus[124]. These results suggest that the epigenetic state of the differentiated nucleus restricts reprogramming of the genome—a notion that is consistent with conclusions from the seminal nuclear transfer experiments done on amphibians 50 years ago[107,125,126].

The widespread dysregulation of genes in clones that survive the postnatal period suggests that mammalian development can tolerate a substantial degree of epigenetic abnormality, and it is likely that epigenetic abnormalities and their associated phenotypes will occur in clones of all species including humans[127]. The available evidence indicates that reprogramming errors that severely impair development of the embryo do not affect the generation of functional cells *in vitro*, which is important for therapeutic applications of the nuclear transfer technology ('therapeutic cloning'). Indeed, it has been shown in a 'proof of principle' experiment that nuclear cloning combined with gene therapy can partially restore the immune defect in *Rag2* mutant mice[128].

Epigenetics and cancer. It is becoming increasingly apparent that the multiple changes in cancer cells, including chromosomal instability, activation of oncogenes, silencing of tumor suppressor genes and inactivation of DNA repair systems, are caused not only by genetic but also by epigenetic abnormalities[129,130]. During carcinogenesis, the genome simultaneously undergoes genome-wide hypomethylation[131,132] and regional hypermethylation of CpG islands[133], which may be of selective advantage for the incipient tumor cell. Table 2 summarizes the possible consequences of altered methylation. Both hypo- and hypermethylation of the genome can have epigenetic and genetic consequences for the cell.

'Epigenetic changes' include the silencing of tumor suppressor genes by hypermethylation, the loss of imprinting and, less likely, the activation of oncogenes by demethylation. Of these, the most important epigenetic change leading to a selective growth advantage of tumor cells is likely to be the hypermethylation-mediated silencing of tumor suppressor genes[129,130].

'Genetic changes' include point mutations at methylated CpGs, which may increase as a consequence of hypermethylation[134]; genome-wide microsatellite instability, owing to the silencing of *MLH1* mismatch repair genes[135]; and impaired genome stability as a consequence of hypomethylation. Consistent with this last possibility is the observation that mitogen-stimulated cells from individuals affected with immunodeficiency, centromere instability and facial anomalies (ICF syndrome) who have mutations of *DNMT3B* (Table 1) show increased chromosomal rearrangements in hypomethylated centromeric regions[136,137].

More direct evidence for a significantly increased rate of mutation owing to *Dnmt1* deficiency has been obtained in ES cells[138] and in fibroblasts (A. Eden, F. Gaudet, A. Waghmare and R.J. manuscript in preparation) that become hypomethylated. The enhanced mutation rate observed at several endogenous loci is caused by chromosomal rearrangements, such as loss of heterozygosity (LOH) owing to increased mitotic recombination. Hypomethylation does not, however, increase the rate of LOH of a transgene in ES cells[139], suggesting that the susceptibility of the genome to rearrangements may be affected by both the chromosomal location of the participating loci and the differentiation state of the cell.

Thus, DNA methylation has an essential role in all three mechanisms by which cancer cells eliminate tumor suppressor gene function: gene mutation, silencing by hypermethylation and deletion by LOH. But the prevalence of each mechanism may vary greatly for different tumor suppressor genes and among different types of cancer. The molecular mechanisms that promote methylation changes in normal or transformed cells are not clear. It has been proposed that *cis*-acting repetitive elements that are dispersed throughout the genome[140] or CpG islands that are situated in transcribed regions[129] may be targets for *de novo* methylation, which then spreads to adjacent promoters of tumor suppressor genes. It is also possible that chromatin alterations[129], which may be associated with prior genome-wide hypomethylation[137], may stimulate *de novo* methylation of specific targets such as the *H19* DMR[141].

Epigenetic states and the environment

Epigenetic modifications of the genome provide a mechanism that allows the stable propagation of gene activity states from one generation of cells to the next. Because epigenetic states are reversible they can be modified by environmental factors, which may contribute to the development of abnormal phenotypes. Normal physiological responses to certain environmental stimuli also may be mediated by epigenetic mechanisms. This has been particularly well demonstrated by the vernalization reaction of plants growing at high altitudes[142].

Vernalization is a quantitative response to low-temperature exposure that causes progressively earlier flowering. The *FLOWERING LOCUS C* (*FLC*) gene is thought to have a principal role in the vernalization response, allowing the mitotically stable inheritance of the vernalized state[143]. Notably, it has been shown that *FLC* activity is controlled by DNA methylation[144], as well as by expression of *VRN2*—a key inhibitory gene that encodes a polycomb group protein[145]. Polycomb proteins are crucial for main-

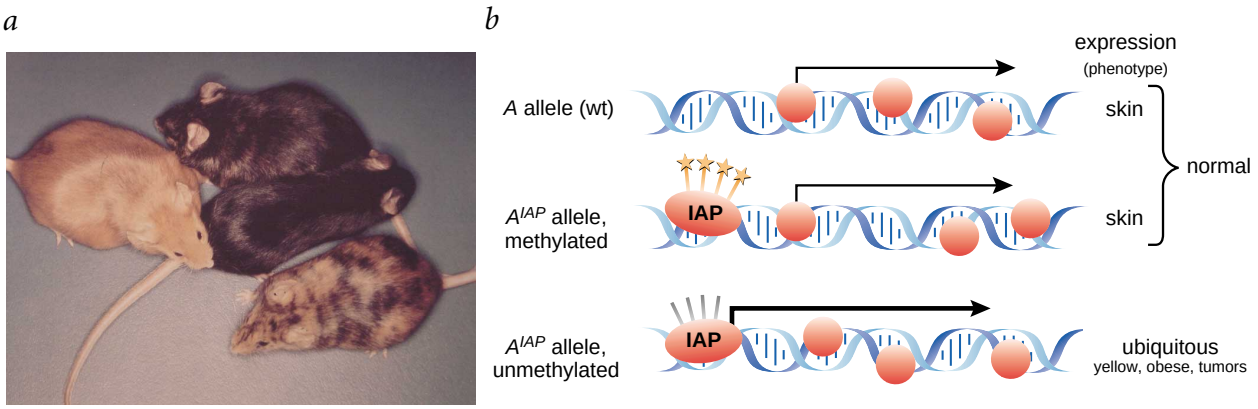

Fig. 2 Epigenetic effects on mouse coat color. ***a***, Mice segregating the A^{hvy} and *a* alleles show variegated coat color. The A^{hvy} allele[186], as well as other dominant *A* alleles, is formed by the insertion of IAP into the *agouti* locus[166–168]. These alleles are also designated as A^{IAP}. Reproduced with permission from ref. 168. ***b***, The methylation status of the IAP element determines expression of the *agouti* gene. When the element is methylated, the gene is expressed only in the skin, similar to expression of the wildtype allele. Hypomethylation of the element generates an ubiquitously expressed transcript that causes the yellow coat color, obesity and tumors.

taining a silent chromatin state in both *Drosophila* and mammals[146]. Thus, vernalization represents a physiological response to environmental stimuli that establishes an appropriate gene expression pattern by altering the epigenetic state of the genome.

DNA methylation, aging and diet. In mammals, the relationship between epigenetic states and environmental signals is less well defined, but both hypo- and hypermethylation have been associated with aging. A progressive loss of overall methylation is seen during the *in vitro* culture of fibroblasts[147] and in aging animals[148,149]; however, the functional significance of age-related loss of methylation, which may affect mostly repeated sequences such as transposable elements[150,151], remains to be determined.

In contrast to hypomethylation of repeated sequences, hypermethylation of specific genes has been observed in tissues of aging individuals. For example, methylation of the CpG islands associated with many genes, including that encoding the estrogen receptor[152], *IGF2* and *MYOD*, is undetectable in young individuals but becomes progressively detectable with age in normal tissues[2]. Because aging is thought to be one of the most important risk factors for cancer, an age-related predisposition to the hypermethylation of CpG islands that can silence tumor suppressor genes[129] may be one of the factors that could increase the risk of developing of malignancies in some individuals[135,153,154].

Diet is known to be a particularly important determinant in the manifestation of late-onset disease. For example, dietary supplements such as folate or vitamins that affect the activity of enzymes supplying methyl groups for the various cellular methylation processes can influence the rate of disease manifestation[155] and can exert marked effects on the incidence of colon cancer[156]. Reduced amounts of folate have been associated with genomic instability[157,158], neural tube defects[159] and genomic hypomethylation[160]. In addition, a methyl-deficient diet has been shown to induce liver cancer associated with both hypomethylation and the enhanced expression of oncogenes such as c-*ras*, c-*myc* or c-*fos*[161–163]. Because there is no evidence that demethylation can cause the increased expression of housekeeping genes such as oncogenes, it is possible that the observed hypomethylation merely reflects a diet-induced increase in the proliferative state of liver tissue[164,165] and is not a causal event in the transformation process.

Principal challenges for the future will be to assess whether environmental conditions such as diet influence methylation changes that occur with age in normal individuals and whether such epigenetic alterations predispose individuals to long-term diseases such as cancer. Because environmental stimuli probably induce stochastic and subtle epigenetic genome-wide changes, a causal relationship between diet-induced methylation changes and altered gene expression has been difficult to obtain.

Diet can affect stable gene expression. To establish a mechanistic link between environmental stimuli and epigenetic states of the genome, a well-defined and sensitive phenotypic readout is required. The coat color gene *agouti* provides such a marker. Several *agouti viable yellow* alleles (A^{hvy} or A^{IAP}) arise spontaneously by the insertion of an intracisternal A particle (IAP) retroviral element into the gene[166–168]. The coat color of mice with such an allele varies from yellow to mottled to wild-type agouti (Fig. 2*a*).Yellow mice become obese and develop tumors with age. The coat color variegation is caused by the methylation state of the IAP element. When methylated, the *agouti* gene is expressed only in the hair follicles just as in wild-type mice. When unmethylated, an ectopic and ubiquitously expressed *agouti* transcript originating in the long terminal repeat (LTR) of the IAP element causes the yellow coat color, obesity and tumors (Fig. 2*b*). In mottled mice, methylation is mosaic, which causes LTR promoter activity in some but not all cells. Thus, the methylation pattern of this element is established early in development and the coat color provides an easy phenotypic readout of the methylation and expression status of the element throughout life.

Wolff *et al.*[169] have investigated whether maternal diet can alter the phenotype of A^{IAP} mice. They found that when pregnant females are fed a diet supplemented with methyl donors, a larger proportion of offspring have a wild-type coat color as compared with the offspring of mothers fed a standard diet. This result argues that an environmental stimulus early in life can change the stable expression of genes and affect the phenotype of the adult. Although the mechanism that shifts coat color in the offspring is not known, the final consequence of the maternal diet-induced epigenetic change is clear: a shift in coat color is correlated with an increase in IAP methylation[170].

Consistent with the idea that hypomethylation of the IAP causes ectopic expression of *agouti* is the observation that inhibition of Dnmt1 activity results in most pups being yellow (F. Gaudet, W.M. Rideout and R.J., unpublished observations). Notably, the methylation status of the IAP element profoundly affects not only the phenotype of the animal itself but also that of the offspring: yellow A^{IAP} females have a higher proportion of yellow pups than do A^{IAP} females that have a wild-type coat

color[171]. This observation indicates that some epigenetic modifications of the genome are not fully erased during oogenesis but influence the epigenetic state of the genome and the activity of genes in the next generation.

It is plausible that the methylation status and/or the chromatin conformation of the whole genome are affected by non-specific external influences such as diet. But changes in global methylation may be so subtle that they are undetectable with current technology, and phenotypic consequences may become apparent only when monitoring a sensitive readout such as the A^{IAP}-dependent phenotype. The observations of Wolff *et al.*[169] raise the intriguing possibility that acquired alterations of the epigenetic state may have long-term health effects. This might contribute to the incidence of diseases such as cancer, which is well known to be affected by diet.

It has been proposed that long-latency ailments of the nervous system, such as multiple sclerosis, major psychoses, ALS (amyotrophic lateral sclerosis) or Huntington disease, may have an epigenetic basis[172]. Consistent with this possibility is a study showing that a diet supplemented with L-methionine affects the methylation and expression of *reelin*[173]. This is of relevance because *reelin* haploinsufficiency in *reeler* mice has a similar neuropathology to that of schizophrenia. Although highly speculative, it is possible that external factors such as diet may result in the accumulation of epigenetic changes over years and accelerate the manifestation of such diseases in genetically compromised individuals. We need better assays for evaluating the effects of environmental stimuli on the epigenetic state of the genome and its long-term phenotypic consequences. In particular, robust microarray-based methods for quantifying DNA methylation throughout the genome[174] are required.

The epigenetic state and therapeutic possibilities

The integration of DNA methylation, histone modification and chromatin remodeling is a complex process that depends on the collaboration of numerous components of the epigenetic machinery (Fig. 1). Transitions between different chromatin states are dynamic and seem to depend on a balance between factors that sustain a silent state (for example, HDACs and histone H3 Lys9 methyltransferases) and those that promote a transcriptionally silent state (for example, HATs and HS Lys4 methyltransferases)[175].

Disturbances of any of these components may shift the balance between an active and a silent chromatin conformation, resulting in an altered transcriptional state (Fig. 3). Indeed, experimental evidence supports the idea that drugs that target different components of the epigenetic machinery can cooperate in restoring normal gene activity. For example, the drug 5-aza-2′-deoxycytidine interferes with the activity of Dnmt1, leading to genomic hypomethylation; it also reactivates silenced tumor suppressor genes[130,176]. Despite its attractiveness as a means of reactivating silenced genes, its toxicity limits its clinical use[177]. Significantly, the combined treatment of cancer cells with 5-aza-2′-deoxycytidine and TSA, a drug that inhibits HDAC activity, results in a more effective reactivation of silenced tumor suppressor genes[178]. This has potential clinical relevance because the combined treatment of cancer with both drugs may reduce the effective drug concentrations and their systemic toxicity.

The treatment of inherited diseases that affect the epigenetic state represents an interesting challenge. An informative example is RTT, which is caused by the mutation of the ubiquitously expressed transcriptional repressor MeCP2 (ref. 81). MeCP2 has no known tissue specificity[73] but when mutated induces a specific neuronal dysfunction. MeCP2 deficiency might result in subtle changes in gene expression that could be the consequence of a slightly disturbed equilibrium between a silent and an active chromatin state. If correct, a therapeutic strategy could be aimed at restoring the appropriate chromatin state by targeting the activity of other components of the epigenetic machinery in individuals affected with RTT. Thus, the inhibition of proteins that induce a transcriptionally competent state, or the enhanced activity of factors that cause a shift towards a silent chromatin state, might counteract the lack of MeCP2 and help to restore a proper epigenetic balance.

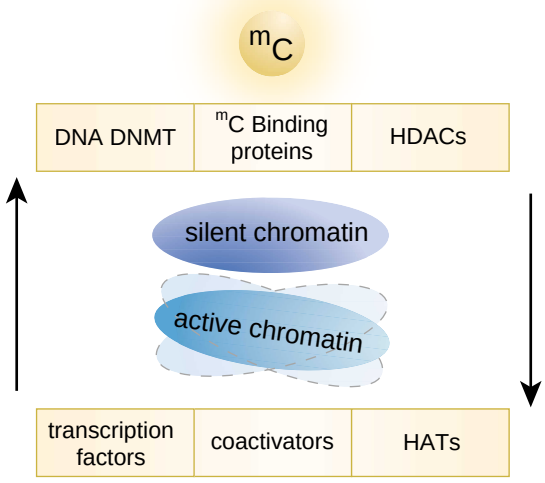

Fig. 3 Transitions between silent and transcriptionally competent chromatin states are dynamic and depend on a balance between factors that sustain a silent state, such as HDACs, and those that promote a transcriptionally active state, such as HATs[175]. Disturbances of any of these components may shift the balance between an active and a silent chromatin conformation, resulting in an altered transcriptional state. mC, methyl-CpG.

Even though the epigenetic misregulations that are involved in the etiology of diseases such as imprinting disorders, RTT, ICF or cancers are potentially reversible, a key issue of any therapeutic strategy that attempts to remedy the abnormal epigenetic state is that of specificity. Interfering with the activity of factors that modify the chromatin state is likely to affect the expression of unwanted genes such as endogenous retroviruses[179]. Before intervening we will need to understand better how the components that establish and maintain different chromatin conformations cooperate to ensure proper gene expression patterns, and how the genome integrates this information with signals from the environment.

Concluding remarks

The genetic information of an organism is differentially expressed in both time and space through mechanisms that we are finally beginning to understand. Epigenetic mechanisms constrain expression by adapting regions of the genome to maintain either gene silencing or gene activity. This is achieved through direct chemical modification of the DNA region itself and by modification of proteins that are closely associated with the locus. The targeting of specific genetic loci ensures that these effects are local. The triggers for this differential marking of the genome are largely mysterious, but are finally yielding to intense study. What needs to be explained is the variety of stimuli that can bring about epigenetic changes, ranging from developmental progression and aging to viral infection and diet. The future will see intense study of the chains of signaling that are responsible for epigenetic programming. As a result, we will be able to understand, and perhaps manipulate, the ways in which the genome learns from its experiences.

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