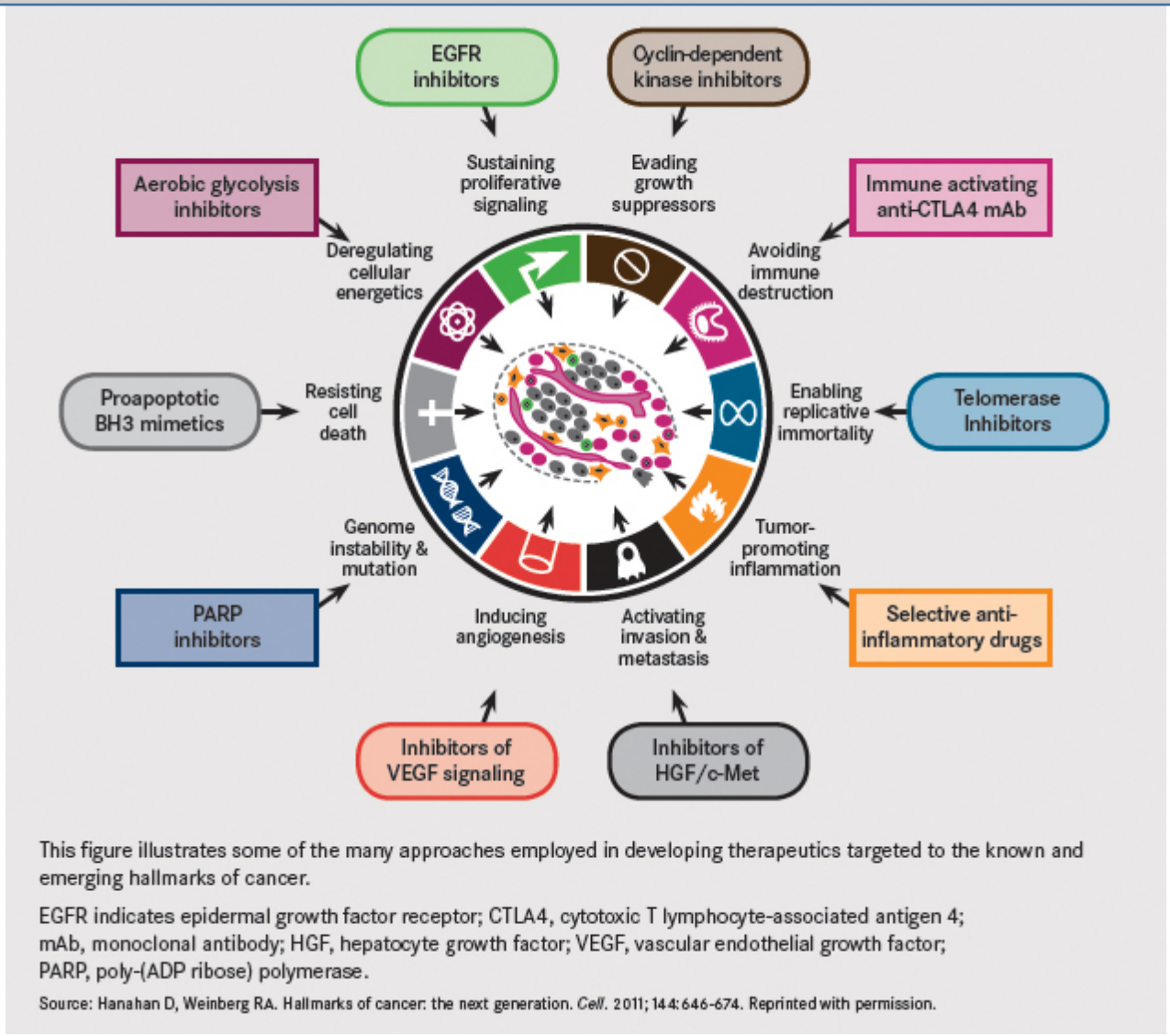
# The Epigenetics of human cancer

# Hallmarks of human cancer

## Cancer cells show Defined key features

Alterations in central biological pathways that Ensure immortality and invasiveness




This figure illustrates some of the many approaches employed in developing therapeutics targeted to the known and emerging hallmarks of cancer.

EGFR indicates epidermal growth factor receptor; CTLA4, cytotoxic T lymphocyte-associated antigen 4; mAb, monoclonal antibody; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; PARP, poly-(ADP ribose) polymerase.

Source: Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144:646-674. Reprinted with permission.

# GENETIC AND EPIGENTIC ALTERATIONS IN HUMAN CANCER

## Classic genetic basis of cancer

**Knudsen hypothesis: Two-hit or multiple hit hypothesis:**
Both alleles of a gene or multiple gene mutations result in cancer formation

## The extended (epi)-genetic basis of cancer

- Mutations in single alleles
- Loss of parts or entire genes
- Loss of one allele; second allele silenced by epigenetic alterations
- Epigenetic silencing of both alleles

## Epigenetic alterations:

- **Mutations in epigenetic writers/chromatin components**
- **Reduced expression of epigenetic regulators**
- **Increased expression of epigenetic regulators**

**The consequence of mutations in epigenetic regulators are difficult to assess**

- **Multiple direct and indirect effects on gene expression (silencing of DNA repair gene MLH1 mediates further mutations, etc)**
- **Important to understand immortalization and transformation**
- **Targets for chemotherapeutic approaches –> effect on multiple genes**

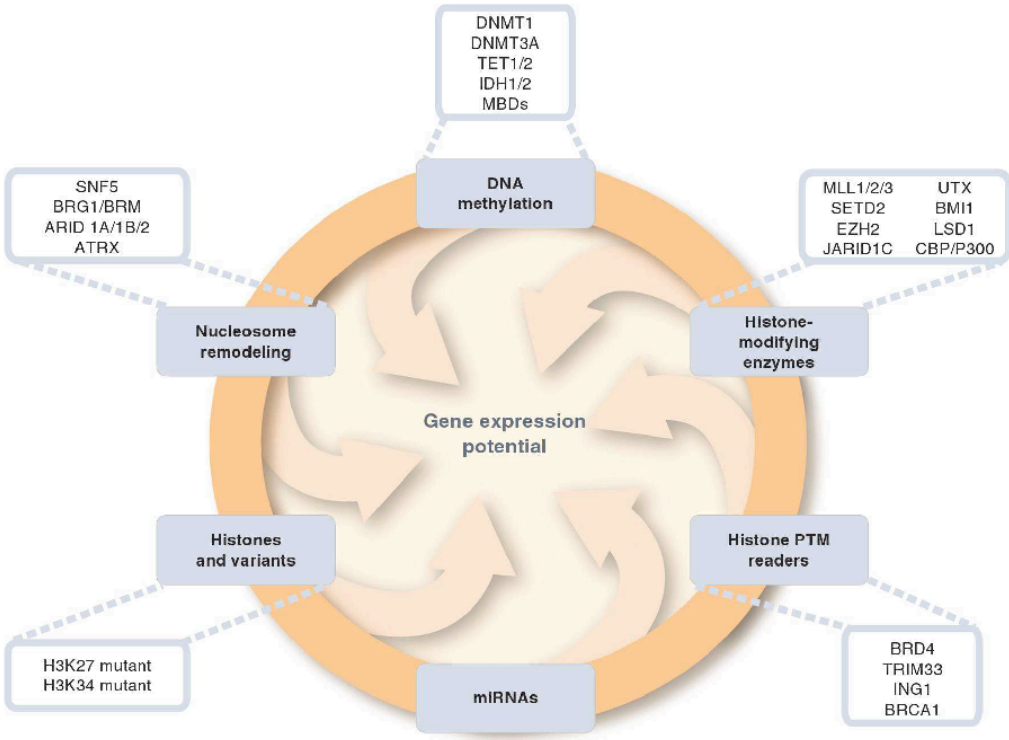



**Figure 1.** Genetic mutations of epigenetic modifiers in cancer. The drawing shows the input of epigenetic processes in specifying gene expression patterns. Recent whole-exome sequencing studies show that mutations in various classes of epigenetic modifiers are frequently observed in many types of cancers, further highlighting the cross talk between genetics and epigenetics. Examples of some, but not all, of these mutations are illustrated here and listed in Table 2. The mutations of epigenetic modifiers potentially cause genome-wide epigenetic alterations in cancer, but, save for isocitrate dehydrogenase (IDH) mutations as discussed in the text, these have yet to be shown on a genome-wide scale. Understanding the relationship of genetic and epigenetic changes in cancer will offer novel insights for cancer therapies. MBDs, methylcytosine-binding proteins; PTM, posttranslational modification. (Adapted from You and Jones 2012.)

# Hallmarks of human cancer – the epigenetics

**ALTERATION OF EPIGENETIC REGULATORS (Mutation/altered expression) CAN SET UP CANCER ASSOCIATED GENE EXPRESSION SIGNATURES**

# THE CANCER EPIGENOME

Epigenetic alterations:

- Can be causal for the alteration of gene expression → epigenetic writer or epigenetic readers are altered (mutated or changed expression
- Can also be a indirect consequence → another mechanism not directly related to epigenetics alters gene expression, epigenetic modifications change as a consequence

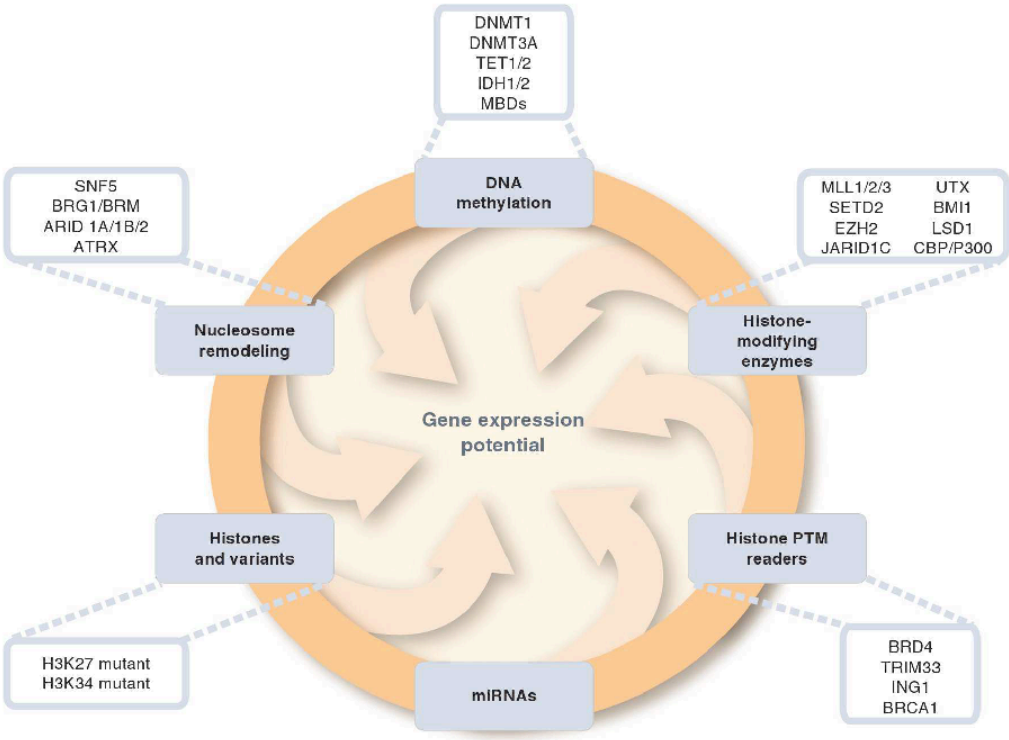



**Figure 1.** Genetic mutations of epigenetic modifiers in cancer. The drawing shows the input of epigenetic processes in specifying gene expression patterns. Recent whole-exome sequencing studies show that mutations in various classes of epigenetic modifiers are frequently observed in many types of cancers, further highlighting the cross talk between genetics and epigenetics. Examples of some, but not all, of these mutations are illustrated here and listed in Table 2. The mutations of epigenetic modifiers potentially cause genome-wide epigenetic alterations in cancer, but, save for isocitrate dehydrogenase (IDH) mutations as discussed in the text, these have yet to be shown on a genome-wide scale. Understanding the relationship of genetic and epigenetic changes in cancer will offer novel insights for cancer therapies. MBDs, methylcytosine-binding proteins; PTM, posttranslational modification. (Adapted from You and Jones 2012.)

# Hallmarks of human cancer – the genetics

**Alterations in central biological pathways that ensure immortality and invasiveness**

**Cancer is caused by key oncogene activation and/or loss of tumorsuppression and paralleled by the acquisition of hundreds of mutations in different genes and also altered gene expression of mutated/non-mutated genes**

**Together this results up a cancer gene expression pattern/genomic instability**

**Genetics - Epigenetics**

**Table 1.** Examples of key cellular pathways disrupted in human cancers by genetic and epigenetic mechanisms

| Pathway | Example of genetic alteration | Example of epigenetic alteration |
|---|---|---|
| Self sufficiency in growth signals | Mutations in *Ras* gene | Methylation of *RASSFIA* gene |
| Insensitivity to antigrowth signals | Mutation in TGF-β receptors | Down-regulation of TGF-β receptors |
| Tissue invasion and metastasis | Mutation in *E-cadherin* gene | Methylation of *E-cadherin* promoter |
| Limitless replicative potential | Mutation in *p16* and pRb genes | Silencing of *p16* or pRb genes by promoter methylation |
| Sustained angiogenesis | | Silencing of thrombospondin-1 |
| Evading apoptosis | Mutation in p53 | Methylation of *DAPK*, ASC/TMS1, and HIC1 |
| DNA repair capacity | Mutations in MLH1, MSH2 | Methylation of *GST Pi*, *O6-MGMT*, *MLH1* |
| Monitoring genomic stability | Mutations in *Chfr* | Methylation of *Chfr* |
| Protein ubiquination functions regulating mitotic control genes | Mutations in *Chfr* | Methylation of *Chfr* |

TGF-β, transforming growth factor β; *DAPK*, death-associated protein kinase.

# CHROMATIN STRUCTURE IS DIFFERENT IN CANCER CELLS

**Staining of chromatin by hematoxylin –> standard procedure in pathology**

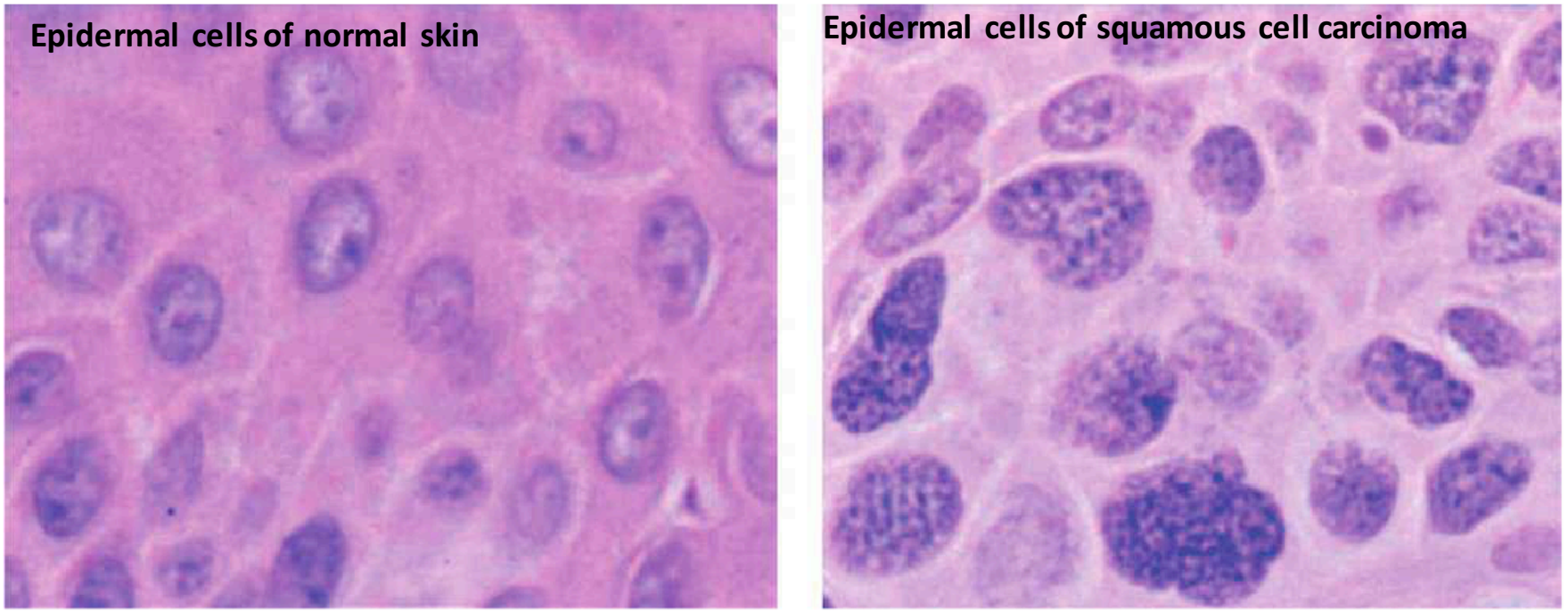
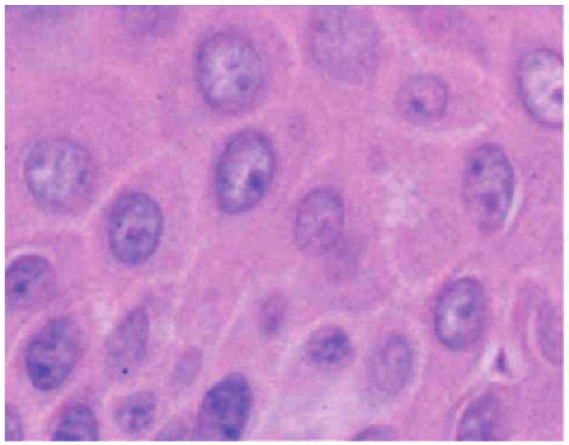
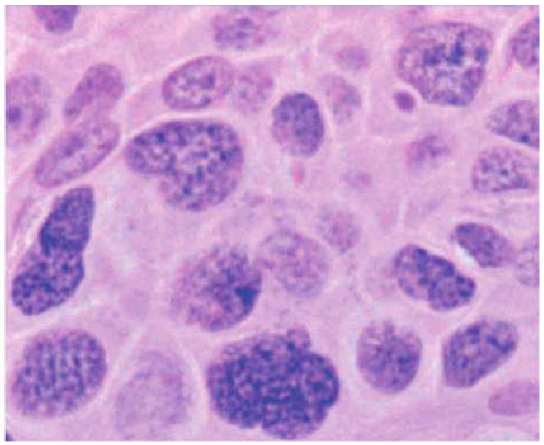



**Figure 2.** Chromatin structural changes in cancer cells. These two photomicrographs were taken from a patient with a squamous cell carcinoma of the skin. The *left* panel shows normal epidermal cells within one millimeter of the contiguous tumor shown at the same magnification on the *right*. The chromatin, which stains purple as a result of its affinity to hematoxylin, appears much more coarse and granular in the cancer cells than in normal epidermis. Such changes in the staining characteristics of chromatin are used by pathologists as diagnostic criteria for cancer.

# Definition of epigenetic determinants in human cancer

## *Classic visual inspection*





**Changes in chromatin conformation**

## *Detailed molecular analysis*

***Genome-wide epigenetic mapping***
***Chromosome conformation capture***
***RNA seq (lncRNA, siRNA, miRNA, etc)***
***DNA methyaltion profiling***
***Genome sequencing***
***Proteiomics***
***Metabolomics***

**High resolution information on Epigenetic alterations on the single nucleotide level**

**Detailed understanding of pathological processes in human cancer**

# Alteration of epigenetic modifiers in human cancer

## DNA methylation

ons in selected epigenetic modifiers in human cancers

| Gene | Function | Tumor type | Alteration |
|---|---|---|---|
| *DNMT1* | DNA methyltransferase | Colorectal, Non–small cell lung, pancreatic, gastric, breast cancer | Mutation (Kanai et al. 2003) Overexpression (Wu et al. 2007) |
| *DNMT3A* | DNA methyltransferase | MDS; AML | Mutation (Ley et al. 2010; Yamashita et al. 2010; Yan et al. 2011) |
| *DNMT3B* | DNA methyltransferase | ICF syndrome, SNPs in breast and lung adenoma | Mutation (Wijmenga et al. 2000) Mutation (Shen et al. 2002) |
| *MBD1/2* | Methyl-binding protein | Lung and breast cancer | Mutation (Sansom et al. 2007) |
| *TET1* | 5′-Methylcytosine hydroxylase | AML | Chromosome translocation (De Carvalho et al. 2010; Wu and Zhang 2010) |
| *TET2* | 5′-Methylcytosine hydroxylase | MDS, myeloid malignancies, gliomas | Mutation/silencing (Araki et al. 2009) |
| *IDH1/2* | Isocitrate dehydrogenase | Glioma, AML | Mutation (Figueroa et al. 2010; Lu et al. 2012; Turcan et al. 2012) |
| *AID* | 5′-Cytidine deaminase | CML | Aberrant expression (De Carvalho et al. 2010) |
| *MLL1/2/3* | Histone methyltransferase H3K4 | Bladder TCC, hematopoietic, non-Hodgkin lymphoma, B-cell lymphoma, prostate (primary) | Translocation, mutation, aberrant expression (Gui et al. 2011; Morin et al. 2011) |

## Histone modifying complexes

| | | | |
|---|---|---|---|
| *EZH2* | Histone methyltransferase H3K27 | Breast, prostate, bladder, colon, pancreas, liver, gastric, uterine tumors, melanoma, lymphoma, myeloma, and Ewing's sarcoma | Mutation, aberrant expression (Chase and Cross 2011; Tsang and Cheng 2011) |
| *BMI-1* | PRC1 subunit | Ovarian, mantle cell lymphomas, and Merkel cell carcinomas | Overexpression (Jiang and Song 2009; Lukacs et al. 2010) |
| *G9a* | Histone methyltransferase H3K9 | HCC, cervical, uterine, ovarian, and breast cancer | Aberrant expression (Varier and Timmers 2011) |
| *PRMT1/5* | Protein arginine methyltransferase | Breast/gastric | Aberrant expression (Miremadi et al. 2007) |
| *LSD1* | Histone demethyltransferase H3K4/H3K9 | Prostate | Mutation (Rotili and Mai 2011) |
| *UTX (KDM6A)* | Histone demethyltransferase H3K27 | Bladder, breast, kidney, lung, pancreas, esophagus, colon, uterus, brain, hematological malignancies | Mutation (Rotili and Mai 2011) |
| *JARID1B/C (KDM5C)* | Histone demethyltransferase H3K4/H3K9 | Testicular and breast, RCCC | Overexpression (Rotili and Mai 2011) |
| *EP300 (P300/KAT3B)* | Histone acetyltransferase | Breast, colorectal, pancreatic cancer | Mutation (Miremadi et al. 2007) |
| *CREBBP (CBP/KAT3A)* | Histone acetyltransferase | Gastric and colorectal, epithelial, ovarian, lung, esophageal cancer | Mutation, overexpression (Miremadi et al. 2007) |
| *PCAF* | Histone acetyltransferase | Epithelial | Mutation (Miremadi et al. 2007) |
| *HDAC2* | Histone deacetyltransferase | Colonic, gastric, endometrial cancer | Mutation (Ropero et al. 2006) |
| *SIRT1, HDAC5/7A* | Histone deacetyltransferase | Breast, colorectal, prostate cancer | Mutation, aberrant expression (Miremadi et al. 2007) |

## Chromatin remodeling complexes

| | | | |
|---|---|---|---|
| Chromatin-remodeling enzymes | *SNF5 (SMARCB1, INI1)* | BAF subunit | Kidney malignant rhabdoid tumors, atypical rhabdoid/teratoid tumors (extrarenal), epithelioid sarcomas, small cell hepatoblastomas, extraskeletal myxoid chondrosarcomas, and undifferentiated sarcomas | Mutation, silencing, loss of expression (Wilson and Roberts 2011) |
| | *BRG1 (SMARCA4)* | ATPase of BAF | Lung, rhabdoid, medulloblastoma | Mutation, low expression (Wilson and Roberts 2011) |
| | *BRM (SMARCA2)* | ATPase of BAF | Prostate, basal cell carcinoma | Mutation, low expression (Sun et al. 2007; de Zwaan and Haass 2010) |
| | *ARID1A (BAF250A)* | BAF subunit | Ovarian clear cell carcinomas, 30% of endometrioid carcinomas, endometrial carcinomas | Mutation, genomic rearrangement, low expression (Jones et al. 2010; Guan et al. 2011) |
| | *ARID2 (BAF200)* | PBAF subunit | Primary pancreatic adenocarcinomas | Mutation (Li et al. 2011) |
| | *BRD7* | PBAF subunit | Bladder TCC | Mutation (Drost et al. 2010) |
| | *PBRM1 (BAF180)* | PBAF subunit | Breast tumors | Mutation (Varela et al. 2011) |
| | *SRCAP* | ATPase of SWR1 | Prostate | Aberrant expression (Balakrishnan et al. 2007) |
| | *P400/Tip60* | ATPase of SWR1, acetylase of SWR1 | Colon, lymphomas, head and neck, breast | Mutation, aberrant expression (Mattera et al. 2009) |
| | *CHD4/5* | ATPase of NuRD | Colorectal and gastric cancer, ovarian, prostate, neuroblastoma, hematopoietic | Mutation (Bagchi et al. 2007; Kim et al. 2011; Wang et al. 2011) |
| | *CHD7* | ATP-dependent helicase | Gastric and colorectal | Mutation (Wessels et al. 2010) |

Adapted from You and Jones 2012.

MDS, myelodysplastic syndromes; AML, acute myeloid leukemia; ICF, immunodeficiency, centromere instability, and facial anomalies; SNPs, single-nucleotide polymorphisms; TCC, transitional cell carcinoma; HCC, hepatocellular carcinoma; RCCC, renal clear cell carcinoma; TET, ten-eleven translocation; NuRD, nucleosome remodeling and deacetylation.

# 1. Alteration of DNA methylation in human cancer

**CANCER IS ASSOCIATED WITH GLOBAL DNA HYPOMETHYLATION and LOCAL DNA HYPERMETHYLATION**

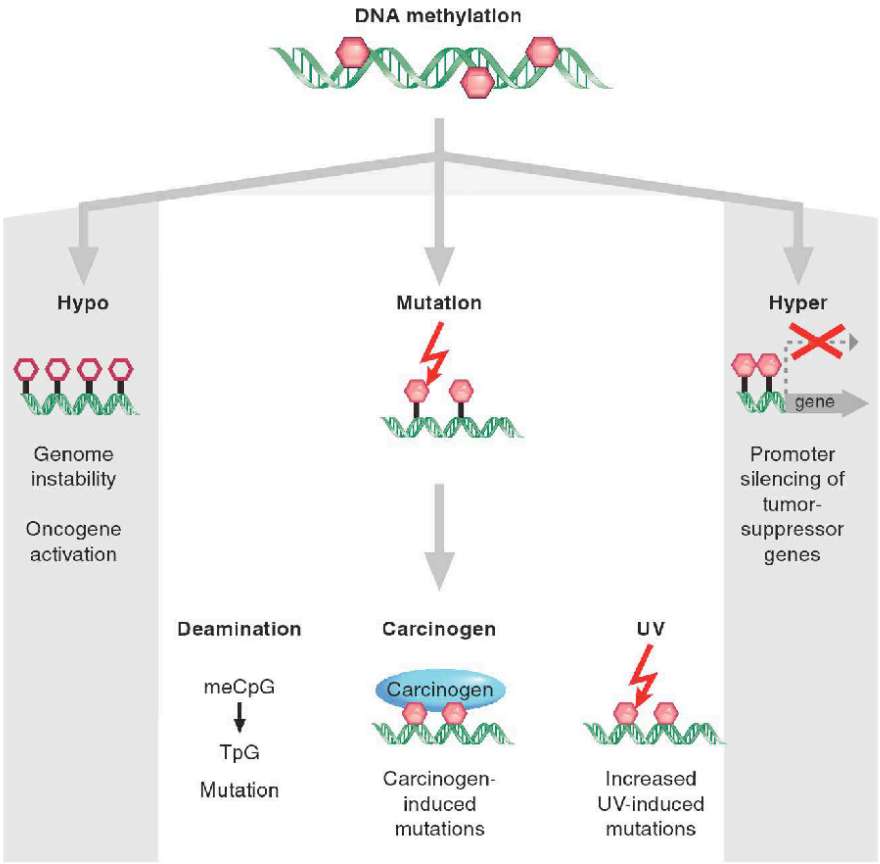



Figure 3. Epigenetic alterations involving DNA methylation can lead to cancer by various mechanisms. Loss of DNA cytosine methylation (white hexagons) illustrated in the hypo column results in genome instability. Focal hypermethylation (pink hexagons) at gene promoters shown in the hyper column causes heritable silencing and, therefore, inactivation of tumor suppressors and other genes. Additionally, methylated CpG sites (pink hexagons) are prone to mutation: They are hot spots for C to T transition mutations caused by spontaneous hydrolytic deamination; or methylation of CpG sites can increase the binding of some chemical carcinogens to DNA; and it increases the rate of UV-induced mutations.

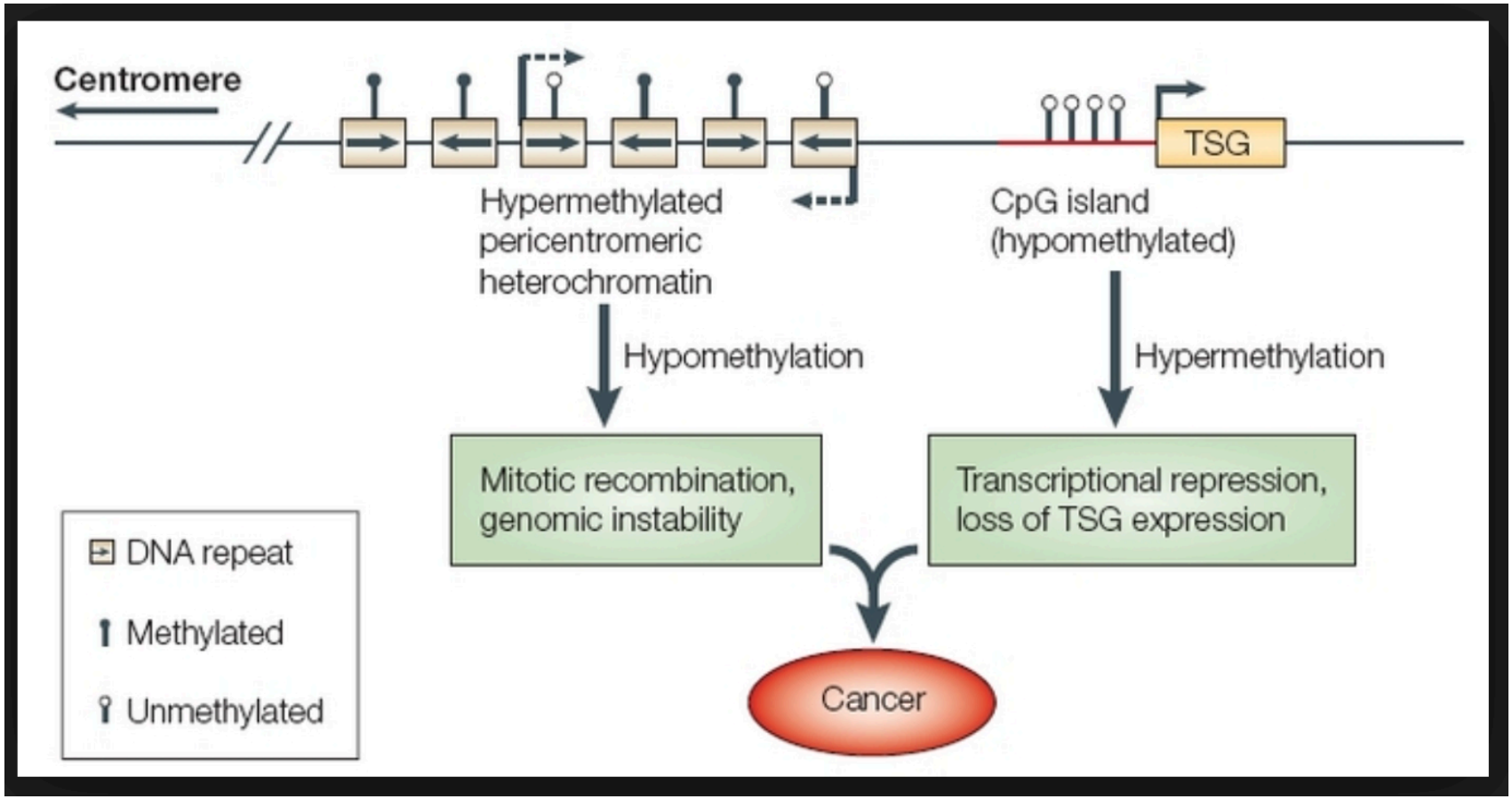
Normal tissue: 80% of CpG methylated
Cancer: 40-60% of CpG methylated

Global DNA methylation is lost from large blocks (28kb – 10Mb)

Local hypermethylation at specific locations such as tumor suppressors

# 1. The role of DNA methylation in human cancer

## *A. HYPOMETHYLATION*



# 1. The role of DNA methylation in human cancer

## A. HYPOMETHYLATION

## Landmark paper introducing differential DNA methylation in cancer research

Prepare DNA from healthy and tumor tissue
Digest with methylation sensitive restriction enzyme (does not cut metylated DNA)
Probe southern blot with probes cDNA probes of different genes
Cancer tissue: smaller fragments → methylation lost



Received 31 August; accepted 26 October 1982

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### Hypomethylation distinguishes genes of some human cancers from their normal counterparts

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**It has been suggested that cancer represents an alteration in DNA, heritable by progeny cells, that leads to abnormally regulated expression of normal cellular genes; DNA alterations such as mutations[1,2], rearrangements[3–5] and changes in methylation[6–8] have been proposed to have such a role. Because of increasing evidence that DNA methylation is important in gene expression (for review see refs 7, 9–11), several investigators have studied DNA methylation in animal tumours, transformed cells and leukaemia cells in culture[8,12–30]. The results of these studies have varied; depending on the techniques and systems used, an increase[12–19], decrease[20–24], or no change[25–29] in the degree of methylation has been reported. To our knowledge, however, primary human tumour tissues have not been used in such studies. We have now examined DNA methylation in human cancer with three considerations in mind: (1) the methylation pattern of specific genes, rather than total levels of methylation, was determined; (2) human cancers and adjacent analogous normal tissues, unconditioned by culture media, were analysed; and (3) the cancers were taken from patients who had received neither radiation nor chemotherapy. In four of five patients studied, representing two histological types of cancer, substantial hypomethylation was found in genes of cancer cells compared with their normal counterparts. This hypomethylation was progressive in a metastasis from one of the patients.**



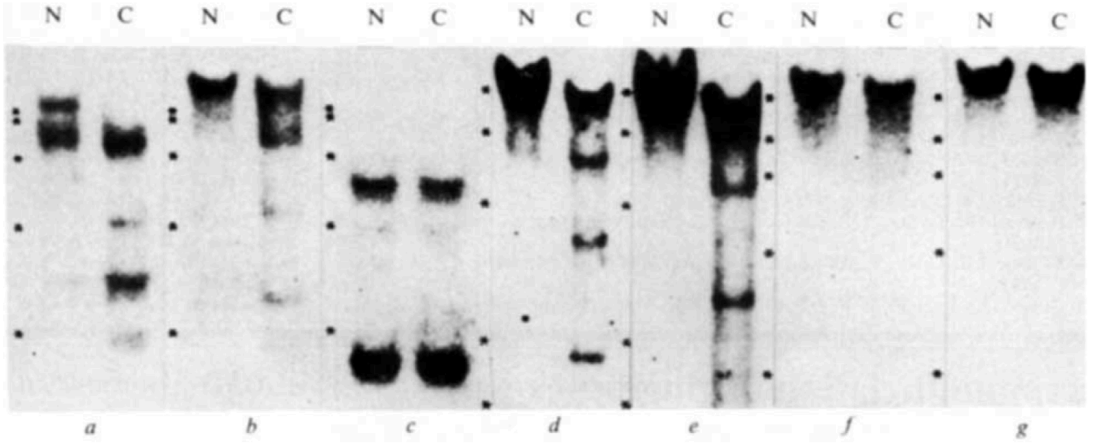



**Fig. 1** Methylation pattern of adenocarcinoma of the colon and normal colonic mucosa from patient 1. Lanes C and N were prepared from DNA digests of the colon carcinoma and normal colonic mucosa, respectively. *a* *Hpa*II digest, HGH probe; *b*, *Hha*I digest, HGH probe; *c*, *Msp*I digest, HGH probe; *d*, *Hpa*II digest, γ-globin probe; *e*, *Hha*I digest, γ-globin probe; *f*, *Hpa*II digest, α-globin probe; *g*, *Hha*I digest, α-globin probe. The asterisks on the left of *a–g* indicate molecular weight markers (*Hin*dIII-digested bacteriophage λ DNA, of sizes 9,500, 6,700, 4,400, 2,000 and 570 bp from top to bottom, respectively).

**Methods:** Tissues were frozen in liquid nitrogen, then pulverized and the DNA extracted essentially as described elsewhere[51]. The DNA was cleaved with 50-fold excess of restriction endonuclease, as assessed by the amount of restriction endonuclease required to digest pBR322 DNA included in a matched duplicate digest of the human DNA. DNA digests (5 μg per lane as assessed by a fluorimetric assay[52]) were electrophoresed on 0.8–1.5% agarose gels at 70 V for 4 h, then transferred to nitrocellulose by the modification of Southern's[39] procedure described by Wahl *et al.*[40]. pBR322 plasmids containing cDNA inserts of human growth hormone (chGH800/pBR322; ref. 41), human γ-globin (JW101; ref. 42) and human α-globin (JW101; ref. 42), were grown in L-broth, and plasmid DNA was isolated by standard techniques[53,54]. The inserts from these plasmids were purified and the DNA labelled with $^{32}$P-dCTP to a specific activity of $10^9$ d.p.m. $\mu g^{-1}$ using a technique described elsewhere[55]. The blots obtained by this method were identical to those obtained when probes were labelled by nick-translation, but exposure times were significantly reduced. The probes were hybridized to the filters for 36–60 h and then washed according to a protocol supplied by K. Peden[56]. The autoradiographs shown were exposed for 2–4 days using pre-exposed Kodak XAR-5 film with DuPont Lightning Plus intensifying screens[57].

# 1. The role of DNA methylation in human cancer

## *A. HYPOMETHYLATION –> alteration in gene expression*

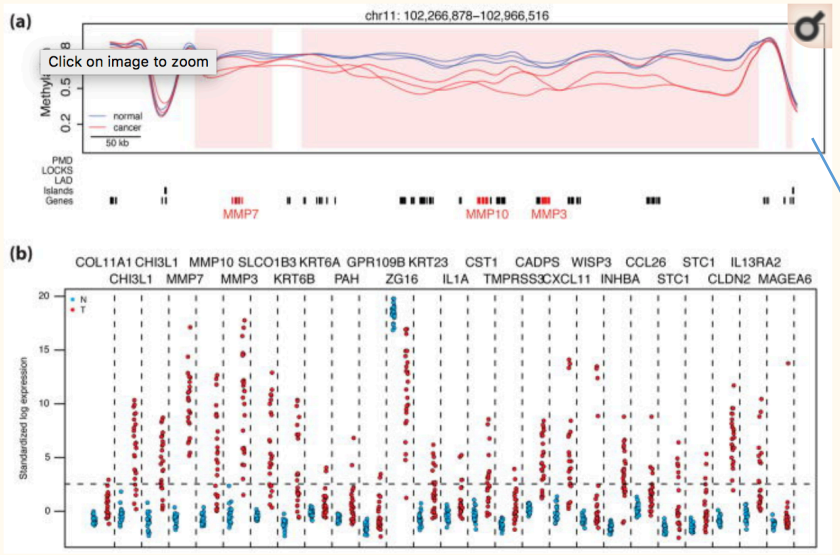



Figure 5

**High variability of gene expression associated with blocks**

**(a)** An example of hypervariably expressed genes contained within a block; note genes *MMP7*, *MMP10*, and *MMP3* highlighted in red. Methylation values for cancer samples (red) and normal samples (blue) with hypomethylated block locations highlighted (pink shading) are plotted against genomic location. Grey bars are as in Fig. 2. **(b)** Standardized log expression values for 26 hypervariable genes in cancer located within hypomethylated block regions (normal samples in blue, cancer samples in red). Standardization was performed using the gene expression barcode. Genes with standardized expression values below 2.54, or the 99.5th percentile of a normal distribution (horizontal dashed line) are determined to be silenced by the barcode method[26]. Vertical dashed lines separate the values for the different genes. Note there is consistent expression silencing in normal samples compared to hypervariable expression in cancer samples. A similar plot drawn from an alternative GEO dataset is shown in Supplementary Figure 18.

Stochastic methylation variation of differentially methylated regions, distinguishing cancer from normal tissue, in colon, lung, breast, thyroid and Wilms' tumors.

Whole-genome bisulfite sequencing shows these variable DMRs are related to loss of sharply delimited methylation boundaries at CpG islands.

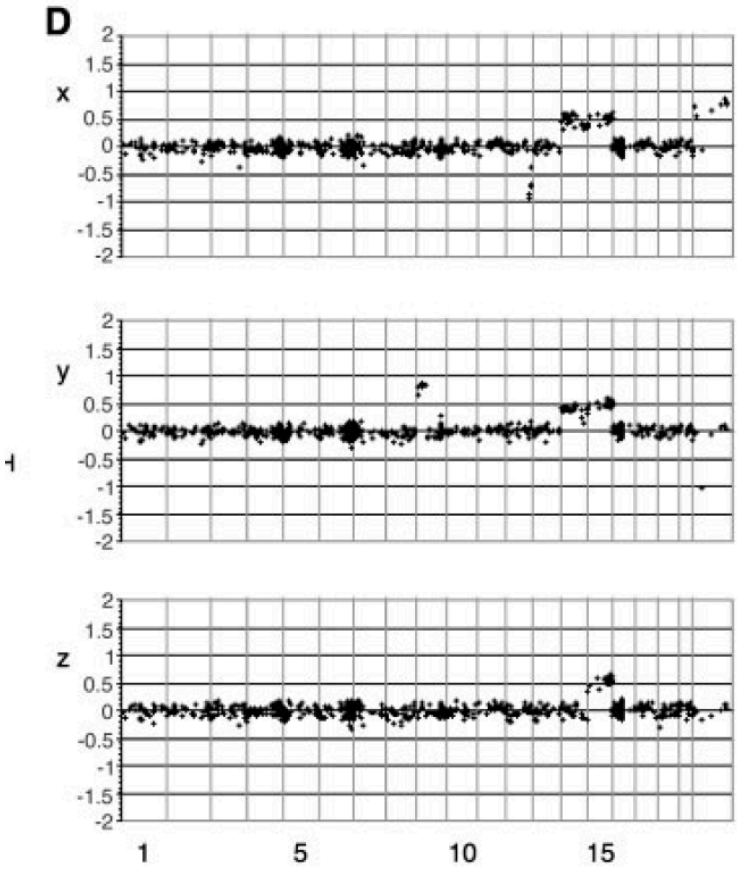
Furthermore, we find hypomethylation of discrete blocks encompassing half the genome, with extreme gene expression variability.

«We suggest a model for cancer involving loss of epigenetic stability of well-defined genomic domains that underlies increased methylation variability in cancer that may contribute to tumor heterogeneity.»

Hansen KD et al. Nat Genet. 2011. Increased methylation variation in epigenetic domains across cancer types.

# 1. The role of DNA methylation in human cancer

## *A. HYPOMETHYLATION –> genomic instability*




**Mouse model for cancer:**

Hypermorph of DNMT1 ($DNMT^{chip}$) reduced DNA methylation $DNMT1^{-/Chip}$ mice are viable; 80% develop aggressive lymphoma

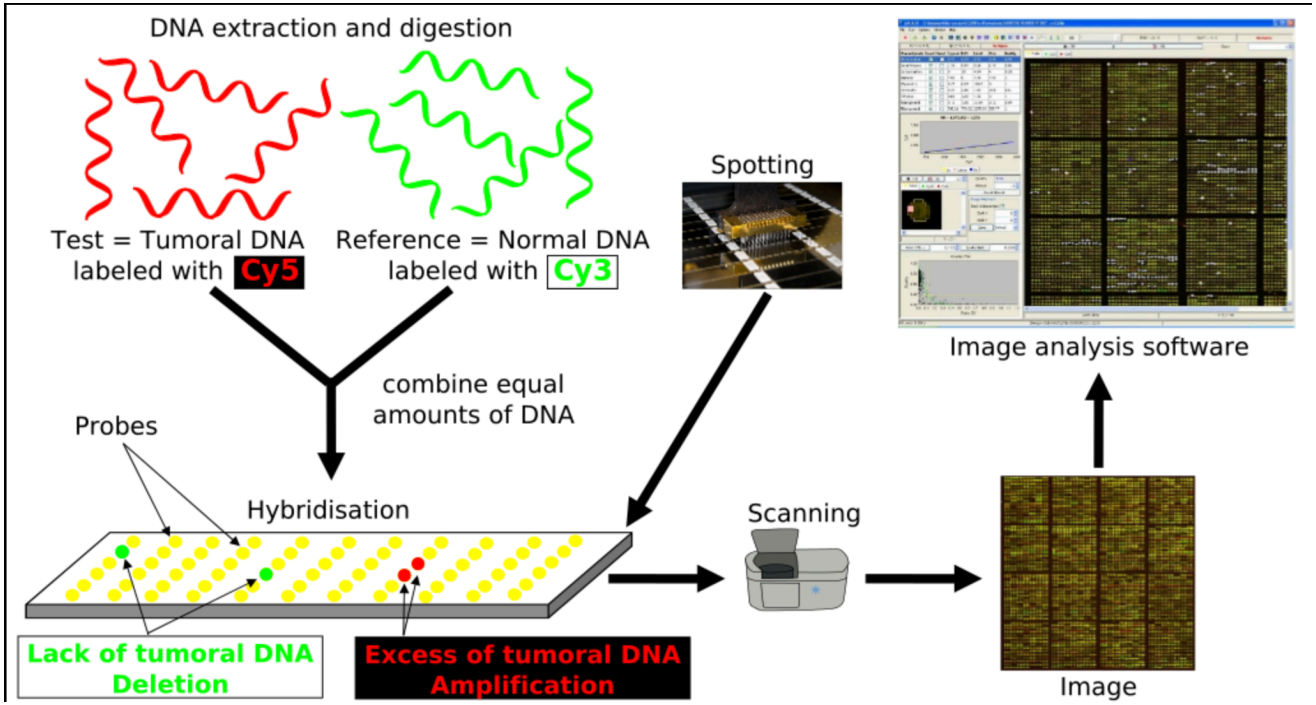
**CGH array shows genomic instability:**

The increased fluorescence ratios observed for chromosomes 14 and 15 are consistent with singlecopy whole-chromosome gains throughout the tumor

(D) Array comparative genome hybridization (CGH) analyses of three Dnmt1chip/–tumors, showing clear single-copy, whole-chromosome gain of chromosome 15 (x, y, and z), whole-chromosome gains of 14and loss on distal 12 (x), andgains of chromosome 14and proximal 9 (y). The X gain (x) reflects a sex difference between tumor and control. Array CGH was performed as in (26). Fluorescence ratios (average of quadruplicate measurements) for each bacterial artificial chromosome are plotted as a function of genome location based onthe February 2002 freeze of the assembled mouse genome sequence (http://genome.ucsc.edu). Verticallines delimit chromosome boundaries.

**Gaudet et al Science 2003**

# Comparative genomic hybridization (CGH)

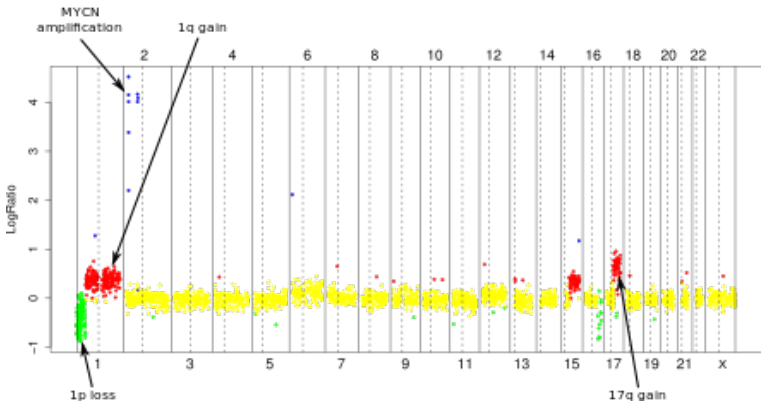



**Methodology**

Array CGH is based on the same principle as conventional CGH. In both techniques, DNA from a reference (or control) sample and DNA from a test (or patient) sample are differentially labelled with two different fluorophores and used as probes that are cohybridized competitively onto nucleic acid targets. (((In conventional CGH, the target is a reference metaphase spread))). In array CGH, these targets can be genomic fragments cloned in a variety of vectors (such as BACs or plasmids), cDNAs, or oligonucleotides.[18]Figure 2.[15] is a schematic overview of the array CGH technique. DNA from the sample to be tested is labeled with a red fluorophore (Cyanine 5) and a reference DNA sample is labeled with green fluorophore (Cyanine 3). Equal quantities of the two DNA samples are mixed and cohybridized to a DNA microarray of several thousand evenly spaced cloned DNA fragments or oligonucleotides, which have been spotted in triplicate on the array. After hybridization, digital imaging systems are used to capture and quantify the relative fluorescence intensities of each of the hybridized fluorophores.[18] The resulting ratio of the fluorescence intensities is proportional to the ratio of the copy numbers of DNA sequences in the test and reference genomes. If the intensities of the flurochromes are equal on one probe, this region of the patient's genome is interpreted as having equal quantity of DNA in the test and reference samples; if there is an altered Cy3:Cy5 ratio this indicates a loss or a gain of the patient DNA at that specific genomic region.[19]

**Technological approaches to array CGH**

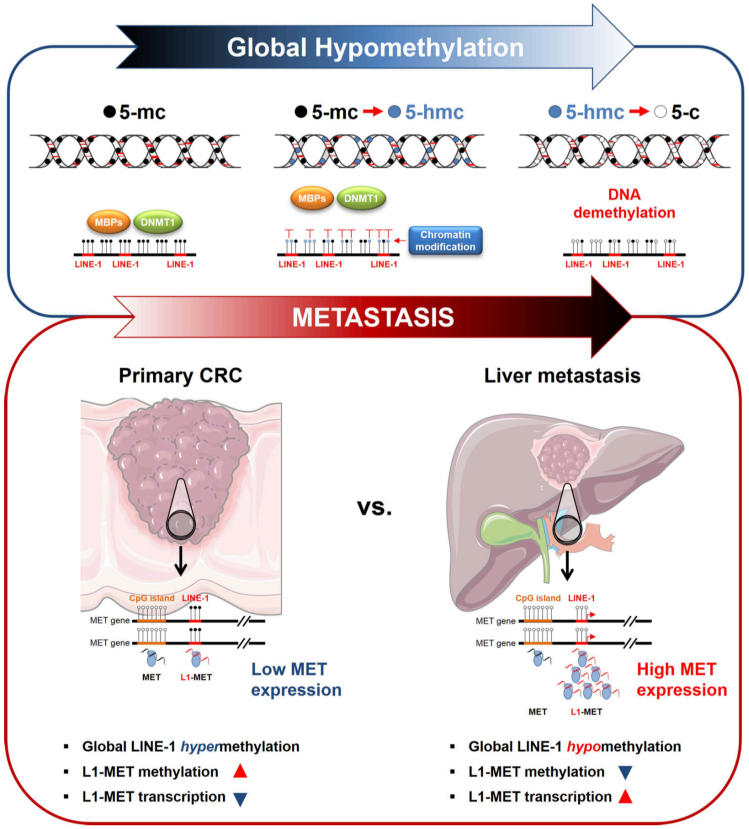
Array CGH has been implemented using a wide variety of techniques. Therefore, some of the advantages and limitations of array CGH are dependent on the technique chosen. The initial approaches used arrays produced from large insert genomic DNA clones, such as BACs. The use of BACs provides sufficient intense signals to detect single-copy changes and to locate aberration boundaries accurately. However, initial DNA yields of isolated BAC clones are low and DNA amplification techniques are necessary. These techniques include ligation-mediated polymerase chain reaction (PCR), degenerate primer PCR using one or several sets of primers, and rolling circle amplification.[20] Arrays can also be constructed using cDNA. These arrays currently yield a high spatial resolution, but the number of cDNAs is limited by the genes that are encoded on the chromosomes, and their sensitivity is low due to cross-hybridization.[15] This results in the inability to detect single copy changes on a genome wide scale.[21] The latest approach is spotting the arrays with short oligonucleotides. The amount of oligos is almost infinite, and the processing is rapid, cost-effective, and easy. Although oligonucleotides do not have the sensitivity to detect single copy changes, averaging of ratios from oligos that map next to each other on the chromosome can compensate for the reduced sensitivity.[22] It is also possible to use arrays which have overlapping probes so that specific breakpoints may be uncovered.




ACGH profile of the IMR32 neuroblastoma cell line

# 1. The role of DNA methylation in human cancer

## *A. HYPOMETHYLATION –> genomic instability, altered gene expression*



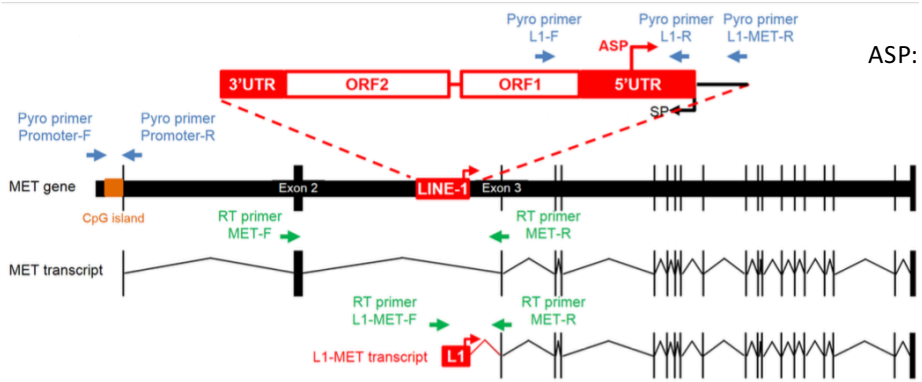

**Coloretal cancer + liver metastasis:**

Reduced DNA methylation in LINE1 retrotransposons

LINE1 elements contain normal promoter but also an additional promoters that can activate downstream genes (i.e. MET oncogene)

LINE1 elements are normally epigenetically silenced by DNA methylation

Metastasis → loss of LINE1 methylation → increases expression
Of MET onocgene.




ASP: L1 antisense promoter

A consolidated model for the activation mechanism of the MET proto-oncogene by LINE-1 hypomethylation in colorectal cancer (CRC) metastasis. In this model, hypomethylation of CpG sites is characterised by enhanced conversion of 5-mc (5-methylcytosine) to 5-c (5-cytosine), with an intermediary generation of 5-hmc (5-hydroylated methylcytosine). Enhanced CpG demethylation with consequent accumulation of 5-hmc prevents the binding of various DNA methyltransferases (DNMTs) and methyl binding proteins (MBPs) to CpG sites, triggering a cascade of events that favour DNA hypomethylation. Occurrence of DNA hypomethylation events in the context of LINE-1 elements can potentially permit activation of certain oncogenes, including the MET proto-oncogene which may result in accelerated CRC metastasis.

Hur et al. 2014

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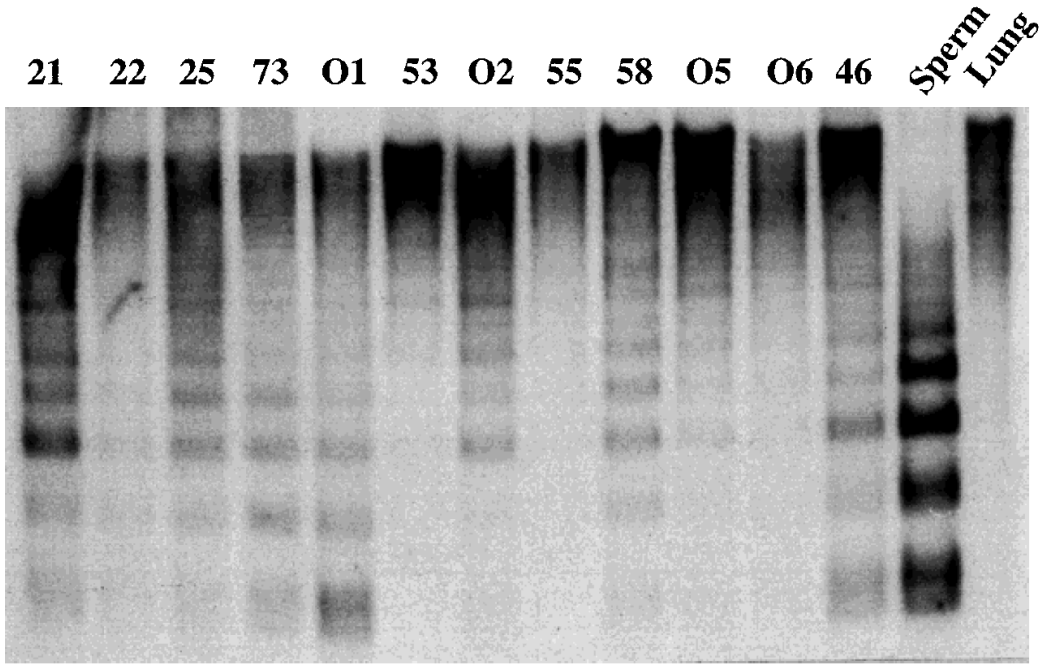



**Prepare DNA from healthy and tumor tissue and sperm**
**Digest with methylation sensitive restriction enzyme (does not cut metylated DNA)**
**Probe southern blot with probes cDNA probes of different genes**
**Cancer tissue: smaller fragments → methylation lost**

**FIGURE 4** – Analysis of methylation of Chr1 Sat2 DNA sequences in malignant breast tissue samples. Breast adenocarcinoma DNA samples, in the first 12 lanes, and sperm and lung DNA samples, in the last 2 lanes, were analyzed for Chr1 Sat2 DNA hypomethylation. The 5 lowest bands in the sperm DNA samples, which were also seen in the breast cancer samples showing considerable hypomethylation (21, 25, 73, O1, 46), had DNA fragments of about 0.8, 1.3, 1.8, 2.2 and 2.7 kb. In addition, a hybridizing fragment migrating at the position for 4.0-kb DNA is seen above the 2.7-kb fragment in samples 21, 25, O2 and 46.

Narayan et al 1998

# 1. Alteration of DNA methylation in human cancer

**CANCER IS ASSOCIATED WITH GLOBAL DNA HYPOMETHYLATION and LOCAL DNA HYPERMETHYLATION**

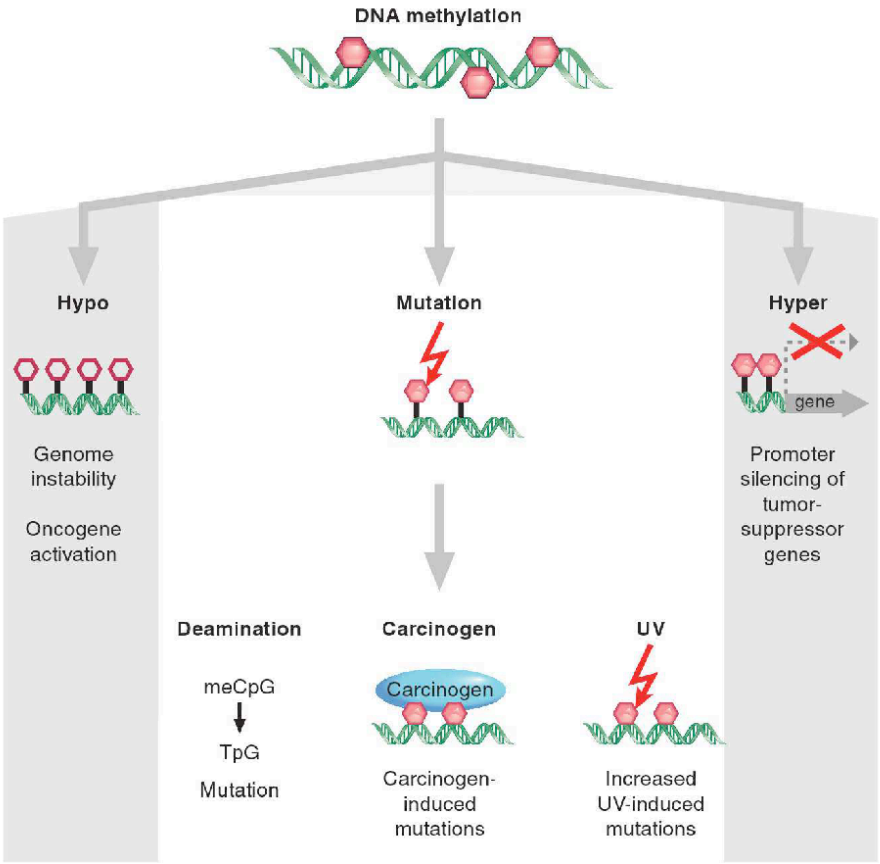



Figure 3. Epigenetic alterations involving DNA methylation can lead to cancer by various mechanisms. Loss of DNA cytosine methylation (white hexagons) illustrated in the hypo column results in genome instability. Focal hypermethylation (pink hexagons) at gene promoters shown in the hyper column causes heritable silencing and, therefore, inactivation of tumor suppressors and other genes. Additionally, methylated CpG sites (pink hexagons) are prone to mutation: They are hot spots for C to T transition mutations caused by spontaneous hydrolytic deamination; or methylation of CpG sites can increase the binding of some chemical carcinogens to DNA; and it increases the rate of UV-induced mutations.

Normal tissue: 80% of CpG methylated
Cancer: 40-60% of CpG methylated

Global DNA methylation is lost from large blocks (28kb – 10Mb)

Local hypermethylation at specific locations such as tumor suppressors

# 1. The role of DNA methylation in human cancer

## *B. UV damage at 5mC*

Thymidine dimers

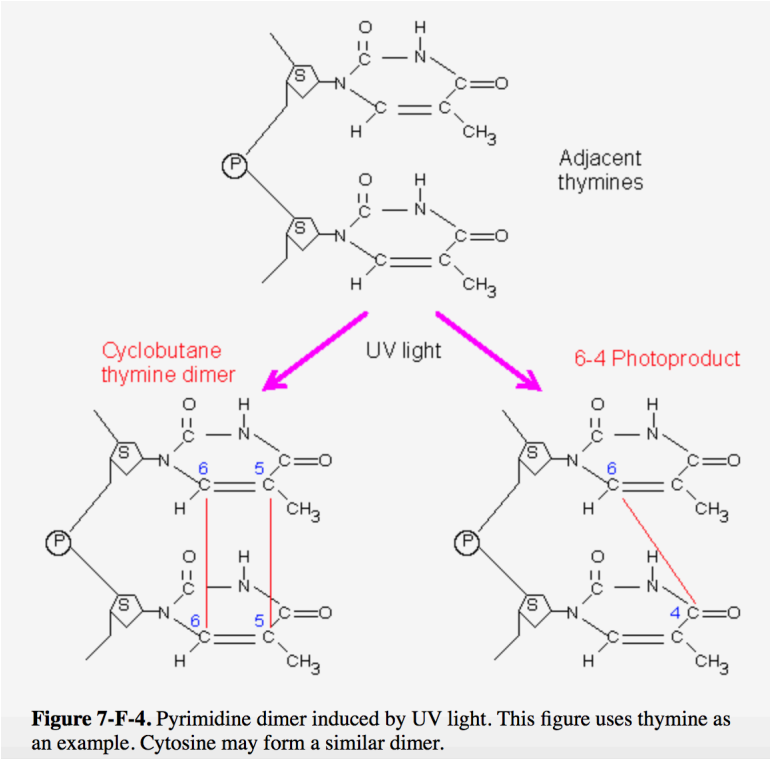
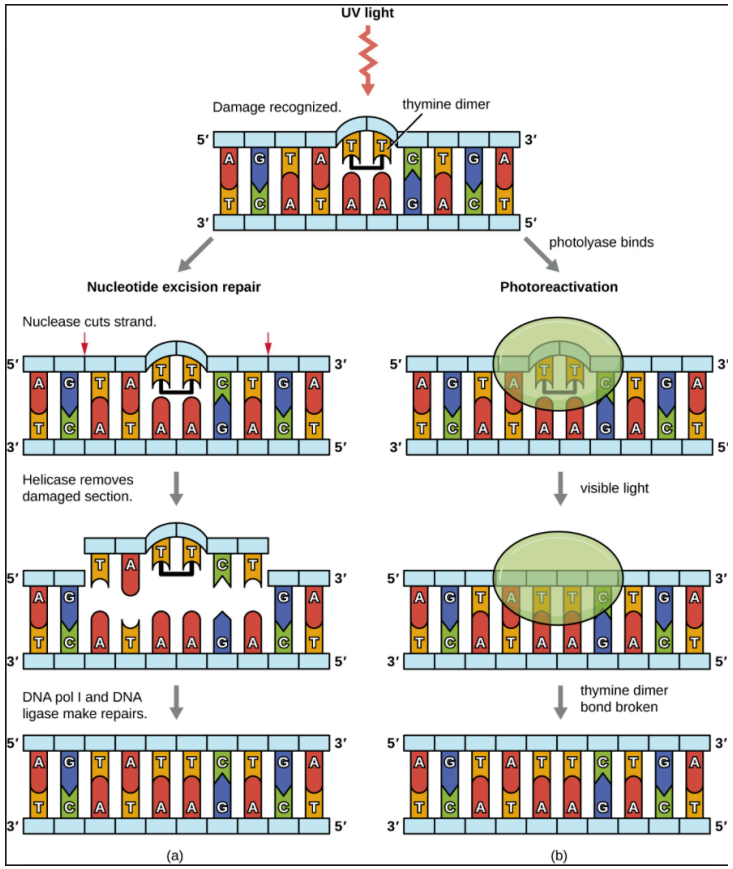


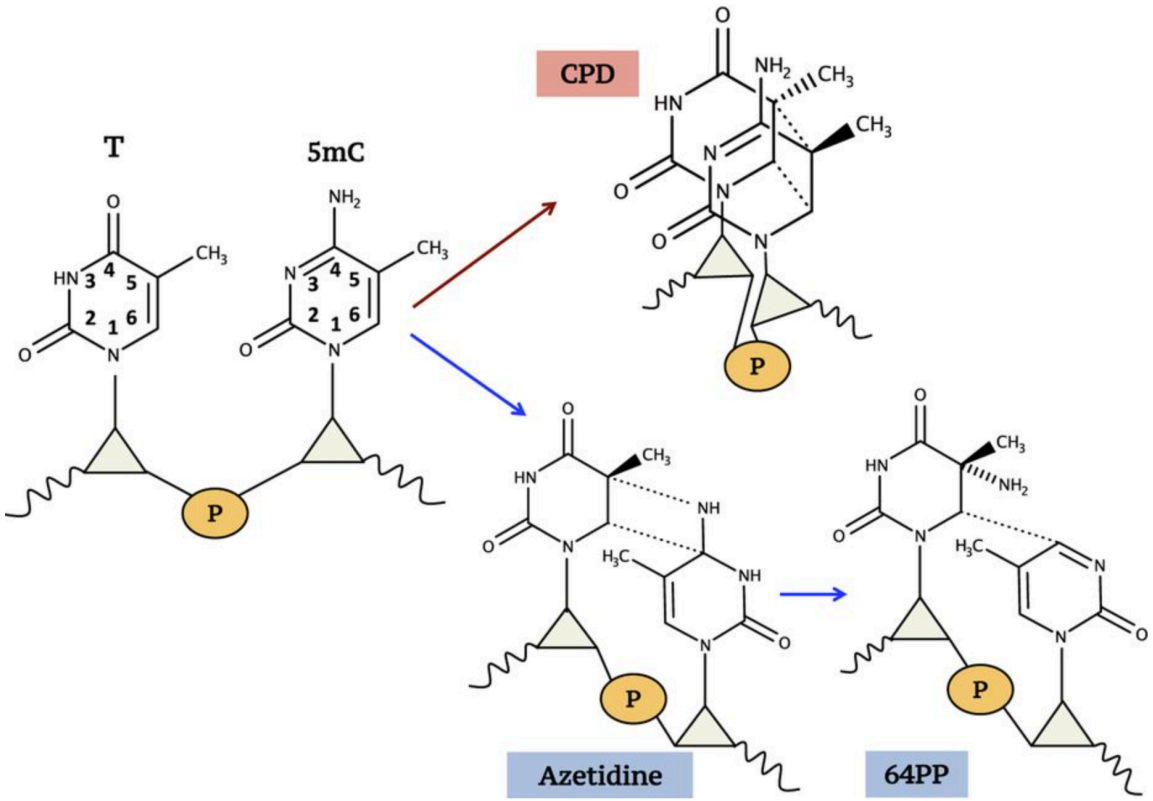

**Figure 7-F-4.** Pyrimidine dimer induced by UV light. This figure uses thymine as an example. Cytosine may form a similar dimer.




Not in placental mammals

# 1. The role of DNA methylation in human cancer

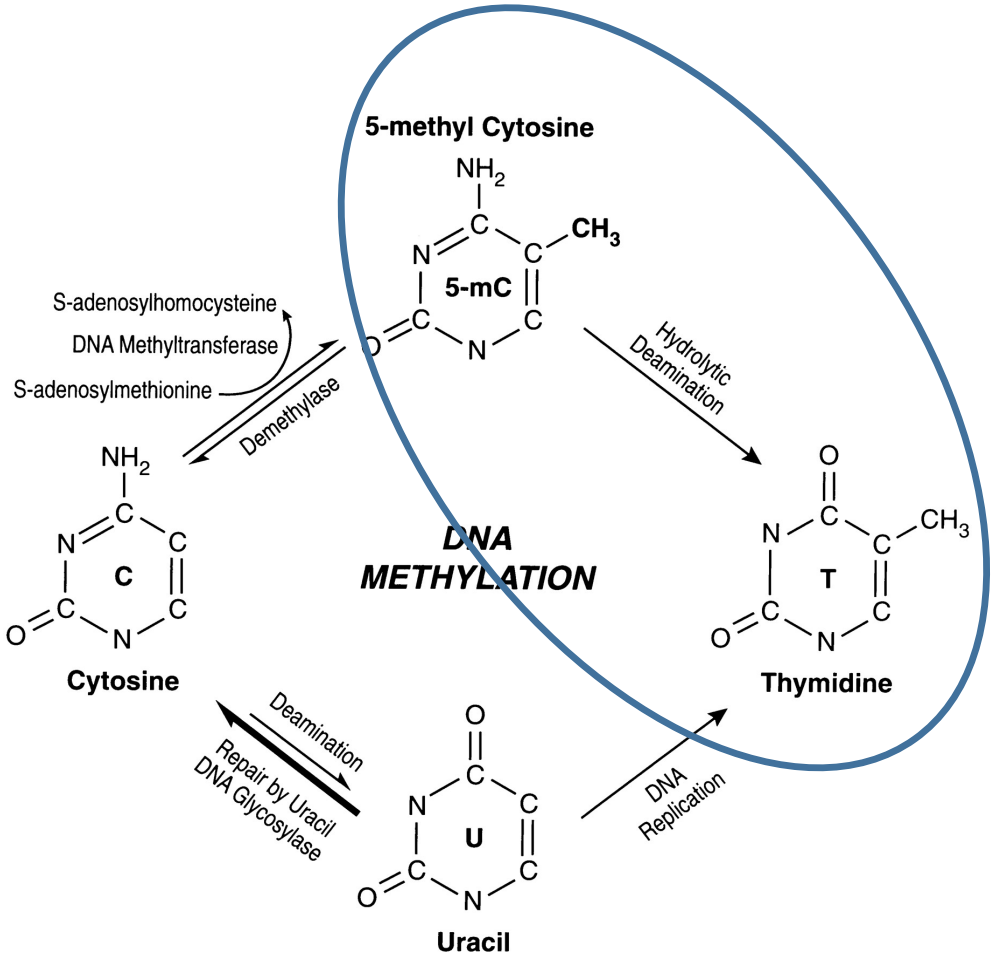
## *B. UV damage at 5mC*




Formation of T5mC dimeric photoproducts. CPD, cyclobutane pyrimidine dimers (c,s stereo isomers); 64PP, pyrimidine (6-4) pyrimidone photoproducts, sugars are represented by triangles, phosphate groups by a circle.

# 1. The role of DNA methylation in human cancer

## *B. INCREASED MUTATION RATE RELATED TO 5mC*




**5mC transition mutation to T is most frequent type of transition mutation (for example 50% of p53 mutations in colorectal cancer occur at sites of DNA methylation)**

**5mC →T is frequent in exons of tumorsuppressor gene bodies**

**5mC →promotes the formation of adducts with benzo(a)pyrene diol epoxide → increased mutation rate in smokers**

# 1. The role of DNA methylation in human cancer

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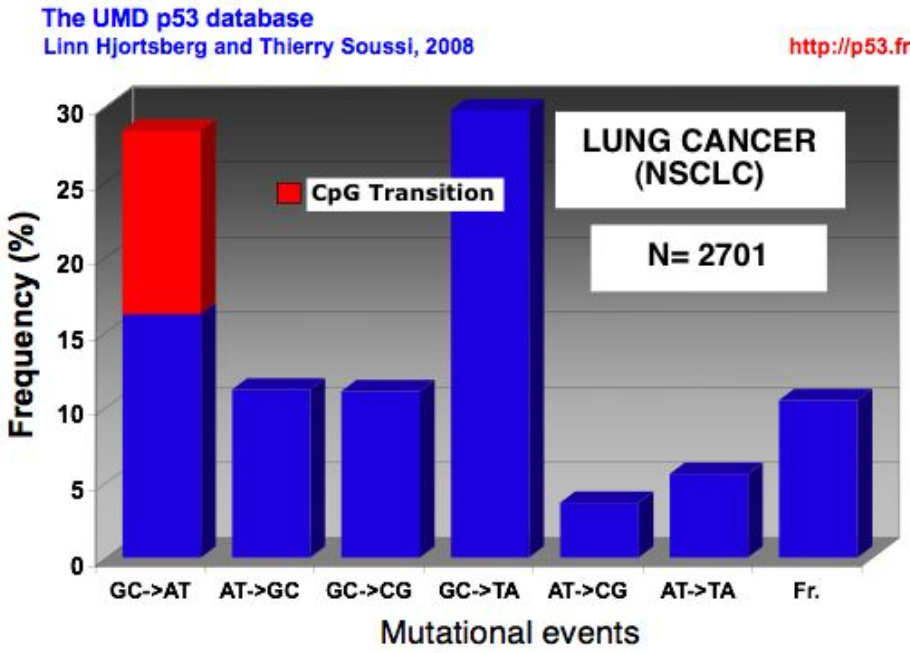
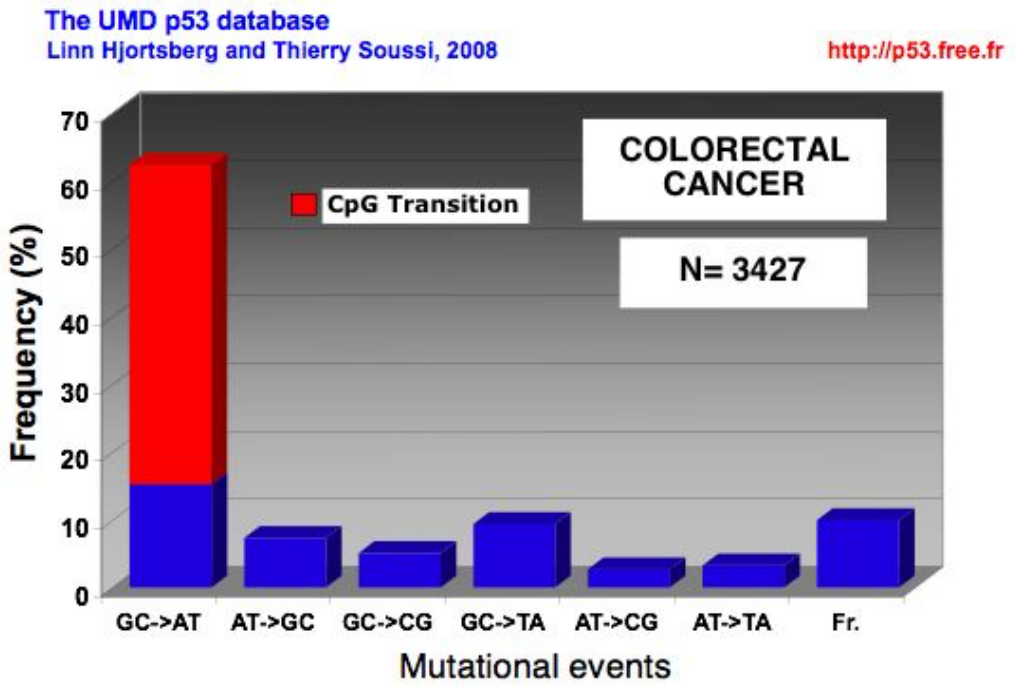
**5mC →T is frequent in exons of the tumorsuppressor gene p53 in colorectal cancer and lung cancer**



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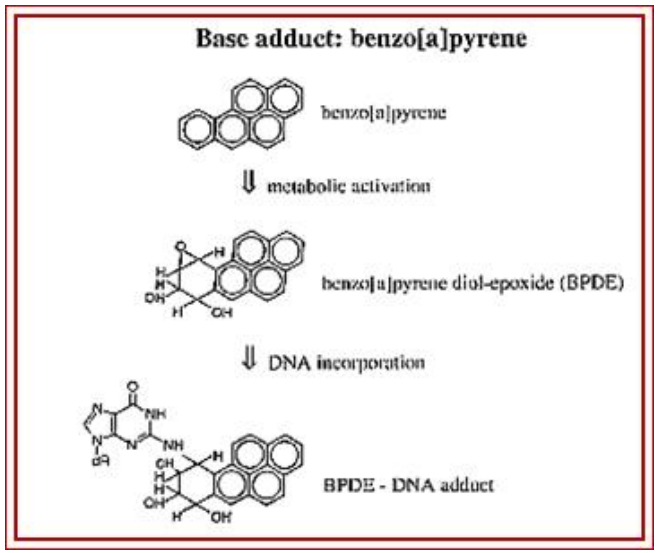


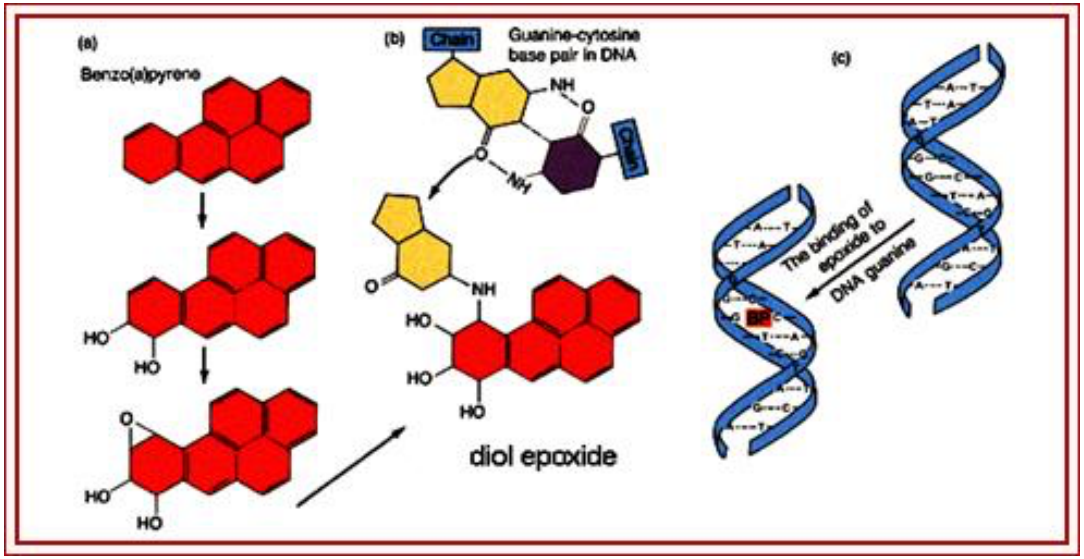

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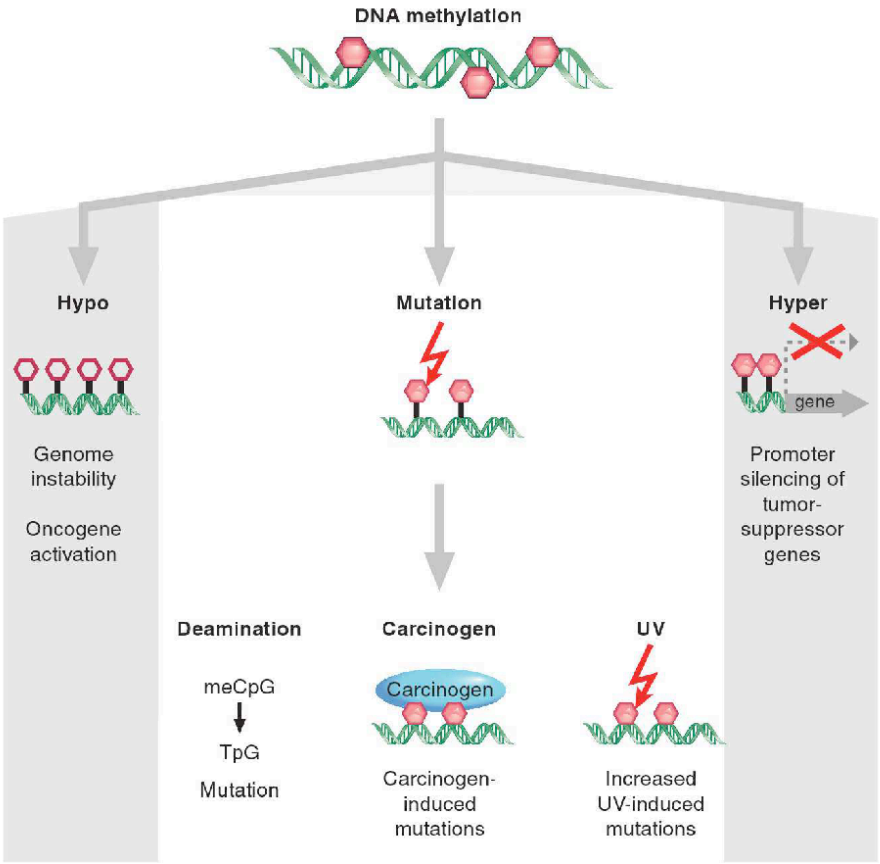


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# 1. The role of DNA methylation in human cancer

## *C. HYPERMETHYLATION*

In human cancer, DNA methylation occurs at CpG islands that are located in and around the transcriptional start site of abnormally silenced genes (in cancer cells)
Typically 5-10% of these CpG island promoters are methylated in cancer.

In an individual tumor hundreds of genes can be subjected to this type of epigenetic alteration.
Apparently, the number of genes with hypermethylated promoters outnumbers the number of genes mutated in the same cancer.

→ **Gene silencing of tumorsuppressors**

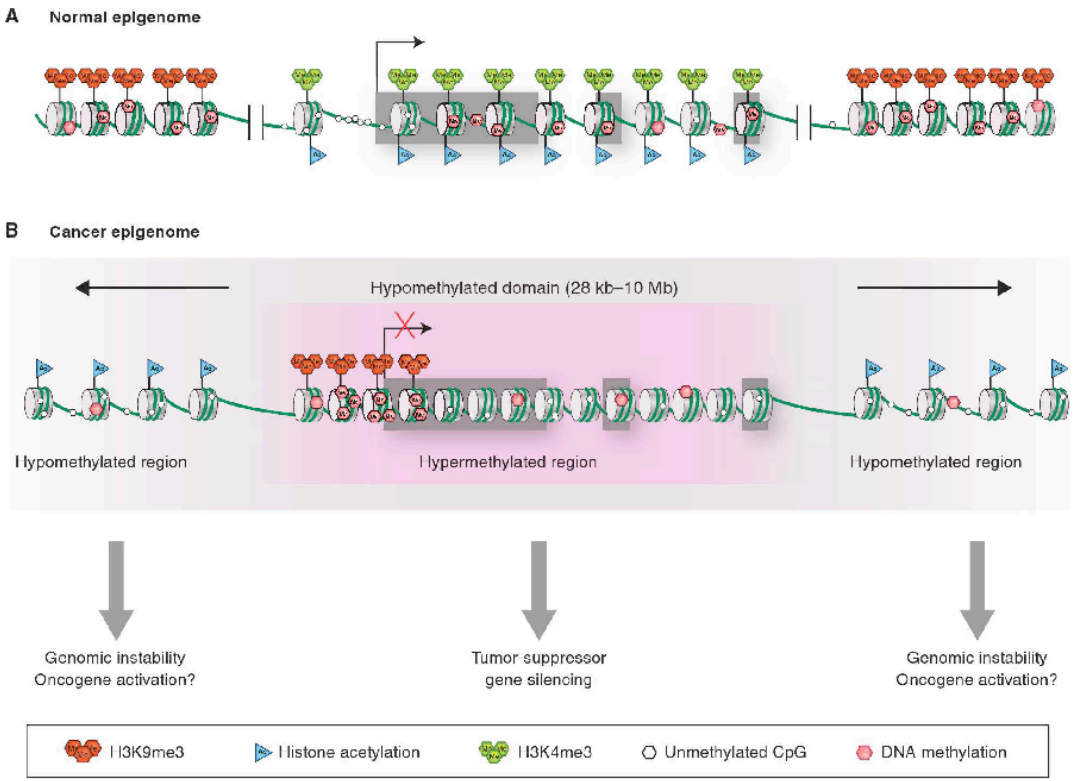


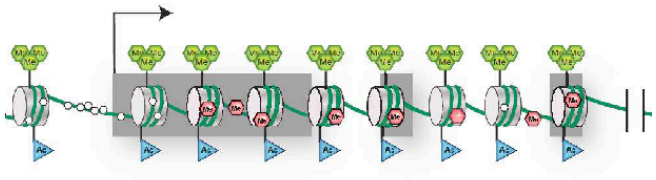

**Figure 4.** Chromatin structural changes in cancer cells. (*A*) In a typical cell, a CpG-island-containing active gene can be recognized by virtue of a nucleosome-depleted promoter, absence of promoter DNA methylation, but marked by H3K4me3 surrounding the promoter and histone acetylation along the locus. Gene body CpG methylation often can be observed. Nongenic regions flanking an active gene are frequently marked by repressive epigenetic marks, such as H3K9me3 and 5mC. (*B*) The cancer epigenome is characterized by simultaneous global losses in DNA methylation (gray shading), interspersed with silenced genes that have abnormal gains of DNA methylation and repressive histone modifications in CpG island promoter regions. These silenced genes may be hypomethylated in their gene body, similar to surrounding chromatin. The hypomethylated regions can have an abnormally open nucleosome configuration and acetylated histone lysines. Conversely, abnormal DNA hypermethylation in promoter CpG islands of silenced genes is associated with nucleosomes positioned over the transcription start sites.

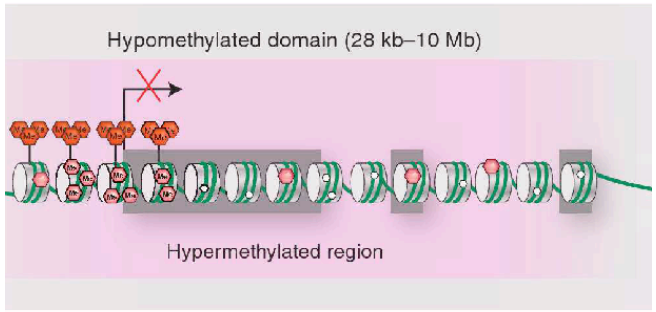
# 2. HYPERMETHYLATION OF GENE PROMOTERS of TSGs (tumor suppressor genes) IN CANCER

**NORMAL DEVELOPMENT:** Promoters of 60% of genes contain CpG islands → vast majority is never DNA methylated during development

**CANCER:** 5–10% of the thousands of promoter CpG islands that never normally contain DNA methylation from embryonic development onwards show increased methylation in human cancer

**Silencing of tumor suppressors ("driver") but also potential "passengers"**

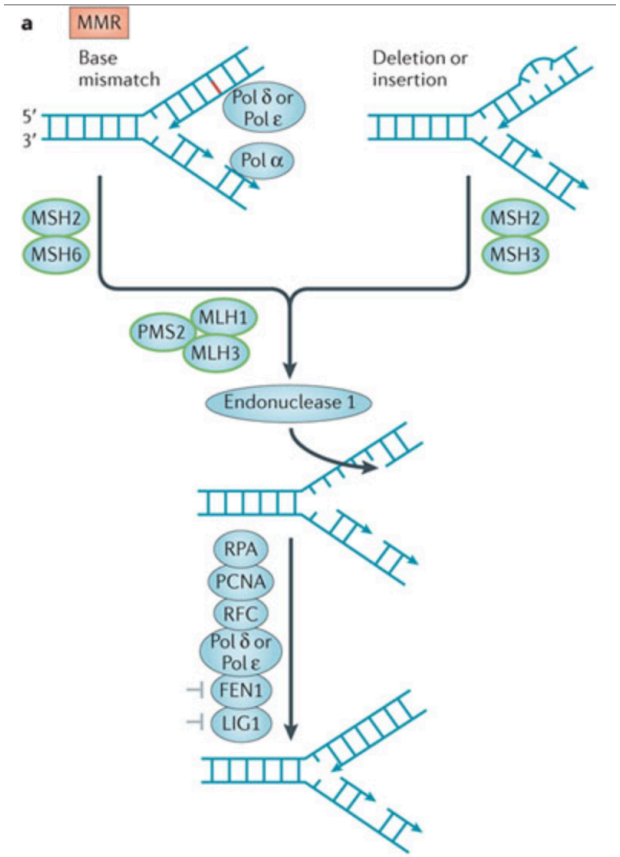





| Class of hypermethylated gene | Examples | |
|---|---|---|
| Known TSG[a] | *VHL*<br>*E-cadherin*<br>*P16Ink4a*<br>*MLH1*<br>*APC*<br>*Stk4*<br>*Rb* | ***Also frequently mutated = driver mutations*** |
| Candidate TSG | *FHIT*<br>*Rassf1a*<br>*O6-MGMT*<br>*Gst-Pi*<br>*GATA4/5*<br>*DAP-kinase* | ***Have role in tumorsuppression, but are not frequently mutated*** |
| Gene discovered through random screens for hypermethylated genes | *HIC-1*<br>*SFRP1, -2, -4, -5*<br>*BMP-3*<br>*SLC5A8*<br>*SSI1* | ***Identified in screens for DNA methylation, but function of gene is not clear*** |

[a] A classic tumor-suppressor gene (TSG) is known to be mutated in the germline of families with hereditary cancer syndromes.

# 2. HYPERMETHYLATION OF GENE PROMOTERS OF TSGs: MLH1 in colorectal cancer

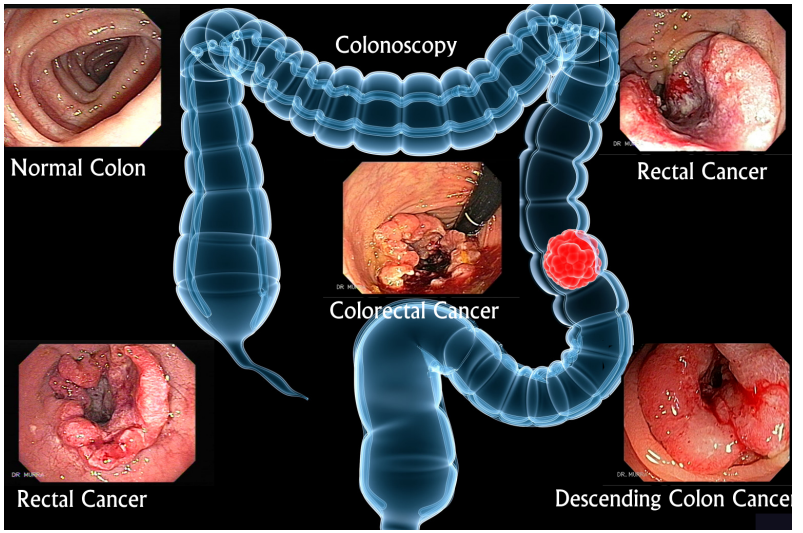



**DNA missmatch repair**

**MLH1: DNA mismatch repair pathway**

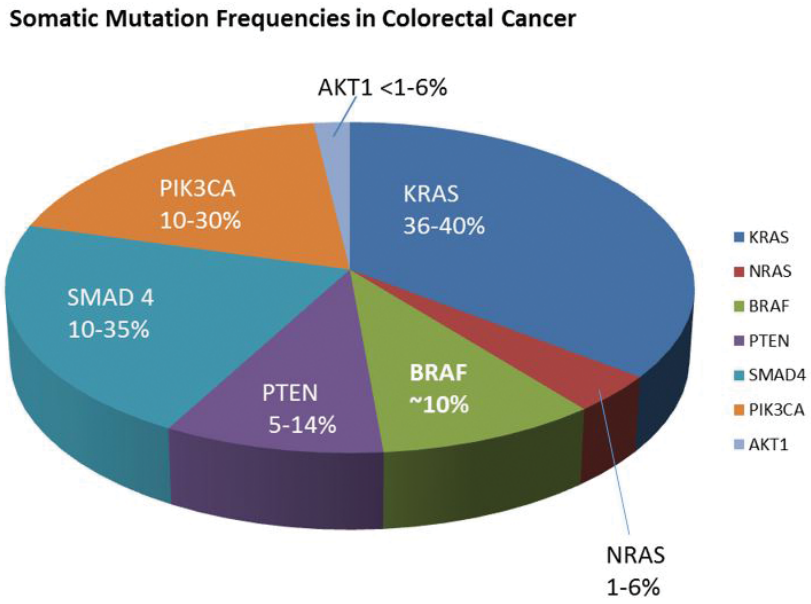
- Defect in DNA damage repair
- Accumulation of mutation
- Genomic instability (microsatellite instability)
- Driver mutation for colon cancer

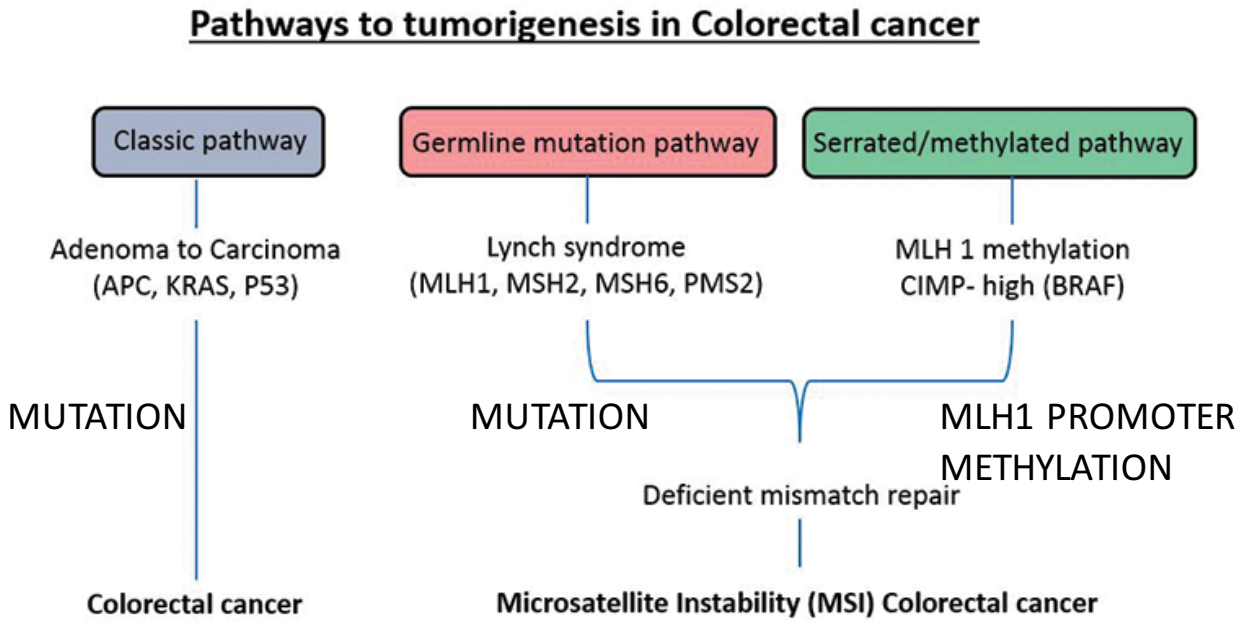
**A subclass of colorectal cancer is characterized by a hypermethylation of CpG islands at the MLH1 promoter**



# 2. HYPERMETHYLATION OF GENE PROMOTERS OF TSGs: MLH1 in colorectal cancer

**MLH1 is not frequently mutated**

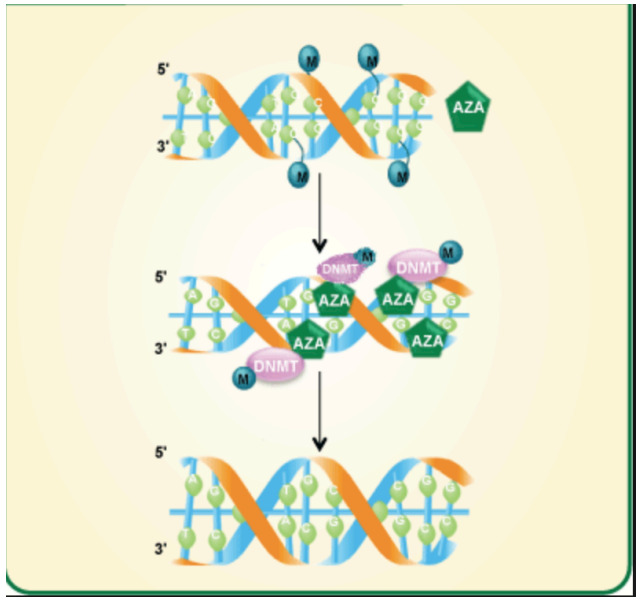


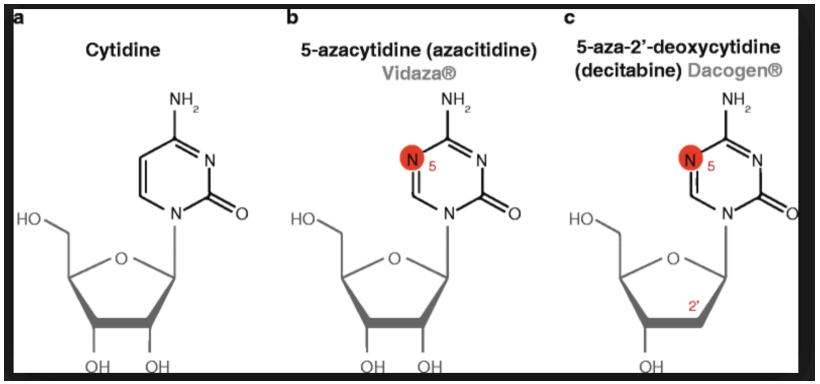




**Lynch syndrome** (HNPCC or hereditary nonpolyposis colorectal cancer) is an autosomal dominant genetic condition that has a high risk of colon cancer[1] as well as other cancers including endometrial cancer (second most common), ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin. The increased risk for these cancers is due to inherited mutations that impair DNA mismatch repair. **It is a type of cancer syndrome**. MUTATIONS in MLH1, MSH2, MSH6, PMS2

**Serrated adenocarcinoma** is a recently described subset of colorectal cancer (CRC), which account for about 10% of all CRCs and follows an alternative pathway in which serrated polyps replace the traditional adenoma as the precursor lesion to CRC. Serrated polyps form a heterogeneous group of colorectal lesions that includes hyperplastic polyps (HPs), sessile serrated adenoma (SSA), traditional serrated adenoma (TSA) and mixed polyps.

# EPIGENETIC DRUGS: DNMT INHIBITORS → loss of DNA methylation




Azacitidine is a **chemical analogue of the nucleoside cytidine**, which is present in DNA and RNA. It is thought to have antineoplastic activity via two mechanisms – at low doses, by **inhibiting of DNA methyltransferase, causing hypomethylation of DNA, and at high doses, by its direct cytotoxicity to abnormal hematopoietic cells** in the bone marrow through its incorporation into DNA and RNA, resulting in cell death. Azacitidine is a ribonucleoside, so it is incorporated into RNA to a larger extent than into DNA. In contrast, **decitabine (5-aza-2'-deoxycytidine) is a deoxyribonucleoside, so it can only incorporate into DNA**. Azacitidine's incorporation into RNA leads to the dissembly of polyribosomes, defective methylation and acceptor function of transfer RNA, and inhibition of the production of proteins**. Its incorporation into DNA leads to covalent binding with DNA methyltransferases, which prevents DNA synthesis and subsequent leads to cytotoxicity.**

After azanucleosides such as **azacitidine have been metabolized to 5-aza-2'-deoxycytidine-triphosphate** (aka, decitabine-triphosphate), they can be incorporated into DNA and azacytosine can be **substituted for cytosine**. Azacytosine-guanine dinucleotides are recognized as substrate by the DNA methyltransferases, which catalyze the methylation reaction by a nucleophilic attack. This **results in a covalent bond between the carbon-6 atom of the cytosine ring and the enzyme. The bond is normally resolved by beta-elimination through the carbon-5 atom, but this latter reaction does not occur with azacytosine because its carbon-5 is substituted by nitrogen, leaving the enzyme remains covalently bound to DNA and blocking its** DNA methyltransferase function.

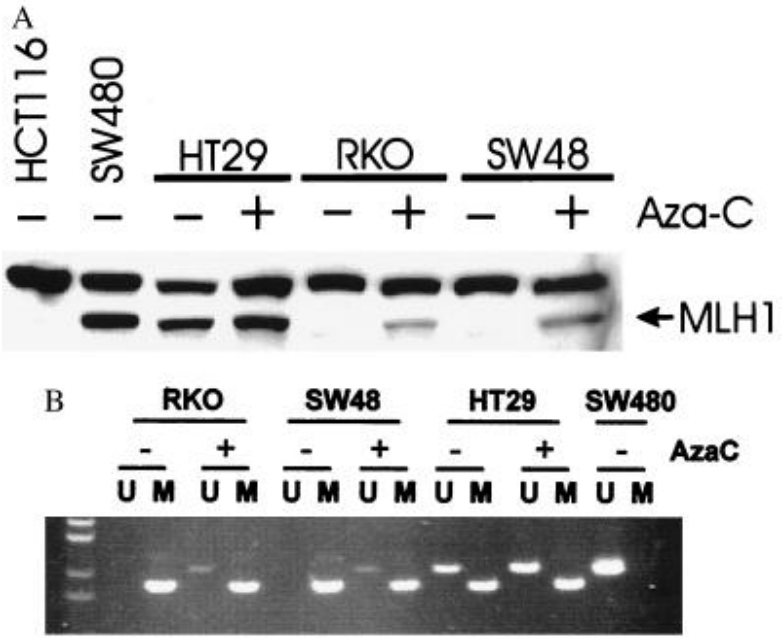
In addition, the **covalent protein adduction also compromises the functionality of DNA and triggers DNA damage signaling, resulting in the degradation of trapped DNA methyltransferases. As a consequence, methylation marks become lost during DNA replication.**

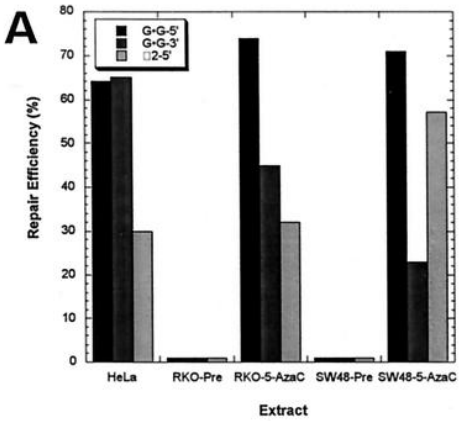
## 2. HYPERMETHYLATION OF GENE PROMOTERS OF TSGs: MLH1 in colorectal cancer

**Reversibly of DNA damage repair defect caused by MLH1 silencing**

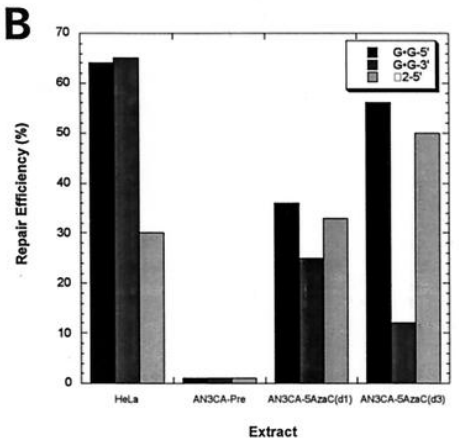
**Treatment of colon cancer cell line (with silenced MLH1) with 5-Aza-2′-deoxycytidine**

**Re-expression of MLH1 and rescue of DNA damage repair**




DNA damage repair assay

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***Also frequently mutated = clear driver mutations***

***Drivers or cancer or passengers??***

[a] A classic tumor-suppressor gene (TSG) is known to be mutated in the germline of families with hereditary cancer syndromes.

**Drivers:** genes that are causal for tumor formation

**Passengers:** genes that are not causal for tumor formation, but support the tumorformation and progression

# 2. HYPERMETHYLATION OF GENE PROMOTERS OF TSGs: WHAT ARE THE IMPORTANT GENES?

***Approaches to identify relevance of potential TSG silenced in human cancer***

**Table 5.** Steps in documenting the importance of a hypermethylated gene for tumorigenesis

| | |
|---|---|
| 1. Document CpG island promoter methylation and correlate with transcriptional silencing of the gene and ability to reverse the silencing with demethylating drugs in culture. | First observation in a caner cell line in vitro<br>→ Bisulfite sequencing, gene expression, 5-aza-2'-deoxycytidine treatment |
| 2. Document correlation of promoter hypermethylation with specificity for this change in tumor cells (cell culture and primary tumors) versus normal cell counterparts and incidence for the hypermethylation change in primary tumors. | Validation of first observation in different type of cancer cells (primary or cancer cell lines and cancer tissue and normal tissue)<br>→ Bisulfite sequencing, gene expression, 5-aza-2'-deoxycytidine treatment, large datasets |
| 3. Document the position of the hypermethylation change for tumor progression of given cancer types. | Fine mapping of critical position<br>→ More detailled methyaltion analysis using cancer progression models (cancer specimen, in vitro models for cancer progression (EMT, oncogenes…) |
| 4. Document the potential significance for the gene silencing in tumorigenesis through gene reinsertion studies in culture and effects on soft agar cloning, growth of tumor cells in nude mouse explants, etc. | Functional experiments<br>→ Reconstitute the expression of the silenced gene (cell models, mouse models), asses key BIOLOGICAL features of cancer cells (proliferation, apoptosis, etc…) |
| 5. Establish function of the protein encoded by the silenced gene, through either known characteristics of the gene or testing for activity of recognized protein motifs in culture systems, etc. | Functional experiments<br>→ Identify the biological pathways the gene is involved |
| 6. Document tumor-suppressor activity and functions of the gene for cell renewal, etc., especially for totally unknown genes, through mouse knockout studies. | Functional experiments<br>→ More complex model systems |
| | Clinical data<br>→ Patient survival according to eventual mutations in the gene of interest, gene expression levels…. |

# 3. THE IMPORTANCE OF EPIGENETIC GENE REGULATION IN EARLY TUMORPROGRESSION

Early phases in colon cancer

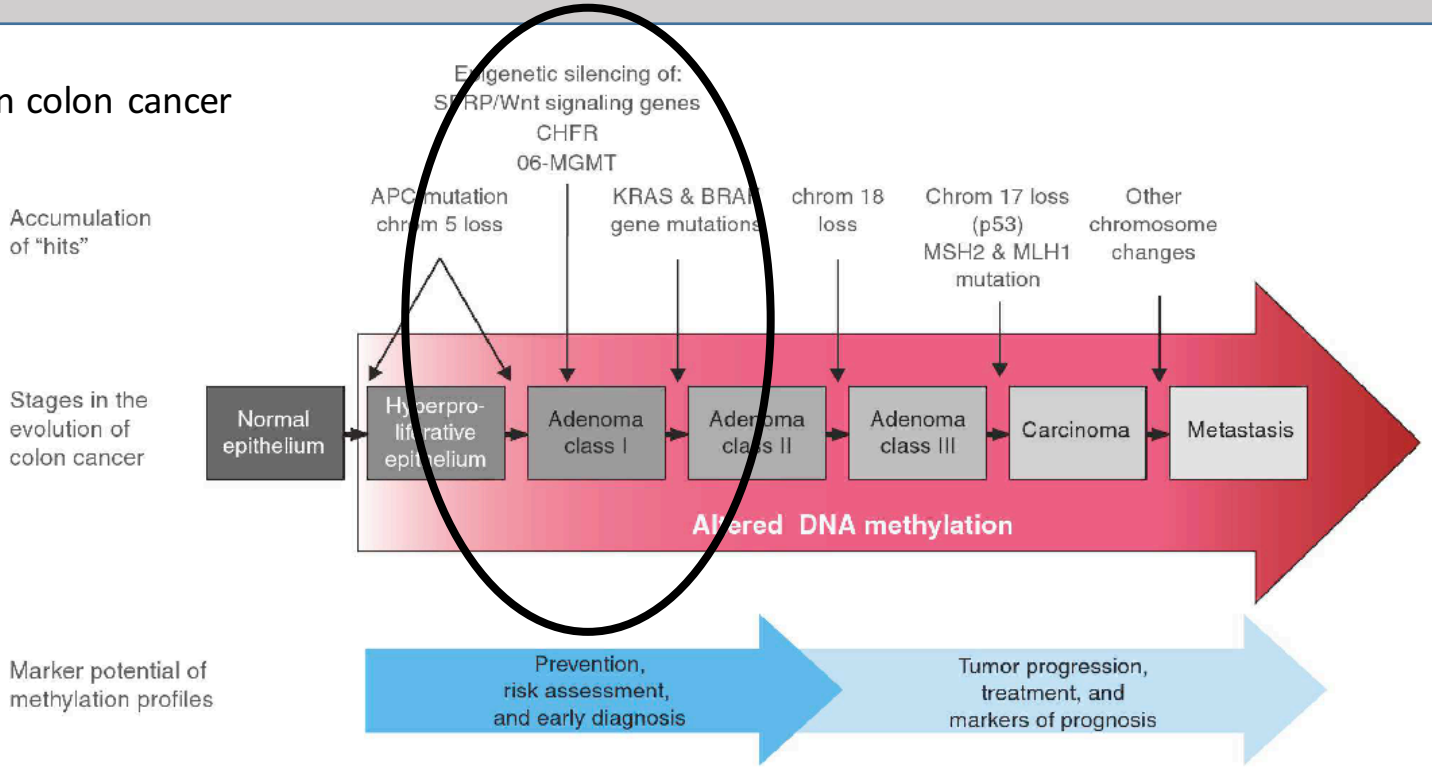



**Figure 5.** The position of abnormal DNA methylation in tumor progression. This is depicted in the classic model (Kinzler and Vogelstein 1997) for genetic alterations during the evolution of colon cancer. Altered DNA methylation is shown to occur from very early on in tumorigenesis (red arrow), as discussed in the text, during the conversion of normal to hyperplastic epithelium, accruing during the progression from noninvasive to invasive and, ultimately, metastatic tumors. This places it in a strategic position for channeling stem cells into abnormal clonal expansion (illustrated in Fig. 6) by cooperating with key genetic alterations. These epigenetic abnormalities also have connotations for cancer treatment and markers of prognosis.

# 3. THE IMPORTANCE OF EPIGENETIC GENE REGULATION IN EARLY TUMORPROGRESSION

Experiments have specifically shown that this leads to the buildup of repressive histone marks in gene promoter regions and, subsequently, DNA hyper-methylation in what may constitute a molecular progression

Engineering the mutations/epigenetic alterations into mice or cells, in vitro, appears to trap stem progenitor cells in states of abnormal self-renewal and/or diminishes their capacity for lineage commitment and differentiation.

Then, inducing DNA de-methylation can partially restore the capacity of cells with the mutations to respond to differentiation cues

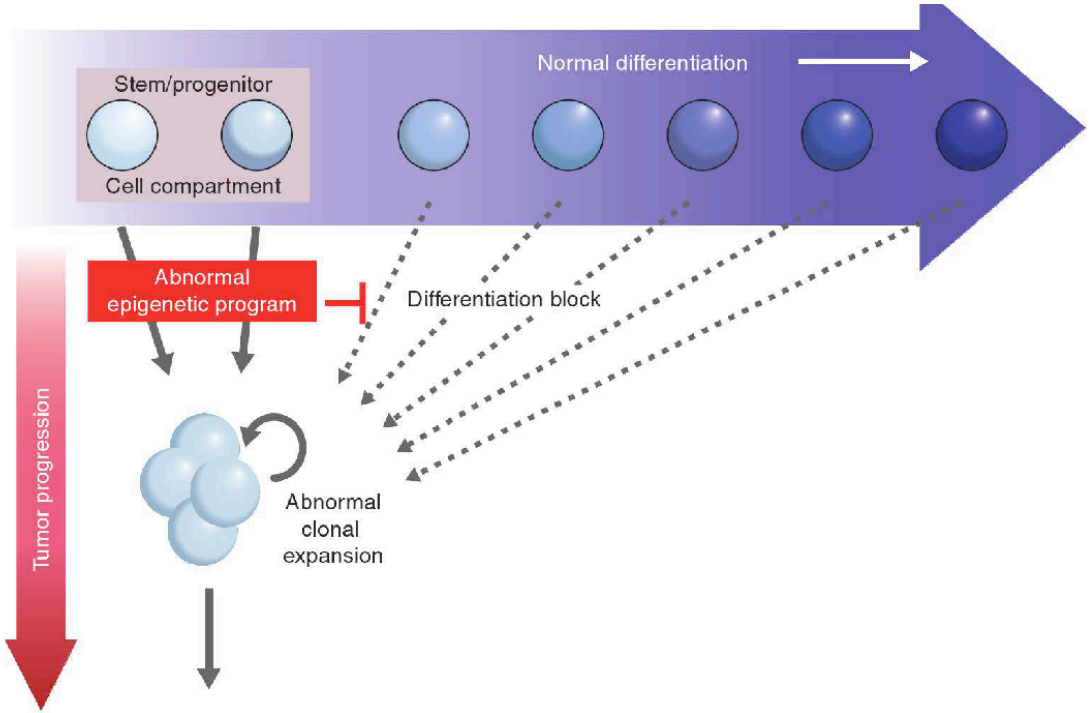



**Figure 6.** Epigenetic gene-silencing events and tumorigenesis. The earliest steps in tumorigenesis are depicted as abnormal clonal expansion, which evolves during the stress of cell renewal. This is caused by factors, such as aging and chronic injury from, for example, inflammation. These cell clones are those at risk of subsequent genetic and epigenetic events that could drive tumor progression. Abnormal epigenetic events, such as the aberrant gene silencing focused on in this chapter, could be the earliest heritable causes, in many instances, for a potential role in inducing the abnormal clonal expansion from within stem/progenitor cell compartments in a renewing adult cell system. The gene silencing is triggered by chromatin modifications that repress transcription, and the DNA hypermethylation of this chromatin serves as the tight lock to stabilize the heritable silencing. The gene silencing, in turn, disrupts normal homeostasis, preventing stem and progenitor cells from moving properly along the differentiation pathway for a given epithelial cell system (blue arrow) and channels them into the abnormal clonal expansion (red arrow).

# 3. THE IMPORTANCE OF EPIGENETIC GENE REGULATION IN EARLY TUMORPROGRESSION

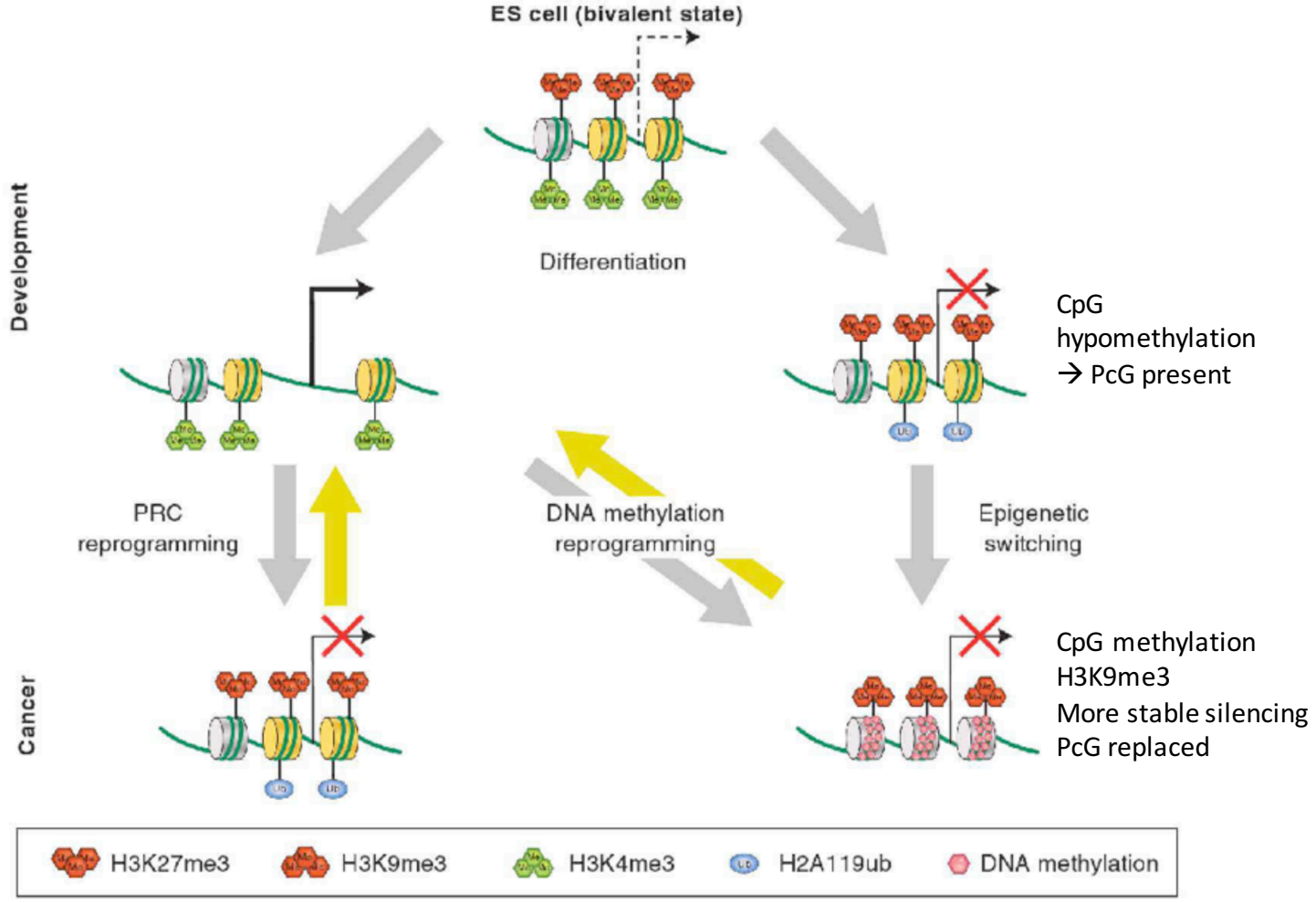
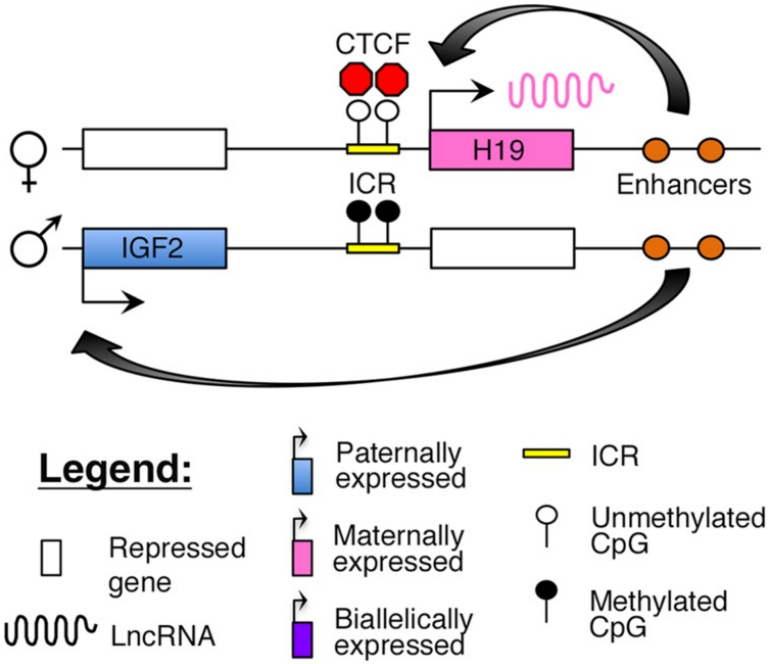



**Figure 7.** Reprogramming of DNA methylation patterns and abnormal modes of gene silencing in cancer. (*A*) Common DNA methylome changes observable in cancer versus a normal somatic cell are illustrated. This is shown in the context of large hypomethylated blocks (gray shading) of the genome seen in cancer interspersed with focal hypermethylation of promoter region CpG-island-containing genes (pink shading). In normal cells, background DNA methylation is high (pink shaded hexagons) with the exception of CpG islands (densely packed white shaded hexagons). In the cancer methylome, overall genome DNA methylation declines, particularly in the hypomethylated blocks, whereas CpG island promoter genes frequently become methylated (pink shading), most of which are located in the hypomethylated blocks. (*B*) The currently suggested routes to abnormally silenced CpG-island-containing genes in cancer are shown. Genes that are active in cells throughout development and adult cell renewal initially have active promoter chromatin, which is characterized by the presence of the bivalent histone modification pattern consisting of H3K4me, the repressive H3K27me3 mark, and a lack of DNA methylation. Genes that become transcriptionally active lose much of their Polycomb-mediated repressive H3K27 methylation, whereas those that become silenced (indicated by a red X) can do so by the loss of H3K4 methylation and acquisition of, or increases in, Polycomb-mediated repressive chromatin (PRC) mark and H2A119 ubiquitination. During tumor progression, active genes may become silenced through either the aberrant PRC-mediated reprogramming (*bottom left*) or DNA methylation and H3K9me marks (*bottom right*). Some normally silent genes may change the way in which they are transcriptionally repressed from H3K27-methylation-type repression to H3K9-methylation-based silencing and/or DNA hypermethylation (epigenetic switching). The reverse yellow arrows indicate the potential for epigenetic abnormalities in cancer to be corrected by epigenetic therapies. Representative of such therapies are DNMT inhibitors, HDAC inhibitors, KMT inhibitors, and others, as discussed in this and other chapters. These inhibitors can all potentially promote gene activation by producing losses of DNA methylation, or deacetylating lysines, or alleviating silencing mediated by histone methylation PTMs, such as H3K27 methylation. (*A*, Adapted from Reddington et al. 2014; *B*, adapted from Sharma et al. 2010.)

# 3. THE IMPORTANCE OF EPIGENETIC GENE REGULATION IN EARLY TUMORPROGRESSION

## Loss of imprinting (LOI): EXAMPLE H19-IGF2 locus

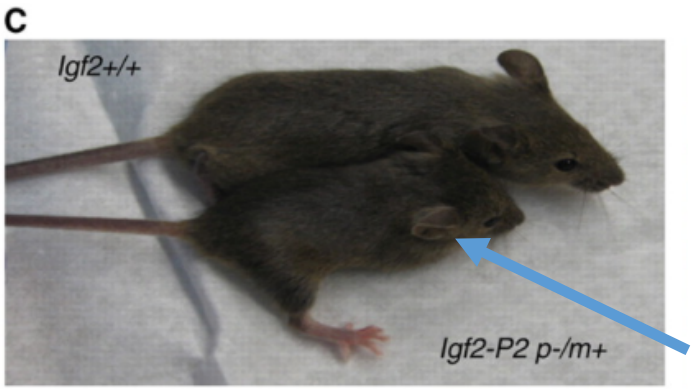



Proposed model of imprinting at the H19–IGF2 locus: the H19–IGF2 locus consists of a paternally methylated ICR (Imprinting Control Region). On the maternal allele, this ICR is unmethylated and is bound by the insulator protein CTCF that prevents access of the IGF2 promoter to downstream enhancers.

***H19–IGF2* LOCUS**

The *H19–IGF2* locus resides adjacent to the *KCNQ1* region on human Chr. 1p15.5. The region consists of the **paternally expressed *insulin-like growth factor 2* (*IGF2*) gene** and **maternally expressed *H19* lncRNA**. The *IGF2* protein functions as a growth factor essential for embryonic development, whereas *H19* may function as a tumor suppressor. Imprinting at this locus is maintained by an ICR, which is selectively methylated on the paternal allele. The insulator protein, CCCTC- binding factor (*CTCF*), binds to critical regulatory regions in the unmethylated ICR (Imprinting control region) on the maternal allele, thus blocking access of downstream enhancers to the *IGF2* promoter.

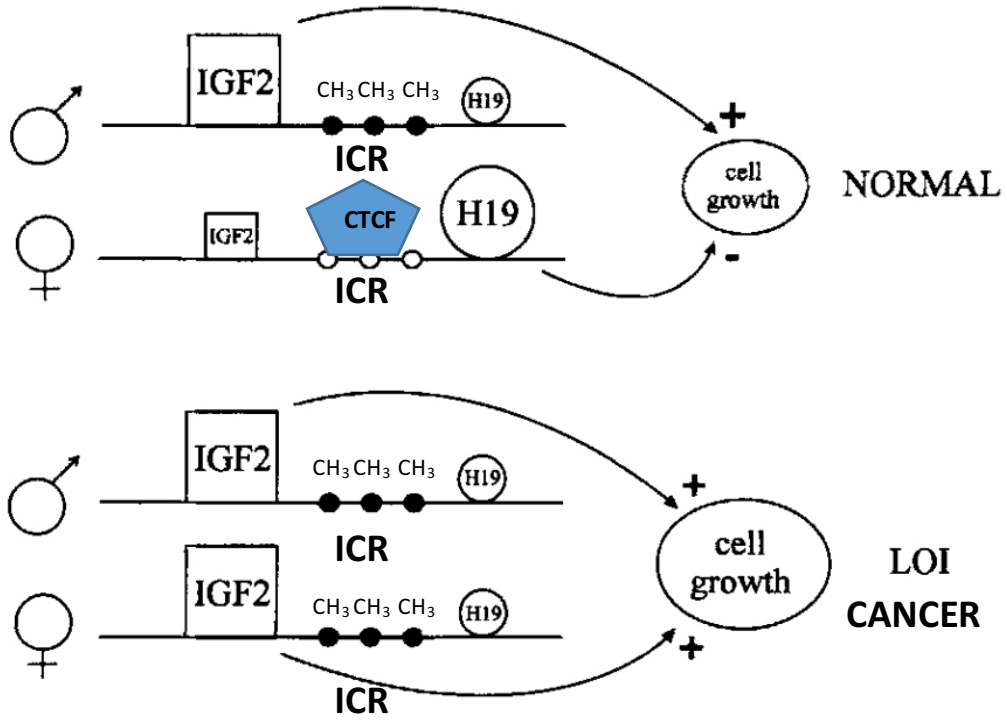
**Loss of methylation at the *H19/IGF2* ICR results in short body length and low birth weight, both in rodent models as well as in humans**




IGF2 knock-out allele was inherited from the father of the mouse

## 3. THE IMPORTANCE OF EPIGENETIC GENE REGULATION IN EARLY TUMORPROGRESSION

# Loss of imprinting (LOI): EXAMPLE H19-IGF2 locus




**In tumors (<u>colon</u>)** : HYPER-methylation of ICR on maternal allele; increased IGF2 expression; increased proliferation potential

**In mouse loss of IGF2 imprinting may be sufficient to initiate tumorigenesis**

## 3. THE IMPORTANCE OF EPIGENETIC GENE REGULATION IN EARLY TUMORPROGRESSION

# IDH1 mutations in glioblastoma lead to epigenetic deregulation in oncogenesis

### Classic oncogenic driver mutations in glioblastoma

| Gene symbol | Gene name | Function of encoded protein | Point of mutation (%) |
|---|---|---|---|
| EGFR | Epidermal growth factor receptor | Regulator of cell signaling involved in cell proliferation and survival | 14–15 |
| ERBB2 | V-erb-b2-erythroblastic leukemia viral oncogene homolog 2 | Regulator of cell signaling involved in cell proliferation and survival | 0–7 |
| IDH1 | Isocitrate dehydrogenase 1 | NADPH production | 12–20 |
| NF1 | Neurofibromin 1 | Regulator of cell signaling involved in cell proliferation and survival | 15–17 |
| PIK3CA | Phosphoinositide-3-kinase catalytic alpha | Regulator of cell signaling involved in cell proliferation and survival | 7–10 |
| PIK3R1 | Phosphoinositide-3-kinase regulatory 1 | Regulator of cell signaling involved in cell proliferation and survival | 7–8 |
| PTEN | Phosphatase and tensin homolog | Regulator of cell signaling involved in cell proliferation and survival | 24–37 |
| PTPRD | Protein tyrosine phosphatase receptor type D | Regulator of cell signaling involved in cell proliferation and survival | 0–6 |
| RB1 | Retinoblastoma 1 | Regulator of cell cycle | 8–13 |
| TP53 | Tumor protein p53 | Apoptosis | 31–38 |

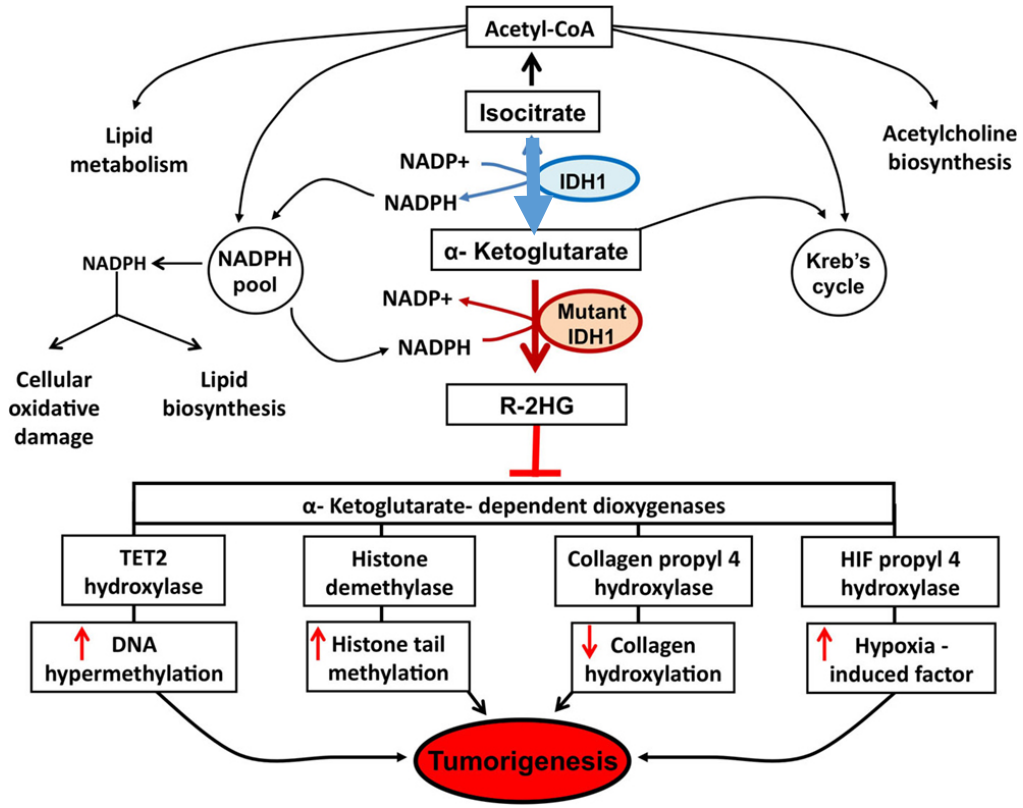
### IDH1 mutations are highly frequent in glioblastoma

| Tumor types | IDH1 mutation frequency (%) |
|---|---|
| Astrocytoma pilocytic (WHO Grade I) | 0.01 |
| Diffuse glioma (WHO Grade II) | 76 |
| Astrocytoma anaplastic (WHO Grade III) | 62.2 |
| Primary glioblastoma (WHO Grade IV) | 5.6 |
| Secondary glioblastoma (WHO Grade IV) | 76.4 |
| **Oligodendroglioma** | |
| WHO Grade II | 78.8 |
| WHO Grade III | 67.5 |
| **Oligoastrocytoma** | |
| WHO Grade II | 79.8 |
| WHO Grade III | 69.7 |

*CNS, central nervous system; WHO, World Health Organisation; AML, acute myeloid leukemia; D-2-HGA, D-2-hydroxyglutaric aciduria; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasms.*

## 3. THE IMPORTANCE OF EPIGENETIC GENE REGULATION IN EARLY TUMORPROGRESSION

# IDH1 mutations in glioblastoma lead to epigenetic deregulation in oncogenesis




IDH1 mutation results result the abberrant production of high levels of **2-hydroxyglutarate (2-HG),** a possible oncometabolite that is thought to influence a range of cellular programs involved in epigenetic control and various processes leading to tumor development.

*IDH1* mutations are heterozygous, typically involving an amino acid substitution in the active site of the enzyme in codon 132. The mutation results in a loss of normal enzymatic function and the abnormal production of 2-hydroxyglutarate (2-HG)

This abnormal **2-hydroxy-glutarate** metabolite, which increases to mM levels in cells with the IDH mutations, constituting a biomarker in and of itself, competes with the necessary **α-ketoglutarate metabolite needed by the TET and lysine (K) demethylase (KDM) enzymes**, which regulate either chromatin demethylase function or levels of DNA methylation.
**α-ketoglutarate metabolite reduced because processed to R-2HG**

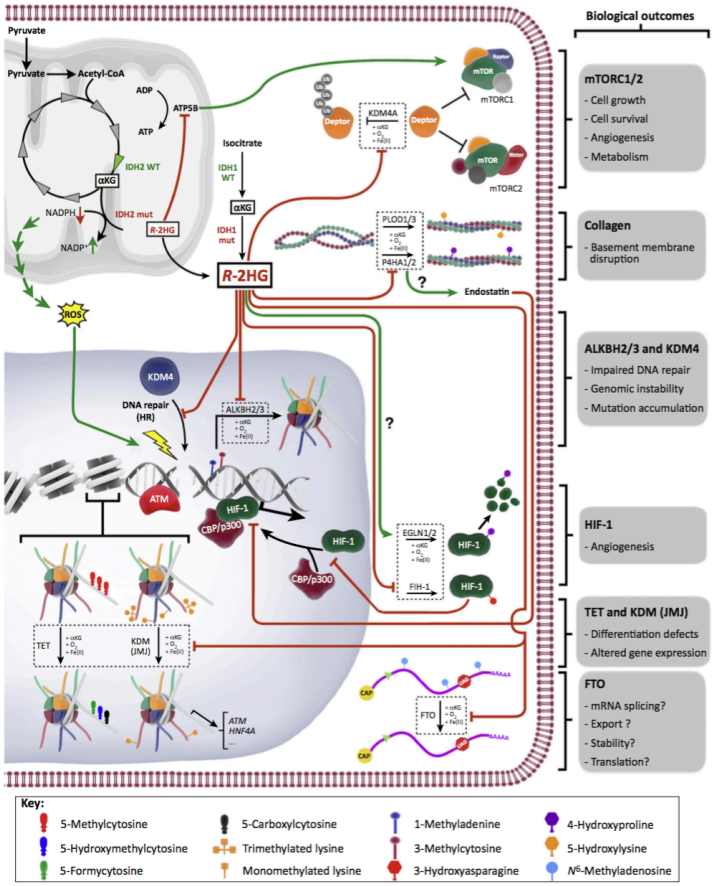



Figure 2. Multiple αKG-dependent dioxygenases family are dependent on αKG for their enzymatic activities to modify gene expression. When associated with αKG, ten-eleven translocation (TET) family catalyze the conversion of the modified DNA base 5-methylcytosine to 5-hydroxymethylcytosine, which can then be further oxidized to form derivatives 5-formylcytosine and 5-carboxylcytosine. While this activity requires αKG, TET methylcytosine hydroxylation is strongly inhibited by *R*-2HG production following *IDH1/2* mutation. Similarly, the activity of Jumonji lysine demethylases, which normally demethylate specific regulatory lysines on histones H3 and H4, is abrogated upon *R*-2HG production, and the ensuing major changes in gene expression impair cell differentiation. (RNA methylation) FTO (fat mass and obesity-associated protein) catalyzes the demethylation of $N^6$-methyladenosine ($m^6A$), an abundant modification present in mRNAs. $m^6A$ regulates several aspects of RNA metabolism such as RNA splicing, transport, stability, and translation. FTO may be inhibited by *R*-2HG, but the consequences of reduced FTO activity in mutant IDH tumors are largely unknown. (DNA damage response and repair) ALKBH2 and ALKBH3 (α-KG-dependent dioxygenase homologs 2 and 3) are involved in reparation of alkylated DNA lesions containing 1-methyladenine and 3-methylcytosine by oxidative demethylation. *R*-2HG production inhibits ALKBH2/3 activity, thus interfering with normal DNA repair processes. Inhibition of KDM4A and KDM4B by *R*-2HG leads to DNA repair defects by impairing homologous recombination (HR). In addition, IDH mutations cause accumulation of the repressive H3K9me3 histone mark at the promoter of gene encoding the DNA damage response kinase ATM, thus decreasing the levels of ATM and altering DNA repair. (mTOR Signaling) KDM4A and ATP5B modulate mTOR activation in the presence of αKG or *R*-2HG. KDM4A stabilizes DEPTOR, the endogenous negative regulator of mTOR, by inhibiting its polyubiquitylation, thus decreasing both mTORC1 and mTORC2 activation. In presence of *R*-2HG, DEPTOR is rapidly degraded, resulting in a massive increase in mTORC1/2 activation in a PI3K/PTEN-independent fashion, thus favoring the proliferation and survival of cancer cells harboring IDH1/2 mutations. In addition, *R*-2HG inhibits ATP synthase activity, leading to reduced ATP content and thus causing mTOR inhibition. *R*-2HG production by *IDH1/2* mutations engages a complex network that likely regulates mTOR activity through regulatory feedback loops. (HIF-1 Signaling) Hypoxia-inducible factor 1α (HIF-1α) is a major regulator of cell proliferation, cell survival, and a promoter of angiogenesis. HIF-1α is a highly unstable protein regulated by $O_2$ availability. EGLN1/2 and FIH are potent regulators of HIF-1α stability and activity, respectively. While FIH is inhibited by *R*-2HG, EGLN1/2 may be activated by the oncometabolite, resulting in a complex modulation of HIF-1α functions in tumors carrying *IDH1/2* mutations. (Collagen maturation) Both prolyl 4-hydroxylases (P4HA1/3) and procollagen-lysine 2-oxoglutarate 5-dioxygenases (PLOD1/3) are enzymes that stabilize the collagen triple helix through hydroxylation of lysine and proline within each collagen chain. Because these enzymes are αKG-dependent, reduced availability of αKG and accumulation of *R*-2HG in *IDH1/2* mutated tumors affect collagen maturation.

Table 1. Enzymes Regulated by Mutated IDH1/2 and the Associated Oncometabolite *R*-2HG

| Enzyme | Enzymatic function | Type of regulation by *R*-2HG | Consequence of *R*-2HG-mediated regulation |
|---|---|---|---|
| TET1 | Methylcytosine dioxygenase | Inhibition [39] | Inhibition of TET1 and TET2 by IDH1/2 mutations caused downregulation of 5-hydroxymethylcytosine (5hmC) [39] |
| TET2 | Methylcytosine dioxygenase | Inhibition [39] | Inhibition of TET1 and TET2 by IDH1/2 mutations caused downregulation of 5hmC [39]; impaired hematopoietic differentiation [47] |
| KDM2A | H3K36me1/2 demethylase | Inhibition ($IC_{50}$ = 106 μM [39]) | Not determined |
| KDM4A | H3K9me2/3, H1.4K26me2/3, and H3K36me2/3 demethylase | Inhibition ($IC_{50}$ from 2.1 μM [97] to 24 μM [38]) | Global increase of H3K9me3 [38]; increased mTOR activity through destabilization of DEPTOR [97]; DNA repair defects [82] |
| KDM4C | H3K9me2/3, H1.4K26me2/3, and H3K36me2/3 demethylase | Inhibition [23] | Global increase of H3K9me3 [38]; differentiation defects [23] |
| KDM4D | H3K9me2/3, H1.4K26me2/3, and H3K36me2/3 demethylase | Inhibition ($IC_{50}$ = 79 μM [38]) | Global increase of H3K9me3 [38] |
| KDM5B | H3K4me2/3 demethylase | Weak inhibition ($IC_{50}$ = 10.9 mM [38]) | Not determined |
| HIF1AN/FIH | HIF asparaginyl hydroxylase | Inhibition ($IC_{50}$ from ~1 mM [123] to 1.5 mM [38]) | HIF-1α accumulation in *R*-2HG-treated cells [39] as well as in $IDH1^{R132H}$-expressing cells and gliomas [116] |
| EGLN1/2 | HIF prolyl 4-hydroxylase | Activation [123] | Activation of EGLN1/2 in $IDH^{R132H}$ mutated cells diminished HIF activity [123] |
| Collagen prolyl hydroxylases (P4HA1/2) and lysine hydroxylases (PLOD1/3) | Collagen prolyl hydroxylases; Collagen lysine hydroxylases | Inhibition ($IC_{50}$ ~2 mM; C-P4H-I [123]) | Defective collagen maturation, impairment of basal membrane structure and function, activation of the ER stress response [92] |
| ALKBH2 | Repair of methylated DNA bases, such as 1-methyladenine and 3-methylcytosine | Inhibition ($IC_{50}$ from 424 μM [38] to ~500 μM [83]) | Accumulation of DNA damage, decreased DNA repair kinetics, and increased sensitivity to alkylating agents of IDH mutated cells [83] |
| ALKBH3 | Repair of methylated DNA bases such as 1-methyladenine and 3-methylcytosine | Inhibition ($IC_{50}$ ~500 μM [83]) | Accumulation of DNA damage, decreased DNA repair kinetics, and increased sensitivity to alkylating agents of IDH mutated cells [83] |
| FTO | $N^6$-methyladenosine and $N^6$,2′-*O*-dimethyladenosine RNA demethylase | Inhibition [70] | Accumulation of $m^6A$ RNAs [70] |
| ATP5B | ATP synthase β subunit | Inhibition [104] | Increased ratio of ADP to ATP, inhibition of mTOR signaling [104] |

# 4. EXAMPLES OF TSG EPIGENETIC SILENCING IN ONCOGENESIS

**Table 1.** Examples of key cellular pathways disrupted in human cancers by genetic and epigenetic mechanisms

| Pathway | Example of genetic alteration | Example of epigenetic alteration |
|---|---|---|
| Self sufficiency in growth signals | Mutations in *Ras* gene | Methylation of *RASSFIA* gene |
| Insensitivity to antigrowth signals | Mutation in TGF-β receptors | Down-regulation of TGF-β receptors |
| Tissue invasion and metastasis | Mutation in *E-cadherin* gene | Methylation of *E-cadherin* promoter |
| Limitless replicative potential | Mutation in *p16* and pRb genes | Silencing of *p16* or pRb genes by promoter methylation |
| Sustained angiogenesis | | Silencing of thrombospondin-1 |
| Evading apoptosis | Mutation in p53 | Methylation of *DAPK*, ASC/TMS1, and HIC1 |
| DNA repair capacity | Mutations in MLH1, MSH2 | Methylation of *GST Pi*, *O6-MGMT*, *MLH1* |
| Monitoring genomic stability | Mutations in *Chfr* | Methylation of *Chfr* |
| Protein ubiquination functions regulating mitotic control genes | Mutations in *Chfr* | Methylation of *Chfr* |

TGF-β, transforming growth factor β; *DAPK*, death-associated protein kinase.

# 4. EXAMPLES OF TSG EPIGENETIC SILENCING IN ONCOGENESIS

## SILENCING OF p16

**Table 1.** Examples of key cellular pathways disrupted in human cancers by genetic and epigenetic mechanisms

| Pathway | Example of genetic alteration | Example of epigenetic alteration |
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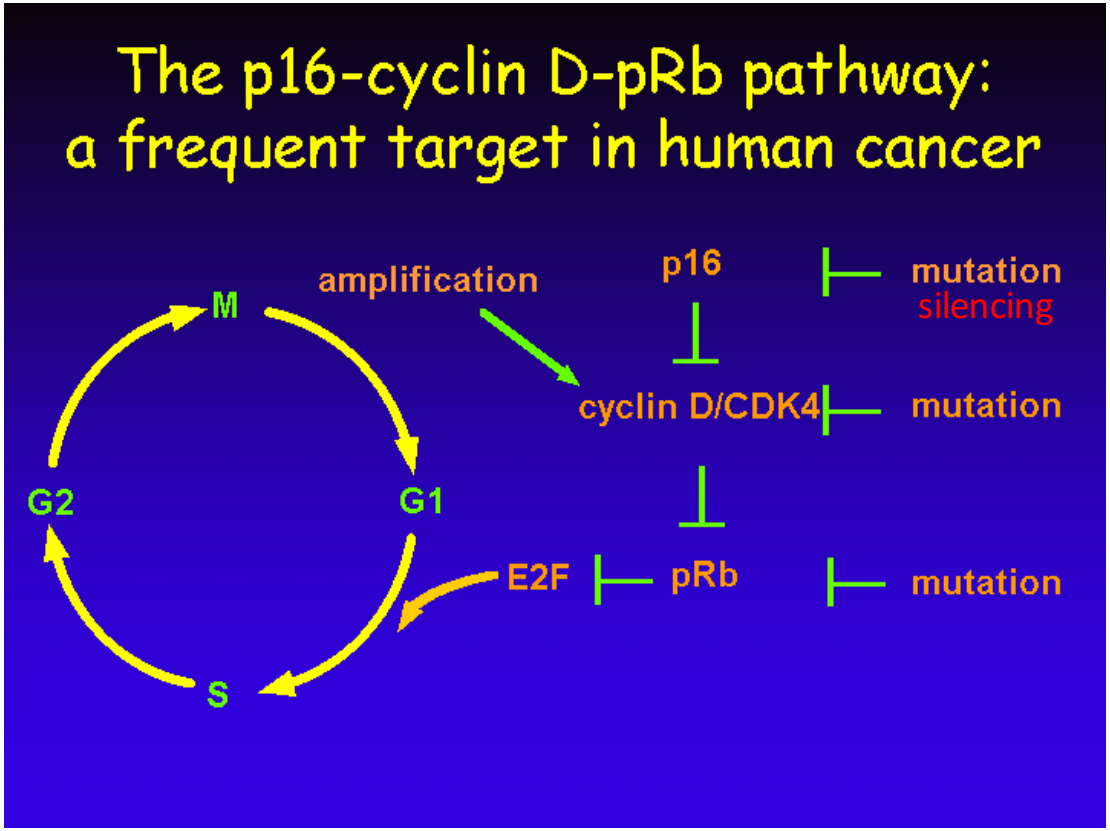
| Monitoring genomic stability | Mutations in *Chfr* | Methylation of *Chfr* |
| Protein ubiquination functions regulating mitotic control genes | Mutations in *Chfr* | Methylation of *Chfr* |

TGF-β, transforming growth factor β; *DAPK*, death-associated protein kinase.

# 3. The importance of epigenetic gene regulation in early tumorprogression

## SILENCING OF p16




Because of its importance in cell proliferation, the human INK4 gene locus, encoding p16, is a frequent target of inactivation by deletion or aberrant DNA methylation in a wide variety of human cancers

p16 silencing is an early event in tumorformation

→ Increased activity of cyclinD/CDK4

→ pRB hyperphoslphorylation

→ E2F activates genes required for the induction of S-Phase

# 4. EXAMPLES OF TSG EPIGENETIC SILENCING IN ONCOGENESIS

## SILENCING OF MLH1

**Table 1.** Examples of key cellular pathways disrupted in human cancers by genetic and epigenetic mechanisms

| Pathway | Example of genetic alteration | Example of epigenetic alteration |
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| Monitoring genomic stability | Mutations in *Chfr* | Methylation of *Chfr* |
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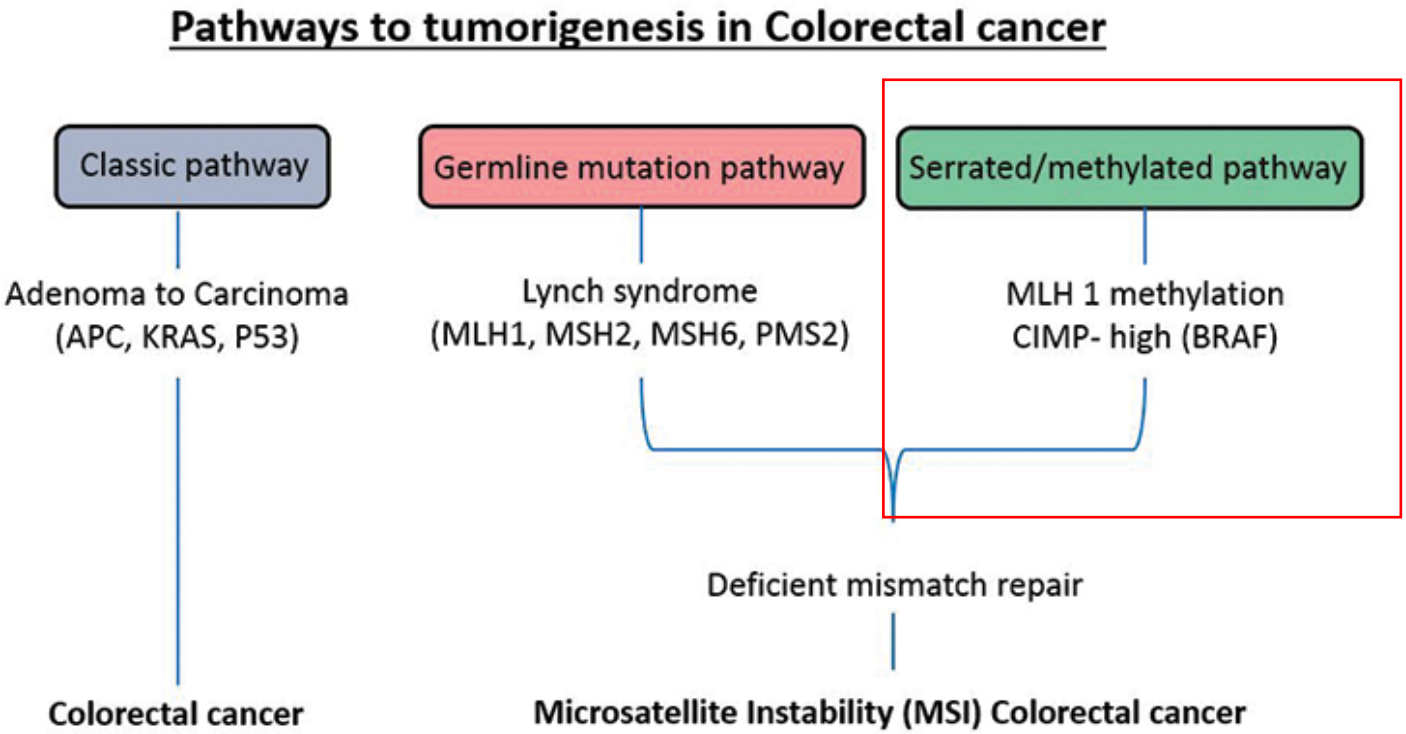
TGF-β, transforming growth factor β; *DAPK*, death-associated protein kinase.

# 4. EXAMPLES OF TSG EPIGENETIC SILENCING IN ONCOGENESIS



# 4. EXAMPLES OF TSG EPIGENETIC SILENCING IN ONCOGENESIS

## SILENCING OF Chfr

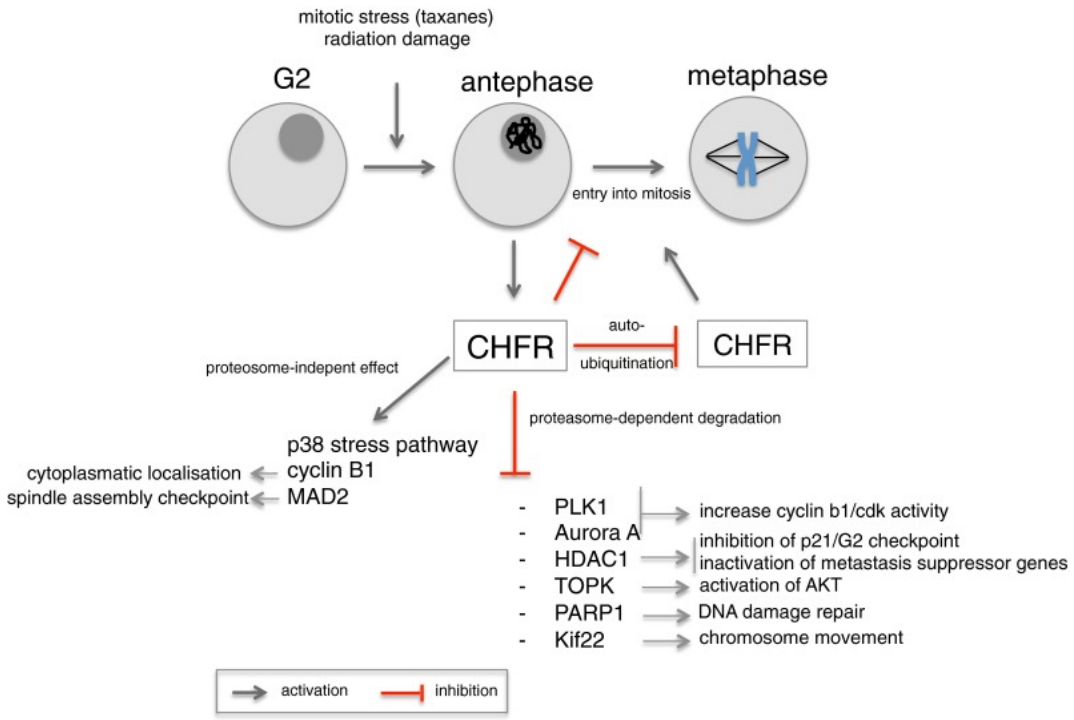
**Table 1.** Examples of key cellular pathways disrupted in human cancers by genetic and epigenetic mechanisms

| Pathway | Example of genetic alteration | Example of epigenetic alteration |
|---|---|---|
| Self sufficiency in growth signals | Mutations in *Ras* gene | Methylation of *RASSFIA* gene |
| Insensitivity to antigrowth signals | Mutation in TGF-β receptors | Down-regulation of TGF-β receptors |
| Tissue invasion and metastasis | Mutation in *E-cadherin* gene | Methylation of *E-cadherin* promoter |
| Limitless replicative potential | Mutation in *p16* and pRb genes | Silencing of *p16* or pRb genes by promoter methylation |
| Sustained angiogenesis | | Silencing of thrombospondin-1 |
| Evading apoptosis | Mutation in p53 | Methylation of *DAPK*, ASC/TMS1, and HIC1 |
| DNA repair capacity | Mutations in MLH1, MSH2 | Methylation of *GST Pi*, *O6-MGMT*, *MLH1* |
| Monitoring genomic stability | Mutations in *Chfr* | Methylation of *Chfr* |
| Protein ubiquination functions regulating mitotic control genes | Mutations in *Chfr* | Methylation of *Chfr* |

TGF-β, transforming growth factor β; *DAPK*, death-associated protein kinase.

# 4. EXAMPLES OF TSG EPIGENETIC SILENCING IN ONCOGENESIS

## Chfr function




CHFR is an early mitotic checkpoint gene that functions as a key player in controlling chromosomal integrity.

CHFR is expressed in the cytoplasm of all normal tissues and accumulates in the nucleus in response to **microtubule poisoning, carcinogen or radiation damaging stress**.

After localization into the nucleus, CHFR becomes phosphorylated by protein kinase B (PKB/AKT), a member of the PI3K signaling pathway.

Microtubule stress/Radiation leads to an elevation of CHFR expression levels and a mitotic arrest.

→ In conditions with increased risk of genomic instability CHFR blocks mitotic progression to allow damage repair

→ Absence of CHFR: mitosis precedes, DNA damage will not be repaired → accumulation of mutations → genomic instability/cancer.

*CHFR is a E3 ubiquitin-protein ligase that delays entry into metaphase in response to microtubular stress/radiation by effecting target proteins in a proteosome-dependent and a proteosome-independent manner* → ***maintains genomic stability***

# 4. EXAMPLES OF TSG EPIGENETIC SILENCING IN ONCOGENESIS

## Chfr knock out mouse model

**Skin carcinogenesis**

(mice are treated with carcinogen (DMBA = DNA damaging agent, TPA cell cycle stimulator

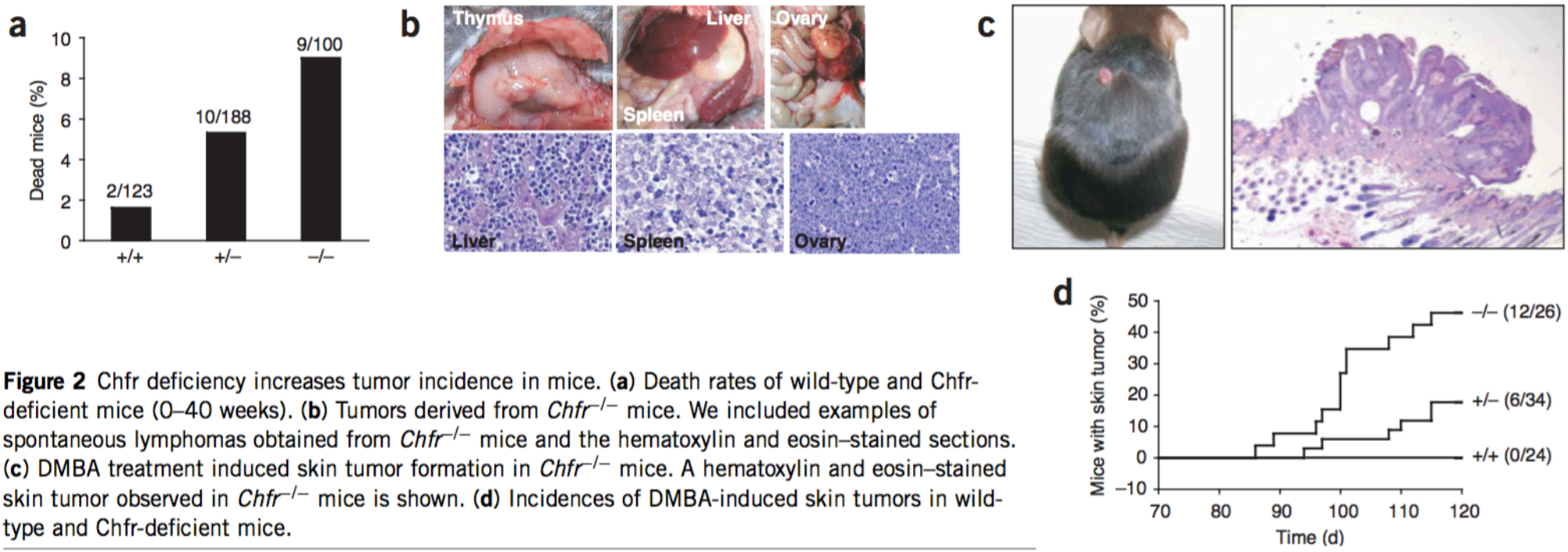



**Figure 2** Chfr deficiency increases tumor incidence in mice. (**a**) Death rates of wild-type and Chfr-deficient mice (0–40 weeks). (**b**) Tumors derived from *Chfr*$^{-/-}$ mice. We included examples of spontaneous lymphomas obtained from *Chfr*$^{-/-}$ mice and the hematoxylin and eosin–stained sections. (**c**) DMBA treatment induced skin tumor formation in *Chfr*$^{-/-}$ mice. A hematoxylin and eosin–stained skin tumor observed in *Chfr*$^{-/-}$ mice is shown. (**d**) Incidences of DMBA-induced skin tumors in wild-type and Chfr-deficient mice.

# 4. EXAMPLES OF TSG EPIGENETIC SILENCING IN ONCOGENESIS

## Chfr knock out mouse model



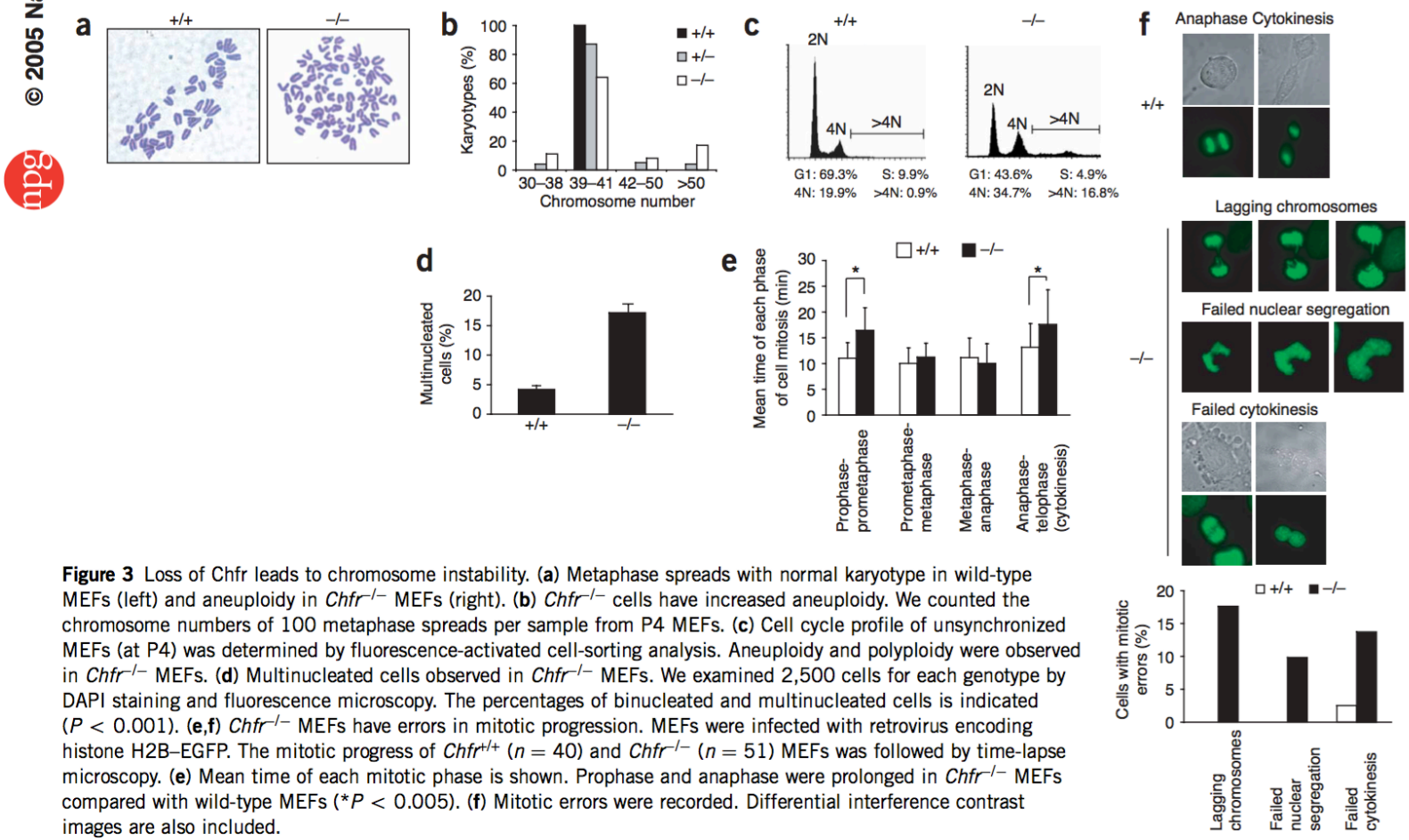



**Figure 3** Loss of Chfr leads to chromosome instability. (**a**) Metaphase spreads with normal karyotype in wild-type MEFs (left) and aneuploidy in *Chfr*$^{-/-}$ MEFs (right). (**b**) *Chfr*$^{-/-}$ cells have increased aneuploidy. We counted the chromosome numbers of 100 metaphase spreads per sample from P4 MEFs. (**c**) Cell cycle profile of unsynchronized MEFs (at P4) was determined by fluorescence-activated cell-sorting analysis. Aneuploidy and polyploidy were observed in *Chfr*$^{-/-}$ MEFs. (**d**) Multinucleated cells observed in *Chfr*$^{-/-}$ MEFs. We examined 2,500 cells for each genotype by DAPI staining and fluorescence microscopy. The percentages of binucleated and multinucleated cells is indicated ($P < 0.001$). (**e**,**f**) *Chfr*$^{-/-}$ MEFs have errors in mitotic progression. MEFs were infected with retrovirus encoding histone H2B–EGFP. The mitotic progress of *Chfr*$^{+/+}$ ($n = 40$) and *Chfr*$^{-/-}$ ($n = 51$) MEFs was followed by time-lapse microscopy. (**e**) Mean time of each mitotic phase is shown. Prophase and anaphase were prolonged in *Chfr*$^{-/-}$ MEFs compared with wild-type MEFs (*$P < 0.005$). (**f**) Mitotic errors were recorded. Differential interference contrast images are also included.

# 4. EXAMPLES OF TSG EPIGENETIC SILENCING IN ONCOGENESIS

## Chfr is frequently inactivated in human cancer

*CHFR* inactivation in multiple cancers

| Cancer | Aberration | Method, region analyzed | Percentage of methylation | Ref |
|---|---|---|---|---|
| Breast cancer | Reduced expression | IHC | 36 % (51/142) | [70] |
| | Methylation | demethylation and northern blot | 8 % (2/24) (cell lines) | [71] |
| Bladder cancer | Methylation | MLPA | 18.7 % (17/91) | [72] |
| Colorectal cancer | Methylation | COBRA | 40 % (25/63) | [73] |
| | | MSP, −281 to +51 bp | 37 % (11/30) | [74] |
| | | COBRA | 53 % (27/51) (adenomas) | [71] |
| | | MSP, −240 to −73 bp | 41 % (29/71) | [75] |
| | | MSP, −226 to −82 bp | 26% (25/98) | [76] |
| | | qMSP, +221 to +325 bp | 24 % (217/888) | [77] |
| | | MSP, −240 to −73 bp | 31 % (19/61) | [67] |
| Gastric cancer | Methylation | COBRA | 39 % (24/61) | [56] |
| | | MSP, −9 to +98 bp | 35 % (25/71) | [78] |
| | | COBRA | 44 % (19/43) | [79] |
| | | MSP, −163 to −8 bp | 52 % (24/46) | [57] |
| Nasopharyngeal cancer | Methylation | MSP, −220 bp to −14 bp | 61 % (22/36) | [80] |
| | | MSP, −220 to −14 bp | 59 % (31/53) | [81] |
| Non-small cell lung cancer | Reduces expression | IHC | 39 % (16/41) | [63] |
| | Methylation | MSP, −220 to −14 bp | 19 % (7/37) | [42] |
| | Mutation | MSP, −220 to −14 bp | 10 % (2/20) | [74] |
| | | MSP, −220 to −14 bp/IHC | 15 % (3/20)/39 % (69/157) | [53] |
| | | MSP −195 to −99 bpm | 32.4 % (100/308) (serum) | [47] |
| | | MSP, −220 to −14 bp | 10 % (16/165) | [52] |
| | | MSP, −220 to −14 bp | 3.1 % (1/32) | [63] |
| | | | 6 % 3/53 | [44] |
| Esophageal cancer | Methylation | MSP, −163 to −8 bp | 16.3 % (7/43) | [46] |
| | Copy number loss | MSP, −227 to −86 bp | 24 % (9/38) | [82] |
| | | bisulfite pyrosequencing | 31 % (18/58) | [45] |
| | | qPCR | 59 % (16/27) | [45] |
| Cervical cancer | Methylation | MSP, +168 to 318 | 12 % (2/14) | [59] |
| Hepatocellular cancer | Methylation | MSP, −225 to −85 bp | 35 % (22/62) | [83] |
| Biliary tract carcinoma | Methylation | MSP, −9 to +98 bp | 16 % (6/37) | [84] |
| Oral squamous cell cancer | Methylation | MSP, −220 bp −14 bp | 31 % (4/13) | [85] |
| | | | 34.7 % (17/49) | [86] |
| Cutaneous T-cell lymphoma | Methylation | CpG island microarray | 19 % (5/28) | [87] |
| Head and neck cancer | | COBRA | 30 % (16/54) | [73] |
| | | MS-MLPA | 25 % (7/28) | [88] |
| Endometrial cancer | Methylation | MSP, +168 to 318 bp | 12 % (6/50) | [60] |

IHC, immunohistochemistry; (MS)-MLPA, (methylation-specific) multiplex ligation-dependent probe amplification; MSP, methylation-specific PCR; COBRA, combined bisulfite restriction analysis

CHFR is more frequently inactivated in cancer than all other mitotic checkpoint control genes together.

Absence of CHFR in these cell lines resulted in a **high mitotic index** when exposed to microtubule stress compared to wild-type cancer cells, which was restored by reintroduction of functional CHFR.

In a breast cancer cell line model, **decreased CHFR expression resulted in an accelerated growth rate, enhanced invasiveness and amplified colony formation.**

In order to study the physiological role of CHFR and its function in tumorigenesis, Chfr knockout mice have been generated.

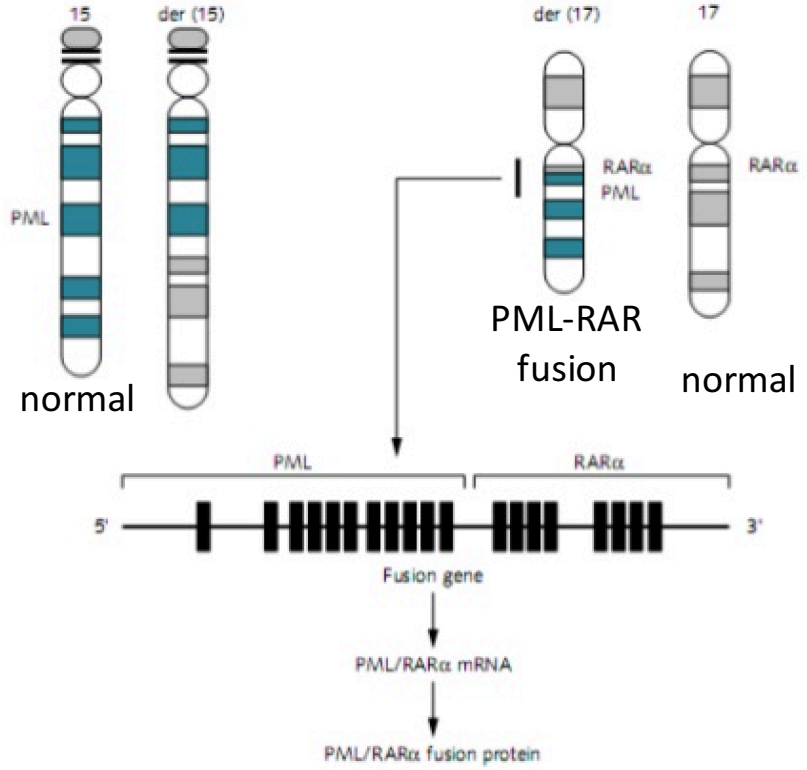
**Chfr knockout mice develop invasive lymphomas and solid tumors** (lung, liver, gastrointestinal) after 40 weeks and have an increased susceptibility to chemical carcinogenesis.
Embryonic fibroblasts from Chfr-deficient mice show substantial aneuploidy and polyploidy.

# 5. EXAMPLES OF MUTATIONS THAT CHANGE THE FUNCTION/USE OF EPIGENETIC REGULATORS

**PML – RAR fusion and aberrant use of HDAC**

**Acute promyelocytic leukemia (APML, APL)** is the M3 subtype of acute myelogenous leukemia (AML), a cancer of the white blood cells. In APL, there is an abnormal accumulation of immature granulocytes called promyelocytes.

The disease is characterized by a chromosomal translocation involving the retinoic acid receptor alpha (RARα or RARA) gene and is distinguished from other forms of AML by its responsiveness to all-trans retinoic acid (ATRA; also known as tretinoin) therapy.



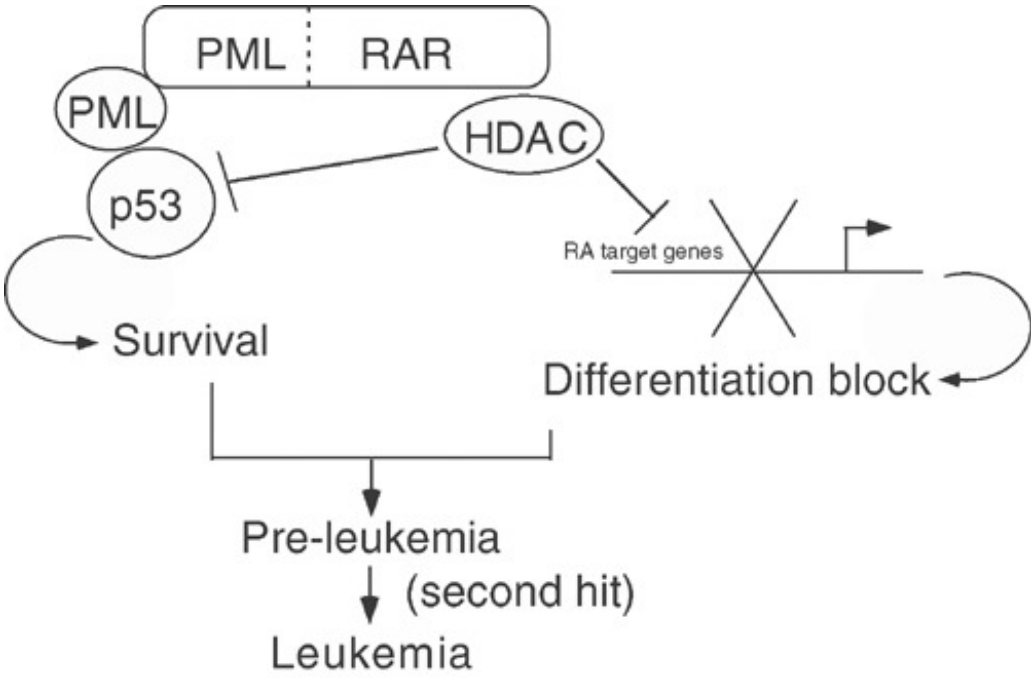

**FUSION OF PML WITH RETINOIC ACID RECEPTOR GENE**

Note: retinoic acid receptor activation by retinoic acid drives differentiation

**Acute promyelocytic leukemia**

# 5. EXAMPLES OF MUTATIONS THAT CHANGE THE FUNCTION OF EPIGENETIC REGULATORS

**PML – RAR fusion and aberrant use of HDAC**




PML–RAR acts as an HDAC-dependent inhibitor of pathways regulated by wild-type PML and RAR.

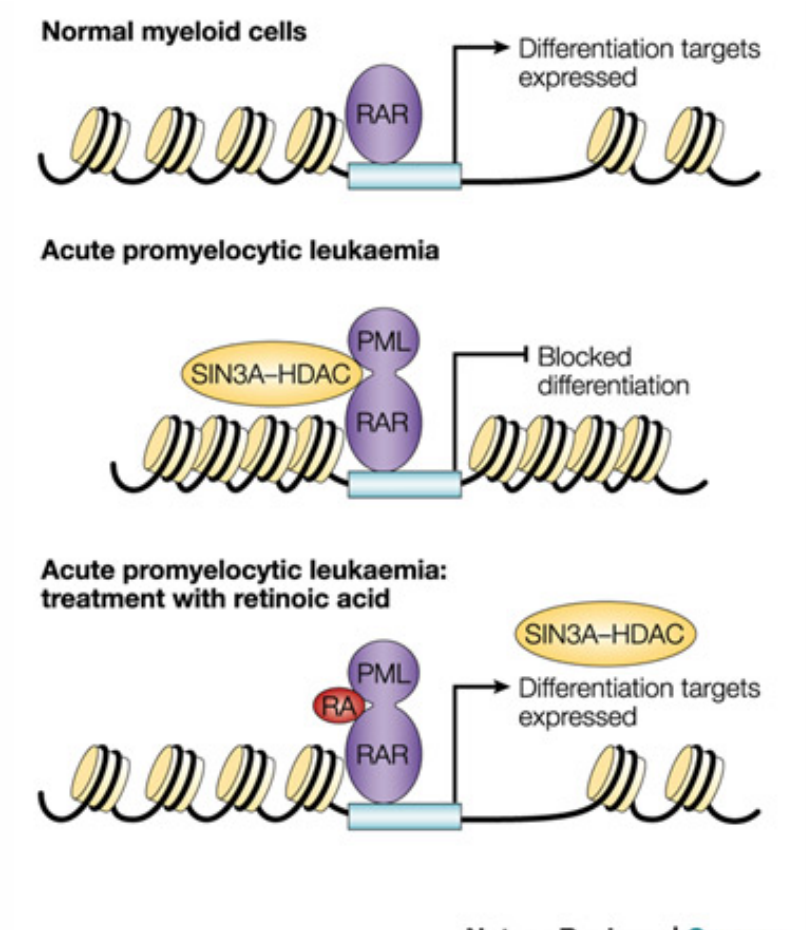
PML–RAR acts as a bifunctional protein:
(i) it inhibits the transcription of RA target genes in the presence of physiological concentrations of ligand, thus blocking myeloid differentiation; and
(ii) it inhibits p53 function (in a manner requiring wild-type PML), allowing enhanced survival of APL blasts.

Both differentiation block and enhanced survival are mediated by recruitment of HDAC by the fusion protein.

**Acute promyelocytic leukemia**

# 5. EXAMPLES OF MUTATIONS THAT CHANGE THE FUNCTION OF EPIGENETIC REGULATORS

## PML – RAR fusion and aberrant use of HDAC




**Acute promyelocytic leukemia**

It is apparent that the multisubunit complexes that are responsible for covalently modifying histones and DNA during chromatin remodelling can contribute to cancer. Histone acetyltransferase (HAT) complexes acetylate histones (leading to relaxation of chromatin structure) and, typically, increase transcription of target genes. CREB-binding protein, which possesses intrinsic HAT activity, is fused — by chromosomal translocation — to the product of the mixed-lineage leukaemia gene (*MLL*) in aggressive paediatric leukaemias and to the histone acetyltransferases MOZ and MORF in other leukaemias.

**Histone deacetylase (HDAC) complexes** have also been implicated in oncogenesis. In acute promyelocytic leukaemia, a recurrent chromosomal translocation results in fusion of the promyelocytic leukaemia gene product (PML), which directly recruits SIN3A–HDAC, to the retinoic-acid receptor (RAR). During normal development of myeloid cells, the RAR transcriptionally activates target genes that promote differentiation into mature myeloid cells. In acute promyelocytic leukaemia, the PML–RAR fusion protein recruits SIN3A–HDAC to RAR targets (see figure). SIN3A–HDAC deacetylates histones, leading to transcriptional repression and to a block in differentiation that contributes to leukaemic growth.

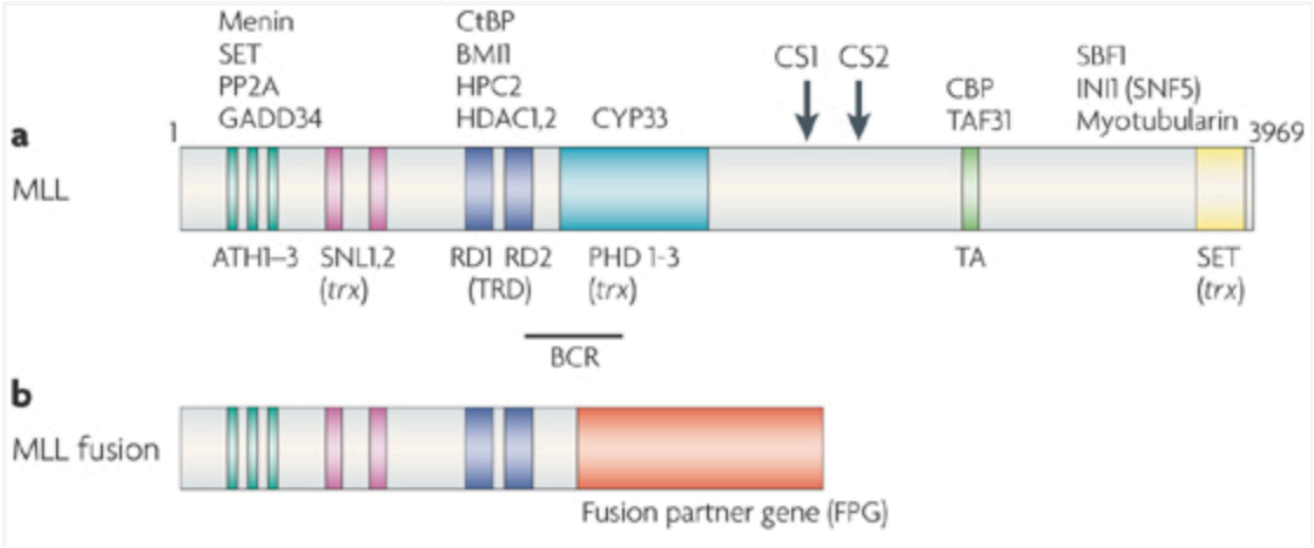
**Treatment of these patients with high-dose retinoic acid (RA) causes dissociation of SIN3A–HDAC and differentiation of immature leukemic blast cells into mature, and benign, myeloid cells.**

# 5. EXAMPLES OF MUTATIONS THAT CHANGE THE FUNCTION OF EPIGENETIC REGULATORS

**MLL fusion with other genes:**

MLL gene on 11q23 is often rearranged with other partner genes in ALL (Acute lymphoblastic leukemia) and AML (Adult Acute Myeloid Leukemia):
accounting for 8% of pediatric and 10% of adult ALL, 15-20% of pediatric AML and <3% of adult AML

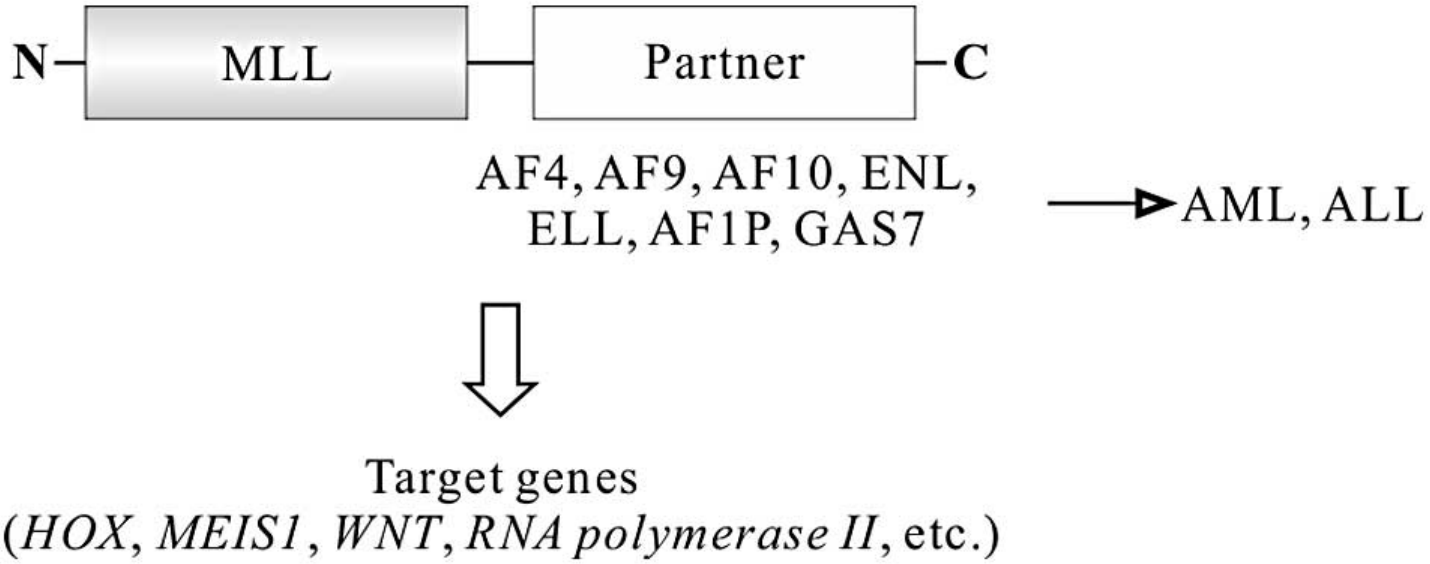
Chromosomal translocations lead to the fusion of 5'-end portion of MLL to one of >60 different partner genes, resulting in the formation of different fusion genes, such as
MLL-AF4 (4q21),
MLL-AF9 (9p22),
MLL-ENL (19p13.3),
MLL-AF10 (10p12),
MLL-AF6 (6q27),
MLL-ELL (19p13.1).
All MLL fusion proteins retain N-terminal AT-hooks, DNA methyltransferase homology domain, thus preserving DNA binding activity whereas the **trithorax PHD domains, TAD and SET domains** are always replaced by the partners.
In these fusions, the original MLL H3K4 methyltransferase activity is replaced by the partners which play a critical role in MLL oncoproteins




**Adult Acute Myeloid Leukemia**

# 5. EXAMPLES OF MUTATIONS THAT CHANGE THE FUNCTION OF EPIGENETIC REGULATORS

**MLL fusion with other genes:**

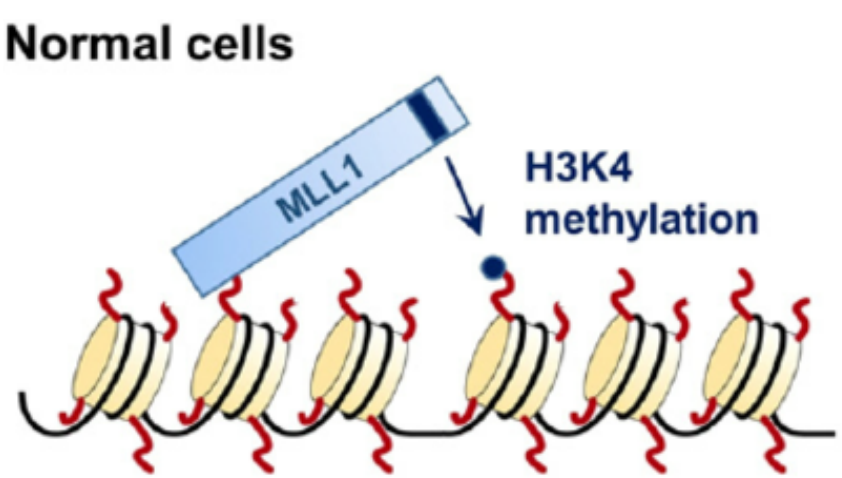



**Aberrant expression of genes that promote cancer formation**
**…classic example: MLL-AF9 (9p22)**

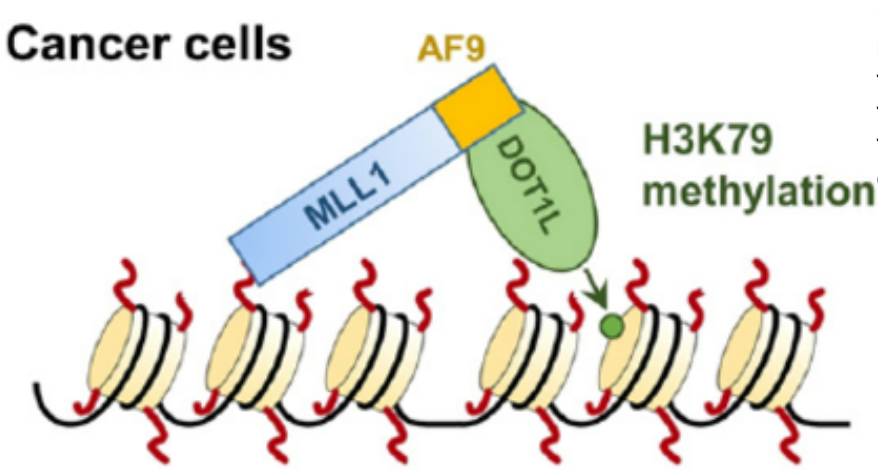
**Adult Acute Myeloid Leukemia**

# 5. EXAMPLES OF MUTATIONS THAT CHANGE THE FUNCTION OF EPIGENETIC REGULATORS

**MLL—AF9 fusion:**




Schematic picture showing the tumor promoting effect of MLL translocations via recruitment of DOT1L. In normal cells, the MLL1 protein binds to its target genes and introduces H3K4 methylation via its catalytic SET domain (highlighted in dark blue).
In the tumor cells MLL1 forms fusions with other protein like AF9, which leads to the loss of the SET domain.




**DOT1-mediated H3K79 methylation is linked to euchromatic gene transcription and transcription elongation.**

Although MLL fusion proteins lose the activity of H3K4 methylation, these fusion proteins gain the activity of H3K79 methylation via recruiting the H3K79 methyltransferase hDOT1L which can cause dysregulation of whole genomic expression and is associated with MLL leukemogenesis. Since hDOT1L plays a key role in the development of MLL leukemia, hDOT1L is an ideal target for MLL leukemia. Several hDOT1L inhibitors are underdeveloped. In particular, EPZ004777, **a special hDOT1L inhibitor, seems to be a promising drug for leukemia with MLL gene translocation**

**Adult Acute Myeloid Leukemia**

# 5. EXAMPLES OF MUTATIONS THAT CHANGE THE FUNCTION OF EPIGENETIC REGULATORS

**MLL—AF9 fusion:**

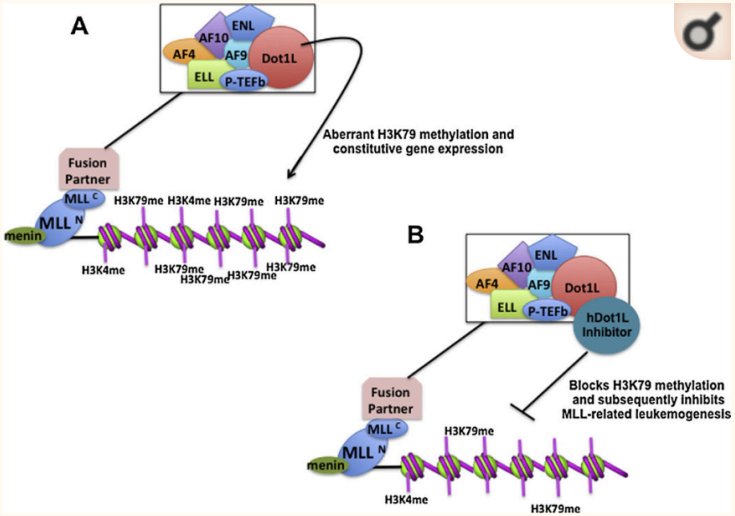
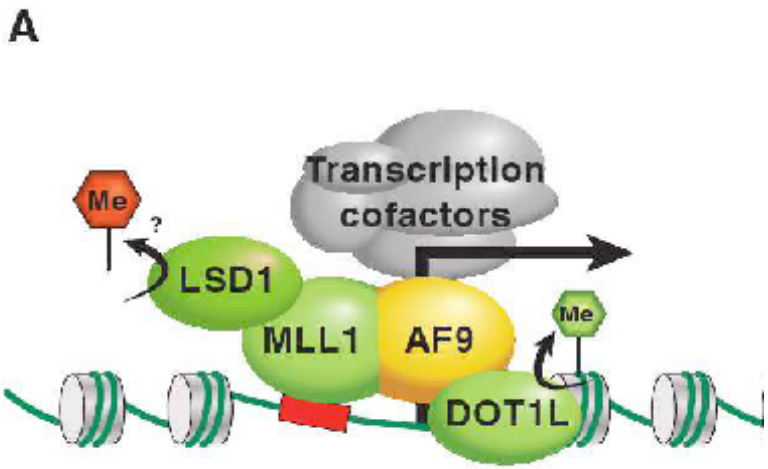



Fig. 3

Model for leukemogenesis mediated by aberrant DOT1L H3K79 methylation. (A) MLL gene undergoes chromosomal translocations and fusion with a number of fusion proteins such as AF9, AF10, and hDot1L. This leads to mistargeting of hDot1L to MLL-regulated genes such as Hox genes. Aberrant H3K79 methylation and the subsequent activation of the target genes result in leukemic transformation. (B) Inhibition of hDot1L HMTase activity with small molecules would prevent aberrant H3K79 methylation and inhibition of MLL-related leukemogenesis.

# 5. EXAMPLES OF MUTATIONS THAT CHANGE THE FUNCTION OF EPIGENETIC REGULATORS

**MLL—AF9 fusion:**




**LSD1: H3K4 de-methylase**

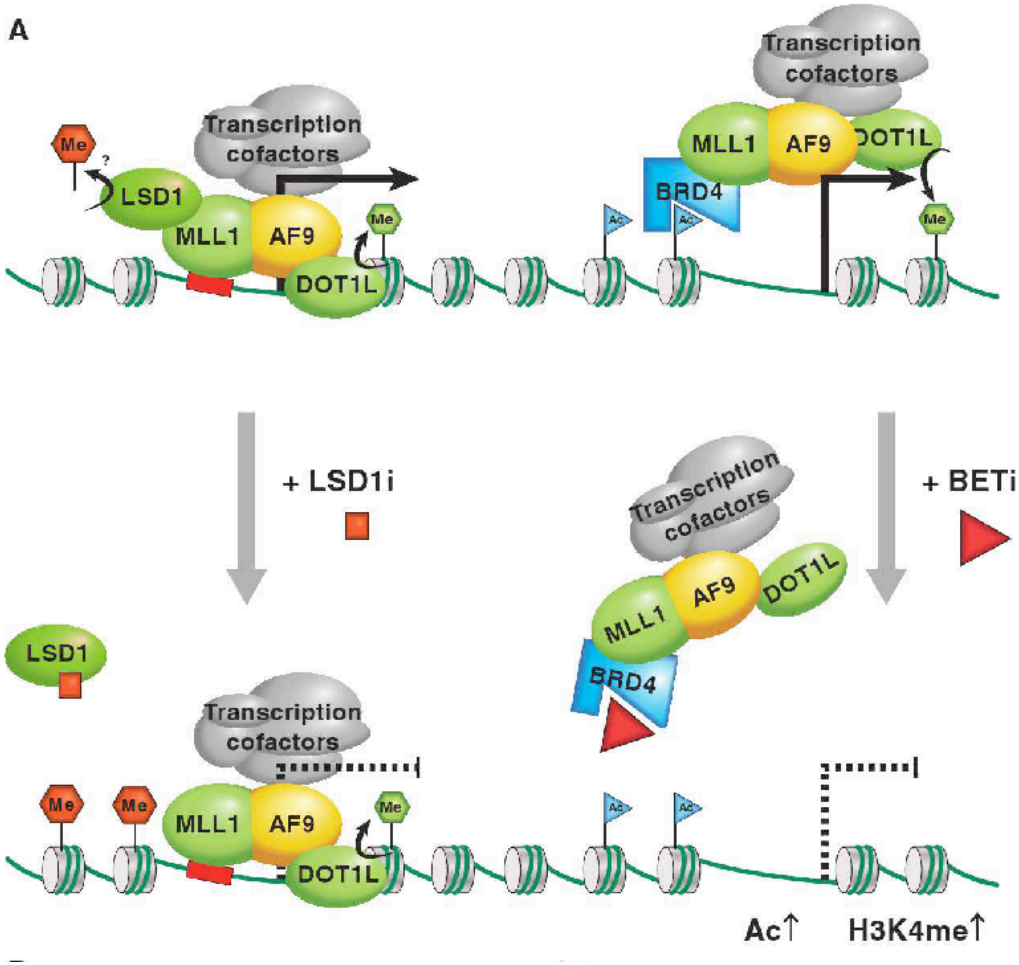
Interaction of LSD1 with MLL-AR9 ensures leukemia stem cells features → oncogenic function

LSD1 inhibitors work in MLL-AR9 fusions models

**Adult Acute Myeloid Leukemia**

# 5. EXAMPLES OF MUTATIONS THAT CHANGE THE FUNCTION OF EPIGENETIC REGULATORS

**MLL—AF9 fusion:**




**Adult Acute Myeloid Leukemia**

# 6. MAJOR EPIGENETIC DRUGS IN CURRENTLY IN DEVELOPMENT

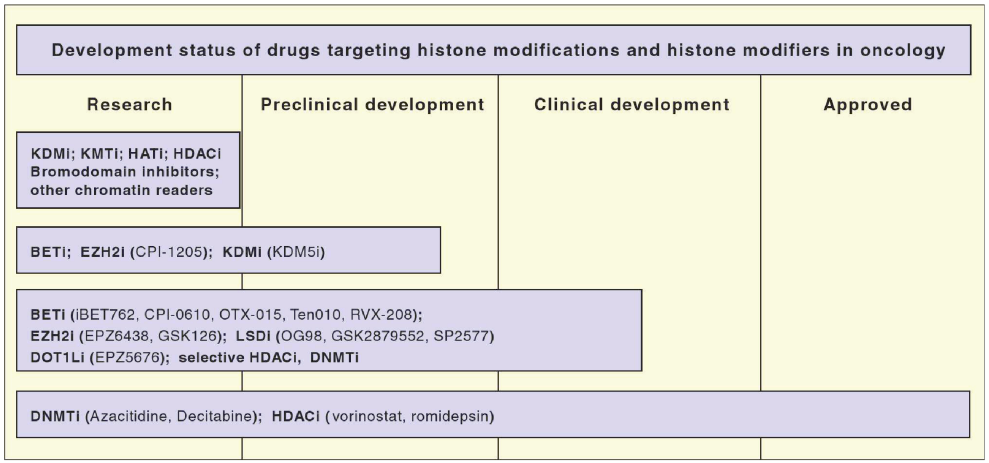



**Figure 5.** The development status of epigenetic drugs. KDMi, lysine demethylase inhibitor; KMTi, lysine methyltransferase inhibitor; HATi, histone acetyltransferase inhibitor; HDACi, histone deacetylase inhibitor; BETi, bromodomain and extracarboxy terminal domain inhibitor; DNMTi, DNA methyltransferase inhibitor.

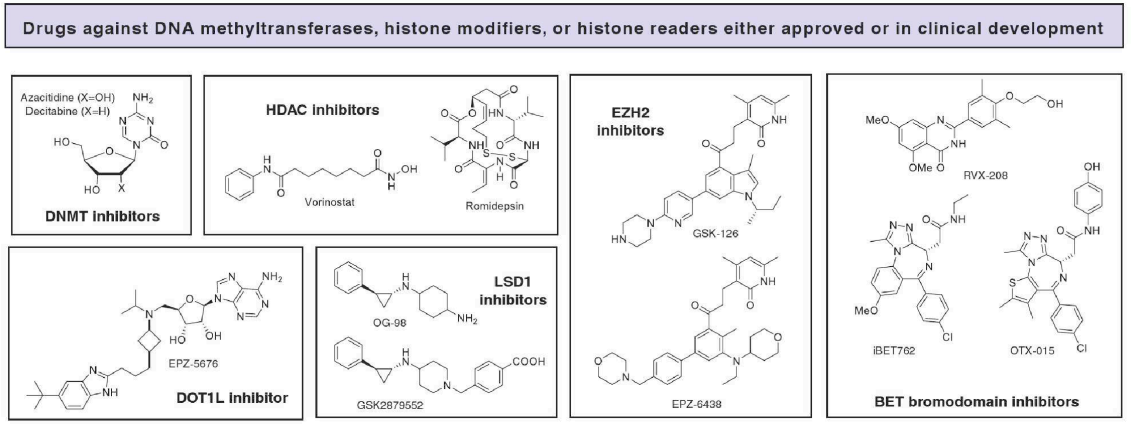
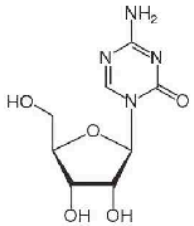
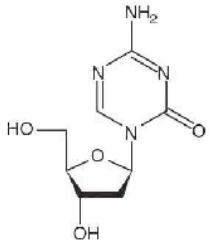
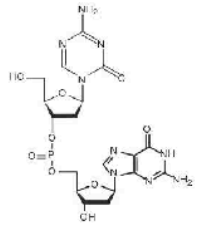



**Figure 6.** Representative structures of drugs targeting DNA methylation, histone modifications, and histone readers in oncology.

# EPIGENETIC DRUGS: DNMT INHIBITORS

| Compound | Structure | Cancer type | Clinical trial status |
|---|---|---|---|
| **DNA methylation inhibitors** | | | |
| 5-Azacytidine<br>5-Aza-CR<br>(Vidaza) |  | Myelodysplastic syndrome; AML | FDA-approved for MDS in 2004 |
| 5-Aza-2′-deoxycytidine<br>5-Aza-CdR<br>Decitabine<br>(Dacogen) |  | Myelodysplastic syndrome; AML | FDA-approved for MDS in 2006 |
| SGI-110 |  | Acute myeloid leukemia; AML | Phase 2 |

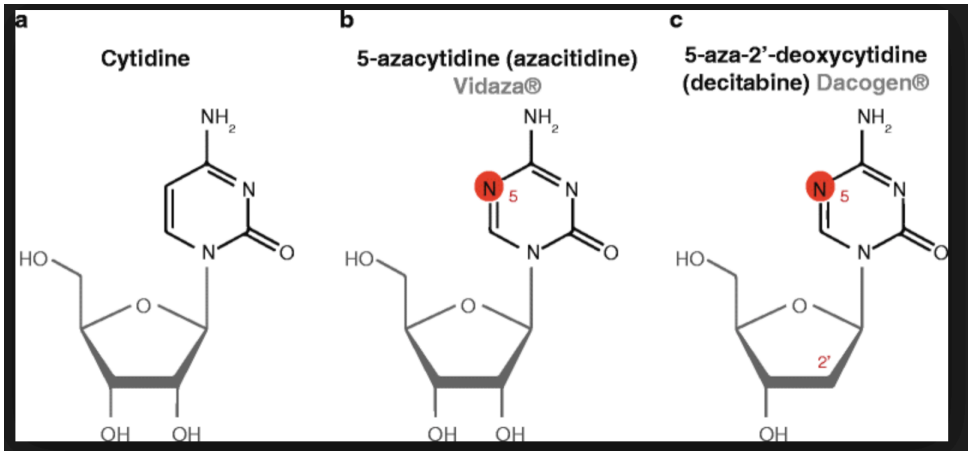
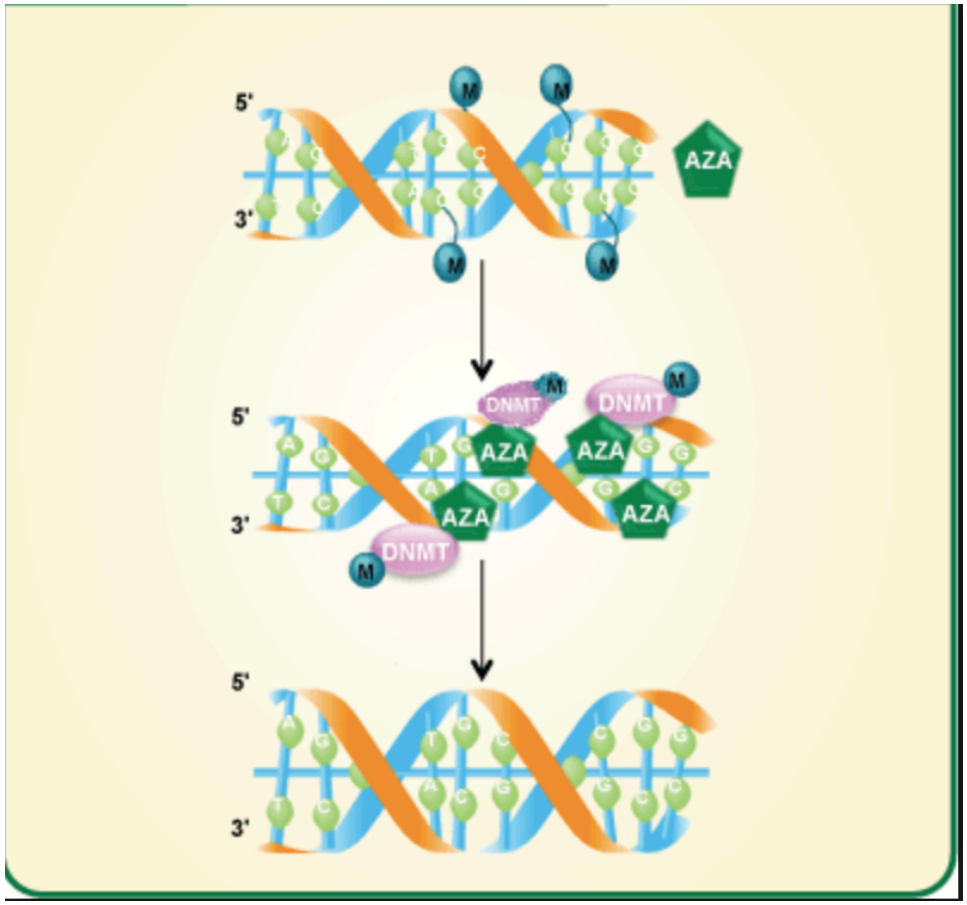



**Figure 8.** Structures of selected epigenetic drugs. Three nucleoside analogs are known that can inhibit DNA methylation after incorporation into DNA. 5-aza-CR (Vidaza) and 5-aza-CdR (decitabine) have been FDA approved for the treatment of the preleukemic disorder, myelodysplasia. Two HDAC inhibitors are also FDA approved for cutaneous T-cell lymphoma and several others are in clinical trials. ]

# EPIGENETIC DRUGS: DNMT INHIBITORS

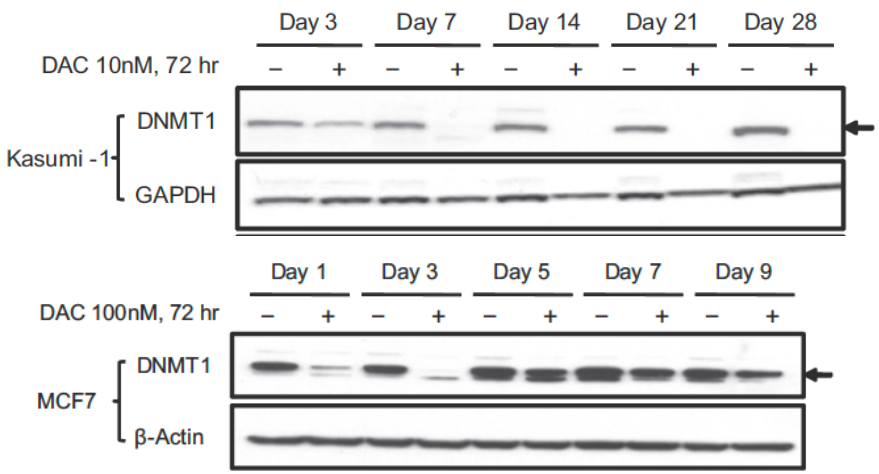



Azacitidine is a chemical analogue of the nucleoside cytidine, which is present in DNA and RNA. It is thought to have antineoplastic activity via two mechanisms – at low doses, by **inhibiting of DNA methyltransferase, causing hypomethylation of DNA, and at high doses, by its direct cytotoxicity to abnormal hematopoietic cells** in the bone marrow through its incorporation into DNA and RNA, resulting in cell death. Azacitidine is a ribonucleoside, so it is incorporated into RNA to a larger extent than into DNA. In contrast, **decitabine (5-aza-2'-deoxycytidine) is a deoxyribonucleoside, so it can only incorporate into DNA**. Azacitidine's incorporation into RNA leads to the dissembly of polyribosomes, defective methylation and acceptor function of transfer RNA, and inhibition of the production of proteins. Its incorporation into DNA leads to covalent binding with DNA methyltransferases, which prevents DNA synthesis and subsequent leads to cytotoxicity.

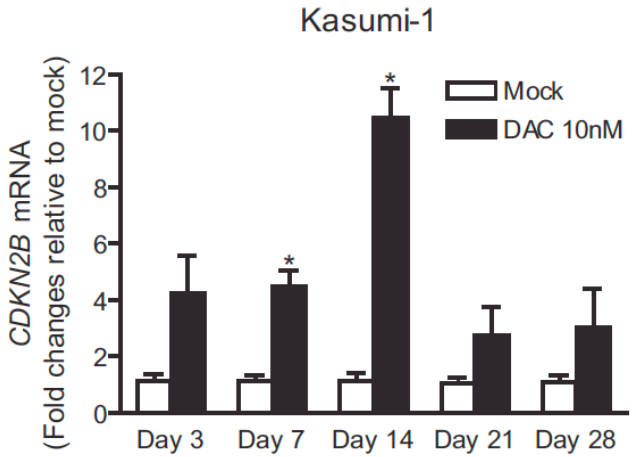
After azanucleosides such as **azacitidine have been metabolized to 5-aza-2'-deoxycytidine-triphosphate** (aka, decitabine-triphosphate), they can be incorporated into DNA and azacytosine can be **substituted for cytosine**. Azacytosine-guanine dinucleotides are recognized as substrate by the DNA methyltransferases, which catalyze the methylation reaction by a nucleophilic attack. This **results in a covalent bond between the carbon-6 atom of the cytosine ring and the enzyme. The bond is normally resolved by beta-elimination through the carbon-5 atom, but this latter reaction does not occur with azacytosine because its carbon-5 is substituted by nitrogen, leaving the enzyme remains covalently bound to DNA and blocking its** DNA methyltransferase function.

In addition, the **covalent protein adduction also compromises the functionality of DNA and triggers DNA damage signaling, resulting in the degradation of trapped DNA methyltransferases. As a consequence, methylation marks become lost during DNA replication.**

# EPIGENETIC DRUGS: DNMT INHIBITORS




Western blot of DNMT1 expression levels in human leukemia cells (Kasumi-1) and breast cancer cell lines (MCF7) after 72 hr daily treatment of 10 nM (leukemia) DACOGEN.




Quantitative real-time PCR analyses of CDKN2B gene expression over time in Kasumi-1 after 72 hr daily treatment of 10 nM DAC. Expression levels are adjusted to GAPDH for each sample and graphed as fold changes relative to mock.

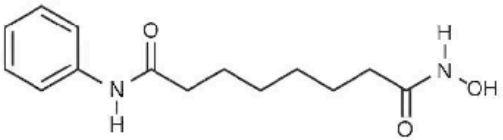
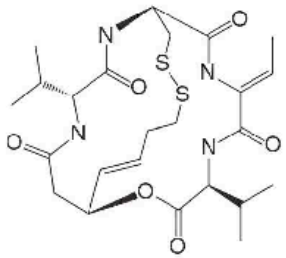
**Special effect in leukemia:**
Treatment with DAC for 72hrs → loss of DNMT1 is mainainted
→ Memory effect «reprogramming of cancer cell

CDKN2B: This gene encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression

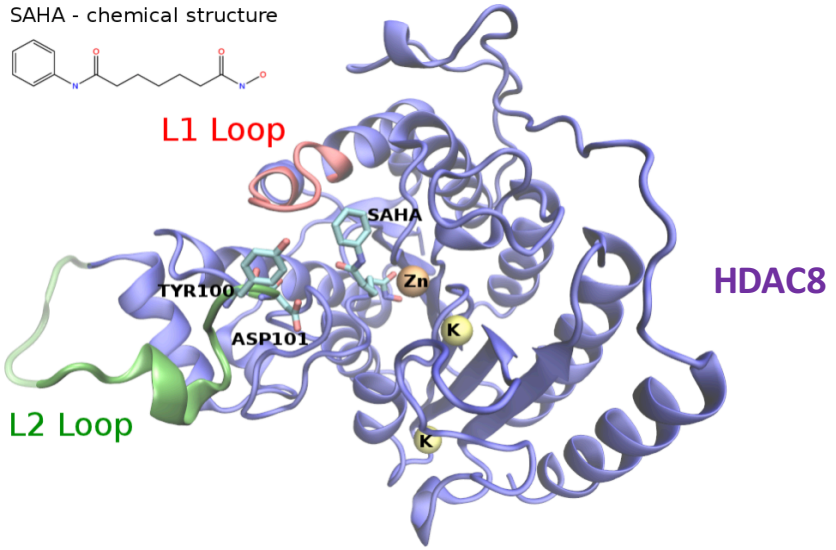
Effect of azanucleosides:
- de-repression of genes
- Other effects: immuneresponse → important anti-tumor effect!

Tsai et al 2012 Cancer Cell

# EPIGENETIC DRUGS: HDAC INHIBITORS

| Histone deacetylase inhibitors | | | |
|---|---|---|---|
| Suberoylanilide hydroxamic acid (SAHA) Vorinostat (Zolinza) |  | T-cell lymphoma | FDA-approved in 2006 |
| Depsipeptide FK-229 FR901228 Romidepsin (Istodax) |  | T-cell lymphoma | FDA-approved in 2009 |

Two HDAC inhibitors are also FDA approved for cutaneous T-cell lymphoma and several others are in clinical trials. Drugs targeting other epigenetic processes are in earlier stages of clinical development (see also Figs. 5 and 6 of Ch. 35 [Audia and Campbell 2014]).

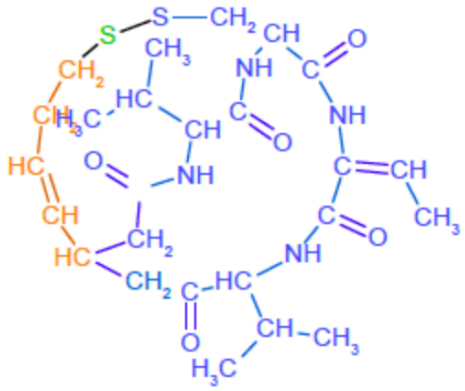



Vorinostat has been shown to bind to the active site of histone deacetylases and act as a chelator for zinc ions also found in the active site of histone deacetylases. Vorinostat's inhibition of histone deacetylases results in the accumulation of acetylated histones and acetylated proteins, including transcription factors crucial for the expression of genes needed to induce cell differentiation. It acts on class I, II and IV of histone deacetylase.

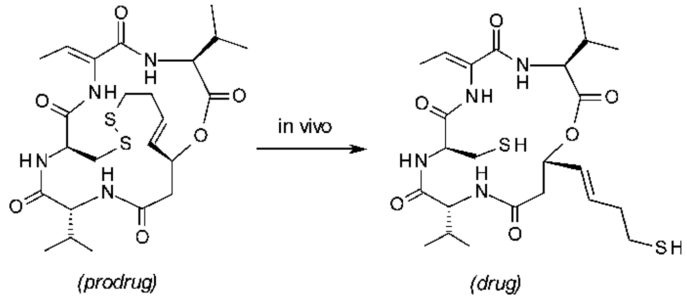
Surface Recognition | Linker | Metal Binding

# EPIGENETIC DRUGS: HDAC INHIBITORS

Romidepsin acts as a prodrug with the disulfide bond undergoing reduction within the cell to release a **zinc-binding thiol**. The thiol **binds to a zinc atom in the binding pocket of Zn-dependent histone deacetylase to block its activity**.



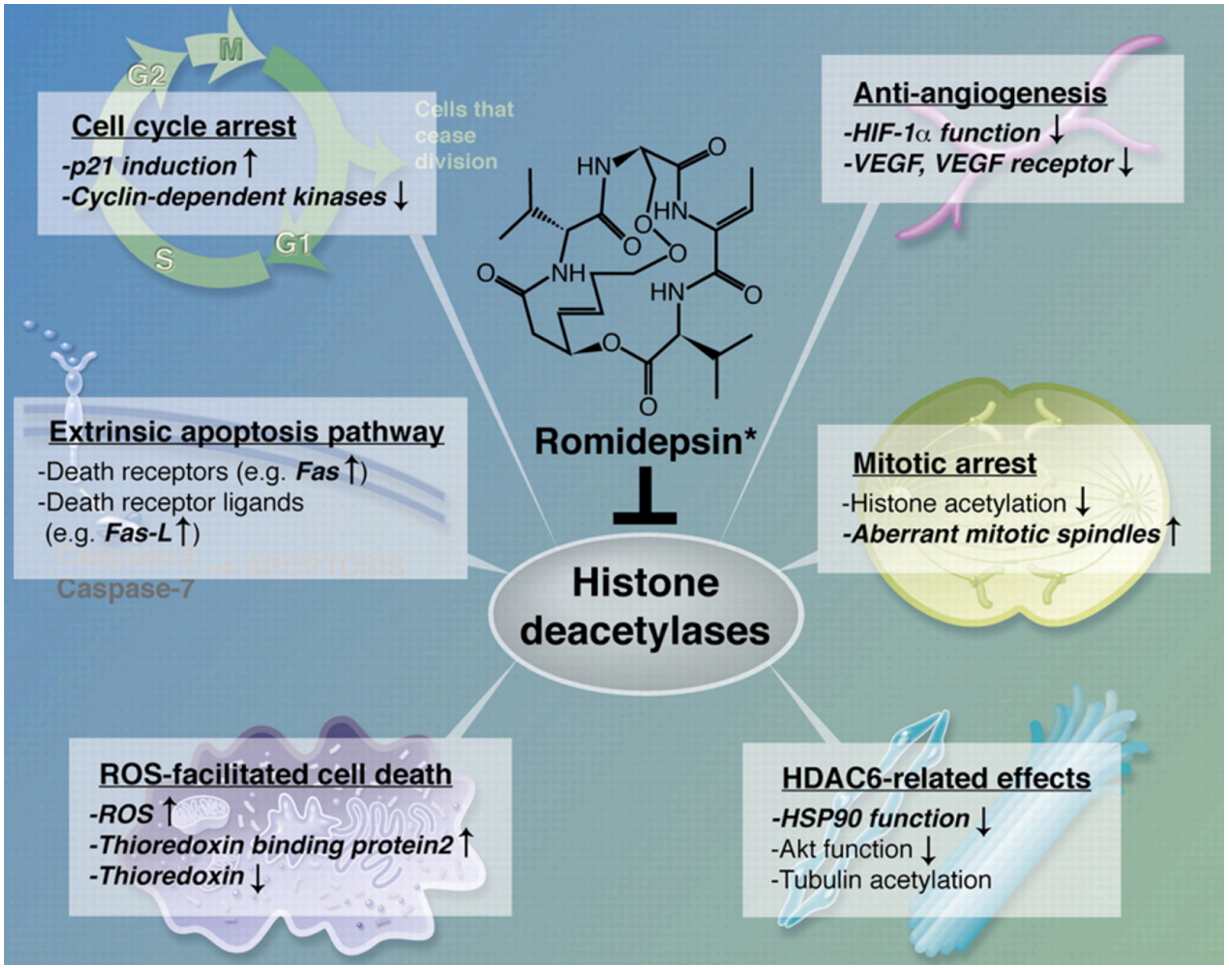
Romidepsin (Istodax®)


**2**
Romidepsin; Istodax
Gloucester Pharmaceuticals, Inc.; now: Celgene Corporation

. Romidepsin (2) is a prodrug, with a disulfide bridge reduced by glutathione upon uptake into the

# EPIGENETIC DRUGS: HDAC INHIBITORS




Work well in lymphoma (T cells) ì but low efficiency in solid tumors when given alone.

Tests: HDAC treatment sensitizes tumors to conventional chemotherapy and newer targeted therapies
i.e. NSCLC: HDACi (entinostat) + EGFRi
i.e. NSCLC: HDACi (vorionostat) + carboplatin and paxitaxel

HDAC inhibiotors increase acetylation levels of genes and cause altered gene expresstion
→ Hypothesis: TSG re-expressed (to be validated)
Many pathways altered → difficult to identify a DIRECT controbution of HDACi