REVIEWS

THE FUNDAMENTAL ROLE OF EPIGENETIC EVENTS IN CANCER

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Patterns of DNA methylation and chromatin structure are profoundly altered in neoplasia and include genome-wide losses of, and regional gains in, DNA methylation. The recent explosion in our knowledge of how chromatin organization modulates gene transcription has further highlighted the importance of epigenetic mechanisms in the initiation and progression of human cancer. These epigenetic changes — in particular, aberrant promoter hypermethylation that is associated with inappropriate gene silencing — affect virtually every step in tumour progression. In this review, we discuss these epigenetic events and the molecular alterations that might cause them and/or underlie altered gene expression in cancer.

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In the mammalian genome, methylation takes place only at cytosine bases that are located 5′ to a guanosine in a CpG dinucleotide[1]. This dinucleotide is actually under-represented in much of the genome, but short regions of 0.5–4 kb in length, known as CpG islands, are rich in CpG content[1,2]. Most CpG islands are found in the proximal promoter regions of almost half of the genes in the mammalian genome and are, generally, unmethylated in normal cells (FIG. 1). In cancer, however, the hypermethylation of these promoter regions is now the most well-categorized epigenetic change to occur in tumours; it is found in virtually every type of human neoplasm and is associated with the inappropriate transcriptional silencing of genes[3,4]. Surprisingly, as shown in FIG. 2, such promoter hypermethylation is at least as common as the disruption of classic tumour-suppressor genes in human cancer by mutation and possibly more so. Nearly 50% of the genes that cause familial forms of cancer when mutated in the germ line are known to undergo methylation-associated silencing in various sporadic forms of cancer (FIG. 2). Additionally, there is a growing list of candidate tumour-suppressor genes that are silenced by promoter hypermethylation in certain cancers. These genes are predicted to be important for tumorigenesis on the basis of their presumed function, but seem not to be frequently mutated in such cancers. Examples of these genes include O[6]-methylguanine-DNA methyl-transferase (*MGMT*)[5], which encodes an important DNA-repair gene; cyclin-dependent kinase inhibitor 2B (*CDKN2B*), which encodes p15, a cell-cycle regulator[6]; and *RASSF1A*[7,8], which encodes a protein of unknown function that can bind to the *RAS* oncogene. Promoter hypermethylation is the only mechanism for the loss of function of many of these genes in tumours (FIG. 2).

During the past few years, it has become increasingly apparent that aberrant promoter methylation is associated with a loss of gene function that can provide a selective advantage to neoplastic cells, as do mutations. For example, the von Hippel–Lindau syndrome (*VHL*)[9], breast cancer 1, early onset (*BRCA1*)[10] and serine/threonine kinase 11 (*STK11*)[11] genes — germ-line mutations of which cause familial forms of renal, breast and colon cancer, respectively — are often epigenetically silenced in the sporadic forms of these tumour types. The importance of epigenetic silencing in the aetiology of non-familial forms of cancer is illustrated by studies of *BRCA1*. This gene was thought to be important only for familial breast cancer (through *BRCA1* germ-line mutations). However, it is now apparent that 10–15% of women with the non-familial form of this cancer have tumours in which this gene is hypermethylated[10]. Furthermore, microarray studies indicate that the overall gene-expression profiles of sporadic breast cancers with hypermethylated *BRCA1* are identical to those of the familial cancers in which *BRCA1* is mutated and are distinct from those of other breast-cancer types[12,13].
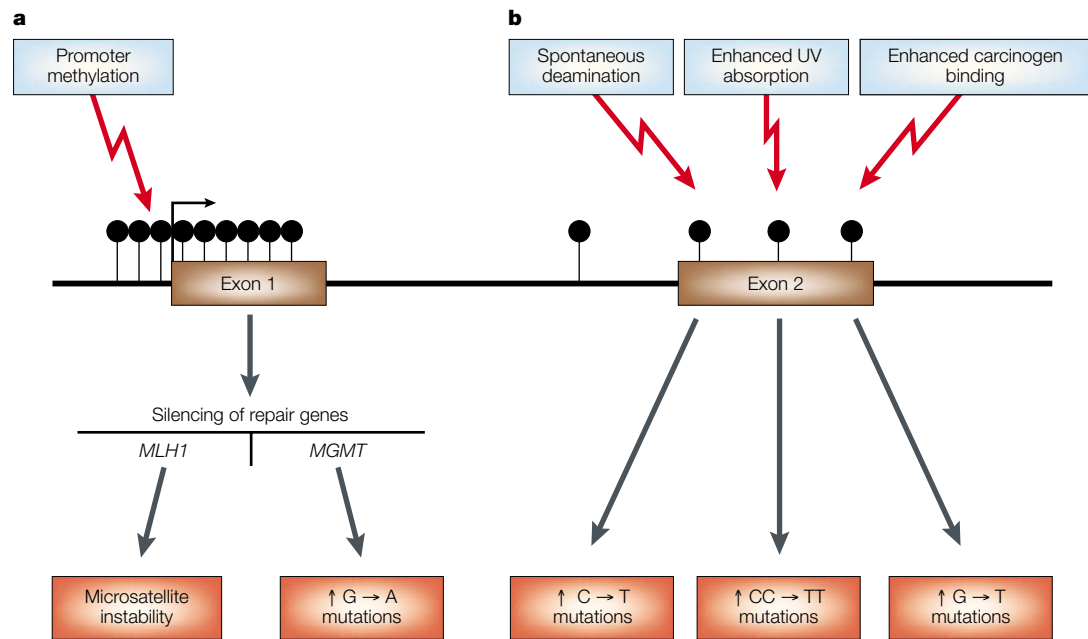
Figure 1 | **How epigenetics affects genetics. a** | How *de novo* hypermethylation of CpG islands in the promoters of DNA-repair genes, such as *MLH1* and *MGMT*, can lead to their inactivation. Hypermethylation of the promoter of *MLH1* can lead to MICROSATELLITE INSTABILITY, and hypermethylation of the promoter of *MGMT* leads to increased G → A mutations. **b** | Cytosine methylation in the coding region of genes can also increase mutation rates because of the spontaneous hydrolytic deamination of methylated cytosine, which causes C → T transition mutations at methylated CpG sites. Methylation also changes the absorption wavelength of cytosine, into the range of incident sunlight, resulting in CC → TT mutations, which commonly occur in skin cancers. Methylated CpGs are also preferred binding sites for benzo(a)pyrene diol epoxide and other carcinogens that are found in tobacco smoke. These cause DNA adducts and G → T transversion mutations, which are often found in the aerodigestive tumours of smokers. *MLH1*, mutL homologue 1, colon cancer, non-polyposis type 2; *MGMT*, O[6]-methylguanine-DNA methyltransferase; UV, ultraviolet.

MICROSATELLITE INSTABILITY
(min). In diploid tumours, genetic instability that is due to a high mutation rate, primarily in short nucleotide repeats. Cancers with the min phenotype are associated with defects in DNA-mismatch-repair genes.

KNUDSON'S TWO-HIT MODEL
In 1971, Alfred Knudson proposed that two successive genetic 'hits' are required to turn a normal cell into a tumour cell and that, in familial cancers, one hit was inherited. Two inactivating 'hits' are therefore required to cause the loss of function of tumour-suppressor genes.

The functional significance of the hypermethylation of key tumour-suppressor gene promoters can also be appreciated by examining the consequences of the inactivation of individual copies of such genes. KNUDSON'S TWO-HIT MODEL predicts that a phenotypic consequence of tumour-suppressor gene loss is not seen unless both alleles of a gene are inactivated in a tumour[14]. Findings from several studies now clearly show that tumours can stably maintain mutations in one allele of a gene while the other allele is hypermethylated, leading to the functional inactivation of the gene[15,16]. In fact, when one of two alleles is mutated in the germ line of a patient with a familial form of cancer, and the resultant tumour retains both alleles of the gene, hypermethylation is commonly seen as the second inactivating change. Moreover, it seems never to be present in the promoter of the mutated gene, but is always associated with the wild-type allele[17].
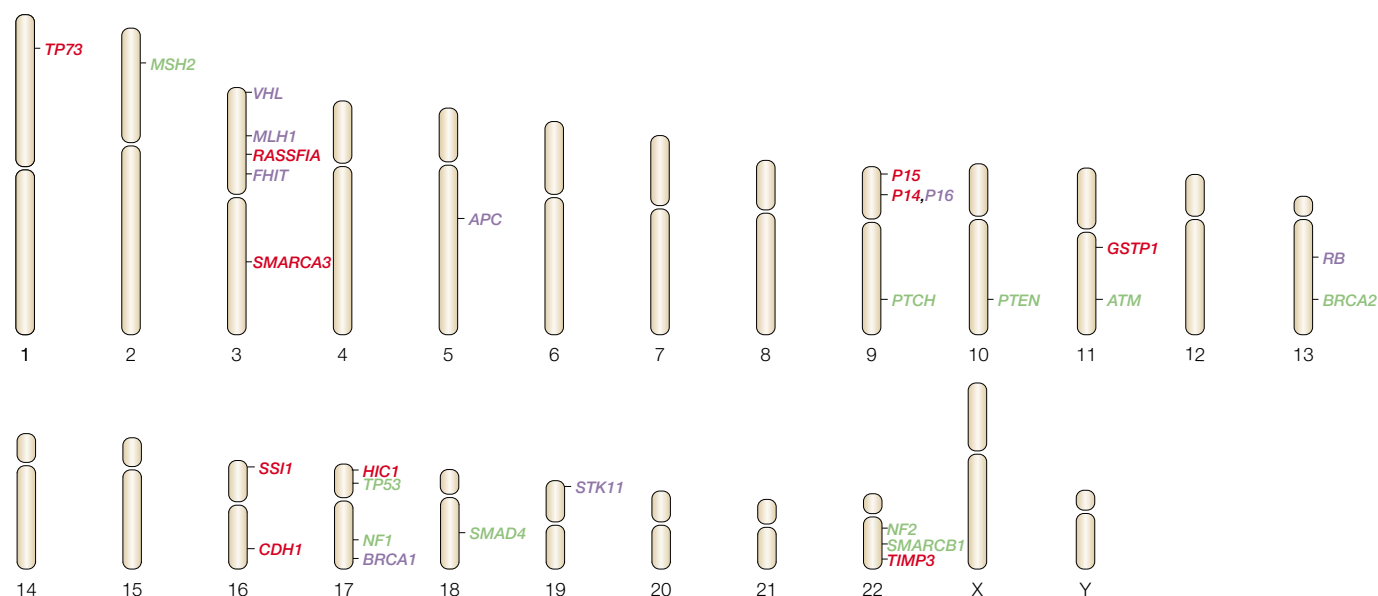
The importance of epigenetic gene silencing in cancer is also highlighted by the growing awareness that such changes can actually predispose to mutational events during tumour progression. This was first shown for the mismatch-repair gene *MLH1* (mutL homologue 1, colon cancer, non-polyposis type 2)[18,19], which is frequently hypermethylated in sporadic tumours that have microsatellite instability (FIG. 1). Importantly, these changes in the methylation of the 5′ region of *MLH1* have

been observed in the apparently normal colonic epithelium of patients that have colorectal cancer with microsatellite instability[20] and, in hyperplastic regions, preceding the development of endometrial cancers that develop this type of genetic change[21]. *MGMT* is another DNA-repair gene that is silenced in association with promoter methylation in colon, lung, lymphoid and other tumours[5,22,23]. O[6]-MGMT protein removes carcinogen-induced O[6]-methylguanine adducts from DNA, which result in G → A transition mutations if left unrepaired. Tumours with silenced *MGMT* alleles do, indeed, seem to be predisposed to mutation in key genes, such as tumour protein p53 (*TP53*)[23] and *K-RAS*[5]. This promoter hypermethylation, like that at *MLH1*, seems to precede genetic changes by occurring in pre-malignant polyps that do not yet harbour gene mutations[5,23].

Another important observation with regard to tumour-suppressor genes that are disrupted epigenetically and/or genetically is that they often reside in genomic regions that are characterized by frequent chromosomal deletions. Virtually every chromosomal location that is shown in FIG. 2 is known to be a region that is frequently deleted in human cancer. These deletions cause loss of heterozygosity (LOH) and are often used to guide searches for tumour-suppressor genes. Interestingly, there are LOH regions in which epigenetic events, rather than genetic alterations, seem to define

Figure 2 | **A map of the human genome.** Genes that are frequently mutated and/or hypermethylated in cancer. Only a subset of the genes that are known to be frequently hypermethylated and silenced in one or more cancer types, and genes that are most often mutated in tumours are shown here. Nearly all of these genes are in chromosome regions that commonly show loss of heterozygosity in cancer. Genes for which only genetic mutations have been reported are shown in green, those that have been reported to be only hypermethylated are shown in red and those for which both changes have been reported are shown in purple. In the 9p region, the individual transcripts *P16^{INK4A}* and *P14^{ARF}* are transcribed from the CDKN2A locus, and are each shown because of the different incidences of hypermethylation and mutation at their promoters. It is noteworthy that as many, if not more, genes are inactivated by promoter hypermethylation and by epigenetic silencing as they are by coding-region mutations. *ATM*, ataxia telangiectasia mutated; *APC*, adenomatosis polyposis coli; *BRCA1/2*, breast cancer1/2, early onset; *CDH1*, E-cadherin; *CDKN2A/B*, cyclin-dependent kinase inhibitor 2A/B; *FHIT*, fragile histidine triad; *GSTP1*, glutathione S-transferase pi; *MLH1*, mutL homologue 1, colon cancer, non-polyposis type 2; *MSH2*, mutS homologue 2, colon cancer, non-polyposis type 1; *NF1/2*, neurofibromin 1/2; *PTCH*, patched homologue; *PTEN*, phosphatase and tensin homologue; *RB1*, retinoblastoma 1; *SMAD4*, mothers against decapentaplegic homologue 4; *SMARCA3/B1*, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 3/subfamily B, member 1; *STK11*, serine/threonine kinase 11; *TIMP3*, tissue inhibitor of metalloproteinase 3; *TP53/73*, tumour protein p53/p73; *VHL*, von Hippel–Lindau syndrome.

CDKN2A
Two tumour-suppressor transcripts are encoded by the *CDKN2A* locus. *P16^{INK4A}* inhibits the cyclin-dependent kinases 4 and 6, blocking them from phosphorylating RB1 and so preventing cells from exiting G1. *P14^{ARF}* is encoded from an alternative reading frame (arf), helps regulate nuclear location of TP53 and putatively causes cell-cycle arrest at G1 and G2. Loss of heterozygosity of either transcript is associated with cancer.

the important genes. For example, *RASSF1A* at 3p21 (REFS 7,8) and hypermethylated in cancer 1 (*HIC1*), which encodes a transcription factor, at 17p13.3 (REF. 24) — which are two chromosomal regions that are characterized by frequent LOH in several tumour types — are often hypermethylated in many important human cancers, such as lung, prostate, colon and breast. These now constitute the principal candidate tumour-suppressor genes in these high-frequency LOH regions, where no gene mutations have been found consistently.

The wide distribution of hypermethylated genes across the human genome, and the finding of hypermethylated candidate tumour-suppressor genes in regions of high-frequency chromosome deletions, has spurred efforts to screen the cancer-cell genome for such genes. There are a growing number of techniques that have been developed for this purpose. Some aim to identify regions of abnormal methylation per se rather than specific genes, whereas others aim to identify genes by discovering aberrantly hypermethylated CpG islands[25–29] (BOX 1). Recently, epigenetically silenced and potentially important genes for colon cancer have been successfully identified using a microarray approach. This approach was used to assay for the re-expression of silenced genes after the treatment of cancer cells with histone deacetylases

(HDACs), which alter gene transcription and chromatin configuration (as discussed in more detail later)[30]. Such genome-scanning procedures could prove to be of great use to cancer-biology research and might also have clinical applications. For example, in the above microarray approach, silencing by promoter hypermethylation of an entire gene family was discovered. This gene family can normally counteract the WNT (wingless-related) signalling pathway that functions in colon cancer; virtually all colorectal cancers have this change in one or more of the genes in this pathway[30].

Although this review focuses predominantly on the gene-silencing events that are associated with methylation changes in cancer, it is important to recognize that cytosine methylation can influence tumorigenicity by other mechanisms. These occur because 5-methylcytosine is itself mutagenic: it can undergo spontaneous hydrolytic deamination to cause C $\rightarrow$ T transitions[31]. This enhanced mutagenesis is seen in the germ line of all organisms that methylate their DNA. Furthermore, as many as 50% of inactivating point mutations in the coding region of the human *TP53* tumour-suppressor gene in somatic cells occur at methylated cytosines[32]. The presence of the methyl group in the CpG dinucleotides in the coding region of this gene strongly

Box 1 | **Techniques for randomly screening cancer-cell genomes for altered methylation loci**

Restriction landmark genomic screening (RLGS)

In this approach[27], genomic DNA is cut with methylation-sensitive restriction enzymes, such as *Not* 1, which recognize large CpG-rich sequences that usually occur in CpG islands. The restricted DNA is electrophoresed in two dimensions to produce a pattern in which a spot will be missing if a particular *Not* 1 site is methylated and has not, therefore, been cut. Although this technique allows researchers to detect large numbers of CpG islands, these are sometimes not in the promoter regions of genes and are, therefore, probably not involved with transcriptional regulation.

MCA–RDA

In methylated CpG island amplification–representational difference analysis (MCA–RDA)[26], DNA is sequentially restricted with two enzymes, which each recognize the same CpG-rich sites that occur predominantly in CpG islands. The first enzyme is methylation sensitive and the second is not. This produces fragments that differ according to the methylation status of the DNA, which will differ between normal and tumour-derived DNA. After PCR amplification of these fragments, the tumour and normal DNA amplicons are subjected to RDA, which exploits the methylation-sensitive restriction-site differences between the normal and tumour-cell DNA to carry out a comparative hybridization subtraction step. The advantages of this approach are very similar to those of RLGS. However, even though many of the CpG islands are associated with genes, defining the start site of a gene and the exact relationship of the island to the transcriptional regulation of a gene can be laborious.

MS-AP-PCR

Methylation-sensitive arbitrarily primed PCR (MS-AP-PCR)[28] also uses methylation-sensitive restriction enzymes to cut DNA before it is amplified with random CpG-rich primers that target CpG islands. The resulting fragments are displayed on gels, and gel-spot patterns between different cell types are compared, which leads to a rapid identification of CpG islands that are differentially methylated in different tissues. The method suffers from the same limitations as those for RLGS and MCA–RDA.

Differential methylation hybridization (DMH)

DMH[25] is an array-based method in which genomic DNA is pre-cut with a methylation-insensitive enzyme, such as *Mse* 1. Linkers are then ligated to the digested DNA before it is cut with a methylation-sensitive enzyme. The resulting digests are amplified by PCR and the products hybridized to an array of immobilized CpG islands.

**Microarray and gene re-expression approach**

In this approach, gene re-expression is induced by treating cells with agents that block both promoter hypermethylation and histone deacetylation[30]. Silenced genes that are re-expressed by this treatment are then surveyed by cDNA microarray analysis. The advantage of this approach is that the detection of hypermethylation sites is linked to the transcriptional status of genes, the promoters of which are affected by this change. A disadvantage is that the CpG island that is hypermethylated, and is associated with the gene promoter, is not always easy to identify in genomic databases.

increases the rate at which mutations are induced by ultraviolet (UV) light during the development of skin cancers[33]. The biochemical mechanisms of this enhancement are well understood; the methyl group shifts the UV absorption spectrum for cytosine to a region in the spectrum that is prevalent in sunlight. Methylated CpG dinucleotides are also the preferred targets of G $\rightarrow$ T transversion mutations, which are induced in mammalian cells by the tobacco carcinogen benzo(a)pyrene diol epoxide[34]. So, the methylation that occurs in the transcribed region of *TP53* (REF. 35) increases its susceptibility to spontaneous deamination, UV-induced mutation and hydrocarbon carcinogenesis. The fact that most coding sequences contain abundant CpG methylation indicates that this epigenetic 'mark' might increase the likelihood that this sequence will undergo heritable genetic changes.

Finally, it has been known for a long time that tumour cells are hypomethylated in comparison to wild-type cells[36,37]. This has led to the suggestion that the hypomethylation of non-promoter regions of DNA and of structural elements, such as centromeric DNAs, might cause enhanced genomic instability. Indeed, germ-line mutations in *DNMT3B* — which encodes

DNA methyltransferase 3β, one of the enzymes that catalyses DNA methylation — lead to immunodeficiency centromeric instability and facial abnormalities (ICF) syndrome[38–40]. ICF patients have a loss of methylation at selected centromeric regions and have profound chromosomal structural changes. Indeed, many human tumours have similar losses of DNA methylation and chromosomal structural changes in these regions[41]. Increased levels of gene deletion have also been reported in mouse embryonic stem (ES) cells that are deficient in DNA methyltransferase 1 (Dnmt1)[42], although, in another study, fewer deletions were observed in *Dnmt1*-deficient ES cells[43]. Therefore, even though it remains an attractive hypothesis that hypomethylation leads to gross chromosomal instability in cancer, the relationship between the two processes is not yet clear.

Given the DNA-methylation changes that can occur in cancer, especially those that are associated with transcriptional silencing and the loss of function of key genes, it seems timely to review our understanding of the molecular mechanisms that might cause these changes. This review explores how our increased understanding of the role of chromatin in organizing and
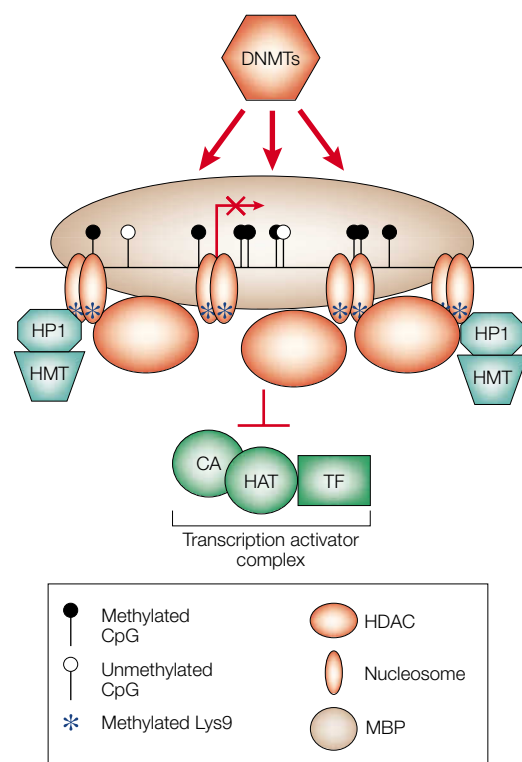
Figure 3 | **Typical chromatin configuration of transcriptionally silent pericentromeric DNA.** In heterochromatin, most candidate CpG sites are methylated and are bound by methyl-cytosine-binding proteins (MBPs), which are present in complexes that include histone deacetylases (HDACs). The histones are deacetylated and organized into regularly spaced, tightly compacted nucleosomes. DNA methyltransferases (DNMTs) can access the area, whereas transcription activator complexes — consisting of a transcription factor (TF), a co-activator protein (CA) and a protein with histone acetyltransferase (HAT) activity — are excluded. The histone 'mark' of a methylated Lys9 residue on the tail of histone 3 (H3) might help to target DNA methylation to the region and signifies, together with deacetylated histones, that this is transcriptionally repressive chromatin (see text for more details). H3 Lys9 methylation is maintained by a histone methyltransferase (HMT) that is recruited by the binding of the chromodomain protein HP1α (HP1) to the methylated H3 Lys9.

regulating the transcription of the mammalian genome is enhancing our understanding of the epigenetic changes that occur in cancer.

Interpreting methylation signals in cells

An important issue in studies of epigenetically mediated gene silencing in cancer is to understand how promoter hypermethylation participates in the loss of gene transcription. In experimental systems, it seems that methylation at promoters does not lead to silenced transcription until chromatin proteins are recruited to the region, which mediate gene silencing[44]. In this setting, methylation seems to initiate the process that results in a loss of transcription. However, in some cellular settings, including in abnormal gene-silencing events in cancer, it is

unclear whether methylation is the initial silencing event or whether it is targeted to the region by earlier chromatin-remodelling events. In some systems, the action of an RNA species is required to initiate gene silencing before promoters in the region become densely methylated, as shown by the role of X (inactive)-specific transcript (*XIST*) in gene silencing on the X chromosome of female mammals[45]. Whatever the sequence of events is at a given promoter, it seems that promoter methylation must be integral to the loss of gene function because drugs that induce DNA de-methylation, such as 5-AZA-2′-DEOXYCYTIDINE[46], can partially reactivate silenced genes in cancer cells to restore their function[3,4].

If chromatin is crucial to aberrant gene silencing in cancer, either primarily or secondarily, which processes are involved? Most of our genome is normally packaged as transcriptionally repressive chromatin of the type found in PERICENTROMERIC HETEROCHROMATIN regions. This type of chromatin is heavily methylated, and the identities of the chromatin-associated protein complexes that might link DNA methylation to transcriptional silencing have been discovered over the past few years through studies of such chromatin[47–49] (FIG. 3). The DNA in these transcriptionally silent regions is packaged into compacted NUCLEOSOMES that contain deacetylated histones, in particular deacetylated histone H3 (H3). These histones are extensively deacetylated through the action of HDACs, and this deacetylated histone state helps to maintain nucleosomes in a compacted and transcriptionally silent state[47–49]. The transcriptional repression status of this chromatin is also facilitated by the participation of key proteins, such as CBX5, the human homologue of the *Drosophila* CHROMODOMAIN protein Hp1α (also known as Suppressor of variegation 205, Su(var)205)[44,46–48], as discussed in more detail below.

DNA methylation itself also seems to be involved crucially in the transcriptionally silent state of pericentromeric heterochromatin. Methyl-cytosine-binding proteins (MBPs) associate with methylated cytosines and also with various chromatin-remodelling complexes. They have also been shown to act as transcriptional repressors *in vitro*[50–53]. Importantly, these MBPs also reside in complexes that contain HDACs; for example, the methyl-binding proteins methyl-CpG-binding protein 2 (MECP2) and methyl-CpG-binding-domain proteins MBD1 and MBD2 have been found to associate with transcriptional co-repressors, such as SIN3, which are known to bind HDACs directly. This presents a method by which the MBPs might recruit histone deacetylation to methylated DNA in regions of transcriptional silencing[50–53].
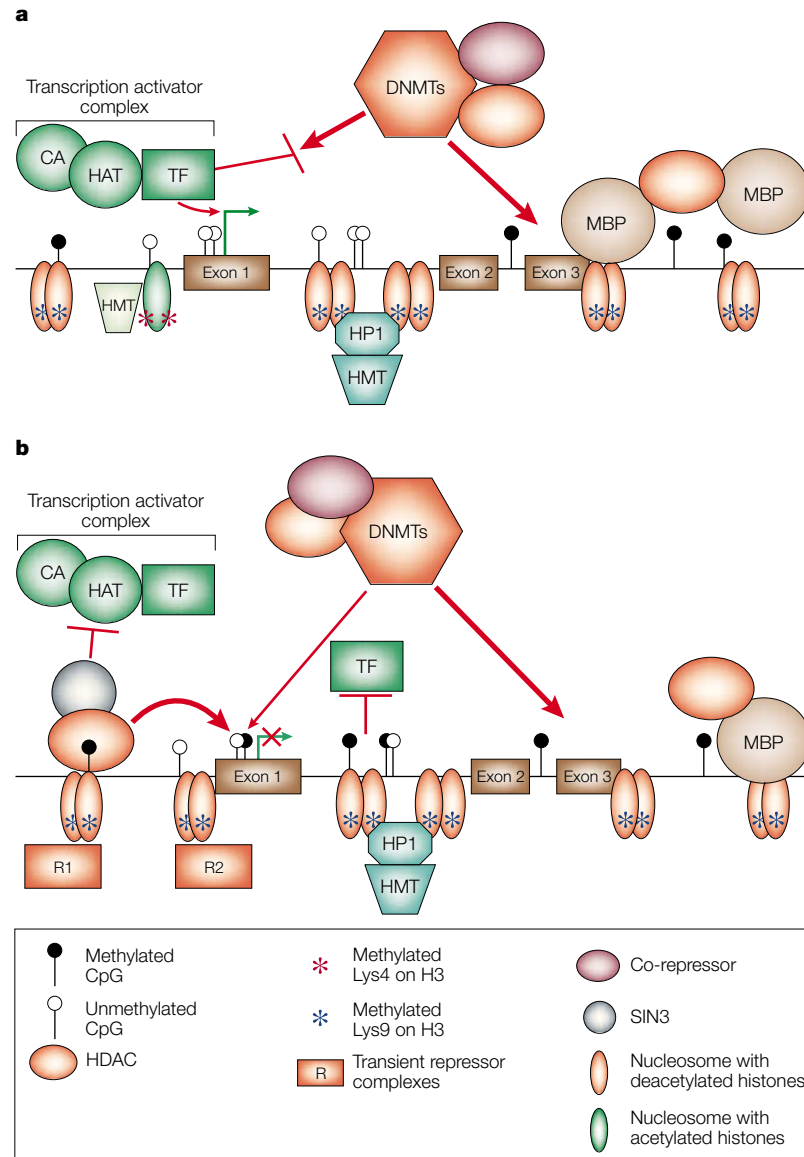
By contrast, only a small fraction of the genome is transcriptionally competent. The state of chromatin in these regions must be dynamic to meet the changing transcriptional requirements of a cell. This balance between EUCHROMATIN and heterochromatin ensures that the gene-expression pattern of a given cell type is stably maintained in daughter cells as a heritable state[1].

In terms of DNA methylation, there are two types of gene promoters[1,3,4] (FIGS 4,5). One type accounts for ~50% of the genes in the mammalian genome and contains unmethylated CpG islands. The other promoter type is CpG poor in composition, as is the rest of the genome. Among the genes that have CpG-poor

Figure 4 | **A CpG-poor promoter in transcriptionally active and transcriptionally repressed states. a** | A CpG-poor promoter in a transcriptionally active state. Individual CpG sites around the transcription start site are unmethylated and are protected from DNA methyltransferases (DNMTs) and transcriptionally repressive complexes that contain histone deacetylases (HDACs) and co-repressor proteins. The nucleosomes around the promoter are more widely spaced than in heterochromatin and contain heavily acetylated histones. The red asterisks depict a histone code 'mark' — methylation of the Lys4 residue of histone 3 — that is typical of transcriptionally permissive chromatin, which is methylated by a different histone methyltransferase (HMT) to that shown in FIG. 3. **b** | The same promoter in a transcriptionally repressed state. Selected CpGs near the transcription start site are methylated. Three possible transcriptionally repressive chromatin changes are shown: methylated CpGs that block the occupation of the target binding site of a transcription factor; a transient repressor complex that might contain SIN3, which is a known transcriptional co-repressor; and transcriptionally repressive MBP complexes that are targeted to methylated CpG regions. CA, co-activator protein; HAT, histone acetyltransferase; HP1, chromodomain protein HP1α; MBP, methyl-cytosine-binding protein; TF, transcription factor.

promoters, it is not known for how many of them CpG methylation might have a modulatory role in their transcription. However, there are examples in which the methylation of individual CpG sites in such promoters can determine the transcriptional status of a gene by blocking the access of certain transcription factors that are sensitive to the methylation of CpG sites in their target binding sites[1,54–56] (FIG. 4). Alternatively, the methylation of sites in these regions might also silence genes by helping to recruit chromatin protein complexes that repress transcription (FIG. 4).

In contrast to CpG-poor promoters, methylation does not normally participate in regulating the transcription of genes with promoters that contain CpG islands, whether such genes are being actively transcribed or not[1,3,4]. These islands reside, especially in active promoters, in chromatin that is composed of widely and irregularly spaced nucleosomes, which contain highly acetylated histones. Such a chromatin conformation and histone-acetylation state is thought to facilitate the accessibility of the promoter to transcription-activating complexes (FIG. 5). Unmethylated CpG-island regions are generally flanked by regions of less-CpG-rich DNA, which is heavily methylated[1,3,57,58], and in a chromatin conformation that, presumably, resembles that of transcriptionally silent pericentromeric heterochromatin (FIG. 5). When the transcription of these CpG-island promoters is downregulated, as required by a given cellular state, the island remains free of methylation[1]. It is likely, in such inactive states, that the promoter is associated with transiently positioned repressor complexes that do not involve methylated DNA or MBP complexes.

The above separation of unmethylated promoter CpG-island regions from immediately flanking areas of methylation seems to involve a functional boundary. Much remains to be determined about how these boundaries for transcriptionally active versus inactive chromatin are established. However, recent exciting discoveries have begun to link a specific histone code to the targeting of methylation to DNA (FIGS 4,5). Specific methylation marks on histone H3 seem to provide a signal that separates regions of transcriptionally active chromatin from regions of transcriptionally inactive chromatin. The methylation of lysine 9 (Lys9) in the tail of histone H3 has been found to be closely associated with transcriptionally repressive chromatin, whereas methylation of lysine 4 (Lys4) on H3 characterizes the transcriptionally active chromatin that immediately flanks the heterochromatin[59–63]. It has been found that the *Drosophila* Hp1α chromodomain protein, as well as its homologues in other species, is important for establishing transcriptionally repressive chromatin. It does this by binding to the methylated-Lys9 residue through its chromodomain and then interacting with histone methyltransferases to recruit them to sites of Lys9 methylation[59–63]. Different histone methyltransferases mediate the Lys4 methylation. Although this histone methylation pattern is found in organisms such as yeast, which do not methylate their DNA, it has also been associated with the establishment of DNA methylation
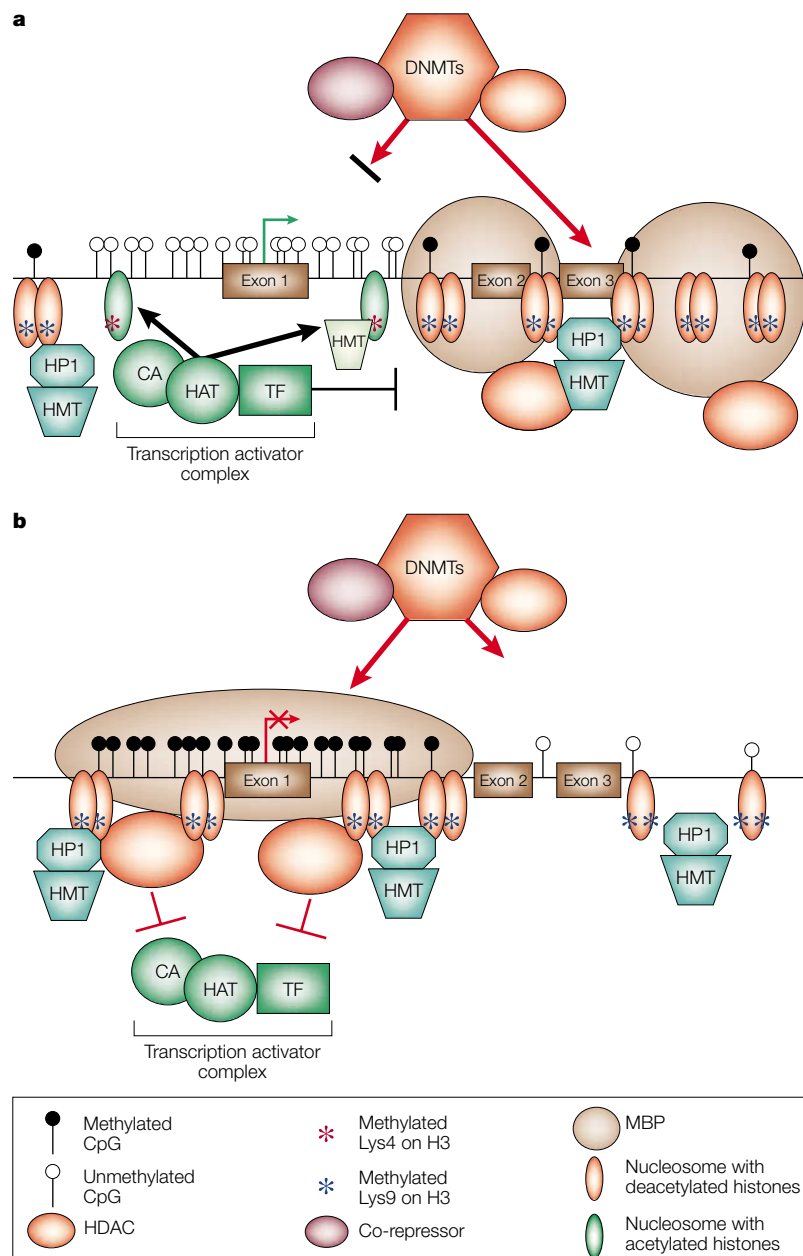
in two organisms — *Neurospora crassa* and *Arabidopsis thaliana*. In these organisms, mutations in a Lys9 histone methyltransferase eliminate all or some DNA methylation[64,65]. These findings are extremely important

Figure 5 | A CpG-rich promoter in transcriptionally active and transcriptionally repressed states. a | A typical, unmethylated and transcribed promoter that contains a CpG island. The island resides in a domain that is protected from the spread of methylated chromatin that flanks it. Around the promoter, there are widely spaced nucleosomes, which contain acetylated histones in which the Lys4 residue of histone H3 is methylated by a Lys4 methyltransferase (HMT). This is in contrast to the flanking transcriptionally repressive chromatin. This chromatin has methylated CpG sites, methyl-cytosine-binding protein (MBP)- and histone deacetylase (HDAC)-containing complexes, is tightly compacted, and has regularly spaced nucleosomes with de-acetylated histones that contain methylated H3 Lys9, as maintained by an HP1α (HP1)–Lys9 HMT complex. DNA methyltransferase (DNMT) complexes are excluded from the promoter. **b |** The same promoter in an aberrantly hypermethylated state that is associated with transcriptional repression in a cancer cell. The promoter region is accessible to DNMT complexes and transcriptionally repressive chromatin occupies the promoter, which is transcriptionally silenced. CA, co-activator protein; HAT, histone acetyltransferase; HP1, chromodomain protein HP1α; TF, transcription factor.

because they indicate that the methylation of H3 Lys9 might somehow be required to establish sites of DNA methylation in these and, perhaps, other organisms. It is not clear how the methylation of H3 Lys9 directs DNA methylation. It has been suggested that it might bring about the binding of DNA methyltransferases to the methylated lysine or to the key proteins that are required for histone methylation, such as Hp1α or the histone methyltransferases themselves. Intriguingly, some histone methyltransferases contain MBP-like domains, which indicate that there might be an additional interaction between the histone and the DNA methylation process[63].

In the light of these findings, how does the gene silencing that is associated with aberrant CpG-island methylation in cancer occur? Although much remains to be proven experimentally, hypermethylated promoters are now known to have certain features that are typical of pericentromeric heterochromatin (FIG. 5). For example, they have a closed chromatin configuration, which is typical of chromatin that is composed of tightly compacted, highly deacetylated nucleosomes[66]. Interestingly, the specific association of MBPs to such promoters is beginning to be described[67–69]. However, studies that have tried to determine which MBP family members, and their associated chromatin-remodelling proteins and HDACs, associate with specific hypermethylated promoters, such as that of the cyclin-dependent kinase inhibitor 2A (p16^{INK4a}; also known as CDKN2A) transcript, differ so far[67–69]. Therefore, this important question will require further characterization. The possibility that a specific histone methylation code, as described above, might be required to initiate and/or maintain the aberrant hypermethylation of gene promoters in cancer must now also be considered and will be a particularly exciting area of future research.

An important characteristic of the interaction between DNA methylation and HDAC activity, particularly in maintaining the aberrant silencing of hypermethylated genes in cancer, is that the methylation seems to function as the dominant event that seals transcriptional repression. This finding stems from studies that have found that the inhibition of HDAC activity alone, by potent and specific drugs such as trichostatin (TSA), does not result in the reactivation of aberrantly silenced and hypermethylated genes in tumour cells[66]. However, TSA can reactivate these same genes if the cells are first treated with demethylating drugs, such as 5-aza-cytidine, which demethylate promoters[66]. This finding not only is important for understanding how DNA methylation works to silence transcription, but also has clinical implications, as discussed in more detail below.

A molecular explanation for the above interaction between DNA methylation and histone deacetylation awaits clarification. However, in a recent study of *MDR1*, which encodes a multidrug transport resistance protein and which is hypermethylated in leukaemia cells, it was found that, although HDAC inhibition alone leads to histone acetylation in the promoter, the

re-expression of the gene did not occur unless the demethylation of its promoter first reduced the presence of MECP2 (REF. 69). Theoretically, the loss of this protein might remove a direct transcriptional repressor of *MDR1*, which might then be recruited by methylated CpGs and/or a complex that contains HDACs. Future studies will be required to verify this possibility and to elucidate the molecular mechanisms that underlie the interplay between DNA methylation and histone acetylation in gene silencing.

Initiating and maintaining methylation

Our ideas of how DNA methylation is established in the mammalian genome are changing as rapidly as is our understanding of how chromatin organization modulates gene expression. Several years ago, only one mammalian DNA methyltransferase (DNMT) enzyme, DNMT1, was thought to be responsible for maintaining DNA-methylation patterns in adult cells, and the mechanism for establishing *de novo* methylation was not known[1]. Now, two other biologically active DNMTs, DNMT3A and DNMT3B have been identified. Targeted disruptions of these Dnmt genes in mice, alone and together, cause embryonic lethality, and studies of these mutant embryos have led to a model in which Dnmt3a and Dnmt3b are responsible for establishing *de novo* methylation patterns, which are then maintained by Dnmt1 (REF. 39).

All three DNMTs, at the mRNA and/or protein level, are modestly overexpressed in many types of tumour cell[70,71]. Furthermore, modest overexpression of exogenous mouse *Dnmt1* in NIH 3T3 cells[72] or the induction of *Dnmt1* expression in these cells by the overexpression of *FOS* (v-fos FBJ mouse osteosarcoma viral oncogene homologue)[73] can promote cellular transformation. This induction of tumorigenic properties by Dnmt1 in cell culture, as well as the fact that the genetic inactivation of *Dnmt1* in mice also decreases the development of gastrointestinal tumours[74] in the *min* mouse model of gastrointestinal cancer, indicate a possible role for at least one DNMT in tumorigenesis. However, the mechanisms that underlie such a role in cancer are still not defined.

Our understanding of aberrant gene silencing in cancer has been expanded recently by studies that indicate that all three biologically active DNMTs are much more complex proteins than has been appreciated. In experimental cell-culture systems, each protein can directly repress transcription in reporter-gene systems by interacting with HDACs and by binding to other proteins with transcriptional-repression activities[75–79]. So, DNMTs could participate in gene silencing with, or without, accompanying DNA methylation, and this has potential ramifications for the abnormal methylation of CpG islands in cancer, especially with respect to the question of which comes first, gene silencing or methylation. The direct link to HDACs provides another mechanism by which they can be targeted to sites of transcriptional repression (FIGS 4,5). Interestingly, DNMT1 co-localizes with HDAC2 at replication foci during late S phase[77], at a time when newly arriving

acetylated histones must be de-acetylated during the formation of late-replicating, transcriptionally repressed and heavily methylated DNA[80].

Several recent investigations indicate that the three active DNMTs, especially in cancer cells, might cooperate, in ways that are not predicted by mouse knockout studies of the Dnmt genes, to maintain the methylated state and transcriptional silencing of genomic regions. In mouse, human and monkey cells, DNMT1 has been found to localize to DNA-replication foci throughout S phase, whereas DNMT3A and DNMT3B co-localize to these sites only during late S phase[78]. Furthermore, in mouse cells, Dnmt3a and Dnmt3b can also be found in non-dividing cells in large pericentromeric heterochromatin-like complexes that contain the mouse homologue of the important silencing protein Hp1α and also, intriguingly, the methyl-cytosine-binding proteins, MECP2 and MBD1 (REF. 78). This cell-cycle-dependent localization seems ideal to facilitate a recently defined pathway in which the error-prone methylating activities of DNMT1 are backed up by DNMT3A and DNMT3B, which might fill in missed methylation sites[81]. In cancer cells, the re-methylation of the promoter CpG island of p16[INK4A] after demethylation induced by 5-aza-2′-deoxycytidine seems to be dependent on active DNA replication, whereas other sites that are not located in CpG islands re-methylate without DNA synthesis[82]. This might indicate a previously unexpected *de novo* role for DNMT1 in methylating CpG islands in cancer cells given its tight association with S-phase activity.

Finally, two genetic changes in cancer cells, one naturally occurring and one experimentally induced, highlight the potential cooperativity between the DNMTs. First, the translocation that produces the PML–RARα (promyelocytic leukaemia–retinoic acid receptor-α) fusion protein in PML cells induces the transcriptional repression and the aberrant methylation of the promoter of a candidate RARα transcriptional target, another retinoic acid receptor gene, retinoic acid receptor-β (*RARB*). This methylation occurs concomitantly with the appearance of complexes that contain each of the DNMTs at the *RARB* promoter[83]. Second, the somatic inactivation of *DNMT1* in human colon cancer cells led to the surprising finding that, although loss of DNMT1 function reduces overall DNMT activity to only 5% that of the wild type, there is surprisingly little effect on the overall DNA methylation of the genome of the cell[84]. Furthermore, the abnormal promoter hypermethylation and the silencing of genes, such as *CDKN2A*, persists in these cells[84]. When *DNMT3B* is inactivated in colon cancer cells, both overall DNMT activity and genomic methylation change very little and abnormal promoter methylation persists[85]. However, when both *DNMT1* and *DNMT3B* are simultaneously deleted, the cells lose most of their DNMT activity and, also, 95% of total genome-wide methylation. Furthermore, promoter hypermethylation and transcriptional silencing were reversed for some genes, such as *CDKN2A* and tissue inhibitor of

metalloproteinase 3 (*TIMP3*)[85]. This finding not only highlights the potential cooperativity and complementary activities of the DNMTs, but also provides genetic evidence of the importance of promoter hypermethylation for the silencing of key tumour-suppressor genes. In fact, growth of the double-knockout cells is markedly slowed, except for one clone in which the *P16* transcript of *CDKN2A* remained hypermethylated[85].

Many of the above observations were unexpected given that knockout studies in mice have indicated that Dnmt1 might predominantly maintain DNA methylation after early embryonic development[39] and given that ES cells from *Dnmt1* knockout mice survive in culture only if kept undifferentiated[86]. How, then, could a cancer cell live without this protein and why should cooperativity between DNMTs be apparent in this setting? The explanation might be that the genetic alterations that

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Box 2 | The dynamics of genetic and epigenetic gene silencing

In cancer, the dynamics of genetically and epigenetically mediated loss of gene function are very different. A somatic genetic mutation (red cross in panel **a**) that occurs in one round of DNA replication is associated with an immediate block in the production of a functional protein from the mutant allele (see panel **a**). If a selective advantage is conferred to cells that carry this mutation, they can expand clonally to give rise to a tumour in which all cells lack the capacity to produce the functional protein.

By contrast, epigenetically mediated silencing of the same gene might begin gradually, starting in the earliest phases of tumour progression. This process might start with a subtle decrease in the level of transcription of a gene (see panel **b**), which leads to a minor decrease in protein production. This decreased transcription might then foster a decrease in protection (shown as red vertical bars) of the CpG island from the spread of flanking heterochromatin and methylation into the island (black angled arrows). This loss results in gradual increases in the methylation of individual CpG sites in the island, the extent of which might vary between copies of the same gene in different cells. In an emerging clone of tumour cells, the result of this progressive and heterogeneous methylation is an increasing degree of transcriptional loss and a variable decrease in

protein production in individual cells of the tumour clone. The degree of loss of protein production is, therefore, not uniform throughout the tumour-cell population, unlike that which is produced by the genetic changes shown in panel **a**. This situation is an ideal one for mediating the dynamic heterogeneity that characterizes important tumour properties, such as metastasis, as discussed in the text. Filled ovals, methylated CpGs; open ovals, unmethylated CpGs.

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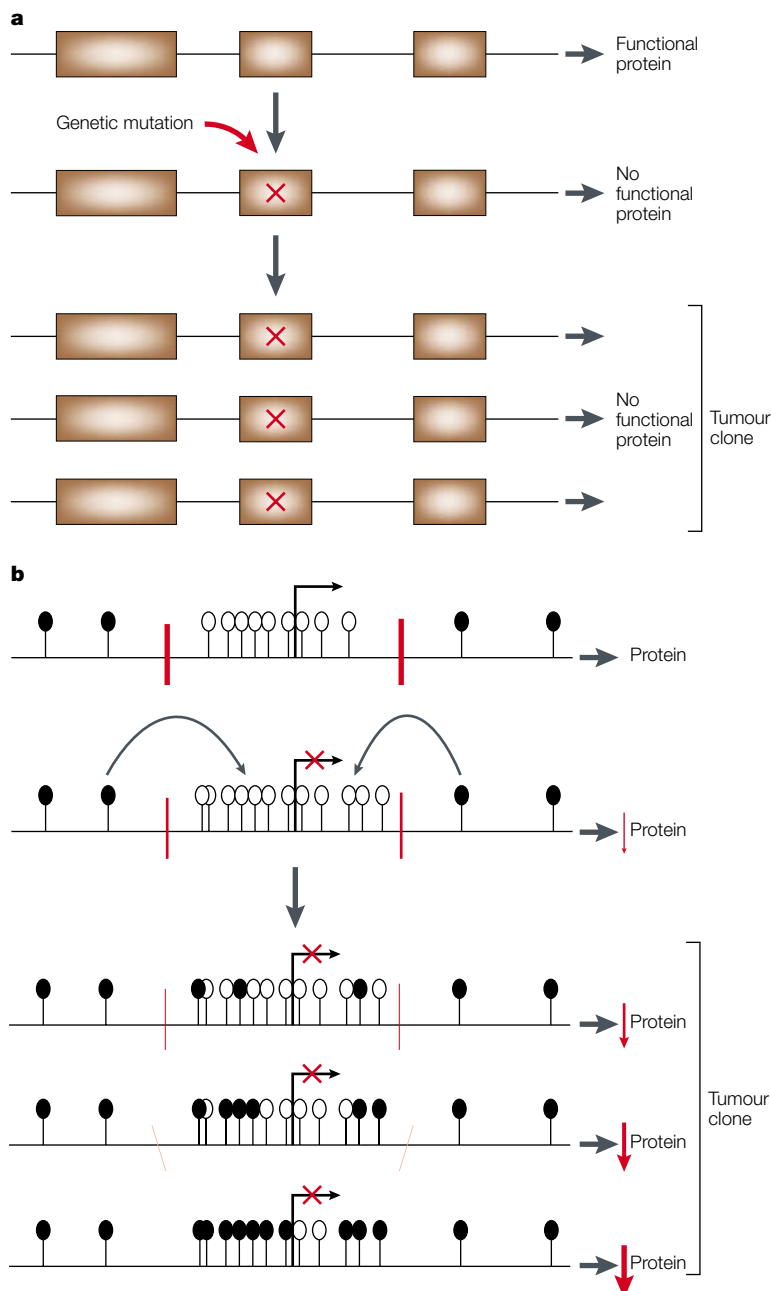
evolve during tumour progression produce such a state of interdependence between DNMTs. For example, mouse *Dnmt1*-knockout cells, which have profoundly decreased genome-wide DNA methylation levels, can live longer in culture, even in a differentiated state, when they are also null for transformation-related protein 53 (*Trp53*)[87]. Although the colon cancer cells used in the above knockout studies are wild type for *TP53*, other genetic and epigenetic abnormalities might produce a state in which DNA-methylation patterns are produced and/or maintained by mechanisms that differ from those seen in normal cells.

In summary, recent experimental data are changing our view of the molecular mechanisms that underlie the establishment and maintenance of DNA methylation. Defining the mechanisms that determine the composition and function of the complexes that link DNMTs in their role in normal and neoplastic cells, and that determine the DNA sites that they affect, are important research priorities. Finding answers to such questions will help to explain how altered chromatin organization and abnormal DNA methylation mediate aberrant gene silencing in cancer.

Spreading of aberrant methylation in cancer

Transcriptionally repressive heterochromatin can spread into adjacent regions of the genome unless 'boundary elements' are present to maintain active and silenced domains[1,88]. One explanation for the widespread *de novo* methylation of CpG islands in cancer might be that the compartmentalization of the genome into euchromatin and heterochromatin, and into unmethylated and methylated components, breaks down during carcinogenesis to allow the spread of heterochromatin to occur[89]. For some genes, such as the oestrogen receptor (*ESR1*) gene, this process seems to be initiated during the course of ageing[90]. The mechanisms that underlie the process are unknown, but they seem to reflect the vulnerability of the protective barriers to CpG-island methylation, which perhaps occurs over the course of cell renewal and seems to affect some genes more than others[91]. Although the molecular link between promoter hypermethylation and ageing awaits further clarification, it is noteworthy that ageing might be the single most important risk factor for cancer[92]; so, age-dependent promoter methylation could explain the association between cancer and ageing.

Despite what we know about how transcriptionally active versus repressive chromatin is formed, we have yet to define the mechanisms that allow normal cells to protect most of their CpG islands from methylation and gene silencing. We are beginning to recognize, however, that the loss of this protection, during tumour progression, is a time-dependent process. During this process, the extent of methylation at any given locus can change dynamically from one cell to another, from one DNA strand to another and even from one CpG site in a CpG island to another. As such, epigenetically mediated silencing might have quite different dynamics to that associated with the loss of protein function caused by genetic mutations and might, therefore, have different phenotypic consequences during tumour progression (BOX 2).

Genetic alterations in cancer can lead to immediate disruptions of protein function, the phenotypic effects of which might not be instantly detectable. However, if a subsequent cell abnormality complements and amplifies the consequences of a given gene mutation, it can provide the cell with a selective tumorigenic advantage. All subsequent clones at that stage of tumour development will harbour that mutation. By contrast, experimental data indicate that promoter CpG-island hypermethylation is a more gradual and progressive process that spreads from the lateral edges of heavily methylated DNA that flanks a CpG island and moves centrally towards the transcription start site of the gene[58,89]. The extent of this spreading in genes — such as in *CDKN2B* in leukaemia[93] and *CDH1* (which encodes E-cadherin) in breast and other tumours — even in long-established cell cultures[58], seems to vary between individual DNA strands and between cells[58,93]. As the degree of transcriptional silencing of CpG-containing promoters is usually dependent on the number of CpG sites in the island that are methylated, this methylation heterogeneity from one DNA strand, and from one cell to another, can lead to heterogeneous populations of cells in a tumour, with respect to their gene-expression levels[58] (BOX 2).

How does such cellular heterogeneity for the density of promoter methylation and its timing affect the cancer phenotype? Tumours consist of cell populations that are functionally heterogeneous for key neoplastic properties[94,95]. How promoter hypermethylation could underlie such processes is outlined in BOX 2. So, the loss of function of the key tumour-suppressor transcript, *P16*, in association with promoter hypermethylation is a cellularly heterogeneous process that can begin early in the neoplastic process, in pre-malignant lesions, and progress to involve virtually all cells in the resulting cancer[96]. The early loss of p16 by this process is probably a fundamental event in the progression of many types of cancer[97,98].

One of the most dynamic, cellularly heterogeneous processes in tumour progression is metastasis. Cells with the most metastatic properties exist as a subpopulation in a heterogeneous cell population in the primary tumour. Once these cells metastasize, they regenerate, at the distant site, the cellular heterogeneity of the metastatic subpopulation[92,99]. This type of cellular diversity is unlikely to be mediated by permanent genetic mutations. However, epigenetically mediated gene silencing is an excellent candidate to support such cellular dynamics. Indeed, the heterogeneous promoter status of a key gene that is related to cell invasion, *CDH1*, supports this idea. Loss of CDH1 function favours tumour cells that acquire the invasive properties of metastatic tumour cells[58,100,101]. As has been shown, cell–cell heterogeneity in the hypermethylation status of the promoter of this gene can underlie the cellular heterogeneity of *CDH1* expression in both primary and metastatic tumours and in cell cultures[58]. Established cultures of prostate and breast cancer cells provide an experimental model in which to study the implications of this heterogeneity for cell invasion. *In vitro* assays for cell invasion actually select for those cells with the most densely methylated

promoters and the lowest expression of *CDH1* (REF. 58). Moreover, a less densely methylated *CDH1* promoter and the re-expression of *CDH1* in these cells occurs concomitantly with their growth in cell clusters[58], an assay that mimics growth at distant metastatic sites.

Reactivating silenced genes

The finding that many genes controlling normal cellular homeostasis can be silenced inappropriately by structural chromatin changes that involve DNA methylation has encouraged a search for agents that might reverse these changes and, therefore, restore principal cellular pathways. The demethylating agent 5-azacytidine and its deoxy derivative 5-aza-2′-deoxycytidine were first synthesized in Czechoslovakia as potential chemotherapeutic agents for cancer[102]. These agents are powerful inhibitors of DNA methylation, as they are incorporated into the nucleic acids of dividing cells, where they act as mechanism-based inhibitors of DNA methyltransferases[46].
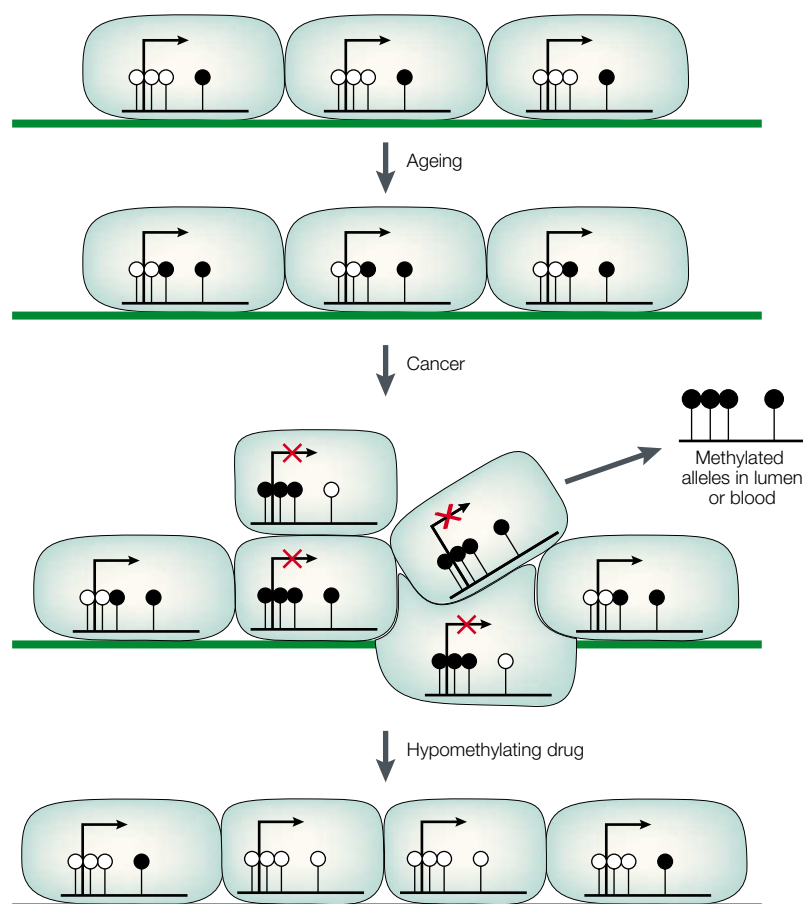
Figure 6 | **Progressive methylation changes in epithelial carcinogenesis.** CpG islands in epithelial cells are generally unmethylated but undergo some *de novo* methylation during ageing. Cancer cells often show focal CpG-island methylation, which occurs coincidently with genome-wide demethylation. This methylation can silence tumour-related genes and have a causal role in carcinogenesis. As tumour cells die and metastasize, methylated alleles are shed into the lumen of the organ or into the plasma, where they can be detected with high sensitivity. This offers opportunities for cancer diagnosis, detection and prognosis. Methylated alleles can be reactivated by drugs such as 5-aza-2′-deoxyazacytidine or procanamide, which leads to gene re-expression and reversion of some aspects of the transformed state. Filled circles, methylated CpGs; open circles, unmethylated CpGs.

Their uses for restoring gene function to treated cells in culture has indicated that they might also be useful for treating patients with malignant disease[103].

The use of aza-nucleoside analogues to treat human diseases is complicated by the fact that the drugs are unstable in aqueous solution, which requires their frequent preparation in fresh solutions for them to remain active. They are also MYELOSUPPRESSIVE, particularly when used at high doses, as is often the case in clinical trials. The efficiency with which genes in cells treated with 5-aza analogues are reactivated is not enhanced necessarily by increasing the dose of these drugs beyond their optimally effective concentration[46]. So, low-dose trials might be more efficacious. In addition, the synergy between demethylating drugs and histone deacetylating agents[66] makes clinical trials that test demethylating agents in combination with deacetylase inhibitors, in both leukaemias and solid tumours, an attractive goal. The results of such clinical investigations will be important for assessing the efficacy of re-activating epigenetically silenced genes as a future therapeutic option for treating cancer.

Although analogues that are more stable in aqueous solution might be of value, another drug that inhibits methylation is procanamide, which is used for the treatment of cardiac arrhythmias. This drug is a noncompetitive inhibitor of the methyltransferase enzymes and can reactivate the transcription of the *P16* transcript from a methylation-silenced promoter in prostate cancer cells that grow in nude mice[104]. Given the therapeutic promise of reversing methylation changes in cancer, there are certain to be more searches to identify better inhibitors of DNA methylation to use in clinical studies. As a result, several companies are pursuing this important goal.

One concern about the use of methylation inhibitors in patients is whether the drugs will cause the inappropriate activation of genes in normal cells. Although important, this might not be an insurmountable problem. This is because transcription from promoter-containing CpG islands does not seem to be commonly controlled by methylation in normal cells. In cases in which the methylation of promoter-associated CpG islands is important, such as in X-chromosome inactivation in mammals, the existence of many levels of gene silencing[1,50] means that these genes cannot be routinely and easily activated by inhibiting DNA methylation in normal cells. So, the inactive X chromosome remains inactive in normal human fibroblasts after 5-azacytidine treatment[105], whereas it can be readily reactivated in rodent–human somatic cells[106], which indicates that some higher level of control is absent in these hybrids. The issue of whether transposable elements, which are normally methylated[107], might be activated by demethylation and cause deleterious effects in normal tissue is a valid concern that requires further study. However, patients who have received 5-aza nucleosides for malignant and non-malignant diseases have generally not shown massive toxicity owing to inappropriate gene activation. Nevertheless, these concerns will need to be

addressed by future studies before these agents can be used routinely. Furthermore, demethylating drugs can activate genes that either do not have CpG islands in their promoters[108] (FIG. 4) or are unmethylated[30,109], and little is known about the spectrum of genes that might be activated in normal cells after exposure to such agents. Another impediment to the use of demethylation therapy is the tendency of methylation to spread back into the demethylated CpG island after the inhibitor is removed. This process has been shown to lead to *de novo* methylation and to the re-silencing of the target gene[110].

Detection and prognosis

The *de novo* methylation of CpG islands occurs early in the process of carcinogenesis and can even be detected in the apparently normal epithelium of patients — a process that is associated with ageing and inflammation[99]. As methylated alleles can be detected with a very high degree of sensitivity, there is great interest in using methylation as a potential early detection system for cancer (FIG. 6). Hypermethylated CpG islands have been detected in cancer patients, in DNA derived from sputum, serum and urine samples (in which the DNA is released presumably after apoptosis or necrosis of the cancer). The results of some of these studies indicate that this approach might offer some promise for early diagnosis and/or risk assessment[111]. However, whether these technologies are of practical use has not yet been shown and might be compromised by the fact that some methylation changes occur in apparently normal epithelial cells. As such, they might indicate a risk for cancer rather than show the presence of cancer in an individual. There also remains the problem of knowing what type of cancer might be releasing methylated DNA into the circulation, where it can be detected against a background of normally methylated lymphocyte DNAs and other DNAs. Nevertheless, although some methylation changes seem to be common to many kinds of cancer, there are clearly tissue-specific differences that exist. For example, the *CDKN2B* gene promoter is frequently methylated in lymphoid, but not in solid, cancers[6], and the glutathione S-transferase (*GSTP1*) gene is methylated in prostate, breast and hepatic cancers, but rarely in others[104]. The use of small panels of hypermethylation markers that almost cover the genomes of individual tumour types has been shown[30,112,113]. These might lead to the development of one of the most powerful molecular strategies for cancer detection.

Conclusions

The contribution of epigenetic silencing to the inactivation of key pathways that are involved in carcinogenesis is only now being realized by the scientific and medical communities. Despite what we know, there are undoubtedly more components in these pathways that are subject to this kind of inactivation. Many of these will be uncovered by the application of candidate-gene approaches and genome-wide screens. Further developments using array-based technologies coupled to informatic approaches are likely to speed the identification of new genes that are silenced by hypermethylation in cancer and to classify the known genes into biologically and clinically relevant hierarchies.

There is tremendous ferment in the fields of chromatin structure and DNA methylation that is just beginning to link the 'histone code' to the 'cytosine methylation code'. It seems almost certain that the methylation of H3 Lys9 and the DNMTs are inexorably linked in mammals. This means that we shall soon begin to understand how cytosine-methylation patterns are established. Knowing this is essential to deciphering what happens during ageing and carcinogenesis to reshuffle the epigenetic deck. The existence of altered cytosine-methylation patterns that are tissue and tumour specific, and that can be readily detected by sensitive PCR methods, holds considerable promise for the detection and diagnosis of cancer and for providing better prognostic information. Unlike mutagenic events, epigenetic events in cancer can also be reversed to restore the function of key control pathways in malignant and premalignant cells.

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