TIMELINE

The history of cancer epigenetics

Andrew P. Feinberg and Benjamin Tycko

Since its discovery in 1983, the epigenetics of human cancer has been in the shadows of human cancer genetics. But this area has become increasingly visible with a growing understanding of specific epigenetic mechanisms and their role in cancer, including hypomethylation, hypermethylation, loss of imprinting and chromatin modification. This timeline traces the field from its conception to the present day. It also addresses the genetic basis of epigenetic changes — an emerging area that promises to unite cancer genetics and epigenetics, and might serve as a model for understanding the epigenetic basis of human disease more generally.

Epigenetic inheritance is defined as cellular information, other than the DNA sequence itself, that is heritable during cell division. There are three main, inter-related types of epigenetic inheritance: DNA methylation, genomic imprinting and histone modification (BOX 1). Epigenetic inheritance accounts for unusual phenomena such as position-effect variegation in flies, telomere and mating-type silencing in yeast, and transgene-induced gene silencing in plants and animals. However, it has become increasingly apparent that epigenetic inheritance is important in many physiological and pathophysiological conditions. It is key to our understanding of the differences between growing, senescent and immortal cells, tumour and normal cells, various differentiated cells, and ageing cells. Epigenetic templates that control gene expression are transmitted to daughter cells independently of the DNA sequence. These metastable patterns can sometimes become abnormal during fetal development, thereby predisposing to paediatric cancers, and they can change during normal ageing and contribute to common cancer risk in adults. They can also support clonal evolution in human cancers, contributing to tumour progression.

But how was this key role for epigenetics in cancer development discovered, how has it come to rival genetics and what else do we need to know?

Hypomethylation and gene activation

Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells. At a symposium at Johns Hopkins in 1982 on tumour-cell heterogeneity, Andy Feinberg and Bert Vogelstein wondered what mechanism accounted for high-frequency 'mutations', adaptation to tumour microenvironment and plasticity in some cancers. The conference was organized by Donald Coffey, who had introduced the two investigators. At the time, many groups were excited by observations that DNA methylation might be linked to tissue-specific gene silencing, so Feinberg and Vogelstein searched for differences between cancers and normal tissues. They used Southern blotting to analyse DNA that had been digested with methylation-sensitive restriction enzymes and found that a substantial proportion of CpGs that were methylated in normal tissues were unmethylated in cancer cells[1]. Ehrlich and colleagues then carried out similar investigations using high-performance liquid chromatography to show that the 5-methylcytosine content was globally reduced[2] (see TIMELINE). The loss of methylation involved every tumour type studied, both benign and malignant; furthermore, pre-malignant adenomas also universally had altered DNA methylation[3,4].

Hypomethylation of DNA has mechanistic implications. First, it can lead to gene activation. It has been found recently that many CpG islands are normally methylated in somatic tissues[5]. These methylated islands can become hypomethylated in cancer and nearby genes become activated. Examples of genes that are affected by hypomethylation include oncogenes such as *HRAS*[6] and the 'CT' genes — those that are expressed normally in the testis and aberrantly in tumours. Their hypomethylation leads, for example, to *MAGE* expression in melanoma — a promising target of immunotherapy[7]. The related cancer/testis antigen CAGE was also shown to be activated by hypomethylation, which was confirmed using 5-aza-2′-deoxycytidine (5-azaCdR), an inhibitor of DNA methylation, as well as promoter reporter transfection experiments; hypomethylation of CAGE was found to precede the development of stomach and liver cancer at high frequency[8]. Although hypomethylation was the originally identified epigenetic change in cancer, it was overlooked in preference of hypermethylation for many years and is only now undergoing a renaissance. This is, in part, because of previous bias in experimental design; if one looks for altered methylation only at sites that are normally unmethylated, then one will only observe hypermethylation. The frequency of hypomethylated sites might be quite high, as indicated by high-throughput genomic-methylation analysis of tumours[9,10], including cancers of the stomach, kidney, colon, pancreas, liver, uterus, lung and cervix[10–18]. Strong support for hypomethylation leading to activation of genes that are important in cancer includes promoter CpG demethylation in the overexpression of *cyclin D2*[11] and *maspin* in gastric carcinoma[12], *MN/CA9* overexpression in human renal-cell carcinoma[13], *S100A4* metastasis-associated gene in colon cancer[14] and human papillomavirus 16 (*HPV16*) expression in cervical cancer[15,16]. Extensive

Box 1 | The three main types of epigenetic information

Cytosine DNA methylation is a covalent modification of DNA, in which a methyl group is transferred from *S*-adenosylmethionine to the C-5 position of cytosine by a family of cytosine (DNA-5)-methyltransferases. DNA methylation occurs almost exclusively at CpG nucleotides and has an important contributing role in the regulation of gene expression and the silencing of repeat elements in the genome.

Genomic imprinting is parent-of-origin-specific allele silencing, or relative silencing of one parental allele compared with the other parental allele. It is maintained, in part, by differentially methylated regions within or near imprinted genes, and it is normally reprogrammed in the germline.

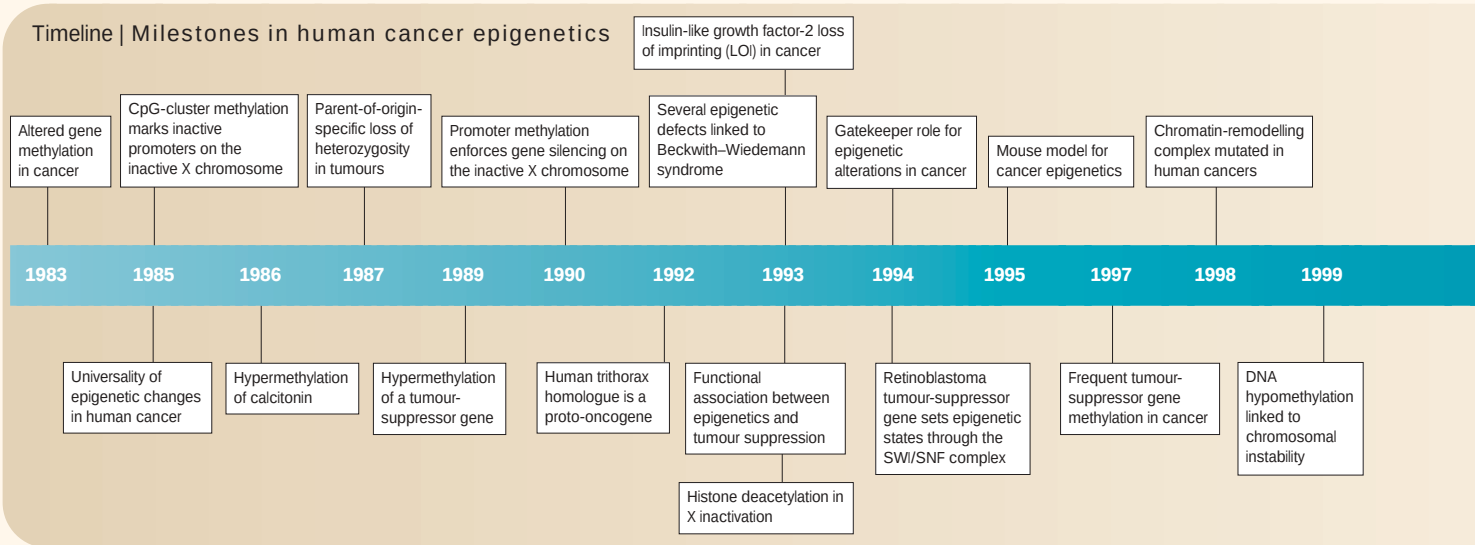
Histone modifications — including acetylation, methylation and phosphorylation — are important in transcriptional regulation and many are stably maintained during cell division, although the mechanism for this epigenetic inheritance is not yet well understood. Proteins that mediate these modifications are often associated within the same complexes as those that regulate DNA methylation.

recent studies of pancreatic cancer by Goggins and colleagues showed widespread hypomethylation associated with proliferation-linked genes, including *14-3-3σ*[10,17].

Second, a cellular 'methylator phenotype' has been linked to mismatch repair, first by Lengauer and colleagues, who showed that cancer cells that are deficient in DNA mismatch repair silenced retroviral construct promoters by DNA methylation[19]. This observation was challenged by Jones and colleagues[20] and the methylator phenotype concept (about which more below) will probably be debated until its genetic basis is elucidated. Nevertheless, the idea makes sense, as hypermethylation of the mismatch-repair gene *MLH1* is commonly found in mismatch-repair-defective tumours, as first described by Kolodner's group[21]. In addition, abnormal imprinting (discussed below) is also more commonly found in mismatch-repair-defective colorectal cancers[22].

Third, Ehrlich and colleagues recently linked tumour hypomethylation in cancer to chromosomal instability. Hypomethylation is particularly severe in pericentromeric satellite sequences, and several cancers (Wilms tumour, ovarian and breast carcinomas) frequently contain unbalanced chromosomal translocations with breakpoints in the pericentromeric DNA of chromosomes 1 and 16 (REF. 23). These rearrangements are specific, and not due to global genomic instability — in Wilms tumours, t(1;16) translocations are sometimes the only detectable abnormality. These unbalanced translocations produce loss of heterozygosity (LOH) for markers on chromosome 16, which, in turn, strongly correlates with tumour anaplasia[24]. Demethylation of satellite sequences might predispose to their breakage and recombination. The presumed causal relationship in these cancers has not been proven, but a developmental disorder — ICF syndrome (immunodeficiency, chromosomal instability and facial anomalies) — was found by several investigators to be caused by loss-of-function mutations in the cytosine DNA methyltransferase *DNMT3B*[25–27]. ICF syndrome has as its cardinal features loss of methylation in classical satellite DNA and mitogen-inducible formation of bizarre multiradial chromosomes that contain arms from chromosomes 1 and 16 (REF. 26). Indeed, this is why DNMT3B was successfully scrutinized as a candidate gene for the disorder by Viegas-Pequignot and Bestor, Gartler, Li and others. Nevertheless, ICF syndrome does not lead to cancer (discussed below). An even more direct link between hypomethylation and chromosomal instability was made by Jaenisch's group, who found that neurofibromatosis 1 (*Nf1*)$^{+/-}$*Trp53*$^{+/-}$ mice showed a 2.2-fold increase in frequency of LOH when a hypomorphic *Dnmt1* allele was introduced[28]. Another potential connection between hypomethylation and chromosomal instability is the hypomethylation of L1 retrotransposons in colorectal cancer, which might promote chromosomal rearrangement[29].

Fourth, hypomethylation is a mechanism of drug, toxin and viral effects in cancer. In addition to gene amplification, hypomethylation of the multidrug-resistance gene *MDR1* correlates with increased expression and drug resistance in acute myelogenous leukaemia[30]. Toxic carcinogens might also act through methylation alterations. For example, cadmium inhibits DNA methyltransferase activity and leads to acute hypomethylation, which is followed by hypermethylation of DNA after chronic exposure to this 'epigenetic carcinogen'[31]. Similarly, arsenic induces *Ras* hypomethylation in mice[32]. Finally,

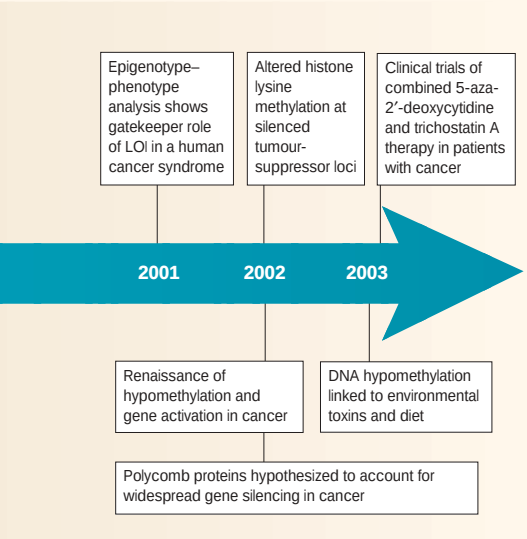


cervical cancer latency seems to be caused, in part, by hypermethylation of the HPV16 genome, and latent Epstein–Barr virus in human lymphoma cells uses a similar strategy to enforce silencing of a subset of its genes[16,33]. In cervical cancer, activation of the HPV genome and progression occur with progressive hypomethylation of the virus in precursor lesions.

An exciting recent development in cancer hypomethylation involves a link to diet. A common polymorphism of methylenetetrahydrofolate reductase (*MTHFR*), which is involved in biosynthesis of the methylation precursor *S*-adenosylmethionine, was associated with increased colorectal cancer prevalence in a population-based study, and cancer incidence was lower in patients with high dietary methionine, which increases methylation content[34]. Reduced MTHFR was also linked to alcohol consumption[34,35], and colonic hypomethylation was found in patients with colorectal cancer[36]. These results are consistent with studies in rodents showing that choline- or choline- and methionine-deficient diets lead to hepatocellular carcinoma, without any added carcinogen, first shown by Poirier[37] and confirmed by many groups.

The mechanism behind global hypomethylation in cancers remains unknown, but an indirect link was indicated by two different discoveries that might turn out to be connected. First, ATP-dependent DNA helicases of the SNF2 family — the catalytic components of SWI/SNF chromatin-remodelling complexes — are essential for maintaining normal DNA methylation. Individuals with the developmental disorder ATRX (α-thalassaemia, myelodysplasia) have mutations in the *ATRX* gene, which encodes a SNF2-family helicase. In ATRX cells, the ribosomal DNA repeats are hypomethylated[38]. Although ATRX is associated with a pre-malignant myelodysplasia, but not cancer *per se*, a second discovery shows that the connection of SWI/SNF complexes with human cancer is unambiguous. As uncovered by Delattre and colleagues in 1998, germline and somatic mutations in *SNF5* (also known as *INI1*) — which encodes a SWI/SNF complex component — cause rare, but lethal, cancer — malignant rhabdoid tumour[39]. It is not yet known if, as might be predicted, this class of neoplasm has more extensive global demethylation than most cancers. The more general question, whether SWI/SNF function is altered in more common types of cancers, is also unanswered, but results from research on the retinoblastoma (*RB*) tumour suppressor (see below) indicate that this is indeed the case.

A second chromatin protein that has been linked to hypomethylation and cancer was recently identified by Muegge and colleagues, who found that Lsh, a SNF2-family member, is required for maintenance of normal methylation. Gene knockout leads to a global defect in genomic methylation, as well as a severe proliferative defect and chromosomal instability[40]. Further support for a link between hypomethylation and tumorigenesis was provided by Hirohashi's group, who identified a common splice variant of *DNMT3B* in patients with liver cancer, which is associated with hypomethylation and causes hypomethylation of pericentromeric satellite sequences when transfected into cells[41].



Hypermethylation and gene silencing

Of course, hypomethylation is not the only way in which methylation can contribute to cancer. Steve Baylin and Barry Nelkin, in conversations at Johns Hopkins with Feinberg and Vogelstein, decided to examine calcitonin, which is a marker of small-cell lung cancer and was their main interest at the time. In 1986, they were surprised to find site-specific hypermethylation of calcitonin, with relative silencing of calcitonin expression[42]. However, calcitonin is not a tumour-suppressor gene, and the first link between hypermethylation and tumour-suppressor genes was made, fittingly enough, on the first known tumour-suppressor gene — the retinoblastoma gene *RB*. This gene might not come to mind as a locus that is frequently inactivated by the epigenetic pathway, but, in fact, the *RB* promoter is methylated in a significant subset of sporadic and even hereditary retinoblastomas. The papers reporting this phenomenon, published by the Dryja and Horsthemke laboratories, were the first to indicate that tumour-suppressor silencing might occur by an epigenetic pathway[43,44]. In the Horsthemke study in 1989, hypermethylation was specifically linked to *RB*, which led his group to suggest it might have a direct role in tumour-suppressor gene silencing[43]. In 1991, Dryja's group showed that the hypermethylation was confined to one allele, again indicating specificity. He argued explicitly that it leads to gene silencing[44]. Direct confirmation of epigenetic silencing of a tumour-suppressor gene was provided by Sakai's group in 1993, who showed a 92% reduction of *RB* expression in tumours with promoter hypermethylation[45] and by Horsthemke's group in 1994 (REF. 46).

Two years later, beginning in 1995, several groups, including the Baylin, Jones and Sidransky laboratories, confirmed promoter hypermethylation at numerous other loci in cancer cells, supporting the principle of epigenetic gene inactivation in cancer. Key tumour-suppressor proteins — including the INK4A (also known as p16; encoded by *CDKN2A*) cyclin-dependent kinase inhibitor, the mismatch-repair enzyme MLH1, the von Hippel–Lindau (VHL) tumour suppressor and E-cadherin — were all shown to be eliminated both in cell lines and in primary cancers by an epigenetic pathway that correlates with dense CpG methylation of their gene promoters. The primary publications that describe these correlations for the *CDKN2A* and *VHL* genes appeared between 1994 and 1995 (REFS 47–50) and were extended to include *MLH1* between 1997 and 1998 (REFS 21,51,52). It is important to note that the basic relationship between CpG-island methylation and gene inactivation, and the identification of CpG islands themselves, came from early studies of X chromosome inactivation (BOX 2).

After leaving the Baylin laboratory, J.-P. Issa (and colleagues) then showed methylation profiling data that indicated a dichotomous classification of human carcinomas into frequent promoter methylation and infrequent methylation groups, and led him to promote the idea of a CpG-island methylator phenotype — termed 'CIMP' — in human cancer[53]. This attractive, but still controversial, concept has stimulated many follow-up studies and, as we discuss below, there are now several candidate biochemical mechanisms that could conceivably account for CIMP. The first functional report showing a relationship between tumour-suppressor activity and DNA methylation was performed by West and Barrett in 1993, in which they examined a model of progressive loss of tumour-suppressor activity in Syrian hamster cells[54].

Box 2 | An X connection

In the background of the discoveries that link CpG-island methylation and gene inactivation is a large body of important correlative and mechanistic data that are related to X chromosome inactivation. For example, an early clue to a role in gene silencing came from studies of Mohandas *et al*., who showed in 1981 that 5-aza-2′-deoxycytidine (5-azaCdR), an inhibitor of DNA methylation[176] could reactivate the inactive X chromosome[176]. The discovery of the functional significance of what are now termed CpG islands also came from studies of the X chromosome. Wolf, Migeon and colleagues showed in 1984 that clusters of CpG dinucleotides are specifically methylated on the inactive X chromosome and reactivated with 5-azaCdR[177]. These were later extensively characterized and termed CpG islands by Adrian Bird and colleagues, who found that they were common in the promoters of autosomal genes[178]. Studies from the Gartler laboratory showed that gene reactivation on the inactive X chromosome is associated with large regions of promoter demethylation after 5-azaCdR treatment, indicating a causal relationship between methylation and gene silencing on the inactive X chromosome[179]. Incidentally, a peculiarity of the DNA methylation literature is the term CpG: what else connects the two sugars but a phosphate? After all, we don't say 'TpApTpA box'!

The connection between epigenetic gene silencing and chromatin modifications, another theme that is increasingly important in the progress of cancer epigenetics, was also highlighted early on in studies of X inactivation — with memorable images of 45 human chromosomes intensely stained by an antibody specific for acetylated histone H4, with the lone inactive X chromosome globally deacetylated and unstained[180].

That DNA methylation is causal in maintaining the silent epigenetic state has been shown by the potency of demethylating drugs in reactivating gene expression and by recent studies that use somatic-cell knockout procedures[55], or antisense and RNA interference[56], to eliminate DNA methyltransferases from cancer cells. These manipulations resulted in tumour-suppressor gene reactivation, but the details differed depending on the experimental system: acute elimination of *DNMT1* in HCT116 cells by antisense or RNA interference was sufficient to reactivate *CDKN2A*, whereas in this same cell line, a double somatic knockout of both *DNMT1* and *DNMT3B* was required to demethylate and reactivate this gene. A possible explanation is that *DNMT3B* can ultimately replace the function of *DNMT1* during cell selection, as occurs in knockout experiments, but *DNMT3B* does not completely substitute for *DNMT1* acutely.

What has not been as clear is that the mechanism of initial silencing of these genes is hypermethylation, and this has been the subject of some debate: see, for example, Bestor[57]. Indeed, Modrich and colleagues showed that activation of *MHL1* by 5-azaCdR is rapidly reversed spontaneously[52]. So, methylation changes could arise secondarily to other epigenetic changes, such as chromatin modification, but then help to maintain the silenced state. Consistent with this idea, although tumour-suppressor gene silencing *per se* can be a dominant trait in somatic-cell genetic experiments[58], a *trans*-acting defect in methylation has not been demonstrated in tumour cells and the same is as true for hypomethylation as for hypermethylation. Indeed, a significant challenge to the causal role of hypermethylation in initiating the process of gene silencing comes from a recent report showing that methylation of histone H3 lysine 9 — that is, chromatin modification — occurred in conjunction with re-silencing of *CDKN2A* in the absence of DNA methylation, in cells in which *CDKN2A* had previously been activated by DNA methyltransferase knockout[59]. Consistent with this observation, Clark and Melki also point out cogently that *CDKN2A* is silenced in proliferating colonies of mammary epithelial cells that escape senescence, even in the absence of promoter methylation, suggesting that hypermethylation is not responsible for silencing, but helps to maintain silencing[60].

Clearly, one must look at methylation in cancer as an example of epigenetic dysregulation, with both hypomethylation and hypermethylation having significant roles. Nicely summarizing this situation, a recent study of Wilms tumours found different unique-gene loci that were affected by hypomethylation or hypermethylation in the same tumour[61]. Moreover, the final epigenetic programme varies strongly by tumour type; an interesting example is a 'counterintuitive' report of *CDKN2A* hypomethylation in some breast cancers compared with normal breast tissue[62].

Loss of imprinting in cancer

Imprinting — which refers to conditioning of the maternal and paternal genomes during gametogenesis, such that a specific parental allele is more abundantly (or exclusively) expressed in the offspring — was discovered in embryological studies published in the mid-1980s[63,64]. But even earlier, cancer cytogeneticists studying two human neoplasms, hydatidiform moles and ovarian teratomas, had produced data that presaged these findings. Moles — which are placenta-derived (that is, extraembryonic) tumours — were found to contain two complete sets of paternal chromosomes with no maternal contribution[65], whereas findings in both mice and humans indicated that ovarian teratomas, which contain many tissue types, but never placental trophoblast, carried a bi-maternal chromosome complement[66].

Evidence for a role for human imprinted genes in development then came from two neurodevelopmental disorders, Prader–Willi syndrome (PWS) and Angelman syndrome (AS). These were found to be caused by uniparental chromosomal disomies of the long arm of chromosome 15, which were maternal in PWS and paternal in AS. More pertinent to this timeline, in the late 1980s, several independent studies reported a strong (in fact, absolute) parent-of-origin bias in LOH for chromosome 11p15 alleles in Wilms tumours and embryonal rhabdomyosarcomas, with invariable loss of maternal and duplication of paternal alleles[67–70]. This striking finding was hard to explain without postulating a role for imprinted gene(s) in these tumours, as was suggested first by Sapienza and colleagues[69] in 1989. An important clue was also provided by studies of the disorder Beckwith–Wiedemann syndrome (BWS), which causes prenatal overgrowth, birth defects (including a large tongue, ear creases and abdominal-wall closure defects) and predisposition to various embryonal tumours of childhood including Wilms tumour. Rare familial cases of BWS also indicated a parent-of-origin effect, as the overgrowth phenotype was only seen after maternal transmission[71]. More direct evidence came from mapping of BWS to 11p15 (REFS 72,73), followed by observations from Mannens *et al*. that chromosomal rearrangements in 11p15 in patients with BWS were all of maternal origin[74].

The discovery of *bona fide* human imprinted genes was made independently in the early 1990s by Tycko, Ohlsson, Feinberg Polychronakos and Reeve (and colleagues)[75–79], following from mouse studies[80–82] of these same genes — *IGF2* and *H19*. Additional imprinted human genes were uncovered in the PWS/AS region of chromosome 15 (REFS 83,84). Studies of the mechanism of imprinting were in full swing by the mid-1990s, with some landmarks being the direct demonstration of a

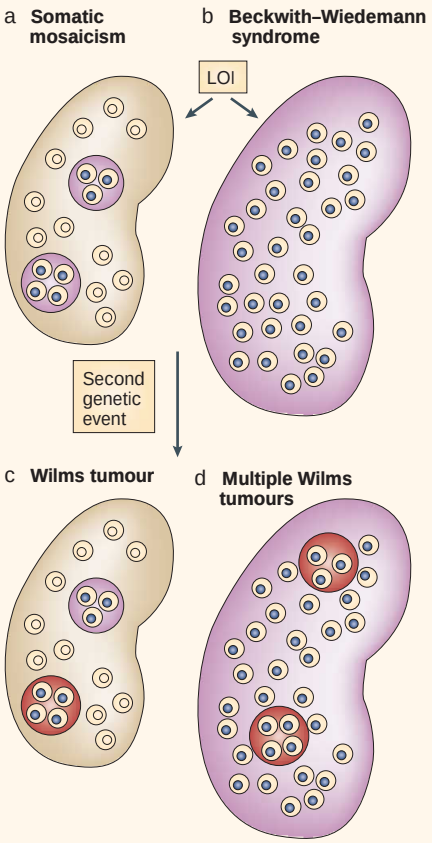


Figure 1 | **Gatekeeper role for loss of imprinting of *IGF2* in Wilms tumour. a** | Loss of imprinting (LOI, dark nuclei) has been shown to arise sporadically as a somatic mosaic epigenetic alteration, because LOI has been found in parenchymal kidney tissue of patients with Wilms tumour, as well as in pre-malignant nephrogenic rests (pink circles). **b** | Alternatively, LOI can arise in the germline or very early in development in Beckwith–Wiedemann syndrome, causing nephromegaly (overgrowth of the whole kidney). In both cases, overgrowth is caused by a double dose of *IGF2* expression and possibly silencing of *H19*. **c** | A second, presumably genetic, event can then lead to Wilms-tumour formation (red circles). **d** | This will be more common in those with Beckwith–Wiedemann syndrome, so multiple tumours arise.

role for DNA methylation in maintaining allele-specific gene expression[85] and the discovery of species-conserved imprinted chromosomal domains, containing several imprinted genes, found by chromosomal-walking experiments done for the chromosome 11p15 BWS region by the Feinberg and Tycko groups, as well as others[86–89].

Loss of imprinting (LOI), leading to pathological biallelic expression of *IGF2* (REFS 78,79) in Wilms tumours, was discovered by the Feinberg and Reeve laboratories in 1993, and in 1994 the Feinberg and Tycko laboratories showed that this abnormality in embryonal tumours is invariably linked to a gain of DNA methylation that is localized to the 5′ sequences and transcribed region of the closely linked and reciprocally imprinted *H19* gene, which is thereby transcriptionally silenced[90,91]. The presence of *H19* hypermethylation (a somatic gain of an epigenetic mark on the previously expressed maternal allele) was found not only in the tumour DNA, but also in the non-neoplastic kidney parenchyma surrounding some of the tumours[90]. These observations were the first to indicate a gatekeeper role for epigenetic alterations in cancer, as they are the earliest observable genetic change (FIG. 1). In 1997, mosaicism for *H19* hypermethylation in patients with Wilms tumour was confirmed in an independent series of cases[92]. The net effect of this epigenetic lesion is silencing of *H19*, a gene that encodes an abundant spliced but non-translated RNA, and a reciprocal increase in expression of *IGF2* (REFS 90,91). The roughly twofold increase in effective *IGF2* gene dosage is now considered the most likely explanation for the associated tumour susceptibility, although *H19* RNA is growth suppressive in some cancer cell lines[93]. The mechanism by which IGF2 promotes tumour formation might be by inhibiting apoptosis, as Hanahan and colleagues found that knockout of one allele (preventing biallelic progression) arrests tumour progression in an activated T-antigen transgenic tumour model[94], and this arrested progression involves increased apoptosis[95].

Epidemiological support for the gatekeeper role of altered methylation and LOI in Wilms tumour came from the discovery, in 2000, that Knudson's hypothesis did not explain the bimodal age distribution of most Wilms tumours. Tumours that are 'late arising', that is, not in infancy, were found to involve epigenetic rather than genetic alterations, and early-arising tumours had classical genetic changes involving Wilms tumour 1 (*WT1*) and LOH[96]. Definitive clinical evidence for a gatekeeper function of the gain of DNA methylation upstream of *H19* and LOI of *IGF2* in Wilms tumours has since come from studies of tumour susceptibility in the pre-neoplastic disorder BWS (see below).

Indicating generality of a role for imprinted genes in cancer, a recent publication reported selective loss of paternal alleles (6/6 cases of LOH) on chromosome 19q in oligodendrogliomas[97]. As indicated by data from the Oshimura laboratory[98], the putative tumour-suppressor gene identified by these data might be *PEG3* — a paternally expressed imprinted gene that maps to band 19q13.4, or perhaps another nearby imprinted gene, as *PEG3* might lie outside the region of LOH[99]. These results are provocative, as they seem to go against the 'paternal allele active→growth promoter' paradigm. In fact, data from *Peg3*-knockout mice[100] indicate the expected growth-promoting function of this paternally expressed gene (the knockout mice are small); but human *PEG3* has tumour-suppressor activity in transfected cells[101] and the gene is epigenetically silenced in gliomas[102]. Selective loss of alleles has also been reported in neuroblastomas[103], but in these tumours the parent-of-origin dependence is by no means absolute and an unequivocally imprinted locus on chromosome 1 has not materialized. Recently, Morison, Reeve and colleagues have found selective loss of maternal alleles on chromosome 9p in childhood acute lymphoblastic leukaemias[104]. There are some data to indicate that LOI can also lead to tumour-suppressor gene silencing; for example, *ARHI* — a candidate breast tumour gene that was found by the Yu laboratory — shows aberrant allele-specific silencing[105]. In addition, *LIT1* — an untranslated RNA found by the Feinberg, Oshimura and Higgins groups to undergo LOI in about half of patients with BWS[106,107] — might cause downregulation of *CDKN1C* (which encodes KIP2, also known as p57).

Chromatin and methylation

The third epigenetic mechanism — histone modification — has been the last to be linked to cancer research. A link between chromatin and DNA methylation, however, dates back to the 1980s, in the elegant observation by the Cedar and Graessmann laboratories that naked DNA templates, pre-methylated *in vitro* and then transfected or microinjected into cells, only became transcriptionally silenced after packaging into a repressive form of chromatin[108,109]. Proteins that bind to methylated CpGs were soon identified by Adrian Bird's group[110,111], and work from that laboratory, along with Steve Baylin's and Alan Wolfe's groups, showed that these proteins (MECP2 and MBD2), as well as DNA methyltransferases themselves (DNMT1, DNMT3A and DNMT3B), physically associate with histone deacetylases[112–115]. DNMT1 maintains patterns of methylation during replication, and DNMT3A and DNMT3B can add methylation to previously unmethylated templates. A recurring theme of DNA methylation is that its machinery co-opts a more fundamental system of chromatin modification. For example, whereas DNMTs associate with chromatin proteins in mammals, the *Drosophila* orthologues of methyl-binding proteins Mbd2 and Mbd3 do not bind methyl-C, but still show conserved

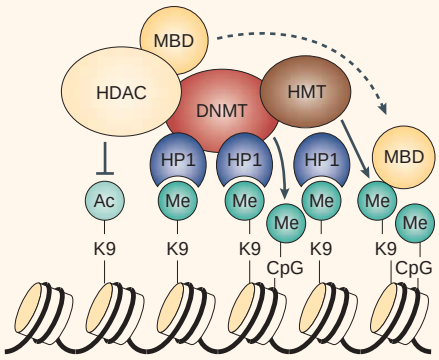


Figure 2 | **Co-operative and self-reinforcing organization of the chromatin and DNA-modifying machinery responsible for gene silencing in normal and malignant cells.** Histone (H3) modifications include lysine (K) acetylation (Ac) and lysine methylation (Me). Lysines at other positions are also modified. The HP1 protein recognizes MeK9 and, as this protein also binds the histone methyltransferase (HMT), heterochromatin can spread. Histone deacetylases (HDAC) deacetylate lysine residues as a prerequisite for their subsequent methylation. DNA methyltransferases (DNMT) participate in multiprotein complexes that contain HDACs and HMTs, and methyl-C binding proteins (MBD) can be loaded onto methylated DNA through their interactions with both HDACs and HMTs. Much of the evidence comes from studies of constitutive heterochromatin, but recent studies indicate similar interactions of genes silenced *de novo* in cancer cells.

transcriptional repressor function/histone deacetylase association[116].

Methylation at lysine residues in histones has been known for many years, but this modification was only recently recognized by Jenuwein and colleagues and Allis and colleagues as crucially important for normal gene regulation[117,118]. Histone methylation is a parsimonious explanation for the perpetuation of silent epigenetic states through cell divisions. The silent state can be maintained by a cycle of histone methylation, which is catalysed by the SUV39H1 histone methyltransferase — an orthologue of a *Drosophila* protein that is involved in suppression of position-effect variegation — followed by recruitment of the binding protein heterochromatin protein-1 (HP1) to the lysine-9-methylated histone, which perpetuates the cycle by recruiting SUV39H1 (FIG. 2). This work was influenced by the knowledge that position-effect variegation is mediated by heterochromatin formation. Indeed, Jones has found that methylation of lysine 9 in histone H3 correlates with silencing of the *CDKN2A* tumour suppressor in cancer cells[119]. Moreover, Vogelstein and colleagues found that when a DNA-methyltransferase-null cell line was followed over prolonged passage in tissue culture, lysine 9 methylation accompanied the cytosine methylation-independent re-silencing of demethylated *CDKN2A* alleles[59]. The histone methylation/ HP1-binding cycle, which is present in organisms as diverse as yeast, *Drosophila* and humans, is an ancient mechanism for propagating epigenetic states, whereas the analogous CpG methylation/histone-deacetylase-binding cycle is evidently a later addition. This fail-safe mechanism, which stabilizes silent chromatin in mammals, keeps parasitic retroelements repressed and might have co-evolved with these invasive sequences[120].

Another very recent advance that might prove relevant comes from observations by Henikoff and Ahmad that histones can be selectively replaced at transcriptionally active loci, in a manner that is independent of DNA replication[121]. This process entails the transcription-dependent accumulation of a highly conserved histone variant — H3.3 — which substitutes for the canonical H3 histone. Histone replacement, which presumably occurs after invasion of promoters by strongly activating transcription factors, offers an explanation for how the cell might reactivate genes that were previously silenced via histone methylation. This is an attractive idea, given that there are no known histone demethylases[122]. Whether histone replacement might be perturbed in cancer cells is an open question.

Very recently, an intriguing relationship between genomic imprinting, DNA methylation and chromatin has been established by the discovery and functional analysis of CTCF, an insulator protein that establishes chromatin boundaries and the binding of which is blocked by DNA methylation, shown by the Lobanenkov, Ohlsson, Felsenfeld and Tilghman laboratories[123–126]. Cui, Feinberg and colleagues have found that LOI in Wilms tumour depends on hypermethylation of CTCF binding sites[127], which reside in the DNA upstream of *H19*, but hypomethylation of *IGF2*, not apparently involving *CTCF*, seems to occur in some colorectal cancers[128], whereas de la Chapelle and colleagues have found hypermethylation of *CTCF* in other colorectal cancers[129].

A paralogue of *CTCF*, termed *BORIS*, was recently discovered by Lobanenkov, Ohlsson and colleagues to be amplified and overexpressed within the 20q amplicon in breast cancer, and its overexpression is thought to impede normal CTCF binding[130]. It is intriguing that *BORIS* is itself a cancer/testis gene and, therefore, its hypomethylation might be linked to hypermethylation at other sites. A similar mechanism has been suggested by Chinnaiyan and colleagues for *EZH2*, an orthologue of the *Drosophila* chromatin-repressor protein 'enhancer of zeste'. Increased expression of *EZH2* is linked to generalized hypermethylation and gene silencing in metastatic prostate cancer[131]. As we discuss in more detail below, another methylation and chromatin connection involves RB, which associates both with DNA methyltransferases and with the SUV39H1 histone methyltransferase and HP1, providing a mechanism for repressing cell-cycle genes, such as cyclin E.

BWS: epigenetic casualty in cancer

A key barrier to the acceptance of epigenetic alterations as a cause rather than a consequence of cancer, has been the lack, until recently, of well-defined human pre-neoplastic disorders that are caused by epigenetic mutations. There are known disorders involving genes that encode the methylation machinery of the cell (for example, *DNMT3B* in ICF syndrome and *MECP2* in Rett syndrome), but these disorders do not predispose to cancer. By contrast, many tumour-suppressor genes, when mutated in the germline, cause cancer predisposition syndromes (*TP53* in Li–Fraumeni syndrome being the original).

However, the discovery of the mechanisms of BWS provides the genetic smoking gun for at least one epigenetic mechanism in cancer, genomic imprinting. The generalized overgrowth characteristic of BWS sometimes includes kidney enlargement, and the affected kidneys can contain persistent nephrogenic blastema — the precursor of Wilms tumour. By no means do all children with BWS develop Wilms tumours, but the relative risk is 816 (REF. 132). Potentially explaining this clinical heterogeneity, in the years between 1993 and 2000, BWS was shown to have various molecular causes, including LOI of *IGF2* (REFS 90,91,133) or, alternatively, point mutations in the *CDKN1C* gene[134] or epigenetic lesions in the nearby antisense RNA *LIT1* (REFS 106,107), which, together, lie within a separate imprinted subdomain of chromosome 11p15. Furthermore, a review of BWS cases in the literature up to 1999 indicated that cancer predisposition might be specifically associated with LOI of *IGF2* and hypermethylation of *H19* (REF. 135). To test this idea, several groups, including Mannens, Weksberg, Reik and Maher, found association of cancer in BWS with hypermethylation of *H19*, although in those studies, because of patient numbers, one could not distinguish statistically an association specific to *H19* from uniparental disomy including *H19* and other genes[136–138]. In the first epigenotype–phenotype study for any disease, DeBaun and Feinberg found

that in a large registry of patients with BWS that was studied prospectively, gain of methylation at *H19*, presumably resulting in biallelic expression of *IGF2*, was specifically and statistically associated with cancer risk, whereas loss of methylation at *LIT1* was specifically associated with birth defects (macrosomia and midline abdominal-wall defects)[139]. This specificity for cancer risk indicates a gatekeeper role of LOI in BWS (FIG. 1). The mechanisms accounting for gain of methylation at *H19* or loss of methylation at *LIT1* are not known, but recent findings by DeBaun, Feinberg, Maher and others[140,141], which associate BWS with *in vitro* fertilization, together with the simple fact that the epigenetic abnormality is widespread in various somatic tissues, indicates that these epigenetic aberrations occur very early in development, before the development of malignancy. In other words, epigenetic lesions in BWS are a cause, not a consequence, of cancer.

LOI might have a causal role in common cancer as well. A recent study indicates that, although BWS is rare, epigenetic alterations affecting *IGF2* might be common in the general population and associated with more prevalent malignancies. LOI of *IGF2* was found by Cui, Cruz-Correa and Feinberg in normal lymphocytes and colonic mucosa in 10% of healthy adults, and the odds ratio for LOI was 5.15 for patients with a positive family history, and 21.7 for patients with a past history, of colorectal cancer[142]. Here too, the epigenetic abnormality is found in normal cells, and so is not an epiphenomenon of the cancer phenotype. Interestingly, the mechanism of LOI in colorectal cancer involves hypomethylation of *IGF2*, rather than hypermethylation of *H19* that is seen in embryonal tumours[128], which is consistent with the idea that cancer is linked to epigenetic disequilibrium rather than hypomethylation or hypermethylation *per se*. The timing of epigenetic lesions in cancer is a topic that cannot be overemphasized. Promoter hypermethylation might also often be an early event. Ominously, in smokers with bronchial epithelial atypia that a pathologist would classify as preneoplastic, Herman and colleagues found already substantial hypermethylation of the *CDKN2A* promoter region[143]. In an intriguing recent study, Sapienza has found familial clustering of epigenetic alterations involving *H19*, indicating that methylation might represent an epigenetic cancer-associated polymorphism in the population[144].

An alternative genetic argument can be derived from mouse models, with the caveat that mouse and human carcinogenesis might differ. Three mouse models potentially link DNA methylation to cancer. The first of these was the demonstration by Jaenisch and colleagues that a *Dnmt1* hypomorphic mutation reduces the frequency of intestinal neoplasia when crossed to *Apc*^{Min} mice[145]. These results indicate that hypomethylation might abate the risk of cancer, but more recent studies from Jaenisch and colleagues indicate just the opposite, with a high frequency of lymphomas in mice with a hypomorphic *Dnmt1* allele[146]. Together, the data indicate that a disruption in the balance of methylation is associated with cancer risk, an idea that is consistent with observations of human cancer. In a third model — heterozygous knockout of the *Hic1* gene, which is hypermethylated in human colorectal cancer — Baylin and colleagues found a modest, but significant, increase in cancer frequency of very late onset, with hypermethylation of the wild-type allele[147].

Tumour suppressors and chromatin

The counter-argument from human genetic studies is that the many tumour-suppressor genes in cancer are not modifiers of DNA methylation, even though they are involved in virtually every other potential growth or regulatory pathway. On the other hand, many tumour-suppressor genes are involved in some aspect of chromatin structure. The first of these involved the cloning by Canaani, Rowley and Cleary of the *ALL1* gene (also known as *MLL* or *HRX*) in 1991. *ALL1* — which is altered by chromosomal translocations involving band 11q23 to produce fusion genes in human leukaemias[148–150] — is the human homologue of *Drosophila trithorax*, which functions to stably maintain active expression states of homeobox genes in growing tissues. The protein encoded by *ALL1* participates in a megadalton-size multiprotein complex that has chromatin remodelling, histone acetylation/deacetylation and histone methylation activities[151].

A suggested candidate for a mechanism of methylation modification was the DNA methyltransferase *DNMT1*, which was originally reported to be overexpressed in cancer cells[152], but these studies have been controversial[153,154], and a well-controlled real-time PCR analysis did not show a significant increase[155]. An alternative hypothesis involves expression of transcriptional repressors or, equivalently, the loss of activators, as the primary event. A recent example, put forward by Pelicci and colleagues to support the first possibility, is the recruitment of DNMT1 and DNMT3A to the retinoic-acid receptor (*RARB2*) locus by binding of these methyltransferases to the oncogenic PML–RARα fusion protein in promyelocytic leukaemia[156]. This 'secondary methylation' scenario was questioned based on the finding of *RARB2* methylation in many leukaemias that lack PML–RARα[157], but these findings can be reconciled if other transcriptional repressors, present in the PML–RARα-negative cases, also recruit methyltransferases. A second type of repressor that might shut down gene promoters before DNA methylation is typified by the SLUG transcription factor, which Fearon and colleagues found binds and silences the E-cadherin promoter — a known target of *de novo* methylation — in breast cancer cell lines[158], although, to our knowledge, there have not yet been reports of methyltransferase recruitment via SLUG.

In an important line of work that was initiated by the Goff laboratory in 1994 and substantially extended by Doug Dean's laboratory, RB has been shown to function as a brake on the cell cycle at least in part by establishing and enforcing stable epigenetic silencing of its target genes. It does this by participating in a multiprotein complex that includes chromatin-remodelling enzymes of the SWI/SNF class[159], as well as histone deacetylases[160,161] and epigenetic silencing proteins of the polycomb class (genetic antagonists of trithorax-group proteins)[162]. Furthermore, based on at least two reports, the DNA methyltransferase DNMT1 also binds to RB[163,164]. Interestingly, the *Riz1* gene, which encodes a histone methyltransferase that can associate with Rb, was found by Huang and colleagues to sometimes be inactivated epigenetically and act as a tumour suppressor in mice[165]. This finding indicates a hypothetical sequence of events in which a cancer-associated epigenetic lesion (including silencing at the *Riz1* locus) exerts downstream effects that abrogate the function of a tumour-suppressor protein (Rb), and this, in turn, erases stable epigenetic silencing at the cyclin E gene and other Rb targets. Involvement of polycomb-group proteins in human cancer is not restricted to their interaction with RB: overexpression of *EZH2* in metastatic prostate cancer, already mentioned above, is another example[131].

Epigenetic chemotherapy

The demethylating drug 5-azaCdR — which inactivates methyltransferases — was first shown to transform cultured cells by Weinstein and colleagues in 1984 (REF. 166), and these studies were stimulated by the earlier finding by Taylor and Jones that 5-azaCdR has reproducible effects on cell

differentiation in tissue culture[167]. This drug is now used in some clinical situations, notably as part of combined chemotherapy regimens for myelodysplastic syndrome and leukaemias[168]. Part of its activity in patients might be due to its ability to reactivate growth suppressors, such as the INK4B (also known as p15) cyclin-dependent kinase inhibitor[168]. However, data that were obtained with *Dnmt1*-knockout cells that are resistant to 5-AzaCdR indicate that the incorporation of the drug into DNA, and the resulting formation of covalent DNA–Dnmt adducts, might contribute to its cytotoxic effects[169]. A necessity for intravenous administration has limited the usefulness of 5-AzaCdR, but an orally active inhibitor, zebularine[170], is entering clinical trials. 5-AzaCdR can also restore a normal pattern of imprinting to cells[171]. The histone deacetylase inhibitor trichostatin A is already in use and seems to have efficacy against leukaemias. Potential synergy between trichostatin A and 5-azaCdR[172] is now being tested in this clinical setting[173].

These promising therapies seek to reactivate tumour-suppressor genes that have been silenced epigenetically, and they are justified in patients with cancer in which other treatments have failed or are expected to fail. Nevertheless, a recent exchange in *Science* has highlighted the concern that genomic instability, because of hypomethylation, might be an adverse long-term consequence[174,175].

Conclusions

In the past 20 years, cancer epigenetics has come full circle, with a renaissance of interest in hypomethylation and its role in activating oncogenes and chromosomal rearrangement, as well as hypermethylation affecting tumour-suppressor genes. In the past 10 years, the discovery of imprinted genes and their role in cancer has added a new dimension to the field, and the impact of the role of chromatin modifications is just beginning to be felt. One of the most intriguing recent advances is the convergence of mechanistic studies linking DNA methylation, genomic imprinting and histone modification. Although cancer epigenetics is now considered to be well within the mainstream, there are several remaining questions that continue to limit its complete acceptance and still stimulate debate in assigning causal relationships. First, the mechanism of epigenetic inheritance other than DNA methylation is still largely unknown, yet it must be important as nonmethylated species handle epigenetic modification quite well. One could argue that the most important open question in molecular genetics is the mode of propagation of the histone code, through disassembly and reassembly during cell division, and the mechanism for this process. Cancer epigenetics will probably advance substantially when that process is better understood. Second, it is remarkable that tumour-suppressor genes are drawn from so many aspects of cell biology except DNA methylation. This indicates that the methylation changes themselves are secondary to other important causal elements, although that need not necessarily be the case. It might simply be that the known mediators of DNA methylation are factors that are essential for mammalian life, but that accessory factors for methylation propagation that are mutated are not known or that their role in methylation is not known. Third, the most compelling evidence for a causal role of epigenetic changes comes from the study of well-defined human genetic and epigenetic syndromes. The main problem is that so few familial disorders seem to involve the epigenetic machinery. One notable exception is BWS, which is caused by epigenetic defects, and those alterations are specifically linked to cancer risk in affected patients. The future might reveal population epigenetic polymorphisms that contribute to cancer risk, but that do not cause a stark definable syndrome; the recent identification of a methylation variant that is linked to colorectal cancer might be such a polymorphism.

Finally, we would argue that age is central to our understanding of cancer epigenetics, an idea that goes all the way back to Holliday's observations of methylation erosion during ageing. The single leading risk factor for cancer is age. Although that has often been attributed to the accumulation of mutations over time, an alternative and complementary interpretation is that age itself disrupts the epigenetic programme, increasing cancer risk. This relationship might be even more true for non-malignant disease than for cancer, as epigenetics might explain why most common disorders that involve complex genetics are of adult onset; after all, the genome has been there since birth, so the genetic factors are presumably there as well. We feel that substantially more attention must be paid to epigenetic variation in the population, epigenetic changes during ageing and the relationship between these changes and common diseases including cancer. For these studies, the best model organisms are humans themselves.

Andrew P. Feinberg is at the Departments of Medicine, Oncology, and Molecular Biology & Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA.

Benjamin Tycko is at the Institute for Cancer Genetics and Department of Pathology, Columbia University College of Physicians and Surgeons, New York 10032, USA.

Correspondence to A.P.F. or B.T.
e-mail: afeinberg@jhu.edu; bt12@columbia.edu
doi10.1038/nrc1279

1. Feinberg, A. P. & Vogelstein, B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* **301**, 89–92 (1983).
2. Gama-Sosa, M. A. *et al.* The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res.* **11**, 6883–6894 (1983).
3. Goelz, S. E., Vogelstein, B., Hamilton, S. R. & Feinberg, A. P. Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science* **228**, 187–190 (1985).
4. Feinberg, A. P., Gehrke, C. W., Kuo, K. C. & Ehrlich, M. Reduced genomic 5-methylcytosine content in human colonic neoplasia. *Cancer Res.* **48**, 1159–1161 (1988).
5. Strichman-Almashanu, L. Z. *et al.* A genome-wide screen for normally methylated human CpG islands that can identify novel imprinted genes. *Genome Res.* **12**, 543–554 (2002).
6. Feinberg, A. P. & Vogelstein, B. Hypomethylation of *ras* oncogenes in primary human cancers. *Biochem. Biophys. Res. Commun.* **111**, 47–54 (1983).
7. De Smet, C. *et al.* The activation of human gene *MAGE-1* in tumor cells is correlated with genome-wide demethylation. *Proc. Natl Acad. Sci. USA* **93**, 7149–7153 (1996).
8. Cho, B. *et al.* Promoter hypomethylation of a novel cancer/testis antigen gene *CAGE* is correlated with its aberrant expression and is seen in premalignant stage of gastric carcinoma. *Biochem. Biophys. Res. Commun.* **307**, 52–63 (2003)
9. Adorjan, P. *et al.* Tumour class prediction and discovery by microarray-based DNA methylation analysis. *Nucleic Acids Res.* **30**, e21 (2002).
10. Iacobuzio-Donahue, C. A. *et al.* Exploration of global gene expression patterns in pancreatic adenocarcinoma using cDNA microarrays. *Am. J. Pathol.* **162**, 1151–1162 (2003).
11. Oshimo, Y. *et al.* Promoter methylation of cyclin D2 gene in gastric carcinoma. *Int. J. Oncol.* **6**, 1663–1670 (2003).
12. Akiyama, Y., Maesawa, C., Ogasawara, S., Terashima, M. & Masuda, T. Cell-type-specific repression of the maspin gene is disrupted frequently by demethylation at the promoter region in gastric intestinal metaplasia and cancer cells. *Am. J. Pathol.* **163**, 1911–1919 (2003).
13. Cho, M. *et al.* Hypomethylation of the *MN/CA9* promoter and upregulated *MN/CA9* expression in human renal cell carcinoma. *Br. J. Cancer* **85**, 563–567 (2001).
14. Nakamura, N. & Takenaga, K. Hypomethylation of the metastasis-associated *S100A4* gene correlates with gene activation in human colon adenocarcinoma cell lines. *Clin. Exp. Metastasis* **16**, 471–479 (1998).
15. Badal, V. *et al.* CpG methylation of human papillomavirus type 16 DNA in cervical cancer cell lines and in clinical specimens: genomic hypomethylation correlates with carcinogenic progression. *J. Virol.* **77**, 6227–6234 (2003).
16. De Capoa, A. *et al.* DNA demethylation is directly related to tumour progression: evidence in normal, pre-malignant and malignant cells from uterine cervix samples. *Oncol. Rep.* **10**, 545–549 (2003).
17. Sato, N. *et al.* Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. *Cancer Res.* **63**, 4158–4166 (2003).
18. Piyathilake, C. J. *et al.* Race- and age-dependent alterations in global methylation of DNA in squamous cell carcinoma of the lung (United States). *Cancer Causes Control* **14**, 37–42 (2003).
19. Lengauer, C., Kinzler, K. W. & Vogelstein, B. DNA methylation and genetic instability in colorectal cancer cells. *Proc. Natl Acad. Sci. USA* **94**, 2545–2550 (1997).
20. Pao, M. M. *et al.* DNA methylator and mismatch repair phenotypes are not mutually exclusive in colorectal cancer cell lines. *Oncogene* **19**, 943–952 (2000).
21. Kane, M. F. *et al.* Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair- defective human tumor cell lines. *Cancer Res.* **57**, 808–811 (1997).
22. Cui, H., Horon, I. L., Ohlsson, R., Hamilton, S. R. & Feinberg, A. P. Loss of imprinting in normal tissue of colorectal cancer patients with microsatellite instability. *Nature Med.* **4**, 1276–1280 (1998).
23. Qu, G. Z., Grundy, P. E., Narayan, A. & Ehrlich, M. Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. *Cancer Genet. Cytogenet.* **109**, 34–39 (1999).

24. Yeh, A. *et al.* Chromosome arm 16q in Wilms tumors: unbalanced chromosomal translocations, loss of heterozygosity, and assessment of the *CTCF* gene. *Genes Chromosomes Cancer* **35**, 156–163 (2002).
25. Hansen, R. S. *et al.* The *DNMT3B* DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *Proc. Natl Acad. Sci. USA* **96**, 14412–14417 (1999).
26. Xu, G. L. *et al.* Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* **402**, 187–191 (1999).
27. Okano, M., Bell, D. W., Haber, D. A. & Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* **99**, 247–257 (1999).
28. Eden, A., Gaudet, F., Waghmare, A. & Jaenisch, R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* **300**, 455 (2003).
29. Suter, C. M., Martin, D. I. & Ward, R. L. Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. *Int. J. Colorectal Dis.* 8 Oct 2003 (doi: 10.1007/s00384-003-0539-3).
30. Nakayama, M. *et al.* Hypomethylation status of CpG sites at the promoter region and overexpression of the human *MDR1* gene in acute myeloid leukemias. *Blood* **92**, 4296–4307 (1998).
31. Takaguchi, M., Achanzar, W. E., Qu, W., Li, G. & Waalkes, M. P. Effects of cadmium on DNA-(Cytosine-5) methyltransferase activity and DNA methylation status during cadmium-induced cellular transformation. *Exp. Cell Res.* **286**, 355–365 (2003).
32. Okoji, R. S., Yu, R. C., Maronpot, R. R. & Froines, J. R. Sodium arsenite administration via drinking water increases genome-wide and Ha-*ras* DNA hypomethylation in methyl-deficient C57BL/6J mice. *Carcinogenesis* **23**, 777–785 (2002).
33. Li, H. & Minarovits, J. Host cell-dependent expression of latent Epstein–Barr virus genomes: regulation by DNA methylation. *Adv. Cancer Res.* **89**, 133–156 (2003).
34. Heijmans, B. T. *et al.* A common variant of the methylenetetrahydrofolate reductase gene (1p36) is associated with an increased risk of cancer. *Cancer Res.* **63**, 1249–1253 (2003).
35. Chen, J. *et al.* A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. *Cancer Res.* **56**, 4862–4864 (1996).
36. Pufulete, M. *et al.* Folate status, genomic DNA hypomethylation, and risk of colorectal adenoma and cancer: a case control study. *Gastroenterology* **124**, 1240–1248 (2003).
37. Poirier, L. A. Folate deficiency in rats bearing the Walker tumor 256 and the Novikoff hepatoma. *Cancer Res.* **33**, 2109–2113 (1973).
38. Gibbons, R. J. *et al.* Mutations in *ATRX*, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. *Nature Genet.* **24**, 368–371 (2000).
39. Versteege, I. *et al.* Truncating mutations of *hSNF5/INI1* in aggressive paediatric cancer. *Nature* **394**, 203–206 (1998).
40. Fan, T. *et al.* Lsh-deficient murine embryonal fibroblasts show reduced proliferation with signs of abnormal mitosis. *Cancer Res.* **63**, 4677–4683 (2003).
41. Saito, Y. *et al.* Overexpression of a splice variant of DNA methyltransferase 3b, *DNMT3b4*, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. *Proc. Natl Acad. Sci. USA* **99**, 10060–10065 (2002).
42. Baylin, S. B. *et al.* DNA methylation patterns of the calcitonin gene in human lung cancers and lymphomas. *Cancer Res.* **46**, 2917–2922 (1986).
43. Greger, V., Passarge, E., Hopping, W., Messmer, E. & Horsthemke, B. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum. Genet.* **83**, 155–158 (1989).
44. Sakai, T. *et al.* Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *Am. J. Hum. Genet.* **48**, 880–888 (1991).
45. Ohtani-Fujita, N. *et al.* CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. *Oncogene* **8**, 1063–1067 (1993).
46. Greger, V. *et al.* Frequency and parental origin of hypermethylated *RB1* alleles in retinoblastoma. *Hum. Genet.* **94**, 491–496 (1994).
47. Gonzalez-Zulueta, M. *et al.* Methylation of the 5′ Cpg island of the *p16/CDKN2* tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. *Cancer Res.* **55**, 4531–4535 (1995).
48. Graff, J. R. *et al.* E-Cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res.* **55**, 5195–5199 (1995).
49. Herman, J. G. *et al.* Silencing of the *VHL* tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc. Natl Acad. Sci. USA* **91**, 9700–9704 (1994).
50. Merlo, A. *et al.* 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor *p16/CDKN2/MTS1* in human cancers. *Nature Med.* **1**, 686–692 (1995).
51. Cunningham, J. M. *et al.* Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res.* **58**, 3455–3460 (1998).
52. Veigl, M. L. *et al.* Biallelic inactivation of *hMLH1* by epigenetic gene silencing, a novel mechanism causing human MSI cancers. *Proc. Natl Acad. Sci. USA* **95**, 8698–8702 (1998).
53. Toyota, M. *et al.* CpG island methylator phenotype in colorectal cancer. *Proc. Natl Acad. Sci. USA* **96**, 8681–8686 (1999).
54. West, R. W. & Barrett, J. C. Inactivation of a tumor suppressor function in immortal Syrian hamster cells by N-methyl-N′-nitro-N-nitrosoguanidine and by 5-aza-2′-deoxycytidine. *Carcinogenesis* **14**, 285–289 (1993).
55. Rhee, I. *et al.* DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* **416**, 552–556 (2002).
56. Robert, M. F. *et al.* DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nature Genet.* **33**, 61–65 (2003).
57. Bestor, T. H. Unanswered questions about the role of promoter methylation in carcinogenesis. *Ann. NY Acad. Sci.* **983**, 22–27 (2003).
58. Hajra, K. M., Ji, X. & Fearon, E. R. Extinction of E-cadherin expression in breast cancer via a dominant repression pathway acting on proximal promoter elements. *Oncogene* **18**, 7274–7279 (1999).
59. Bachman, K. E. *et al.* Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell* **3**, 89–95 (2003).
60. Clark, S. J. & Melki, J. DNA methylation and gene silencing in cancer: which is the guilty party? *Oncogene* **21**, 5380–5387 (2002).
61. Ehrlich, M. *et al.* Hypomethylation and hypermethylation of DNA in Wilms tumors. *Oncogene* **21**, 6694–6702 (2002).
62. Van Zee, K. J., Calvano, J. E. & Bisogna, M. Hypomethylation and increased gene expression of $p16^{INK4a}$ in primary and metastatic breast carcinoma as compared to normal breast tissue. *Oncogene* **16**, 2723–2727 (1998).
63. Surani, M. A., Barton, S. C. & Norris, M. L. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* **308**, 548–550 (1984).
64. McGrath, J. & Solter, D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179–183 (1984).
65. Kajii, T. & Ohama, K. Androgenetic origin of hydatidiform mole. *Nature* **268**, 633 (1977).
66. Linder, D., McCaw, B., Kaiser, X. & Hecht, F. Parthenogenetic origin of benign ovarian teratomas. *N. Engl. J. Med.* **292**, 63–66 (1975).
67. Pal, N. *et al.* Preferential loss of maternal alleles in sporadic Wilms' tumor. *Oncogene* **5**, 1665–1668 (1990).
68. Schroeder, W. T. *et al.* Nonrandom loss of maternal chromosome 11 alleles in Wilms tumors. *Am. J. Hum. Genet.* **40**, 413–420 (1987).
69. Scrable, H. *et al.* A model for embryonal rhabdomyosarcoma tumorigenesis that involves genome imprinting. *Proc. Natl Acad. Sci. USA* **86**, 7480–7484 (1989).
70. Williams, J. C., Brown, K. W., Mott, M. G. & Maitland, N. J. Maternal allele loss in Wilms' tumor. *Lancet* **1**, 283–284 (1989).
71. Brown, K. W., Williams, J. C., Maitland, N. J. & Mott, M. G. Genomic imprinting and the Beckwith–Wiedemann syndrome. *Am. J. Hum. Genet.* **46**, 1000–1001 (1990).
72. Koufos, A. *et al.* Familial Wiedemann–Beckwith syndrome and a second Wilms tumor locus both map to 11p15.5. *Am. J. Hum. Genet.* **44**, 711–719 (1989).
73. Ping, A. J. *et al.* Genetic linkage of Beckwith–Wiedemann syndrome to 11p15. *Am. J. Hum. Genet.* **44**, 720–723 (1989).
74. Mannens, M. *et al.* Parental imprinting of human chromosome region 11p15.3-pter involved in the Beckwith–Wiedemann syndrome and various human neoplasia. *Eur. J. Hum. Genet.* **2**, 3–23 (1994).
75. Zhang, Y. & Tycko, B. Monoallelic expression of the human H19 gene. *Nature Genet.* **1**, 40–44 (1992).
76. Giannoukakis, N., Deal, C., Paquette, J., Goodyer, C. G. & Polychronakos, C. Parental genomic imprinting of the human *IGF2* gene. *Nature Genet.* **4**, 98–101 (1993).
77. Ohlsson, R. *et al. IGF2* is parentally imprinted during human embryogenesis and in the Beckwith–Wiedemann syndrome. *Nature Genet.* **4**, 94–97 (1993).
78. Rainier, S. *et al.* Relaxation of imprinted genes in human cancer. *Nature* **362**, 747–749 (1993).
79. Ogawa, O. *et al.* Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature* **362**, 749–751 (1993).
80. Barlow, D. P., Stoger, R., Herrmann, B. G., Saito, K. & Schweifer, N. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the *Tme* locus. *Nature* **349**, 84–87 (1991).
81. Bartolomei, M., Zemel, S. & Tilghman, S. M. Parental imprinting of the mouse *H19* gene. *Nature* **351**, 153–155 (1991).
82. DeChiara, T. M., Robertson, E. J. & Efstratiadis, A. Parental imprinting of the mouse insulin-like growth factor-2 gene. *Cell* **64**, 849–859 (1991).
83. Glenn, C. C., Porter, K. A., Jong, M. T., Nicholls, R. D. & Driscoll, D. J. Functional imprinting and epigenetic modification of the human *SNRPN* gene. *Hum Mol. Genet.* **2**, 2001–2005 (1993).
84. Leff, S. E. *et al.* Maternal imprinting of the mouse *Snrpn* gene and conserved linkage homology with the human Prader–Willi syndrome region. *Nature Genet.* **2**, 259–264 (1992).
85. Li, E., Beard, C. & Jaenisch, R. Role for DNA methylation in genomic imprinting. *Nature* **366**, 362–365 (1993).
86. Onyango, P. *et al.* Sequence and comparative analysis of the mouse 1 megabase region orthologous to the human 11p15 imprinted domain. *Genome Res.* **10**, 1697–1710 (2000).
87. Paulsen, M. *et al.* Syntenic organization of the mouse distal chromosome 7 imprinting cluster and the Beckwith–Wiedemann syndrome region in chromosome 11p15.5. *Hum. Mol. Genet.* **7**, 1149–1159 (1998).
88. Qian, N. *et al.* The *IPL* gene on chromosome 11p15.5 is imprinted in humans and mice and is similar to *TDAG51*, implicated in Fas expression and apoptosis. *Hum. Mol. Genet.* **6**, 2021–2029 (1997).
89. Dao, D. *et al. IMPT1*, an imprinted gene similar to polyspecific transporter and multi-drug resistance genes. *Hum. Mol. Genet.* **7**, 597–608 (1998).
90. Moulton, T. *et al.* Epigenetic lesions at the H19 locus in Wilms' tumour patients. *Nature Genet.* **7**, 440–447 (1994).
91. Steenman, M. J. *et al.* Loss of imprinting of *IGF2* is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour. *Nature Genet* **7**, 433–439 (1994).
92. Okamoto, K., Morison, I. M., Taniguchi, T. & Reeve, A. E. Epigenetic changes at the insulin-like growth factor II/H19 locus in developing kidney is an early event in Wilms tumorigenesis. *Proc. Natl Acad. Sci. USA* **94**, 5367–5371 (1997).
93. Hao, Y., Crenshaw, T., Moulton, T., Newcomb, E. & Tycko, B. Tumor-suppressor activity of *H19* RNA. *Nature* **365**, 764–767 (1993).
94. Christofori, G., Naik, P. & Hanahan, D. Deregulation of both imprinted and expressed alleles of the insulin-like growth factor 2 gene during β-cell tumorigenesis. *Nature Genet.* **10**, 196–201 (1995).
95. Christofori, G., Naik, P. & Hanahan, D. A second signal supplied by insulin-like growth factor II in oncogene-induced tumorigenesis. *Nature* **369**, 414–418 (1994).
96. Ravenel, J. D. *et al.* Loss of imprinting of insulin-like growth factor-II (*IGF2*) gene in distinguishing specific biologic subtypes of Wilms tumor. *J. Natl Cancer Inst.* **93**, 1698–1703 (2001).
97. Sanson, M., Leuraud, P., Marie, Y., Delattre, J. Y. & Hoang-Xuan, K. Preferential loss of paternal 19q, but not 1p, alleles in oligodendrogliomas. *Ann. Neurol.* **52**, 105–107 (2002).
98. Maegawa, S. *et al.* Epigentic silencing of *PEG3* gene expression in human glioma cell lines. *Mol. Carcinogen.* **31**, 1–9 (2001).
99. Jenkins, R. B., Curran, W., Scott, C. B. & Cairncross, G. Pilot evaluation of 1p and 19q deletions in anaplastic oligodendrogliomas collected by a national cooperative cancer treatment group. *Am. J. Clin. Oncol.* **24**, 506–508 (2001).
100. Li, L. *et al.* Regulation of maternal behavior and offspring growth by paternally expressed *Peg3*. *Science* **284**, 330–333 (1999).
101. Kohda, T. *et al.* Tumour suppressor activity of human imprinted gene *PEG3* in a glioma cell line. *Genes Cells* **6**, 237–247 (2001).
102. Maegawa, S. *et al.* Epigenetic silencing of *PEG3* gene expression in human glioma cell lines. *Mol. Carcinogen.* **31**, 1–9 (2001).

103. Caron, H. *et al.* Chromosome bands 1p35-36 contain two distinct neuroblastoma tumor suppressor loci, one of which is imprinted. *Genes Chromosomes Cancer* **30**, 168–174 (2001).
104. Morison, I. M., Ellis, L. M., Teague, L. R. & Reeve, A. E. Preferential loss of maternal 9p alleles in childhood acute lymphoblastic leukemia. *Blood* **99**, 375–377 (2002).
105. Yuan, J. *et al.* Aberrant methylation and silencing of *ARHI*, an imprinted tumor suppressor gene in which the function is lost in breast cancers. *Cancer Res.* **63**, 4174–4180 (2003).
106. Lee, M. P. *et al.* Loss of imprinting of a paternally expressed transcript, with antisense orientation to *KVLQT1*, occurs frequently in Beckwith–Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. *Proc. Natl Acad. Sci. USA* **96**, 5203–5208 (1999).
107. Smilinich, N. J. *et al.* A maternally methylated CpG island in *KvLQT1* is associated with an antisense paternal transcript and loss of imprinting in Beckwith–Wiedemann syndrome. *Proc. Natl Acad. Sci. USA* **96**, 8064–8069 (1999).
108. Buschhausen, G., Wittig, B., Graessmann, M. & Graessmann, A. Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. *Proc. Natl Acad. Sci. USA* **84**, 1177–1181 (1987).
109. Keshet, I., Lieman-Hurwitz, J. & Cedar, H. DNA methylation affects the formation of active chromatin. *Cell* **44**, 535–543 (1986).
110. Lewis, J. D. *et al.* Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* **69**, 905–914 (1992).
111. Meehan, R. R., Lewis, J. D. & Bird, A. P. Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic Acids Res.* **20**, 5085–5092 (1992).
112. Jones, P. L. *et al.* Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature Genet.* **19**, 187–191 (1998).
113. Nan, X. *et al.* Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**, 386–389 (1998).
114. Rountree, M. R., Bachman, K. E. & Baylin, S. B. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nature Genet.* **25**, 269–277 (2000).
115. Wade, P. A. *et al.* Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nature Genet.* **23**, 62–66 (1999).
116. Roder, K. *et al.* Transcriptional repression by *Drosophila* methyl-CpG-binding proteins. *Mol. Cell Biol.* **20**, 7401–7409 (2000).
117. Rea, S. *et al.* Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**, 593–599 (2000).
118. Strahl, B. D., Ohba, R., Cook, R. G. & Allis, C. D. Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in *Tetrahymena*. *Proc. Natl Acad. Sci. USA* **96**, 14967–14972 (1999).
119. Nguyen, C. T. *et al.* Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2′-deoxycytidine. *Cancer Res.* **62**, 6456–6461 (2002).
120. Yoder, J. A., Walsh, C. P. & Bestor, T. H. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* **13**, 335–340 (1997).
121. Ahmad, K. & Henikoff, S. Histone H3 variants specify modes of chromatin assembly. *Proc. Natl Acad. Sci. USA* **99**, S16477–S16484 (2002).
122. Bannister, A. J., Schneider, R. & Kouzarides, T. Histone methylation: dynamic or static? *Cell* **109**, 801–806 (2002).
123. Lobanenkov, V. V., Nicolas, R. H., Plumb, M. A., Wright, C. A. & Goodwin, G. H. Sequence-specific DNA-binding proteins which interact with (G + C)-rich sequences flanking the chicken c-*myc* gene. *Eur. J. Biochem.* **159**, 181–188 (1986).
124. Holmgren, C. *et al.* CpG methylation regulates the Igf2/H19 insulator. *Curr. Biol.* **11**, 1128–1130 (2001).
125. Hark, A. T. *et al.* CTCF mediates methylation-sensitive enhancer-blocking activity at the *H19/Igf2* locus. *Nature* **405**, 486–489 (2000).
126. Bell, A. C., West, A. G. & Felsenfeld, G. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell* **98**, 387–396 (1999).
127. Cui, H. *et al.* Loss of imprinting of insulin-like growth factor-II in Wilms' tumor commonly involves altered methylation but not mutations of CTCF or its binding site. *Cancer Res.* **61**, 4947–4950 (2001).
128. Cui, H. *et al.* Loss of imprinting in colorectal cancer linked to hypomethylation of *H19* and *IGF2*. *Cancer Res.* **62**, 6442–6446 (2002).
129. Nakagawa, H. *et al.* Loss of imprinting of the insulin-like growth factor II gene occurs by biallelic methylation in a core region of H19-associated CTCF-binding sites in colorectal cancer. *Proc. Natl Acad. Sci. USA* **98**, 591–596 (2001).
130. Loukinov, D. I. *et al.* BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. *Proc. Natl Acad. Sci. USA* **99**, 6806–6811 (2002).
131. Varambally, S. *et al.* The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* **419**, 624–629 (2002).
132. DeBaun, M. R. & Tucker, M. A. Risk of cancer during the first four years of life in children from The Beckwith–Wiedemann Syndrome Registry. *J. Pediatr.* **132,** 398–400 (1998).
133. Weksberg, R., Shen, D. R., Fei, Y. L.,Song, Q. L., & Squire, J. Disruption of insulin-like growth factor 2 imprinting in Beckwith–Wiedemann syndrome. *Nature Genet.* **5**,143–150 (1993).
134. Hatada, I. *et al.* An imprinted gene $p57^{KIP2}$ is mutated in Beckwith–Wiedemann syndrome. *Nature Genet.* **14**, 171–1733 (1996).
135. Tycko, B. Genomic imprinting and cancer. *Results Probl. Cell. Differ.* **25**, 133–169 (1999).
136. Engel, J. R. *et al.* Epigenotype-phenotype correlations in Beckwith–Wiedemann syndrome. *J. Med. Genet.* **37**, 921–926 (2000).
137. Bliek, J. *et al.* Increased tumour risk for BWS patients correlates with aberrant *H19* and not *KCNQ1OT1* methylation: occurrence of *KCNQ1OT1* hypomethylation in familial cases of BWS. *Hum. Mol. Genet.* **10**, 467–476 (2001).
138. Weksberg, R. *et al.* Tumor development in the Beckwith–Wiedemann syndrome is associated with a variety of constitutional molecular 11p15 alterations including imprinting defects of KCNQ1OT1. *Hum. Mol. Genet.* **10**, 2989–3000 (2001).
139. DeBaun, M. R. *et al.* Epigenetic alterations of H19 and LIT1 distinguish patients with Beckwith–Wiedemann syndrome with cancer and birth defects. *Am. J. Hum. Genet.* **70**, 604–611 (2002).
140. DeBaun, M. R., Niemitz, E. L. & Feinberg, A. P. Association of *in vitro* fertilization with Beckwith–Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am. J. Hum. Genet.* **72**, 156–160 (2002).
141. Maher, E. R. *et al.* Beckwith–Wiedemann syndrome and assisted reproduction technology (ART). *J. Med. Genet.* **40**, 62–64 (2003).
142. Cui, H. et al. Loss of *IGF2* imprinting: a potential marker of colorectal cancer risk. *Science* **299**, 6442–6446 (2003).
143. Belinsky, S. A. *et al.* Aberrant methylation of *p16*(*INK4a*) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc. Natl Acad. Sci. USA* **95**, 11891–11896 (1998).
144. Sandovici, I. *et al.* Familial aggregation of abnormal methylation of parental alleles at the *IGF2*/*H19* and *IGF2R* differentially methylated regions. *Hum. Mol. Genet.* **12**, 1569–1578 (2003).
145. Laird, P. W. *et al.* Suppression of intestinal neoplasia by DNA hypomethylation. *Cell* **81**, 197–205 (1995).
146. Gaudet, F. *et al.* Induction of tumors in mice by genomic hypomethylation. *Science* **300**, 489–492 (2003).
147. Chen, W. Y. *et al.* Heterozygous disruption of *Hic1* predisposes mice to a gender-dependent spectrum of malignant tumors. *Nature Genet.* **33**, 197–202 (2003).
148. Ziemin-van der Poel, S. *et al.* Identification of a gene, *MLL*, that spans the breakpoint in 11q23 translocations associated with human leukemias. *Proc. Natl Acad. Sci. USA* **88**, 10735–10739 (1991).
149. Tkachuk, D. C., Kohler, S. & Cleary, M. L. Involvement of a homolog of *Drosophila* trithorax by 11q23 chromosomal translocations in acute leukemias. *Cell* **71**, 691–700 (1992).
150. Gu, Y. *et al.* The t(4;11) chromosome translocation of human acute leukemias fuses the *ALL-1* gene, related to *Drosophila* trithorax, to the *AF-4* gene. *Cell* **71**, 701–708 (1992).
151. Nakamura, T. *et al.* ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. *Mol. Cell* **10**, 1119–1128 (2002).
152. El-Deiry, W. S. *et al.* High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. *Proc. Natl Acad. Sci. USA* **88**, 3470–3474 (1991).
153. Lee, P. J. *et al.* Limited upregulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation. *Proc. Natl Acad. Sci. USA* **93**, 10366–10370 (1996).
154. De Marzo, A. M. *et al.* Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. *Cancer Res.* **59**, 3855–3860 (1999).
155. Eads, C. A. *et al.* CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res.* **59**, 2302–2306 (1999).
156. Di Croce, L. *et al.* Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* **295**, 1079–1082 (2002).
157. Esteller, M. *et al.* Cancer epigenetics and methylation. *Science* **297**, 1807–1808 (2002).
158. Hajra, K. M., Chen, D. Y. & Fearon, E. R. The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res.* **62**, 1613–1618 (2002).
159. Dunaief, J. L. *et al.* The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell* **79**, 119–130 (1994).
160. Luo, R. X., Postigo, A. A. & Dean, D. C. Rb interacts with histone deacetylase to repress transcription. *Cell* **92**, 463–473 (1998).
161. Magnaghi-Jaulin, L. *et al.* Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391**, 601–605 (1998).
162. Dahiya, A., Wong, S., Gonzalo, S., Gavin, M. & Dean, D. C. Linking the Rb and polycomb pathways. *Mol. Cell* **8**, 557–569 (2001).
163. Pradhan, S. & Kim, G. D. The retinoblastoma gene product interacts with maintenance human DNA (cytosine-5) methyltransferase and modulates its activity. *EMBO J.* **21**, 779–788 (2002).
164. Robertson, K. D. *et al.* DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nature Genet.* **25**, 338–342 (2000).
165. Steele-Perkins, G. *et al.* Tumor formation and inactivation of RIZ1, an Rb-binding member of a nuclear protein-methyltransferase superfamily. *Genes Dev.* **15**, 2250–2262 (2001).
166. Hsiao, W.-L., Gattoni-Celli, S. & Weinstein, I. B. Effects of 5-azacytidine on the progressive nature of cell transformation. *Mol. Cell. Biol.* **5**, 1800–1803 (1985).
167. Taylor, S. M. & Jones, P. A. Multiple new phenotypes induced in 10T 1/2 and 3T3 cells treated with 5-azacytidine. *Cell* **17**, 771–779 (1979).
168. Daskalakis, M. *et al.* Demethylation of a hypermethylated *P15*/*INK4B* gene in patients with myelodysplastic syndrome by 5-aza-2′-deoxycytidine (decitabine) treatment. *Blood* **100**, 2957–2964 (2002).
169. Juttermann, R., Li, E. & Jaenisch, R. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc. Natl Acad. Sci. USA* **91**, 11797–11801 (1994).
170. Cheng, J. C. *et al.* Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *J. Natl Cancer Inst.* **95**, 399–409 (2003).
171. Barletta, J. M., Rainier, S. & Feinberg, A. P. Reversal of loss of imprinting in tumor cells by 5-aza-2′-deoxycytidine. *Cancer Res.* **57**, 48–50 (1997).
172. Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G. & Baylin, S. B. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nature Genet.* **21**, 103–107 (1999).
173. Shaker, S., Bernstein, M., Momparler, L. F. & Momparler, R. L. Preclinical evaluation of antineoplastic activity of inhibitors of DNA methylation (5-aza-2′-deoxycytidine) and histone deacetylation (trichostatin A, depsipeptide) in combination against myeloid leukemic cells. *Leuk. Res.* **27**, 437–444 (2003).
174. Eden, A., Gaudet, F. & Jaenisch, R. Response to comment on "Chromosomal instability and tumors promoted by dna hypomethylation" and "Induction of tumors in mice by genomic hypomethylation". *Science* **302**, 1153 (2003).
175. Yang, A. S., Estecio, M. R., Garcia–Manero, G., Kantarjian, H. M. & Issa, J. P. Comment on "Chromosomal instability and tumors promoted by DNA hypomethylation" and "Induction of tumors in nice by genomic hypomethylation". *Science* **302**, 1153 (2003).

176. Mohandas, T., Sparkes, R. S. & Shapiro, L. J. Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. *Science* **211**, 393–396 (1981).
177. Wolf, S. F., Jolly, D. J., Lunnen, K. D., Friedmann, T., & Migeon, B. R. Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X chromosome: implications for X-chromosome inactivation. *Proc. Natl Acad. Sci. USA* **81**, 2806–2810 (1984).
178. Antequera, F., Macleod, D. & Bird, A. P. Specific protection of methylated CpGs in mammalian nuclei. *Cell* **58**, 509–517 (1989).
179. Hansen, R. S. & Gartler, S. M. 5-azacytidine-induced reactivation of the human X chromosome-linked *PGK1* gene is associated with a large region of cytosine demethylation in the 5′ CpG island. *Proc. Natl Acad. Sci. USA* **87**, 4174–4178 (1990).
180. Jeppesen, P. & Turner, B. M. The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell* **74**, 281–289 (1993).

Acknowledgements
We thank E. Fearon, B. Vogelstein, R. Ohlsson and G. Klein for reading the manuscript. This work was supported by grants from the National Institutes of Health. We apologize in advance for any errors of omission or commission.

Competing interests statement
The authors declare that they have no competing financial interests.

Online links

DATABASES
The following terms in this article are linked online to:
Cancer.gov: http://cancer.gov/
acute lymphoblastic leukaemias | acute myelogenous leukaemia | cervical cancer | colon cancer | liver cancer | lung cancer | malignant rhabdoid tumour | pancreatic cancer | stomach cancer | uterine cancer | Wilms tumour
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
ALL1 | *ARHI* | *ATRX* | *BORIS* | CAGE | calcitonin | *CDKN1C* | CTCF | DNMT1 | DNMT3A | DNMT3B | *EZH2* | *H19* | HP1 | HRAS | *IGF2* | *LIT1* | Lsh | MBD2 | MDR1 | MECP2 | *MLH1* | *Nf1* | *PEG3* | PML | RARα | *RARB2* | *RB* | SLUG | SNF5 | SUV39H1 | *Trp53* | WT1

FURTHER INFORMATION
DNA Methylation Society:
http://dnamethsoc.server101.com/
Imprinted Gene Catalogue:
http://cancer.otago.ac.nz/IGC/Web/home.html
Imprinting: http://www.geneimprint.com
Access to this interactive links box is free online.

GUIDELINES

Testing guidelines for hereditary non-polyposis colorectal cancer

Asad Umar, John I. Risinger, Ernest T. Hawk and J. Carl Barrett

Hereditary non-polyposis colorectal cancer is almost always associated with microsatellite instability, so what is the best way to identify the disorder at an early-stage, and what should the next step be in preventing the development of colorectal cancer? Different clinical and molecular diagnostic guidelines have recently been proposed in the context of recent scientific advances, but how are these criteria interpreted and modified across the world?

Hereditary non-polyposis colorectal cancer (HNPCC) was originally called 'cancer family syndrome'[1], as this autosomal dominant disease predisposes carriers of mutation to the development of several tumour types[2]. In 1913, the pathologist Alfred Warthin published the first known case report of a family with characteristics of HNPCC. Clinical clues in similar families accumulated for many years and it was clearly delineated as a hereditary cancer syndrome that was distinct from familial adenomatous polyposis — which is caused by an inherited mutation of the tumour-suppressor gene *APC* — in the mid-1960s by Henry T. Lynch. For this reason, it is also known as Lynch syndrome[1].

HNPCC has an incidence of 1:1,000 in the general population and up to 1:100 in individuals with colorectal cancer, which accounts for 1–5% of colorectal cancer[3–5]. It is characterized by an 80% lifetime risk for colorectal cancer and a 60% lifetime risk for endometrial cancer. It is important to emphasize that the lifetime risk of developing endometrial cancer in affected women is higher than their lifetime risk of developing colorectal cancer, so 'Lynch syndrome' might be a better choice of name than HNPCC[6]. Incidentally, individuals with HNPCC have also been shown to have an increased risk of developing extracolonic cancers, including those of the stomach, ovaries, small bowel, biliary tract, uroepithelium, kidney and central nervous system. Individuals with HNPCC colon cancers differ from those with sporadic colorectal cancer in several ways: the tumours are diagnosed at an earlier age; they are frequently located in the proximal colon (60–70%); they have an increased risk of developing synchronous or metachronous colon cancers; and they have a better prognosis[7–10].

Advances in the understanding of the genetic basis of HNPCC carcinogenesis have led to efforts to exploit this knowledge clinically, primarily in the form of predictive diagnostic criteria. In conjunction with family history, molecular tests have been designed to improve cancer-risk assessment of individuals. Diagnostic guidelines for HNPCC that were used previously were somewhat confusing and could now be outdated, as the advances in our understanding of the disease have progressed substantially over the past decade. Here, we discuss diagnostic guidelines for HNPCC across the world; these have been developed over time and several modifications have been made.

At an international workshop that was held recently in Bethesda, Maryland, in the United States[6], we discussed the recent advances in the understanding of the genetic basis of HNPCC and the Bethesda guidelines were revised. These guidelines are intended to be used to make a decision as to whether genetic testing of individuals should be performed in an attempt to detect HNPCC early. The early knowledge that an individual carries a defective allele of one of the DNA mismatch-repair (MMR) genes could allow individuals to realize preventive measures to delay and/or reduce the chance of getting the malignant disease.

MMR defects cause HNPCC

HNPCC[10,11] is caused by germline mutations in any one of five DNA MMR genes — *MSH2*, *MLH1*, *MSH6*, infrequently *PMS2* and, rarely, *PMS1* (REFS 12–16; FIG. 1; BOX 1). Genetic testing for HNPCC is therefore defined as the determination of the primary DNA sequence of *MSH2*, *MLH1* or *MSH6* to detect heritable disease-related genotypes or mutations for clinical purposes.

Defective MMR leads to an inability to repair base–base mismatches and small insertions and deletions, so causing an increased genomic mutation rate, which can lead to cancer. Paradoxically, however, as many as 50% of the suspected cases of HNPCC are not confirmed by a genetic defect (that is, mutation in one of the known MMR genes), so it remains a key issue to define the genotype–phenotype relationship between these and confirmed cases[17,18]. Epigenetic silencing of *MLH1* is also common in non-hereditary cancers that resemble HNPCC[19], which further confuses diagnosis of HNPCC.

Microsatellite DNA comprises repetitive sequences of 1–6 bases that are scattered throughout the human genome — most commonly as $(CA)_n$. The replication machinery slips more frequently on repetitive sequences than on non-repetitive sequences, so microsatellite sequences accu-