De Novo DNA Methyltransferases in Tumorigenesis

***De novo* DNA methyltransferases bij tumorigenese**

Eveline J. Steine

De Novo DNA Methyltransferases in Tumorigenesis

***De novo* DNA methyltransferases bij tumorigenese**

Eveline J. Steine

ISBN: 978-94-6108-305-0

Lay-out & printing: Gildeprint Drukkerijen, Enschede, The Netherlands.

Cover images: Mount Denali, Denali National Park, AK, USA (front); Bryce Canyon National Park, UT, USA (back); Eveline Steine.

The research described in this thesis was performed at the Whitehead Institute for Biomedical Research affiliated with Massachusetts Institute of Technology (MIT) in Cambridge, MA, USA.

The author of this thesis was supported by the Banning-de Jong Fonds - Prins Bernhard Cultuurfonds. Financial support by the Jurriaanse Stichting for the publication of this thesis is gratefully acknowledged.

De Novo DNA Methyltransferases in Tumorigenesis

***De novo* DNA methyltransferases bij tumorigenese**

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 27 juni 2012 om 17:30 uur

door

Eveline Josephine Steine
geboren te Rotterdam

Promotiecommissie

Promotoren	Prof.dr. J.A. Grootegoed
	Prof.dr. J. Gribnau
	Prof.dr. R. Jaenisch
Overige leden	Prof.dr. F.G. Grosveld
	Prof.dr. C.P. Verrijzer
	Prof.dr. D.N. Meijer

Table of contents

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Epigenetics

Multicellular organisms develop from a single cell, the zygote, that starts to divide and gives rise to daughter cells that subsequently differentiate into specific tissues. The differentiated cells will fulfill specific functions in the body such as the formation of blood, skin or intestine, and become restricted in their potential to develop into various tissues during differentiation. When a cell has differentiated to its final cell type, heritable changes of the DNA are being maintained during cell division, in the form of epigenetic information (described below).

Genetic information is stored in the cells in the form of long DNA strands that are folded into chromatin. Chromatin is composed of repeating units of 146 bp of DNA wrapped around a nucleosome. The DNA connecting the nucleosomes is called linker DNA. A nucleosome consists of an octamer of four different histones, two molecules of each histone 2A (H2A), histone 2B (H2B), histone 3 (H3), and histone 4 (H4) (Figure 1). Other histone variants are H3.3, which can be found on active genes, pericentric heterochromatin and telomeres and can replace H3 [1]; H2A.X is required for efficient DNA double strand break repair, and H2A.Z is reducing chromatin stability and is involved in transcriptional control. Both H2A.X and H2A.Z can replace H2A [2]. Histones are important for chromatin organization and regulating gene expression.

Chromatin exists as euchromatin, which is loosely packaged and easily accessible for transcription, and heterochromatin, which is densely packaged and inaccessible for transcription [3]. Heterochromatin can be divided into constitutive heterochromatin and facultative heterochromatin, with the former being comprised of DNA repetitive elements such as satellite repeats and transposons located at centromeres and telomeres, and the latter being less condensed and transcriptionally active under specific developmental or environmental cues. The chromatin state is dynamically mediated by epigenetic mechanisms.

Epigenetics was first defined by Waddington [4] in the context of embryonic development, as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being.” [5] Currently, epigenetics is defined as a heritable change in gene expression that is not accompanied by changes in DNA sequence [6]. These heritable changes include DNA methylation, histone modifications, non-covalent mechanisms and RNA silencing, and restrict cells to a certain cell type in the developing embryo. Epigenetics plays an important role in embryonic development, genomic imprinting,

X chromosome inactivation, and chromosomal stability. Consequently, dysregulation of the epigenetic machinery can lead to disease and has been found to be a causative factor in cancers, pediatric syndromes, auto-immune diseases and aging [7,8].

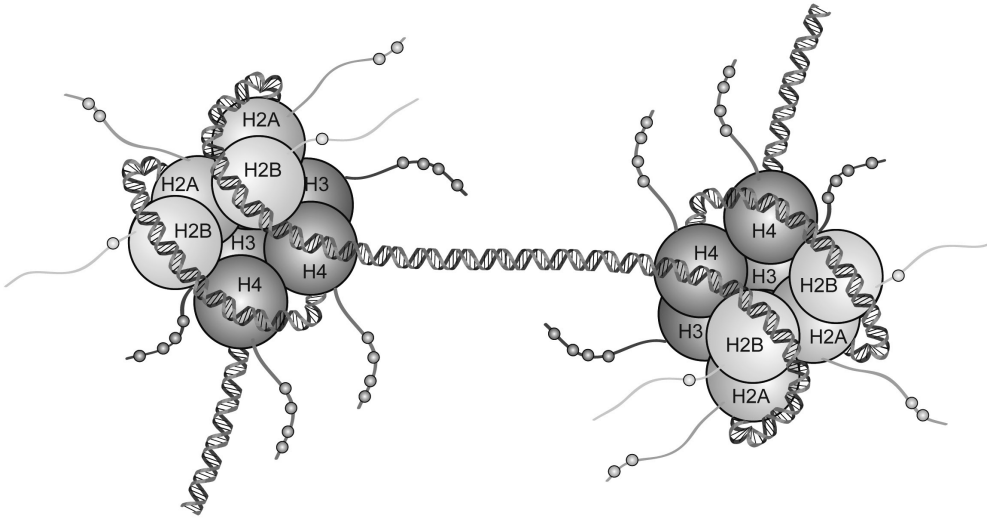


Figure 1. **Nucleosomes**.
A nucleosome is composed of a DNA strand wrapped around an octamer of histones, which consists of H2A, H2B, H3 and H4. The tails of the histones can be modified by methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and ADP ribosylation. These modifications are depicted as circles on the histone tails. The DNA between two nucleosomes is called linker DNA.

1.1.1 DNA methylation

One epigenetic mechanism is DNA methylation, the process in which a methyl group is added to the five position of the pyrimidine ring of cytosine or adenine. DNA methyltransferases (Dnmts) catalyze this reaction by using S-adenosyl-methionine (SAM) as the source of methyl groups, by converting it to S-adenosyl-homocysteine (SAH) (Figure 2). In 1925, Johnson and Coghill first reported about the nucleo-acid 5-methylated cytosine [9]**;** two decades later, in 1948, methylated cytosine was purified from a mammalian tissue, calf thymus [10]. Much later, in 1975, Riggs and Holiday first proposed a model in which methylated cytosine plays an important role in gene regulation, suggesting that a pattern of methylation that is inherited early in development can be propagated indefinitely [11,12].

Although DNA methylation is conserved among species, there are interesting differences. For example in invertebrates like *Ciona intestinalis*, DNA methylation is mosaic, meaning that confined regions of the genome are heavily methylated interspersed with methylation

free regions, whereas in vertebrates it is globally distributed over the whole genome [13,14]. In eukaryotes, DNA methylation plays a role in gene expression and occurs only at cytosines, whereas in prokaryotes, DNA methylation occurs on cytosines and adenines and is important for distinguishing invading phage DNA from the host DNA and regulation of the cell cycle [15,16].

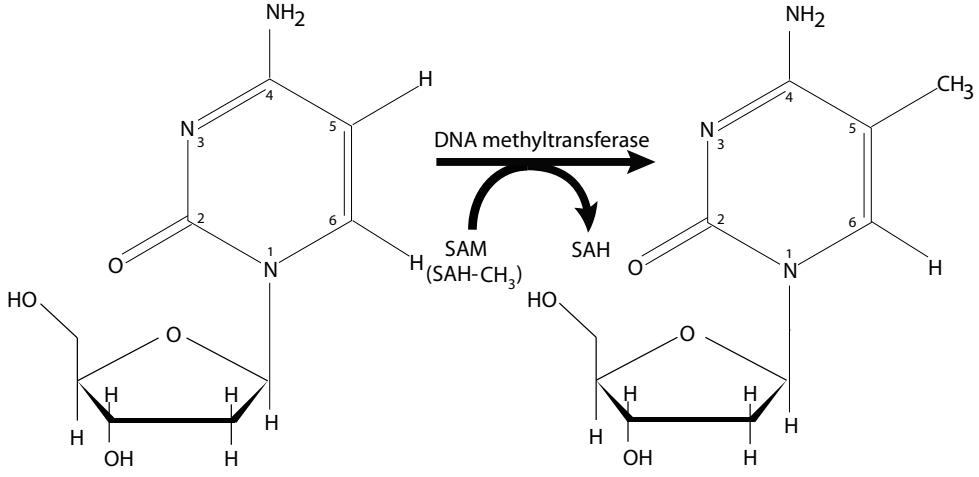


Figure 2. Chemical basis of cytosine methylation.
DNA methyltransferases (Dnmts) catalyze the addition of a methyl group to the 5 position of the pyrimidine ring of the cytosine. S-adenosyl-methionine (SAM) is the donor of the methyl group, which becomes S-adenosyl-homocysteine (SAH) upon losing its methyl group.

Methylation of cytosine

In mammals, methylation of cytosines occurs mainly at CpG nucleotides, while in mammalian embryonic stem cells (ES cells) cytosine methylation also takes place at CpA dinucleotides although to a much lower extent when compared to CpG methylation [17-22]. In plants, however, cytosine methylation occurs at CpG dinucleotides and CpNpG and CpNpN (N=A, C, T) sequences [23,24], and in *Drosophila melanogaster* cytosine methylation occurs at CpT and CpA [25,26]. In addition to differences in sequence specificity, the density of DNA methylation varies widely between species. For example, *Schizosaccharomyces pombe* [27] and *Saccharomyces cerevisiae* [28] have less than 0.1% methylated cytosines in their genome, while *Neurospora crassa* has 1.5% methylated cytosines in its genome [29]. At the lower end of the spectrum, only 0.05-0.1% of cytosines of the *Drosophila melanogaster* genome are methylated [26,30]. The present thesis is focused on DNA methylation in mammals.

Interestingly, in the genome of placental mammals, CpG dinucleotides occur at a much lower frequency than would be expected due to random chance. The methylated genome with a 42% GC content [31] is expected to have C followed by G at 4% of the respective sequences. Yet in the human genome, the frequency of CpG dinucleotides is only 1% [32]. It has been proposed that CpG depletion is due to the high mutability of methylated cytosine, causing a CpG to TpG transition, if not repaired by a DNA repair mechanism, based on a special DNA glycosylase, which recognizes a mismatched T-G base pair and removes the T [32-34]. Seventy to eighty percent of the CpG cytosines are methylated, which corresponds to only 4-6% of total cytosines in the genome [35-37]. In other words, less than 1% of the genome is composed of methylated cytosine [36]. Even though this seems to be a small percentage of the genome, methylated cytosines play a highly important role, as described below, in controlling gene expressing and genomic stability.

Methylation often occurs at CpG-poor regions and less frequently at CpG-rich areas [38]. For example repetitive non-coding DNA such as transposable elements tend to be methylated [39-41]. Transposable elements are transposons and retrotransposons, which are DNA sequences that transposition through the genome without or with a RNA intermediate, respectively. Transposition could cause genome instability and disrupt the function of a gene. DNA methylation of these elements inhibits their transposition [39,41].

CpG islands were first described as CpG-rich regions in 1985 by Bird et al. [38]. Later, in 2002, Taka and Jones modified the definition of a CpG island, to specify that the islands comprise 500 bp regions that contain 55% CpG or GpC with an (observed CpG)/ (expected CpG) ratio of greater than 0.6 [42]. CpG islands are usually located at the 5' terminus (promoter, untranslated region and 1st exon) of a gene [38,43,44] and correlate with gene silencing. Approximately 40% of the human genes contain a CpG island [42,45]. Gene promoters with CpG islands can be subdivided into promoters with high density CpGs (HCP), including genes regulating embryonic development and ubiquitously expressed housekeeping genes, and low density CpG (LCP) including mainly tissue specific expressed genes [38,46-48]. Thus, upon differentiation a subset of the promoters acquires DNA methylation [49], thereby inhibiting transcription of the corresponding genes (Figure 3).

Furthermore some CpG islands are also located in differentially methylated promoter or regulatory regions the so called imprinted control regions (ICR), which are important for genomic imprinting. Genomic imprinting describes a process in which certain genes are expressed in a parent-of-origin specific manner [50]. More specifically, some genes are only expressed from the paternal allele, since the maternal allele is methylated, while other

genes are only expressed from the maternal allele due to methylation of the paternal allele [50]. Additionally, there are also imprinted genes that are expressed from the allele of which the ICR is fully methylated [50].

While DNA methylation in promoters is associated with gene silencing, it has been shown that DNA methylation in the gene body correlates with active transcription [21,22,51,52]. Additionally, it has been suggested that DNA methylation might play a role in tissue-specific transcript splicing since it was observed that DNA methylation sharply changes at intron-exon junctions [21,53]. However, the weight of the evidence indicates that the majority of methylated cytosines detected in CpG-poor repetitive elements function to maintain genome stability; methylated cytosines detected in CpG-rich promoter regions promote gene silencing**.**

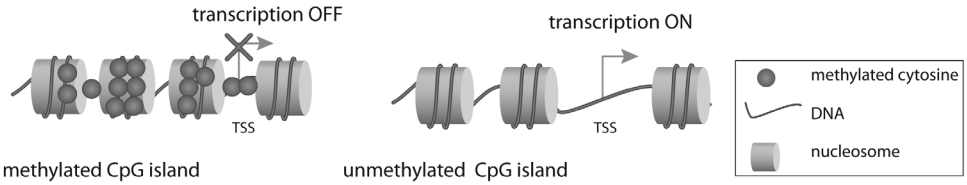


Figure 3. **DNA methylation at CpG islands.**
Schematics of a gene with a CpG island promoter region. When the CpG nucleotides are methylated, the chromatin structure is condensed, thus the transcriptional start site (TSS) is not available for transcription and the gene is inactive. When the CpG nucleotides are unmethylated the gene is active, since the chromatin is less condensed and the TSS is available for transcription. Adapted from:Rodríguez-Paredes and Esteller (2011) [138].

DNA methylation and embryonic development

For proper development of an organism it is important that the developmental genes are available for transcription. The methylation patterns of both the paternal and maternal genomes are therefore erased when the sperm fertilizes the egg and the zygote is being formed (Figure 4). The paternal genome rapidly loses its DNA methylation through active demethylation [54,55], while the maternal genome loses its methylation due to passive demethylation during cleavage divisions. Some genomic regions are resistant to this wave of demethylation, i.e. the ICR [56], intracisternal A particle (IAP) retrotransposons [57,58] and pericentromeric heterochromatin [59]. Post-implantation, a new methylation pattern is established on the embryonic genome by DNA *de novo* methyltransferases; this methylation is important for regulating gene expression and to ensure proper embryonic development. A second wave of demethylation occurs during E10.5 and E12.5, but only in the male and female primordial germ cells. This second wave is more complete than the first wave, and

includes the ICRs; however, IAP retrotransposons remain methylated [57,60]. The mechanism of DNA demethylation, at either one of the two waves, is unclear. Methylated cytosine can be hydroxylated to hydroxymethylated cytosine, which in turn may not be recognized by DNA methyltransferase Dnmt1, thereby resulting in loss of the methyl mark [61]. During fetal development and postnatal life, differentiating somatic cells gain new DNA methylation marks in a stepwise manner, and the germ line cells acquire maternal and paternal genomic imprints at sex-specific steps of gametogenesis [62].

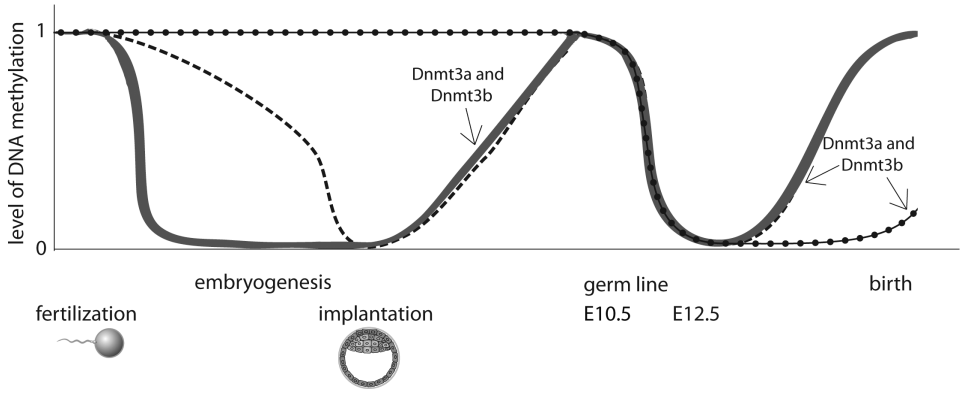


Figure 4. **DNA methylation during development.**
Demethylation of DNA takes places twice during embryonic development. The relative mean level of methylation of genes is shown, meaning that the respective starting level in mature gametes is set at 1, for different groups of genes. Imprinted genes are represented by the dotted line, non-imprinted genes of the paternal genome by the grey line, and non-imprinted genes of the maternal genome by the striped line. When the sperm cell fertilizes the egg to form the zygote, both the paternal and maternal DNA becomes demethylated with the exception of the imprinted genes. Demethylation happens rapidly through active demethylation for the non-imprinted paternal sequences, and slowly for the maternal genome due to passive demethylation during cleavage divisions. During primordial germ cell development (E10.5-E12.5 in the mouse) the genome undergoes a second wave of demethylation, which includes the imprinted genes. The new DNA methylation patterns are established by the *de novo* methyltransferases Dnmt3a and Dnmt3b, both in somatic cells and germ line cells.

DNA methyltransferases

DNA methyltransferases (Dnmts) are enzymes responsible for catalyzing the DNA methylation process. In mammals, there are three DNA methyltransferase; *Dnmt1*, *Dnmt2* and *Dnmt3*. Dnmt1 and isoforms of Dnmt3 are catalytically active DNA methyltransferases [63]. Dnmt1 is a maintenance methyltransferase. During replication Dnmt1 copies the DNA methylation pattern from the mother strand onto the daughter strand, ensuring the maintenance of the DNA methylation. *Dnmt3* enzymes are *de novo* methyltransferase, which are important for establishing a DNA methylation pattern during embryonic

development (Figure 5). The schematic structure of the Dnmts is depicted in Figure 6. The N-terminal domain is important for regulating nuclear localization and binding to interacting proteins and DNA. The size of this domain varies widely between the different Dnmts, with Dnmt1 having a large N-terminus, Dnmt3 having a small N-terminus, and Dnmt2 lacking it completely. The C-terminus, which is conserved between prokaryotes and eukaryotes, contains the catalytically active domain. It contains the ADoMet-dependent MTase fold, consisting of 10 amino acid motifs, by which all Dnmts can be recognized. Motifs 1 and 10 of this DNA cytosine-5-methyltransferase domain are important for co-factor binding, whereas motifs 4, 6 and 8 are important for catalysis. The region between motifs 8 and 9 is not conserved and is known to be important for DNA recognition and specificity (see review by Lan et al. [63]).

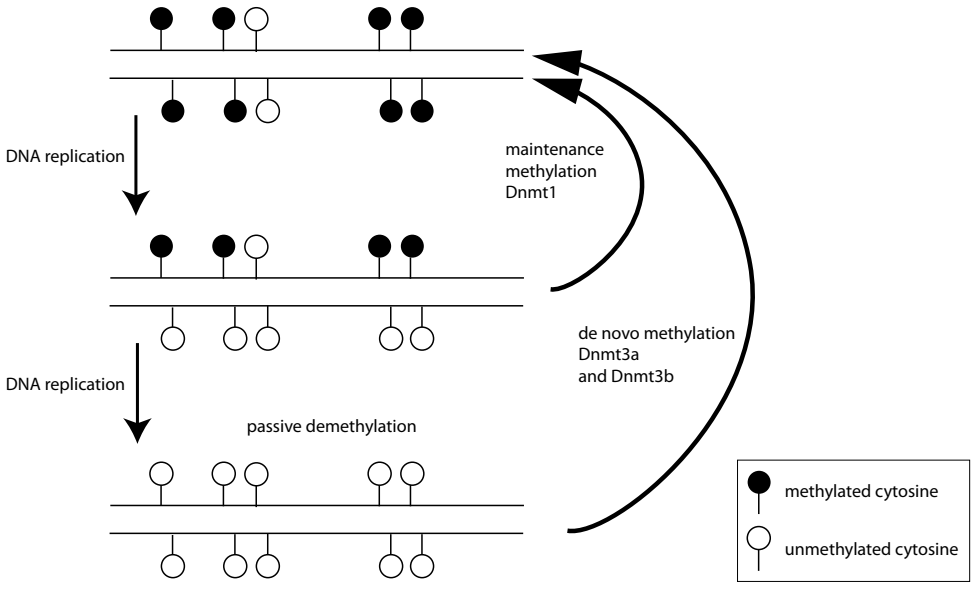


Figure 5. **Establishing and maintaining DNA methylation.**
During embryonic development, *de novo* DNA methylation is established by the *de novo* methyltransferases Dnmt3a and Dnmt3b. During DNA replication, the DNA methylation is maintained on the daughter strand by maintenance methyltransferase Dnmt1, which prefers to bind to hemimethylated DNA. In the absence of Dnmt1, passive demethylation occurs.

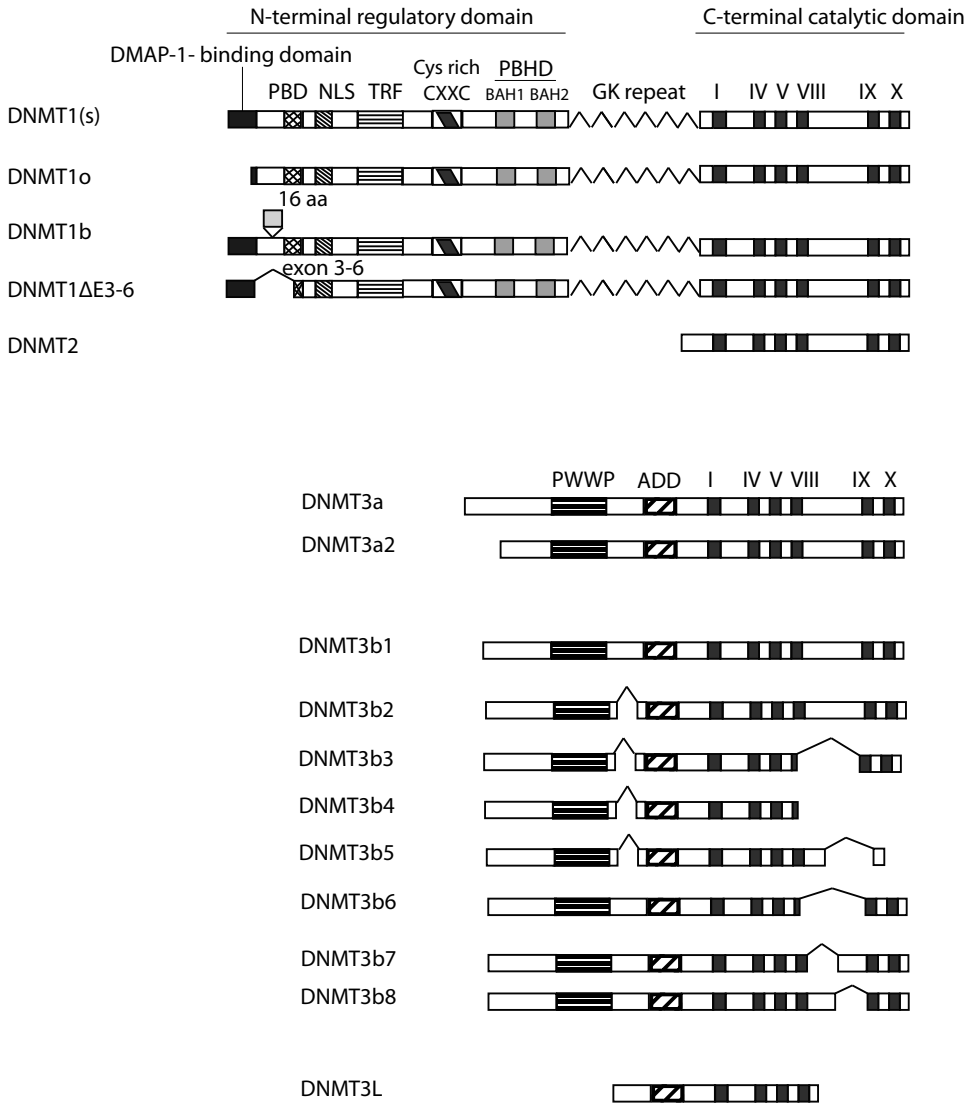


Figure 6. **Schematic representation of the primary protein structures of human DNA methyltransferases Dnmt1, Dnmt2, and Dnmt3, and their isoforms**.
DMAP-1-binding domain: DNA methyltransferase 1-associated protein 1 binding domain, PBD: PCNA binding domain, NLS: nuclear localization signal, TRF: target replication foci, Cys rich CXXC: cysteine rich CCXC domain, PBHD: polybromo homology domain, BAH1: bromo adjacent homology 1 domain, BAH2: bromo adjacent homology 2 domain, GK-repeat: glycine-lysine repeat, PWWP: proline-tryptophan-tryptophan-proline, ADD ATRX-DNMT3-DNMT3L Domain. I, IV, VI, VIII, IX, X conserved amino acid motifs 1, 4, 6, 8, 9 and 10 respectively [63,206,207].

DNA methyltransferase 1

Dnmt1, the first characterized mammalian DNA methyltransferase 64, is a maintenance methyltransferase. As described above, a maintenance methyltransferase copies the pre-existing methylation pattern from the mother strand on the newly synthesized daughter strand during DNA replication (Figure **5**), which is key to its role in epigenetic inheritance. More specifically, during S phase, Dnmt1 is located in the replication foci [65] and recognizes hemimethylated DNA in a complex with UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1) [66,67]. Dnmt1 has a higher affinity to hemimethylated DNA compared to unmethylated DNA [68,69]***.*** In humans, DNMT1 has four isoforms and one untranslated transcript (Figure 6) (reviewed by Lan et al. 2010 [63]). The most common isoform is DNMT1 (also called DNMT1s), which is expressed in somatic tissues. DNMT1b has similar catalytic activity as DNMT1, but it is expressed at much lower levels than DNMT1. DNMT1$^{\Delta E3\text{-}6}$ is another isoform that lacks the region encoded by exons 3-6. This results in a truncated DNA methyltransferase 1-associated protein 1 (DMAP1) binding domain and a deleted proliferating cell nuclear antigen (PCNA) binding domain. Despite the loss of exons 3-6, the encoded DNMT1$^{\Delta E3\text{-}6}$ is catalytically active. The fourth isoform is DNMT1o, an oocyte specific isoform. Additionally, untranslated transcript *DNMT1p* is expressed in pachytene spermatocytes [63,70]. The most common isoform Dnmt1 has been extensively studied.

Dnmt1 plays an important role in development, as demonstrated by three knockout studies. The first study showed that deletion of 900bp encoding the N-terminus of Dnmt1 (*Dnmt1^{n}*) results in a hypomorphic allele, with only partial loss of function. *Dnmt1$^{n/n}$* embryos die in midgestation at the 20-somite stage, and show a 60% reduction of DNA methylation [71]. In a second study, the authors mutated the domain important for binding to the DNA replication fork (*Dnmt1^{s}*) creating a more severe loss of function allele than the *Dnmt1^{n}* allele [72,73]. The third study reported the generation of a null allele for *Dnmt1*, by deleting the catalytic domain in the C terminus (*Dnmt1^{c}*) resulting in hypomethylation and a more severe phenotype [72]. The homozygous *Dnmt1$^{s/s}$* or *Dnmt1$^{c/c}$* embryos die earlier than *Dnmt1$^{n/n}$* embryos and do not develop further than the 8-somite stage [72,73]. Moreover, DNA methylation levels in the homozygous *Dnmt1$^{s/s}$* or *Dnmt1$^{c/c}$* knockout embryos are significantly lower than observed for the *Dnmt1$^{n/n}$* embryos [72,73]. The exact cause of embryonic lethality has not been elucidated. It has been hypothesized that loss of Dnmt1 may destabilize the replication complex at the DNA replication fork, thereby reducing proliferation and inducing apoptosis through p53 [74]. Similar to *Dnmt1$^{c/c}$* embryos, the genome of *Dnmt1$^{c/c}$* ES cells is hypomethylated compared to the wild type ES cells; however, *Dnmt1$^{c/c}$* ES cells grow

normally when kept in an undifferentiated state [72]. It has further been shown that Dnmt1 plays a role in several other processes, discussed next.

Dnmt1 is required for X chromosome inactivation in female cells, which results in an equal dose of expression of X-linked genes in male and female cells. Inactivation of the X chromosome is regulated by the *Xist* gene, which is silent and methylated on the active X chromosome and active and unmethylated on the inactive X chromosome. The *Xist* gene product is an untranslated RNA. *Xist* expression is low in undifferentiated wild type ES cells but is upregulated on the inactive X chromosome. In *Dnmt1* knockout ES cells the Xist promoter is not properly methylated on the active X chromosome and therefore *Xist* is ectopically expressed in *Dnmt1* knockout female ES cells and mutant female embryos. Consequently, *Dnmt1* mutant embryos and differentiating mutant ES cells have aberrant X-inactivation, indicating that Dnmt1 is important for X-inactivation [75,76].

Dnmt1 is also known to be necessary for maintenance of genomic imprinting. Genomic imprints, which are differentially methylated regions between maternal and paternal alleles, are established during germ cell development. It has been shown that Dnmt1s and Dnmt1o is essential for maintaining imprints, since *Dnmt1*$^{-/-}$ ES cells and heterozygous embryos from *Dnmt1o*$^{-/-}$ females show loss of imprinting [73].

In addition, Dnmt1 has been demonstrated to be important for maintaining genome stability and silencing of transposons like IAPs. *Dnmt1* mutant cells display increased mutation rates, mostly due to deletions either by mitotic recombination or due to chromosomal loss [77]. Mice with only 10% Dnmt1 activity developed thymic lymphomas with hypomethylated centromeric repeats and trisomy 15 [78]. Moreover, Eden et al. (2003) showed that reduction of Dnmt1 levels in fibroblasts derived from mice prone to develop soft tissue sarcoma leads to an increased loss of heterozygosity (LOH) of entire chromosome arms [79]. Additionally, *Dnmt1* mutant embryos were demethylated for the IAPs, and expression of these transposons is increased [80]. Furthermore, mice with only 10% Dnmt1 activity develop thymomas that have transposed IAPs [81]. In summary, maintenance DNA methyltransferase Dnmt1 activity is indispensable for embryonic development and important for X-inactivation, genomic imprinting, and maintaining chromosomal stability.

DNA methyltransferase 2

Dnmt2 was identified as a DNA methyltransferase since it contains all the sequence motifs of a DNA (cytosine-5)-methyltransferases, except the N-terminal domain (Figure 6) [82-84]. Although Dnmt2 is ubiquitously expressed in mouse and human tissues [83], no maintenance

or *de novo* DNA methylase activity has been detected [83,84]. However, Dnmt2 methylates three transfer RNAs at cytosine 38 in flies [85,86]. Two of these three tRNAs have been studied more extensively in mice, where they are protected from ribonuclease cleavage when methylated [86]. *Dnmt2* knockout mouse ES cells grow normally and show no abnormalities in their DNA methylation status compared to wild type ES cells [84]. In accordance with these data, *Dnmt2* mutant mice are phenotypically normal with an unchanged DNA methylation pattern, suggesting that Dnmt2 is not essential for mammalian development.

DNA methyltransferase 3

Dnmt3 includes two catalytically active enzymes, Dnmt3a and Dnmt3b, and catalytically inactive Dnmt3L. *De novo* methyltransferases Dnmt3a and Dnmt3b are important for establishing a methylation pattern in the post-implantation embryo and in germ cells (Figures 4 and 5) [87,88]. Dnmt3a and Dnmt3b have partially redundant *de novo* methyltransferase capacity. For example, proviral DNA becomes methylated *in Dnmt3a* or *Dnmt3b* knockout ES cells, but it remains unmethylated in *Dnmt3a* and *Dnmt3b* double knockout ES cells [88]. It has further been shown that Dnmt3a and Dnmt3b are important to maintain methylation on repetitive elements and genomic imprinted loci, since *Dnmt3a* and *Dnmt3b* double knockout ES cells lose methylation on these loci over time [89-91].

DNA methyltransferase 3a

Two isoforms of the *de novo* methyltransferase *Dnmt3a* have been described, Dnmt3a and Dnmt3a2 (Figure 6) [92]. Dnmt3a2, expressed from an intronic promoter, is shorter than Dnmt3a and localizes with euchromatin, while Dnmt3a localizes with heterochromatin. Dnmt3a2 is predominantly expressed in mouse ES cells, germ cells, embryonal carcinomas, spleen and thymus, and Dnmt3a is ubiquitously expressed at low levels [88,92,93]. *Dnmt3a* knockout mice are runted at birth and die within 4 weeks after birth; nevertheless, the global methylation pattern of these animals seems to be normal [88]. Dnmt3a and Dnmt3a2 are important for establishing and maintaining methylation at imprinted loci. For example, *Dnmt3a*$^{-/-}$*, Dnmtb*$^{-/-}$ ES cells cultured for a long period are demethylated for the ICR of *H19/Igf2* and the *Xist* promoter regions [89]. Both loci are methylated upon re-introduction of *Dnmt3a2* expression, and *Xist* promoter region is also methylated upon re-introduction of *Dnmt3a* expression. In addition, deletion of *Dnmt3a* after day 9.5 of embryonic development results in animals that develop to adulthood but these mice cannot deliver live pups, due to impaired maternal and paternal imprinting in their germ cells [43]. Additionally, it has been shown that

the interaction of Dnmt3a with Dnmt3L is important for establishing genomic imprints in oocytes [43,94]. Further, Dnmt3a methylates major satellite repeats in the pericentromeric regions [89]. In summary, *de novo* DNA methyltransferase Dnmt3a and Dnmt3a2 are important for embryonic development and are necessary for genomic imprinting.

DNA methyltransferase 3b

Dnmt3b has 8 different isoforms, Dnmt3b1 – 8, which are the result of alternative splicing involving exons 10, 11, 21 and 22, as shown in Figure 6 [63]. Of these isoforms, only Dnmt3b1 and Dnmt3b2 are catalytically active [95]. Dnmt3b isoforms 4 through 8 are catalytically inactive [89,92] and it has been suggested that Dnmt3b3, the most ubiquitously expressed isoform, has a regulatory function [96]. Further discussion will focus on Dnmt3b1.

Dnmt3b1 (further referred to as Dnmt3b) is highly expressed in ES cells and in the developing mouse embryo [84,88]. Moreover, *Dnmt3b* knockout mice die around 9.5 days post coitum (dpc) with multiple developmental defects [88]. Additionally, *Dnmt3b* knockout E9.5 embryos and *Dnmt3b* ES cells have demethylated minor pericentromeric satellite repeats in contrast to *Dnmt3a* knockout embryos, which are demethylated in the major pericentromeric satellite repeats. *Dnmt3a* and *Dnmt3b* double knockout mice die at 8.5 dpc, with lower methylation levels than wild type embryos. Their global methylation, however, is not reduced to the extent as observed for *Dnmt1* knockout mice. Similarly, ES cells deficient for Dnmt3a and Dnmt3b have mildly reduced global methylation, which is less severe than observed for *Dnmt1* knockout ES cells.

It is known that mutations in *Dnmt3b* are connected to human disorders. For example, a mutation in *Dnmt3b* causes immunodeficiency, centromeric instability and facial anomalies syndrome (ICF) [88,97,98]. The clinical symptoms of this syndrome are severe recurrent infections in early childhood, facial abnormalities and mental retardation in 30% of the patients. Notably, these patients have hypomethylated pericentromeric satellite repeats 2 and 3 on chromosomes 1, 9 and 16 [99]. Thus, *de novo* methyltransferase Dnmt3b is essential for embryonic development and important for methylation of pericentromeric elements, both in mouse and human.

DNA methyltransferase 3-Like

Dnmt3-like (Dnmt3L) is a catalytically inactive Dnmt3 that plays an important role in regulating DNA methylation. More precisely, Dnmt3L and Dnmt3a form a tetrameric complex, which preferentially methylate CpG dinucleotides with 8-10 bp separation[100,101].

Dnmt3L knockout mice are viable and appear to develop normally; yet, male *Dnmt3L* knockout mice are infertile [94,102,103]. Specifically, one week after birth the seminiferous tubuli in the testes of *Dnmt3L* knockout males contain spermatogonia similar to wild type males. However, when the *Dnmt3L* knockout animals become adult, the spermatogonia fail to develop into spermatocytes and spermatids, possibly due to demethylation and reactivation of retrotransposons elements and meiotic failure [104]. Female *Dnmt3L* knockout mice are fertile, but their embryos die around 9.5-10.5 dpc with developmental defects and a smaller chorion placenta. Interestingly, the global methylation pattern is unaffected in *Dnmt3L* knockout embryos and ES cells; however, the maternally imprinted loci are demethylated in the male and female germ cells as was found for retrotransposons in male germ cells [94,102-104]. Thus, although Dnmt3L has no catalytic activity, it has important regulatory functions and it is essential for establishing maternal genomic imprints.

Directing *de novo* Dnmts to DNA

DNA methylation mainly occurs on CpG dinucleotides; however, not all cytosines next to a guanine are methylated. The exact mechanism behind the target specificity of *de novo* Dnmts is not well understood. Different groups reported that *de novo* Dnmts have sequence specificity beyond the CpG dinucleotides when methylating CpG dinucleotides. Dnmt3a prefers to methylate CpG as RCGY (R=purine, Y=pyrimidine), and Dnmt3b preferably methylates cytosines with YCGR as flanking sequence [105,106]. It has been shown that not all RCGY and YCGR sequences are methylated, indicating that the DNA sequence is not the only factor determining which cytosine gets methylated by the Dnmt3a and Dnmt3b [105], other factors which maybe involved will be discussed later in this thesis.

1.1.2 Histone modifications

Histone modifications, in addition to DNA methylation, are a part of the epigenetic machinery and play regulatory roles in gene expression. Furthermore, there are indications that histone modifications and DNA methylation regulate each other. The histones contain histone tails, which protrude from the nucleosomes. These tails can be post-translationally modified by methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and ADP ribosylation (Figure 1). Consequently, the expression of the genes encoded in the DNA in close proximity of these histone modifications is regulated. For example, acetylation opens up the chromatin for transcription. More specifically, deacetylated histones 3 and 4 are positively charged and form a tight configuration with negatively charged DNA,

consequently this DNA is not accessible for transcription. Acetylation of lysine residues in histone tails, mediated by histone acetylation transferase (HAT), neutralizes the positive charge of the histones, making chromatin accessible for the transcription machinery [3].

Histone methylation may promote or inhibit gene expression depending on which amino acid residue is methylated. For example, methylation of lysine 4 and 36 of histone 3 (H3K4 and H3K36) are associated with gene expression, while methylation of lysine 9 and 27 (H3K9 and H3K27) are associated with gene silencing [107]. The histone methylation can occur in three flavors, mono-, di- and trimethylation, which is mediated by histone methylases [3]. Interestingly, histone methylation is reversible, which is mediation by histone demethylases[3].

Histone modifications and DNA methylation are highly correlated, and it has been suggested that one attracts the other. Certain histone marks may repulse or attract Dnmts and thus direct DNA methylation to certain loci. First, DNA methylation positively correlates with unmethylated H3K4. Genome wide studies have shown that DNA methylation and H3K4 methylation are inversely correlated [108-111]. H3K4 trimethylation (H3K4me3) is mostly observed at the transcriptional start site (TSS) of active genes, which lack DNA methylation. Conversely, chromatin regions which lack H3K4 methylation contain methylated DNA. DNA methyltransferases Dnmt3a, Dnmt3b and Dnmt3L contain an ATRX-Dnmt3a-Dnmt3L (ADD) domain that binds to unmethylated H3K4 and stimulates Dnmt3a methylation activity *in vitro* [112-115]. Additionally, Dnmt3a2 methylates the linker DNA upon binding to unmethylated H3K4 [112,114]. Further evidence that H3K4 methylation inhibits DNA methylation comes from studies using a knockout mouse model for Kmd1b, a H3K4 demethylase primarily expressed in oocytes [116]. *Kmd1b*$^{-/-}$ oocytes have increased levels of H3K4 dimethylation and *Kmd1b*$^{-/-}$ embryos show impaired imprinting. The studies demonstrated that four out of seven maternal imprinted genes analyzed were not methylated in these embryos, suggesting that increased H3K4 methylation inhibits DNA methylation. Taken together these data support that unmethylated H3K4 promotes DNA methylation, and that H3K4 methylation inhibits DNA methylation.

Another histone modification, H3K36trimethylation (H3K36me3), correlates positively with DNA methylation as shown by genome wide studies [52,53,117]. Additionally, it has been shown that the ATRX-DNMT3-DNMT3L (ADD) domain of Dnmt3a binds to the tail of histone 3 and that the proline-tryptophan-tryptophan-proline (PWWP) motif of Dnmt3a is involved in targeting Dnmt3a to H3K36me3 (Figure 7A) [118]. The observations indicate that H3K36me3 directs DNA methylation.

Moreover, DNA methylation overlaps with histone 3 lysine 9 trimethylation (H3K9me3). For example, H3K9me3 methylation and DNA methylation are both located at ICRs, centrosomes, transposons, and tandem repeats [117,119]. Additionally, major satellite repeats are methylated at H3K9 by histone methylases Suv39h1 and Suv39h2 [120]. Similarly, IAP elements, murine leukemia virus and Line1 elements, retrotransposons, major satellite repeats and high density CpG promoters (HCP) are methylated at H3K9 by Ga9, which is involved in DNA methylation at these loci [121-124]. Collectively, these findings indicate that enzymes that modify the methylation status of H3K9 promote DNA methylation [120-124].

Several studies have shown that histone 3 lysine 27 trimethylation (H3K27me3) or polycomb repressive complex 2 (PRC2), the complex which establishes this histone mark, are correlated with DNA methylation in cancer cells. For example, Vire et al. (2006) showed that enhancer of zeste homologue 2 (EZH2), a core component of PRC2, recruits Dnmts to certain loci (Figure 7B) [125]. Additionally, three studies showed that loci that harbor H3K27me3 or a component of the PRC2 complex in ES cells are more likely to be hypermethylated in cancer cell lines or tumors [126-128]. Taken together, a large series of experimental results have provided evidence that DNA methylation correlates with specific DNA sequences, and that Dnmts interact with or are instructed by histone modifications.

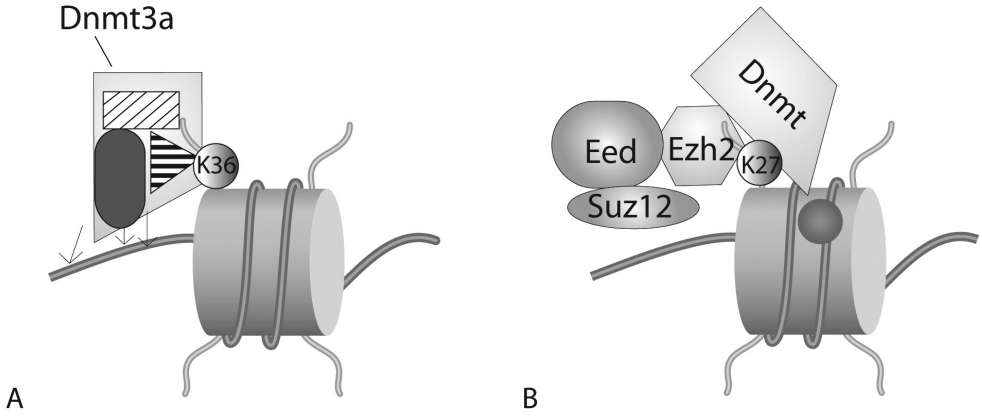


Figure 7. **Dnmt complexes.**
A) Schematic drawing showing binding of Dnmt3a to the tail of histone 3 with its ADD domain and to H3K36me3 (circle on histone tail) with its PWWP domain (triangle), respectively. Consequently, the catalytic domain of Dnmt3a (oval) is in proximity of the linker DNA, where it may methylate the DNA. B) Schematic drawing showing that Ezh2, as part of the PRC2 complex, recruits Dnmt. The PRC2 complex, consisting of Eed, Suz12 and Ezh12, establishes trimethylation on lysine 27 (K27; trimethylation is depicted as circle on histone tail).

1.1.3 Nucleosome positioning and histone variants

Another epigenetic mechanism affecting gene expression is nucleosome positioning. More specifically, active genes have nucleosome-free transcription start sites, enabling assembly of the transcriptional machinery (reviewed by Hargreaves and Crabtree, 2011 [129]). Conversely, nucleosomes are located at the transcriptional start site (TSS) of silenced genes, inhibiting the assembly of the transcriptional machinery and consequently inhibiting gene expression. Nucleosome positioning is regulated by the SWI/SNF chromatin remodeling complex through nucleosome remodeling and nucleosome sliding.

Nucleosome positioning is an epigenetic mechanism, in view of an involvement of histone modifications and histone variants. Histone variants H3.3, H2A.Z and H2A.X influence nucleosome position and consequently gene expression. H3.3 and H2A.Z are associated with active promoters [1,2]. Importantly, histone variants may be modified adding an extra layer of gene regulation. For example, ubiquinated H2A.Z associates with facultative heterochromatin [2]. Thus, location of nucleosomes and the composition of histones in the nucleosome regulate gene expression in a non-covalent manner.

1.2 Epigenetics and disease

Epigenetics plays an important role in normal and malignant development. Beckwith-Wiedemann syndrome (BWS) is characterized by prenatal overgrowth, malformations and increased risk for developing embryonal tumors such as Wilms tumor. This syndrome is associated with microdeletions in one of both imprinted subdomains on chromosome 11p15; the ICR of *H19*/*IGF2* and the ICR of *CDKN1c*. For most BWS patients, it has been shown that deletions in these regions caused dysregulation of imprinting, resulting in either biallelic expression of *IGF2* and/or silencing of *CDKN1c* [130]. Other examples of imprinting syndromes are Angelman syndrome (AS) and Prader-Willi syndrome (PWS), which are caused by microdeletions or epimutations on chromosome 15q11-13 [130,131]. This region codes for the imprinted genes ubiquitin-protein ligase E3A *(UBE3A*), which is normally expressed from the maternal allele, in addition to small nuclear ribonucleoprotein polypeptide N (*SNRPN)* and a cluster of small nucleolar RNAs (*SnoRNA*s), which are normally expressed from the paternal allele. Angelman syndrome patients have lost the maternal allele, resulting in loss of Ube3a expression. Conversely, the paternal allele is deleted in PWS patients, and loss of *SNORD116* (one of SnoRNAs) expression is suggested to be the major cause of the symptoms of PWS patients [132]. AS and PWS are both characterized by mental retardation. Collectively, these

syndromes are caused by deletions of a chromosomal region, resulting in misregulated DNA methylation.

Besides epigenetic diseases caused by dysregulation of imprinting, diseases can also be the direct consequence of a mutation in genes of the epigenetic machinery. Immunodeficiency, centromeric instability and facial anomalies syndrome (ICF) is the result of mutations in the *DNMT3B* gene, usually in the C-terminus (ICF is reviewed by Matarazzo, 2009) [88,97,98,133]. These patients have reduced serum immunoglobulin levels and only immature B-cells, and they suffer from severe immunodeficiency, which is usually lethal during childhood. In addition, satellites 2 and 3 of chromosomes 1 and 16 and less often chromosome 9 are hypomethylated in these patients, with rearrangements of these chromosomes as consequence [98,99]. Strikingly, these rearrangements have also been found in cancer (reviewed in [134,135]). Conversely, cancer is not a common symptom of ICF, although it should be kept in mind that ICF patients have a very short life span [134]. Furthermore, microarray analysis on lymphocytes of ICF patients showed that some genes were misregulated, which could explain the symptoms of ICF. Strikingly the corresponding gene promoters were not hypomethylated. The authors suggested that the hypomethylation of the repetitive sequences resulted in changes in the nuclear architecture, causing the altered gene expression.

DNA methylation and cancer

Aberrant DNA methylation is implicated in cancer. Generally, cancer is considered as a disease caused by genetic mutations. Knudson (1971) proposed the 'two- hit' model for cancer, which requires that both alleles of a tumor suppressor gene must be inactivated before a cell can grow out to a cancer (reviewed in Knudson 2000 [136]). These hits could be the result of a genetic mutation, followed by loss of heterozygosity (LOH), which is the loss of a normal allele when the other allele is already inactive. Alternatively, the hits could involve an epigenetic mutation, either as a first or a second hit (Figure 8) [137]. Epigenetic mutations, also named epimutations, could silence tumor suppressor genes or activate/ increase oncogene expression, serving as hits in cancer initiation. Cancer is associated with a high rate of epigenetic changes and involve global hypomethylation and regional hypermethylation (Figure 9) [138]. The next paragraphs describe in more detail our knowledge of global and gene specific hypomethylation in cancer.

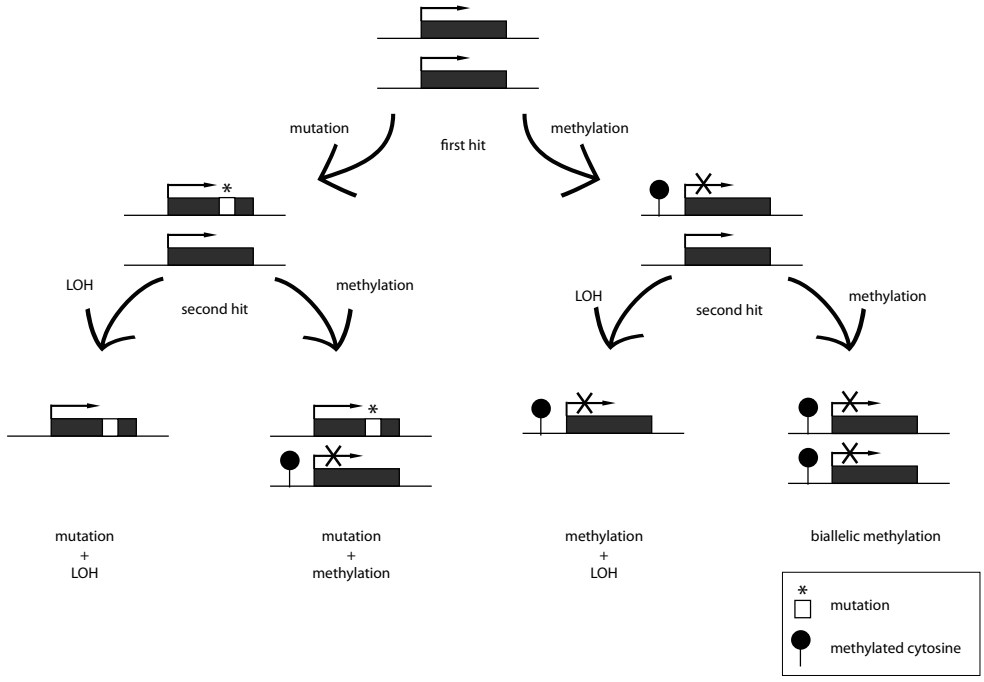


Figure 8. **Knudson's 'two- hit' model for cancer.**
This model could involve epigenetic mutation, as shown in this schematic drawing. The boxes at the top represent two active tumor suppressor genes. The first hit of tumor suppressor gene inactivation could be a genetic mutation (left) or promoter DNA methylation leading to gene silencing (right). The second hit of tumor suppressor inactivation could be a result of LOH or methylation. Adapted from Jones and Laird (1999) [137].

Hypomethylation

Global hypomethylation

In 1983, Feinberg and Vogelstein first reported hypomethylation of certain genes in cancer [139]. Subsequently, global hypomethylation of repetitive elements and transposable elements have been reported, leading to genome instability due to activation and translocation of the transposable elements and chromosomal rearrangements between repeat elements (Figure 9) [138,140,141]. For example, in mice, the reduction of Dnmt1 to 10% of its wild type enzymatic activity results in global hypomethylation [78,81]. These mice develop thymomas with trisomy 15, and the hypomethylated IAPs in these tumors are activated and transposed into the *Notch1* locus inducing oncogenic Notch1 expression [78,81]. Another example of genomic instability was shown when $Nf1^{+/-}$,$p53^{+/-}$ (NPcis) mice that are prone to develop soft tissue sarcomas, were found to have a more severe phenotype when Dnmt1

activity was reduced to 10% of its wild type levels [79]. More specifically, the tumors arise at a younger age and the mutant mice die earlier than mice with normal Dnmt1 activity levels. Additionally, fibroblasts of these mice have increased loss of heterozygosity of whole chromosome arms. Taken together, these studies suggest that hypomethylation-induced genomic instability promotes tumor formation.

Conversely, reduced methylation in *Apc*$^{min/+}$ mice, a colorectal cancer mouse model [142], resulted in fewer tumors compared to control mice [143-146]. Mutations in the *APC* gene have been found in both sporadic and hereditary human colorectal cancer. Normally, the APC protein targets beta-catenin for degradation in the cytoplasm. However, when the APC gene is mutated, beta-catenin is not targeted for degradation but is translocated to the nucleus where it induces transcription of genes of the Wnt pathway, which causes tumor formation in the intestine (reviewed by Clevers 2006 [147]). The *Apc*$^{min/+}$ (min = multiple intestinal neoplasia) mouse carries one wild type *Apc* allele and one allele with a mutation in codon 850, which corresponds to the mutation causing familial adenomatous polyposis (FAP), a form of hereditary colorectal cancer [148]. Loss of the wild type *Apc* allele by spontaneous homologous recombination in the intestinal mucosa leads to development of microadenomas, which may grow out to a macroscopic adenoma. *Apc*$^{min/+}$ mice with 10% Dnmt1 activity of the wild type levels have a higher incidence of microadenomas, likely caused by increased LOH of *Apc* [143]. However, the outgrowth of these microadenomas to macroscopic adenomas is reduced due to decreased cell proliferation. Strikingly, these mutant *Dnmt1 Apc*$^{min/+}$ mice develop hepatocarcinomas, whereas *Apc*$^{min/+}$ mice that are wild type for *Dnmt1* do not develop any tumors other than intestinal tumors. The development of hepatocarcinomas in the above model is likely due to LOH of *Apc*. As described above, Gaudet (2003) observed increased thymoma incidence due to trisomy [78]. These observations demonstrated that loss of global methylation has different effects in different tissues; global hypomethylation promotes the incidence of thymic and liver tumors by increased genome instability, but suppresses outgrowth of macroscopic intestinal tumors [78,143].

Although, various cancers are characterized by global hypomethylation, the timing of global hypomethylation in tumor development may differ. Global hypomethylation seems to be an early event in colon cancer since hypomethylation of long interspersed nuclear element 1 (LINE1) retrotransposons is observed in both the colon tumors and in the intestinal mucosa of these patients, while other tissues are unaffected [149]. The phenotypically normal liver of hepatocellular carcinoma patients has no aberrant methylation, and the degree of

hypomethylation correlates with progression of the disease [150]. Thus, when hepatocarcinomas increase in size or the histological grade progresses, global hypomethylation of the genome of the tumor cells is more severe compared to the genome of cells of a smaller tumor or a lower histological-graded tumor. For more details about global hypomethylation patterns in cancer, the review by Wilson (2007) provides much information [135].

Gene specific hypomethylation

In addition to global hypomethylation, cancer is associated with aberrant gene specific hypomethylation, resulting in gene activation. Regions that are demethylated differ between cancers, although certain genes are more commonly hypomethylated than others. Examples are *MASPIN,* a gene coding for serine protease inhibitor; *S100A4,* encoding a calcium-binding protein; *R-RAS*, *CYCLIN D2,* and microRNA *let-7a-3* [151-157]. Another group of genes that is found hypomethylated in cancer cells are the so-called cancer/testis (C/T) genes or tumor specific antigen genes. These genes, which are normally only expressed in healthy testis, are hypomethylated and aberrantly expressed in tumors. Melanoma antigen family a1 (*MAGE1*) belongs to this group of genes and this gene is hypomethylated and expressed in melanoma and glioblastoma [158,159]. In conclusion, DNA hypomethylation in cancer can be divided into global hypomethylation that causes chromosomal instability and gene specific hypomethylation resulting in gene activation.

DNA hypermethylation

In addition to hypomethylation, aberrant promoter specific hypermethylation is frequently observed in the cancer epigenome that leads to silencing of the corresponding gene (Figure 9). In 1989, Greger et al. showed the first link between hypermethylation of a tumor suppressor gene and carcinogenesis [160]. In retinoblastomas, the tumor suppressor gene retinoblastoma (*RB*) was found to be frequently hypermethylated in the promoter region. Later, the same group and others showed that methylation of the promoter region of *RB* results in reduced *RB* expression [161,162]. Since then, many examples of promoter-hypermethylation of tumor suppressor genes have been described for cancer cells, i.e. cyclin dependent kinase inhibitor *p16/INK4A*, von Hippel–Lindau (*VHL*), *MLH1*, *BRCA1* and *RASSF1A* [161-170]. Interestingly, some tumor suppressor genes, such as *p16*, are found methylated in many types of cancers [168], whereas others such as *BRCA1* [164] and *RASSF1A* [163] correlate with breast and lung cancer, respectively. Hypermethylation can also affect genes encoding transcription factors such as *GATA4* and *GATA5*, which are important for

gastrointestinal development [171]. In general, hypermethylation in cancer occurs at genes, which are important for suppression of tumor development.

Hypermethylation in cancer cells is mostly restricted to small regions of DNA, in contrast to global DNA hypomethylation. Frigola et al. (2006) reported an exception to this; they found regions of DNA up to 1MB that were hypermethylated [172]. Consequently, genes located in these stretches were silenced. Interestingly, unmethylated genes flanking the hypermethylated region also showed reduced gene expression. One hypothesis is that this may be due to chromatin remodeling, such as caused by H3K9 dimethylation, which is also associated with gene silencing [172]. In conclusion, hypermethylation in cancer quite frequently occurs at gene promoters, specifically resulting in gene silencing.

Loss of imprinting

Loss of imprinting (LOI) is the aberrant expression of a normally silent copy of an imprinted gene or the aberrant silencing of a normally active copy of an imprinted gene, and has also been implicated in cancer. In tumors, LOI occurs primarily at genes encoding growth factors and tumor suppressor genes. Chimeric mice derived from imprint-free ES cells (IF-ES cells) develop tumors within 12 months, indicating that LOI can cause cancer [173]. IGF2 expression is controlled by the ICR *H19*/*IGF2*. LOI of *IGF2* has been found Wilms tumor, which is the most common cancer in children with Beckwith-Wiedemann syndrome (BWS), a syndrome associated with deletions in the *H19/IGF2* ICR [7,130]. In several other cancers LOI of IGF2 has been found such as breast cancer, colon cancer, ovarian cancer [174-178]. Moreover, individuals with LOI of *IGF2* have a higher risk of developing colorectal cancer [179]. Other examples where LOI appears to play a role in cancer are *MEST1* in breast cancer and *GTL2* neuroblastoma and Wilm's tumors [180,181].

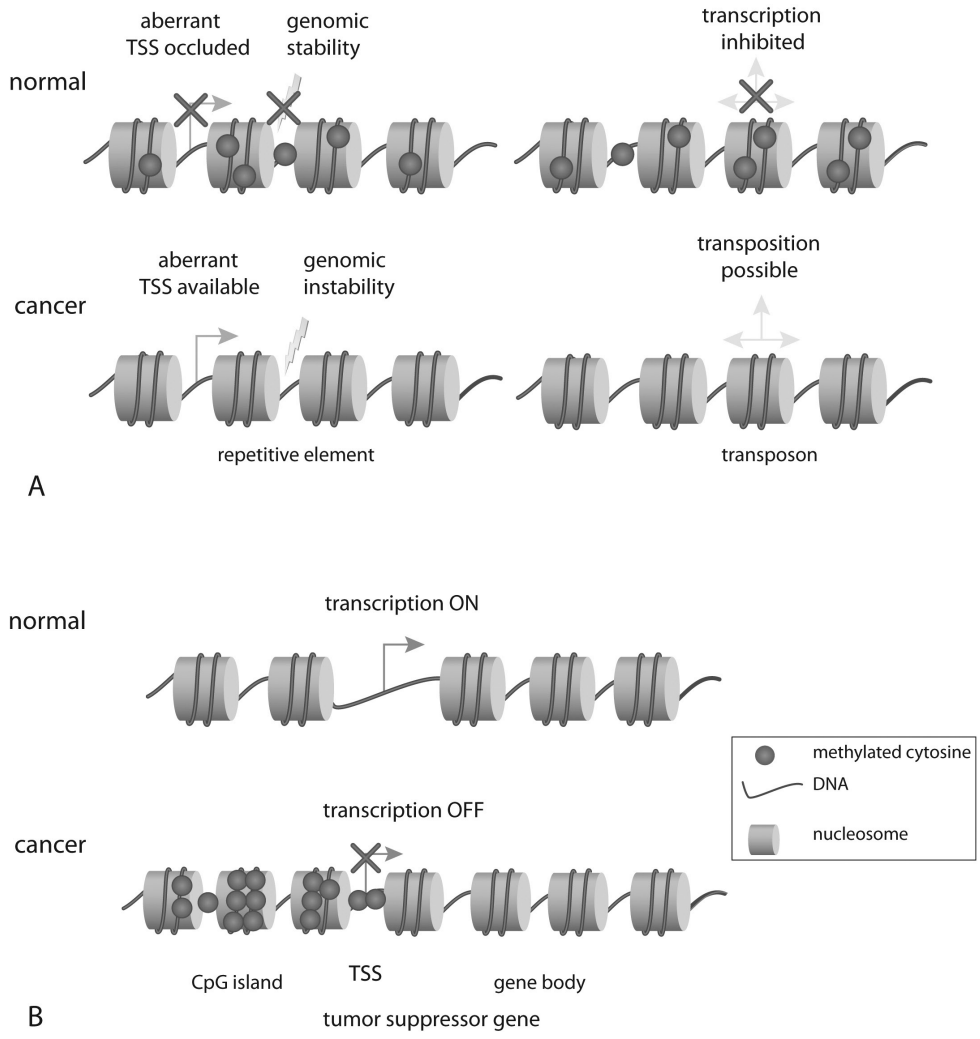


Figure 9. DNA methylation in normal and cancer cells.
A) In normal cells, methylation of repetitive elements inhibits transcription and transposition of transposons, and lowers chromosomal instability. In cancer cells, hypomethylation of the repetitive elements results in aberrant transcription, a higher rate of transposition of transposons, and marked chromosomal instability.
b) In normal cells, promoters (CpG islands) of tumor suppressor genes are unmethylated, consequently the genes are available for transcription. In cancer cells, promoters of tumor suppressor genes are often methylated; consequently, the TSS of the genes are not available for transcription and the genes are inactive. Adapted from Rodríguez-ParedesEsteller (2011) [138].

Aberrant expression of methyltransferases in cancer

The aberrant DNA methylation patterns observed in cancer cells prompted several research groups to investigate whether the levels of Dnmts changed in tumors. One group reported early on that the total DNA methylase activity was increased in cancer cells [182]. DNMT1 overexpression has been observed in many cancers, such as colon cancer, endometrial cancer, gastric cancer, pancreatic cancer, leukemia, hepatocarcinoma, bladder cancer and kidney cancer [183-190]. However, cancers are highly proliferative tissues and DNMT1 is expressed higher in proliferating tissues. When the proliferation status of the tumor tissue is taken into account, DNMT1 overexpresion in tumor tissue is moderate [191 191]. Additionally, since DNMT1 is a maintenance methyltransferase that preferentially methylates hemimethylated DNA, it is reasonable to assume that *de novo* methyltransferases, rather than DNMT1, are responsible for the new methylation patterns observed in cancer cells.

Indeed, *de novo* methyltransferases DNMT3a and DNMT3b are overexpressed in several cancers such as endometrial cancer, bladder cancer, hepatocarcinoma, colon cancer and kidney cancer, and leukemia [185,186,188-190,192]. Interestingly, it has recently been shown that 20% of acute monocytic leukemia (AML) patients carry a mutation in *DNMT3A* [193,194], which may suggest it acts as a tumor suppressor. The correlation between DNMT3b and cancer has been supported by several studies. For example, there is a positive correlation between the differentiation state of tumors and the level of DNMT3b expression [190,192]. Another study showed that *Dnmt3b* deficient fibroblasts transduced with *SV40 large T antigen* and *RAS* are impaired for colony formation in soft agar or tumors when injected into mice, in contrast to wild type *Dnmt3b* fibroblasts [195]. Moreover, Lin et al. (2006) showed that $Apc^{min/+}$ mice deficient for Dnmt3b develop a similar number of microadenomas as wild type mice but the number of macroadenomas was reduced [196]. Collectively, these studies indicate that Dnmt3b promotes tumorigenesis.

DNA methylation as biomarker

Besides the mere observations of aberrant epigenetic changes in cancer, these changes can be of significance in the clinic. The methylation status of loci can be easily determined in biopsy specimens and body fluids such as sputum, saliva, serum, urine and stool, and the methylation status of loci can be used as biomarkers as reviewed by Taby and Issa (2010) and Rodriguez-Paredes and Esteller (2011) [138,197]. For example, epigenetic biomarkers have some predictive value regarding the risk of lung cancer [198]. Moreover, prostate cancer can be diagnosed on the basis of the hypermethylation of a single gene, namely (pi)-class

glutathione S-transferase *GSTP1* [199]. Importantly, glioma patients with a hypermethylated O6-methylguanine-DNA methyltransferase (*MGMT*) promoter have prolonged survival when treated with an alkylating agent, temozolomide in addition to radiotherapy, while patients with an unmethylated *MGMT* promoter do not benefit from temozolomide treatment in addition to radiotherapy [200]. In conclusion, epigenetic biomarkers can be a very useful tool in the clinic, and should be further developed to try to identify individuals with increased risk to develop tumors, to diagnose patients, to predict the patients' prognosis and to determine a treatment plan for patients.

Cancer therapy by epigenetic modulation

Since a large number of hypermethylated genes are silenced in cancer cells, demethylation of these loci should reactivate transcription and thus reverse the epigenetic mutation and re-establish the gene expression profile of healthy tissue. Therefore, the possibility of using demethylating drugs in cancer treatment has been investigated. In 2004 and 2005, two methylation inhibitors, vidaza (5-azacytidine, azacitidine) and decitabine (5-aza-2-deoxycytidine), were approved by the FDA [201,202]. Both drugs are used as treatment against myelodysplastic syndrome (MDS), which is a heterogeneous group of bone-marrow disorders with impaired blood cell formation [201-204]. Vidaza and decitabine are nucleoside analogues that incorporate into the DNA (vidaza also is incorporated into RNA) and induce demethylation by forming an irreversible covalent bond with the Dnmts, consequently depleting Dnmts from the cell. In subsequent rounds of replication, the loss of Dnmts will lead to demethylation of the DNA in the cell, which reverses the methylation of the hypermethylated promoters and thereby restores the normal expression of these genes. There is, however, concern that demethylation of imprinted genes, repetitive elements and oncogenes could lead to misexpression, chromosomal instability and activation of oncogenes. Thus far, data are lacking to conclude whether this is indeed the case. Another potential limitation of using these drugs for cancer treatment is the cellular uptake of these compounds. Vidaza and decitabine are administrated to leukemia patients, but not to patients with solid tumors, although efforts are under way to improve the uptake of these drugs into solid tumors [205]. As of today, treatment against aberrant DNA methylation is directed to reverse the DNA methylation on the whole genome rather than targeted to aberrantly methylated loci or to cells with aberrant methylation. Thus, treatment to reverse DNA methylation could be improved, and understanding the role of Dnmts and DNA methylation in cancer is important for potentially developing new therapies.

1.3 Aim and scope of this thesis

Based on the research described in this Introduction, it can be concluded that Dnmt expression and DNA methylation are frequently misregulated in cancer cells. However, it has not been resolved if aberrant expression of *de novo* methyltransferases Dnmt3a and Dnmt3b and the aberrant DNA methylation are a cause or a consequence of tumorigenesis. Further, it has not been elucidated whether DNA methylation occurs either at random loci with tumor outgrowth as consequence of clonal selection, or at specific loci. My thesis work focused on addressing these questions. In order to study whether ectopic Dnmt expression plays a causal role in cancer formation, we generated mouse models that could be induced for ectopic *de novo* methyltransferase expression. We then crossed these mice with an intestinal cancer mouse model to analyze the tumor phenotype. Additionally, we performed the reciprocal experiment by crossing conditional deletion *Dnmt3a* or *Dnmt3b* mice with a lung cancer model to study the effect of loss of Dnmt activity. To address whether Dnmts methylate loci specifically or randomly, we subjected the tumors and mucosa of the ectopic Dnmt3b expressing mice to DNA methylation analysis. These experiments allow us to gain insight whether *de novo* Dnmts have a distinct role in tumorigenesis.

1.4 Outline of this thesis

Chapter 1 introduces the basic concepts of epigenetics, with a focus on DNA methylation in healthy tissues and in cancer cells. The study described in Chapter 2 addressed the question whether gain of function of Dnmts would play a causal role in tumor formation. To this end, transgenic mouse models that can be induced for ectopic *de novo* Dnmt expression were generated and crossed with $Apc^{min/+}$ mice, which is an intestinal cancer mouse model. In humans, familial adenomatous polyposis (FAP) is caused by mutation of the *APC* gene and such *APC* mutations have also been found in non-familial cases of colorectal cancer. Our mice ectopically expressing Dnmt3b had larger and more adenomas than mice wild type for Dnmt3b1 expression. Subsequently, intestinal adenomas and mucosa were isolated from these mice and analyzed by loci specific bisulfite sequencing to address whether Dnmt3b methylates certain loci specifically, or whether the tumor outgrowth was a result of clonal selection. Chapter 3 describes an expanded DNA methylation analysis of tumors and intestinal mucosa from $Apc^{min/+}$ mice and mucosa from *Apc* wild type mice. The tissues were harvested from mice that were induced for ectopic Dnmt3b expression and from control mice. DNA methylation was analyzed by Mass Array platform from Sequenom and by reduced representation bisulfite sequencing (RRBS). Our data showed that DNA methylation

is not influenced by the genetic background of the mice and that loci methylated upon Dnmt3b induction resemble the DNA methylation pattern found in human colon cancer.

The role of *de novo* Dnmts in tumorigenesis was further investigated using a lung cancer mouse model, *K-ras*$^{lox\text{-}stop\text{-}lox\text{-}G12D}$ that was crossed with a conditional knockout mouse for the *Dnmt3a* gene or the *Dnmt3b* gene (Chapter 4). This mouse model allows simultaneous *de novo Dnmt* deletion and induction of oncogenic *K-ras* expression upon Cre-recombinase treatment. The data in Chapter 4 suggest that Dnmt3a functions as tumor suppressor in lung cancer. In order to investigate whether Dnmt3a plays a similar role in other cancer types, we are currently studying the effect of *Dnmt3a* deletion in intestinal tumorigenesis (Addendum 1).

Addendum 2 describes the progress of another ongoing project, which addresses loci specificity of Dnmt3b mediated DNA methylation: i) does ectopic *Dnmt3b* expression induce a cancer type specific methylation pattern in tissues other than the mouse colon? and ii) is the methylation pattern directed by histone modifications? To address these questions, I isolated small intestinal epithelial cells and hepatocytes from mice induced for ectopic Dnmt3b expression. These samples were analyzed for their DNA methylation pattern by RRBS and histone modifications by ChIP-Seq. Finally, Chapter 6 discusses the results summarized in the present thesis.

References

1 Szenker, E., Ray-Gallet, D. & Almouzni, G. The double face of the histone variant H3.3. *Cell research* **21**, 421-434, doi:10.1038/cr.2011.14 (2011).
2 Redon, C. *et al.* Histone H2A variants H2AX and H2AZ. *Current opinion in genetics & development* **12**, 162-169 (2002).
3 Bannister, A. J. & Kouzarides, T. Regulation of chromatin by histone modifications. *Cell research* **21**, 381-395, doi:10.1038/cr.2011.22 (2011).
4 Waddington, C. H. The Epigenotype. *Endeavour*, 18-20 (1942).
5 Waddington, C. H. The basic Ideas of Biology. *Towards a Theoretical Biology*, 1-32 (1968).
6 Berger, S. L., Kouzarides, T., Shiekhattar, R. & Shilatifard, A. An operational definition of epigenetics. *Genes & development* **23**, 781-783, doi:10.1101/gad.1787609 (2009).
7 Egger, G., Liang, G., Aparicio, A. & Jones, P. A. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* **429**, 457-463, doi:10.1038/nature02625 (2004).
8 Feinberg, A. P. Phenotypic plasticity and the epigenetics of human disease. *Nature* **447**, 433-440, doi:10.1038/nature05919 (2007).
9 Johnson, T. B. & Coghill, R. D. Researches on pyrimidines. C111. The discovery of 5-methyl-cytosine in tuberculinic acid, the nucleic acid of the tubercle bacillus. *Journal of the American Chemical Society* **47**, 2838-2844 (1925).
10 Hotchkiss, R. D. The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. *Journal of Biological Chemistry* **175**, 315-332 (1948).
11 Riggs, A. D. X inactivation, differentiation, and DNA methylation. *Cytogenetics and cell genetics* **14**, 9-25 (1975).
12 Holliday, R. & Pugh, J. E. DNA modification mechanisms and gene activity during development. *Science (New York, NY)*, 226-232 (1975).
13 Tweedie, S., Charlton, J., Clark, V. & Bird, A. Methylation of genomes and genes at the invertebrate-vertebrate boundary. *Molecular and cellular biology* **17**, 1469-1475 (1997).
14 Suzuki, M. M. & Bird, A. DNA methylation landscapes: provocative insights from epigenomics. *Nature Reviews Genetics* **9**, 465-476, doi:10.1038/nrg2341 (2008).
15 Noyer-Weidner, M. & Trautner, T. A. Methylation of DNA in prokaryotes. *EXS* **64**, 39-108 (1993).
16 Collier, J. Epigenetic regulation of the bacterial cell cycle. *Current opinion in microbiology* **12**, 722-729, doi:10.1016/j.mib.2009.08.005 (2009).
17 Dodge, J. E., Ramsahoye, B. H., Wo, Z. G., Okano, M. & Li, E. De novo methylation of MMLV provirus in embryonic stem cells: CpG versus non-CpG methylation. *Gene* **289**, 41-48 (2002).
18 Haines, T. R., Rodenhiser, D. I. & Ainsworth, P. J. Allele-specific non-CpG methylation of the Nf1 gene during early mouse development. *Developmental biology* **240**, 585-598, doi:10.1006/dbio.2001.0504 (2001).
19 Ramsahoye, B. H. *et al.* Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 5237-5242 (2000).
20 Gowher, H. & Jeltsch, A. Enzymatic properties of recombinant Dnmt3a DNA methyltransferase from mouse: the enzyme modifies DNA in a non-processive manner and also methylates non-CpG [correction of non-CpA] sites. *Journal of molecular biology* **309**, 1201-1208, doi:10.1006/jmbi.2001.4710 (2001).
21 Laurent, L. *et al.* Dynamic changes in the human methylome during differentiation. *Genome research* **20**, 320-331, doi:10.1101/gr.101907.109 (2010).
22 Lister, R. *et al.* Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* **462**, 315-322, doi:10.1038/nature08514 (2009).
23 Feng, S. *et al.* Conservation and divergence of methylation patterning in plants and animals. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 8689-8694, doi:10.1073/pnas.1002720107 (2010).
24 Cokus, S. J. *et al.* Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. *Nature* **452**, 215-219, doi:10.1038/nature06745 (2008).

25 Salzberg, A., Fisher, O., Siman-Tov, R. & Ankri, S. Identification of methylated sequences in genomic DNA of adult Drosophila melanogaster. *Biochemical and biophysical research communications* **322**, 465-469, doi:10.1016/j.bbrc.2004.07.134 (2004).

26 Lyko, F., Ramsahoye, B. H. & Jaenisch, R. DNA methylation in Drosophila melanogaster. *Nature* **408**, 538-540, doi:10.1038/35046205 (2000).

27 Antequera, F., Tamame, M., Villanueva, J. R. & Santos, T. DNA methylation in the fungi. *The Journal of biological chemistry* **259**, 8033-8036 (1984).

28 Proffitt, J. H., Davie, J. R., Swinton, D. & Hattman, S. 5-Methylcytosine is not detectable in Saccharomyces cerevisiae DNA. *Molecular and cellular biology* **4**, 985-988 (1984).

29 P J Russell, K. D. R. E. M. R. J. A. M. Differential DNA methylation during the vegetative life cycle of Neurospora crassa. *Journal of Bacteriology* **169**, 2902 (1987).

30 Gowher, H., Leismann, O. & Jeltsch, A. DNA of Drosophila melanogaster contains 5-methylcytosine. *The EMBO Journal* **19**, 6918-6923, doi:10.1093/emboj/19.24.6918 (2000).

31 Vinogradov, A. E. Measurement by flow cytometry of genomic AT/GC ratio and genome size. *Cytometry* **16**, 34-40, doi:10.1002/cyto.990160106 (1994).

32 Bird, A. P. DNA methylation and the frequency of CpG in animal DNA. *Nucleic acids research*, 1499-1503 (1980).

33 Parsons, B. L. MED1: a central molecule for maintenance of genome integrity and response to DNA damage. *PNAS* **100**, 14601-14602, doi:10.1073/pnas.2637169100 (2003).

34 Bellacosa, A. Role of MED1 (MBD4) Gene in DNA repair and human cancer. *Journal of Cellular Physiology* **187**, 137-144, doi:10.1002/jcp.1064 (2001).

35 van der Ploeg, L. H. & Flavell, R. A. DNA methylation in the human gamma delta beta-globin locus in erythroid and nonerythroid tissues. *Cell* **19**, 947-958 (1980).

36 Ehrlich, M. *et al.* Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic acids research* **10**, 2709-2721 (1982).

37 Gruenbaum, Y., Stein, R., Cedar, H. & Razin, A. Methylation of CpG sequences in eukaryotic DNA. *FEBS letters* **124**, 67-71 (1981).

38 Bird, A., Taggart, M., Frommer, M., Miller, O. J. & Macleod, D. A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. *Cell* **40**, 91-99 (1985).

39 Yoder, J. A., Walsh, C. P. & Bestor, T. H. Cytosine methylation and the ecology of intragenomic parasites. *Trends in genetics : TIG* **13**, 335-340 (1997).

40 Yoder, J. A., Soman, N. S., Verdine, G. L. & Bestor, T. H. DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. *Journal of molecular biology* **270**, 385-395, doi:10.1006/jmbi.1997.1125 (1997).

41 Robertson, K. D. & Wolffe, A. P. DNA methylation in health and disease. *Nature Reviews Genetics* **1**, 11-19, doi:10.1038/35049533 (2000).

42 Takai, D. & Jones, P. A. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 3740-3745, doi:10.1073/pnas.052410099 (2002).

43 Kaneda, M. *et al.* Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* **429**, 900-903, doi:10.1038/nature02633 (2004).

44 Wang, Y. & Leung, F. C. C. An evaluation of new criteria for CpG islands in the human genome as gene markers. *Bioinformatics (Oxford, England)* **20**, 1170-1177, doi:10.1093/bioinformatics/bth059 (2004).

45 Larsen, F., Gundersen, G., Lopez, R. & Prydz, H. CpG islands as gene markers in the human genome. *Genomics* **13**, 1095-1107 (1992).

46 Illingworth, R. *et al.* A novel CpG island set identifies tissue-specific methylation at developmental gene loci. *PLoS biology* **6**, e22, doi:10.1371/journal.pbio.0060022 (2008).

47 Eckhardt, F. *et al.* DNA methylation profiling of human chromosomes 6, 20 and 22. *Nature genetics* **38**, 1378-1385, doi:10.1038/ng1909 (2006).

48 Saxonov, S., Berg, P. & Brutlag, D. L. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 1412-1417, doi:10.1073/pnas.0510310103 (2006).

49 Song, F. *et al.* Association of tissue-specific differentially methylated regions (TDMs) with differential gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 3336-3341, doi:10.1073/pnas.0408436102 (2005).

50 Kaneda, M. Genomic imprinting in mammals—Epigenetic parental memories. *Differentiation* **82**, 51-56, doi:10.1016/j.diff.2011.05.004 (2011).

51 Wu, H. *et al.* Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. *Science (New York, NY)* **329**, 444-448, doi:10.1126/science.1190485 (2010).

52 Ball, M. P. *et al.* Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nature biotechnology* **27**, 361-368, doi:10.1038/nbt.1533 (2009).

53 Hodges, E. *et al.* High definition profiling of mammalian DNA methylation by array capture and single molecule bisulfite sequencing. *Genome research* **19**, 1593-1605, doi:10.1101/gr.095190.109 (2009).

54 Mayer, W., Niveleau, A., Walter, J., Fundele, R. & Haaf, T. Demethylation of the zygotic paternal genome. *Nature* **403**, 501-502, doi:10.1038/35000654 (2000).

55 Oswald, J. *et al.* Active demethylation of the paternal genome in the mouse zygote. *Current biology : CB* **10**, 475-478 (2000).

56 Olek, A. & Walter, J. The pre-implantation ontogeny of the H19 methylation imprint. *Nature genetics* **17**, 275-276, doi:10.1038/ng1197-275 (1997).

57 Lane, N. *et al.* Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. *Genesis (New York, NY : 2000)* **35**, 88-93, doi:10.1002/gene.10168 (2003).

58 Howlett, S. K. & Reik, W. Methylation levels of maternal and paternal genomes during preimplantation development. *Development (Cambridge, England)* **113**, 119-127 (1991).

59 Rougier, N. *et al.* Chromosome methylation patterns during mammalian preimplantation development. *Genes & development* **12**, 2108-2113 (1998).

60 Lee, J. *et al.* Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. *Development (Cambridge, England)* **129**, 1807-1817 (2002).

61 Chen, Z.-x. & Riggs, A. D. DNA methylation and demethylation in mammals. *Journal of Biological Chemistry* **286**, 18347-18353, doi:10.1074/jbc.R110.205286 (2011).

62 He, X.-J., Chen, T. & Zhu, J.-K. Regulation and function of DNA methylation in plants and animals. *Cell research* **21**, 442-465, doi:10.1038/cr.2011.23 (2011).

63 Lan, J., Hua, S., He, X. & Zhang, Y. DNA methyltransferases and methyl-binding proteins of mammals. *Acta Biochimica et Biophysica Sinica* **42**, 243-252, doi:10.1093/abbs/gmq015 (2010).

64 Bestor, T., Laudano, A., Mattaliano, R. & Ingram, V. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *Journal of molecular biology* **203**, 971-983 (1988).

65 Leonhardt, H., Page, A. W., Weier, H. U. & Bestor, T. H. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* **71**, 865-873 (1992).

66 Bostick, M. *et al.* UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science (New York, NY)* **317**, 1760-1764, doi:10.1126/science.1147939 (2007).

67 Sharif, J. *et al.* The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. *Nature* **450**, 908-912, doi:10.1038/nature06397 (2007).

68 Fatemi, M., Hermann, A., Pradhan, S. & Jeltsch, A. The activity of the murine DNA methyltransferase Dnmt1 is controlled by interaction of the catalytic domain with the N-terminal part of the enzyme leading to an allosteric activation of the enzyme after binding to methylated DNA. *Journal of molecular biology* **309**, 1189-1199, doi:10.1006/jmbi.2001.4709 (2001).

69 Goyal, R., Reinhardt, R. & Jeltsch, A. Accuracy of DNA methylation pattern preservation by the Dnmt1 methyltransferase. *Nucleic acids research* **34**, 1182-1188, doi:10.1093/nar/gkl002 (2006).

70 Mertineit, C. *et al.* Sex-specific exons control DNA methyltransferase in mammalian germ cells. *Development (Cambridge, England)* **125**, 889-897 (1998).

71 Li, E., Bestor, T. H. & Jaenisch, R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**, 915-926 (1992).

72 Lei, H. *et al.* De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development (Cambridge, England)* **122**, 3195-3205 (1996).
73 Li, E., Beard, C. & Jaenisch, R. Role for DNA methylation in genomic imprinting. *Nature* **366**, 362-365, doi:10.1038/366362a0 (1993).
74 Jackson-Grusby, L. *et al.* Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. *Nature genetics* **27**, 31-39, doi:10.1038/83730 (2001).
75 Beard, C., Li, E. & Jaenisch, R. Loss of methylation activates Xist in somatic but not in embryonic cells. *Genes & development* **9**, 2325-2334 (1995).
76 Panning, B. & Jaenisch, R. DNA hypomethylation can activate Xist expression and silence X-linked genes. *Genes & development* **10**, 1991-2002, doi:10.1101/gad.10.16.1991 (1996).
77 Chen, R. Z., Pettersson, U., Beard, C., Jackson-Grusby, L. & Jaenisch, R. DNA hypomethylation leads to elevated mutation rates. *Nature* **395**, 89-93, doi:10.1038/25779 (1998).
78 Gaudet, F. *et al.* Induction of tumors in mice by genomic hypomethylation. *Science (New York, NY)* **300**, 489-492, doi:10.1126/science.1083558 (2003).
79 Eden, A., Gaudet, F., Waghmare, A. & Jaenisch, R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science (New York, NY)* **300**, 455, doi:10.1126/science.1083557 (2003).
80 Walsh, C. P., Chaillet, J. R. & Bestor, T. H. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nature genetics* **20**, 116-117, doi:10.1038/2413 (1998).
81 Howard, G., Eiges, R., Gaudet, F., Jaenisch, R. & Eden, A. Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. *Oncogene* **27**, 404-408, doi:10.1038/sj.onc.1210631 (2008).
82 Van den Wyngaert, I., Sprengel, J., Kass, S. U. & Luyten, W. H. Cloning and analysis of a novel human putative DNA methyltransferase. *FEBS letters* **426**, 283-289 (1998).
83 Yoder, J. A. & Bestor, T. H. A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. *Human molecular genetics* **7**, 279-284 (1998).
84 Okano, M., Xie, S. & Li, E. Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic acids research* **26**, 2536-2540 (1998).
85 Goll, M. G. *et al.* Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science (New York, NY)* **311**, 395-398, doi:10.1126/science.1120976 (2006).
86 Schaefer, M. *et al.* RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. *Genes & development* **24**, 1590-1595, doi:10.1101/gad.586710 (2010).
87 Lyko, F. *et al.* Mammalian (cytosine-5) methyltransferases cause genomic DNA methylation and lethality in Drosophila. *Nature genetics* **23**, 363-366, doi:10.1038/15551 (1999).
88 Okano, M., Bell, D. W., Haber, D. A. & Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**, 247-257 (1999).
89 Chen, T., Ueda, Y., Dodge, J. E., Wang, Z. & Li, E. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Molecular and cellular biology* **23**, 5594-5605 (2003).
90 Dodge, J. E. *et al.* Inactivation of Dnmt3b in mouse embryonic fibroblasts results in DNA hypomethylation, chromosomal instability, and spontaneous immortalization. *The Journal of biological chemistry* **280**, 17986-17991, doi:10.1074/jbc.M413246200 (2005).
91 Liang, G. *et al.* Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Molecular and cellular biology* **22**, 480-491 (2002).
92 Chen, T., Ueda, Y., Xie, S. & Li, E. A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. *The Journal of biological chemistry* **277**, 38746-38754, doi:10.1074/jbc.M205312200 (2002).
93 Okano, M., Xie, S. & Li, E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nature genetics* **19**, 219-220, doi:10.1038/890 (1998).
94 Hata, K., Okano, M., Lei, H. & Li, E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development (Cambridge, England)* **129**, 1983-1993 (2002).
95 Aoki, A. *et al.* Enzymatic properties of de novo-type mouse DNA (cytosine-5) methyltransferases. *Nucleic acids research* **29**, 3506-3512 (2001).

96 Weisenberger, D. J. *et al.* Role of the DNA methyltransferase variant DNMT3b3 in DNA methylation. *Molecular cancer research : MCR* **2**, 62-72 (2004).
97 Hansen, R. S. *et al.* The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 14412-14417 (1999).
98 Xu, G. L. *et al.* Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* **402**, 187-191, doi:10.1038/46052 (1999).
99 Jeanpierre, M. *et al.* An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. *Human molecular genetics* **2**, 731-735 (1993).
100 Jia, D., Jurkowska, R. Z., Zhang, X., Jeltsch, A. & Cheng, X. Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. *Nature* **449**, 248-251, doi:10.1038/nature06146 (2007).
101 Jurkowska, R. Z. *et al.* Formation of nucleoprotein filaments by mammalian DNA methyltransferase Dnmt3a in complex with regulator Dnmt3L. *Nucleic acids research* **36**, 6656-6663, doi:10.1093/nar/gkn747 (2008).
102 Webster, K. E. *et al.* Meiotic and epigenetic defects in Dnmt3L-knockout mouse spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 4068-4073, doi:10.1073/pnas.0500702102 (2005).
103 Bourc'his, D., Xu, G. L., Lin, C. S., Bollman, B. & Bestor, T. H. Dnmt3L and the establishment of maternal genomic imprints. *Science (New York, NY)* **294**, 2536-2539, doi:10.1126/science.1065848 (2001).
104 Bourc'his, D. & Bestor, T. H. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature* **431**, 96-99, doi:10.1038/nature02886 (2004).
105 Oka, M., Rodić, N., Graddy, J., Chang, L.-J. & Terada, N. CpG sites preferentially methylated by Dnmt3a in vivo. *The Journal of biological chemistry* **281**, 9901-9908, doi:10.1074/jbc.M511100200 (2006).
106 Handa, V. & Jeltsch, A. Profound flanking sequence preference of Dnmt3a and Dnmt3b mammalian DNA methyltransferases shape the human epigenome. *Journal of molecular biology* **348**, 1103-1112, doi:10.1016/j.jmb.2005.02.044 (2005).
107 Barski, A. *et al.* High-resolution profiling of histone methylations in the human genome. *Cell* **129**, 823-837, doi:10.1016/j.cell.2007.05.009 (2007).
108 Edwards, J. R. *et al.* Chromatin and sequence features that define the fine and gross structure of genomic methylation patterns. *Genome research* **20**, 972-980, doi:10.1101/gr.101535.109 (2010).
109 Meissner, A. *et al.* Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* **454**, 766-770, doi:10.1038/nature07107 (2008).
110 Weber, M. *et al.* Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature genetics* **39**, 457-466, doi:10.1038/ng1990 (2007).
111 Wu, S. C. & Zhang, Y. Active DNA demethylation: many roads lead to Rome. *Nature reviews Molecular cell biology* **11**, 607-620, doi:10.1038/nrm2950 (2010).
112 Ooi, S. K. T. *et al.* DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature* **448**, 714-717, doi:10.1038/nature05987 (2007).
113 Otani, J. *et al.* Structural basis for recognition of H3K4 methylation status by the DNA methyltransferase 3A ATRX-DNMT3-DNMT3L domain. *EMBO reports* **10**, 1235-1241, doi:10.1038/embor.2009.218 (2009).
114 Zhang, Y. *et al.* Chromatin methylation activity of Dnmt3a and Dnmt3a/3L is guided by interaction of the ADD domain with the histone H3 tail. *Nucleic acids research* **38**, 4246-4253, doi:10.1093/nar/gkq147 (2010).
115 Li, B.-Z. *et al.* Histone tails regulate DNA methylation by allosterically activating de novo methyltransferase. *Cell research* **21**, 1172-1181, doi:10.1038/cr.2011.92 (2011).
116 Ciccone, D. N. *et al.* KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints. *Nature* **461**, 415-418, doi:10.1038/nature08315 (2009).
117 Mikkelsen, T. S. *et al.* Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**, 553-560, doi:10.1038/nature06008 (2007).

118 Dhayalan, A. *et al.* The Dnmt3a PWWP domain reads histone 3 lysine 36 trimethylation and guides DNA methylation. *Journal of Biological Chemistry* **285**, 26114-26120, doi:10.1074/jbc.M109.089433 (2010).

119 Delaval, K. *et al.* Differential histone modifications mark mouse imprinting control regions during spermatogenesis. *The EMBO Journal* **26**, 720-729, doi:10.1038/sj.emboj.7601513 (2007).

120 Lehnertz, B. *et al.* Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Current biology : CB* **13**, 1192-1200 (2003).

121 Dong, K. B. *et al.* DNA methylation in ES cells requires the lysine methyltransferase G9a but not its catalytic activity. *The EMBO Journal* **27**, 2691-2701, doi:10.1038/emboj.2008.193 (2008).

122 Leung, D. C. *et al.* Lysine methyltransferase G9a is required for de novo DNA methylation and the establishment, but not the maintenance, of proviral silencing. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 5718-5723, doi:10.1073/pnas.1014660108 (2011).

123 Tachibana, M., Matsumura, Y., Fukuda, M., Kimura, H. & Shinkai, Y. G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. *The EMBO Journal* **27**, 2681-2690, doi:10.1038/emboj.2008.192 (2008).

124 Epsztejn-Litman, S. *et al.* De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes. *Nature structural & molecular biology* **15**, 1176-1183, doi:10.1038/nsmb.1476 (2008).

125 Viré, E. *et al.* The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439**, 871-874, doi:10.1038/nature04431 (2006).

126 Ohm, J. E. *et al.* A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nature genetics* **39**, 237-242, doi:10.1038/ng1972 (2007).

127 Schlesinger, Y. *et al.* Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nature genetics* **39**, 232-236, doi:10.1038/ng1950 (2007).

128 Widschwendter, M. *et al.* Epigenetic stem cell signature in cancer. *Nature genetics* **39**, 157-158, doi:10.1038/ng1941 (2007).

129 Hargreaves, D. C. & Crabtree, G. R. ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. *Cell research*, 1-25, doi:10.1038/cr.2011.32 (2011).

130 Butler, M. G. Genomic imprinting disorders in humans: a mini-review. *Journal of Assisted Reproduction and Genetics* **26**, 477-486, doi:10.1007/s10815-009-9353-3 (2009).

131 Buiting, K. *et al.* Epimutations in Prader-Willi and Angelman syndromes: a molecular study of 136 patients with an imprinting defect. *American journal of human genetics* **72**, 571-577, doi:10.1086/367926 (2003).

132 Duker, A. L. *et al.* Paternally inherited microdeletion at 15q11.2 confirms a significant role for the SNORD116 C/D box snoRNA cluster in Prader–Willi syndrome. *European Journal of Human Genetics* **18**, 1196-1201, doi:10.1038/ejhg.2010.102 (2010).

133 Matarazzo, M. R., De Bonis, M. L., Vacca, M., Della Ragione, F. & D'Esposito, M. Lessons from two human chromatin diseases, ICF syndrome and Rett syndrome. *The International Journal of Biochemistry & Cell Biology* **41**, 117-126, doi:10.1016/j.biocel.2008.07.026 (2009).

134 Ehrlich, M. *et al.* ICF, an immunodeficiency syndrome: DNA methyltransferase 3B involvement, chromosome anomalies, and gene dysregulation. *Autoimmunity* **41**, 253-271, doi:10.1080/08916930802024202 (2008).

135 Wilson, A. S., Power, B. E. & Molloy, P. L. DNA hypomethylation and human diseases. *Biochimica et biophysica acta* **1775**, 138-162, doi:10.1016/j.bbcan.2006.08.007 (2007).

136 Knudson, A. G. Chasing the cancer demon. *Annual review of genetics* **34**, 1-19, doi:10.1146/annurev.genet.34.1.1 (2000).

137 Jones, P. A. & Laird, P. W. Cancer epigenetics comes of age. *Nature genetics* **21**, 163-167, doi:10.1038/5947 (1999).

138 Rodríguez-Paredes, M. & Esteller, M. Cancer epigenetics reaches mainstream oncology. *Nature medicine* **17**, 330-339, doi:10.1038/nm.2305 (2011).

139 Feinberg, A. P. & Vogelstein, B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* **301**, 89-92 (1983).

140 Tsai, H.-C. & Baylin, S. B. Cancer epigenetics: linking basic biology to clinical medicine. *Cell research*, 1-16, doi:10.1038/cr.2011.24 (2011).

141 Feinberg, A. P., Gehrke, C. W., Kuo, K. C. & Ehrlich, M. Reduced genomic 5-methylcytosine content in human colonic neoplasia. *Cancer research* **48**, 1159-1161 (1988).

142 Moser, A. R., Pitot, H. C. & Dove, W. F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science (New York, NY)* **247**, 322-324 (1990).

143 Yamada, Y. *et al.* Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 13580-13585, doi:10.1073/pnas.0506612102 (2005).

144 Cormier, R. T. & Dove, W. F. Dnmt1N/+ reduces the net growth rate and multiplicity of intestinal adenomas in C57BL/6-multiple intestinal neoplasia (Min)/+ mice independently of p53 but demonstrates strong synergy with the modifier of Min 1(AKR) resistance allele. *Cancer research* **60**, 3965-3970 (2000).

145 Eads, C. A., Nickel, A. E. & Laird, P. W. Complete genetic suppression of polyp formation and reduction of CpG-island hypermethylation in Apc(Min/+) Dnmt1-hypomorphic Mice. *Cancer research* **62**, 1296-1299 (2002).

146 Laird, P. W. *et al.* Suppression of intestinal neoplasia by DNA hypomethylation. *Cell* **81**, 197-205 (1995).

147 Clevers, H. Wnt/β-Catenin Signaling in Development and Disease. *Cell* **127**, 469-480, doi:10.1016/j.cell.2006.10.018 (2006).

148 Su, L. K. *et al.* Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science (New York, NY)* **256**, 668-670 (1992).

149 Suter, C. M., Martin, D. I. & Ward, R. L. Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. *International Journal of Colorectal Disease* **19**, 95-101, doi:10.1007/s00384-003-0539-3 (2004).

150 Lin, C. H. *et al.* Genome-wide hypomethylation in hepatocellular carcinogenesis. *Cancer research* **61**, 4238-4243 (2001).

151 Akiyama, Y., Maesawa, C., Ogasawara, S., Terashima, M. & Masuda, T. Cell-type-specific repression of the maspin gene is disrupted frequently by demethylation at the promoter region in gastric intestinal metaplasia and cancer cells. *The American journal of pathology* **163**, 1911-1919, doi:10.1016/S0002-9440(10)63549-3 (2003).

152 Brueckner, B. *et al.* The Human let-7a-3 Locus Contains an Epigenetically Regulated MicroRNA Gene with Oncogenic Function. *Cancer research* **67**, 1419-1423, doi:10.1158/0008-5472.CAN-06-4074 (2007).

153 Nakamura, N. & Takenaga, K. Hypomethylation of the metastasis-associated S100A4 gene correlates with gene activation in human colon adenocarcinoma cell lines. *Clinical & experimental metastasis* **16**, 471-479 (1998).

154 Nishigaki, M. *et al.* Discovery of aberrant expression of R-RAS by cancer-linked DNA hypomethylation in gastric cancer using microarrays. *Cancer research* **65**, 2115-2124, doi:10.1158/0008-5472.CAN-04-3340 (2005).

155 Oshimo, Y. *et al.* Promoter methylation of cyclin D2 gene in gastric carcinoma. *International journal of oncology* **23**, 1663-1670 (2003).

156 Sato, N., Fukushima, N., Matsubayashi, H. & Goggins, M. Identification of maspin and S100P as novel hypomethylation targets in pancreatic cancer using global gene expression profiling. *Oncogene* **23**, 1531-1538, doi:10.1038/sj.onc.1207269 (2004).

157 Sato, N. *et al.* Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. *Cancer research* **63**, 4158-4166 (2003).

158 De Smet, C. *et al.* The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 7149-7153 (1996).

159 Liu, G. *et al.* HER-2, gp100, and MAGE-1 are expressed in human glioblastoma and recognized by cytotoxic T cells. *Cancer research* **64**, 4980-4986, doi:10.1158/0008-5472.CAN-03-3504 (2004).

160 Greger, V., Passarge, E., Höpping, W., Messmer, E. & Horsthemke, B. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Human genetics* **83**, 155-158 (1989).
161 Greger, V. *et al.* Frequency and parental origin of hypermethylated RB1 alleles in retinoblastoma. *Human genetics* **94**, 491-496 (1994).
162 Ohtani-Fujita, N. *et al.* CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. *Oncogene* **8**, 1063-1067 (1993).
163 Burbee, D. G. *et al.* Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *Journal of the National Cancer Institute* **93**, 691-699 (2001).
164 Esteller, M. Promoter Hypermethylation and BRCA1 Inactivation in Sporadic Breast and Ovarian Tumors. *Journal of the National Cancer Institute* **92**, 564-569, doi:10.1093/jnci/92.7.564 (2000).
165 Gonzalez-Zulueta, M. *et al.* Methylation of the 5' CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. *Cancer research* **55**, 4531-4535 (1995).
166 Herman, J. G. *et al.* Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 9700-9704 (1994).
167 Kane, M. F. *et al.* Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer research* **57**, 808-811 (1997).
168 Merlo, A. *et al.* 5|[prime]| CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nature medicine* **1**, 686-692, doi:doi:10.1038/nm0795-686 (1995).
169 Veigl, M. L. *et al.* Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8698-8702 (1998).
170 Cunningham, J. M. *et al.* Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer research* **58**, 3455-3460 (1998).
171 Akiyama, Y. *et al.* GATA-4 and GATA-5 transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer. *Molecular and cellular biology* **23**, 8429-8439 (2003).
172 Frigola, J. *et al.* Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. *Nature genetics* **38**, 540-549, doi:10.1038/ng1781 (2006).
173 Holm, T. M. *et al.* Global loss of imprinting leads to widespread tumorigenesis in adult mice. *Cancer cell* **8**, 275-285, doi:10.1016/j.ccr.2005.09.007 (2005).
174 Cui, H. *et al.* Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2. *Cancer research* **62**, 6442-6446 (2002).
175 Murphy, S. K. Frequent IGF2/H19 Domain Epigenetic Alterations and Elevated IGF2 Expression in Epithelial Ovarian Cancer. *Molecular Cancer Research* **4**, 283-292, doi:10.1158/1541-7786.MCR-05-0138 (2006).
176 Rainier, S. *et al.* Relaxation of imprinted genes in human cancer. *Nature* **362**, 747-749, doi:10.1038/362747a0 (1993).
177 Uyeno, S. *et al.* IGF2 but not H19 shows loss of imprinting in human glioma. *Cancer research* **56**, 5356-5359 (1996).
178 van Roozendaal, C. E. *et al.* Loss of imprinting of IGF2 and not H19 in breast cancer, adjacent normal tissue and derived fibroblast cultures. *FEBS letters* **437**, 107-111 (1998).
179 Cui, H. *et al.* Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. *Science (New York, NY)* **299**, 1753-1755, doi:10.1126/science.1080902 (2003).
180 Astuti, D. *et al.* Epigenetic alteration at the DLK1-GTL2 imprinted domain in human neoplasia: analysis of neuroblastoma, phaeochromocytoma and Wilms' tumour. *British Journal of Cancer* **92**, 1574-1580, doi:10.1038/sj.bjc.6602478 (2005).
181 Pedersen, I. S. *et al.* Frequent loss of imprinting of PEG1/MEST in invasive breast cancer. *Cancer research* **59**, 5449-5451 (1999).

182 Kautiainen, T. L. & Jones, P. A. DNA methyltransferase levels in tumorigenic and nontumorigenic cells in culture. *The Journal of biological chemistry* **261**, 1594-1598 (1986).

183 el-Deiry, W. S. *et al.* High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 3470-3474 (1991).

184 Etoh, T. *et al.* Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. *The American journal of pathology* **164**, 689-699, doi:10.1016/S0002-9440(10)63156-2 (2004).

185 Miyake, T., Endo, K., Honjo, S., Hirooka, Y. & Ikeguchi, M. Expression of DNA Methyltransferase (DNMT) 1, 3a and 3b Proteins in Human Hepatocellular Carcinoma. *Yonago Acta medica*, 1-7 (2010).

186 Mizuno, S. *et al.* Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood* **97**, 1172-1179 (2001).

187 Peng, D.-F. *et al.* Increased DNA methyltransferase 1 (DNMT1) protein expression in precancerous conditions and ductal carcinomas of the pancreas. *Cancer Science* **96**, 403-408, doi:10.1111/j.1349-7006.2005.00071.x (2005).

188 Robertson, K. D. *et al.* The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic acids research* **27**, 2291-2298 (1999).

189 Xie, S. *et al.* Cloning, expression and chromosome locations of the human DNMT3 gene family. *Gene* **236**, 87-95 (1999).

190 Xiong, Y. *et al.* Opposite alterations of DNA methyltransferase gene expression in endometrioid and serous endometrial cancers. *Gynecologic Oncology* **96**, 601-609, doi:10.1016/j.ygyno.2004.11.047 (2005).

191 Lee, P. J. *et al.* Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 10366-10370 (1996).

192 Jin, F. *et al.* Up-regulation of DNA methyltransferase 3B expression in endometrial cancers. *Gynecologic Oncology* **96**, 531-538, doi:10.1016/j.ygyno.2004.10.039 (2005).

193 Ley, T. J. *et al.* DNMT3A mutations in acute myeloid leukemia. *The New England journal of medicine* **363**, 2424-2433, doi:10.1056/NEJMoa1005143 (2010).

194 Yan, X.-J. *et al.* Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nature genetics* **43**, 309-315, doi:10.1038/ng.788 (2011).

195 Soejima, K., Fang, W. & Rollins, B. J. DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. *Oncogene* **22**, 4723-4733, doi:10.1038/sj.onc.1206510 (2003).

196 Lin, H. *et al.* Suppression of intestinal neoplasia by deletion of Dnmt3b. *Molecular and cellular biology* **26**, 2976-2983, doi:10.1128/MCB.26.8.2976-2983.2006 (2006).

197 Taby, R. & Issa, J.-P. J. Cancer epigenetics. *CA: a cancer journal for clinicians* **60**, 376-392, doi:10.3322/caac.20085 (2010).

198 Belinsky, S. A. *et al.* Promoter hypermethylation of multiple genes in sputum precedes lung cancer incidence in a high-risk cohort. *Cancer research* **66**, 3338-3344, doi:10.1158/0008-5472.CAN-05-3408 (2006).

199 Lee, W. H. *et al.* Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 11733 (1994).

200 Hegi, M. E. *et al.* MGMT gene silencing and benefit from temozolomide in glioblastoma. *The New England journal of medicine* **352**, 997-1003, doi:10.1056/NEJMoa043331 (2005).

201 Gore, S. D., Jones, C. & Kirkpatrick, P. in *Nature Reviews Drug Discovery* Vol. 5 891-892 (2006).

202 Kaminskas, E. *et al.* Approval summary: azacitidine for treatment of myelodysplastic syndrome subtypes. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 3604-3608, doi:10.1158/1078-0432.CCR-04-2135 (2005).

203 Fenaux, P. *et al.* Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *The lancet oncology* **10**, 223-232, doi:10.1016/S1470-2045(09)70003-8 (2009).
204 Kantarjian, H. *et al.* Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood* **109**, 52-57, doi:10.1182/blood-2006-05-021162 (2007).
205 Belinsky, S. A. *et al.* Combination therapy with vidaza and entinostat suppresses tumor growth and reprograms the epigenome in an orthotopic lung cancer model. *Cancer research* **71**, 454-462, doi:10.1158/0008-5472.CAN-10-3184 (2011).
206 Jurkowska, R. Z., Jurkowski, T. P. & Jeltsch, A. Structure and function of mammalian DNA methyltransferases. *ChemBioChem* **12**, 206-222, doi:10.1002/cbic.201000195 (2011).
207 Latham, T., Gilbert, N. & Ramsahoye, B. DNA methylation in mouse embryonic stem cells and development. *Cell and Tissue Research* **331**, 31-55, doi:10.1007/s00441-007-0537-9 (2007).

CHAPTER 2

DNMT3B PROMOTES TUMORIGENESIS *IN VIVO* BY GENE-SPECIFIC *DE NOVO* METHYLATION AND TRANSCRIPTIONAL SILENCING

Heinz G. Linhart[1], Haijiang Lin[1], Yasuhiro Yamada[2], Eva Moran[1], Eveline Steine[1], Sumita Gokhale[1], Grace Lo[1], Erika Cantu[1], Mathias Ehrich[3], Timothy He[4], Alex Meissner[1] and Rudolf Jaenisch[1]*

[1] Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Nine Cambridge Center, Cambridge, MA 02142, USA

[2] Department for Tumor Pathology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan

[3] Sequenom, Inc., 3595 John Hopkins Court, San Diego, CA 92121, USA

[4] Novartis Institutes for BioMedical Research, Inc., 250 Massachusetts Avenue, Cambridge, MA 02139, USA

* To whom correspondence should be addressed.

Published in *Genes & Development* 2007; 21:3110-3122.

Abstract

Increased methylation of CpG islands and silencing of affected target genes is frequently found in human cancer, however, *in vivo* the question of causality has only been addressed by loss of function studies. To directly evaluate the role and mechanism of *de novo* methylation in tumor development we overexpressed the *de novo* DNA methyltransferases Dnmt3a and b1 in *Apc*$^{Min/+}$ mice. We found that Dnmt3b1 enhanced the number of colon tumors in *Apc*$^{Min/+}$ mice approximately twofold and increased the average size of colonic microadenomas, whereas Dnmt3a had no effect. The overexpression of Dnmt3b1 caused loss of imprinting and increased expression of *Igf2* as well as methylation and transcriptional silencing of the tumor suppressor genes *Sfrp2, 4* and *5*. Importantly we found that Dnmt3b1 but not Dnmt3a1 efficiently methylates the same set of genes in tumors and in non-tumor tissues demonstrating that *de novo* methyltransferases can initiate methylation and silencing of specific genes in phenotypically normal cells. This suggests that DNA methylation patterns in cancer are the result of specific targeting of at least some tumor suppressor genes rather than of random, stochastic methylation followed by clonal selection due to a proliferative advantage caused by tumor suppressor gene silencing.

Introduction

Cancer cells show widespread epigenetic changes when compared to their normal parental tissue including changes in DNA methylation and chromatin modification (Jones and Baylin 2007). The first epigenetic abnormality reported for human cancer was a global decrease in genomic cytosine methylation (Feinberg and Vogelstein 1983) most often seen in repetitive sequences and intergenic regions. It promotes genetic instability, increases the mobility of transposable elements (Walsh et al. 1998) and induces tumorigenesis in several different mouse models (Eden et al. 2003; Gaudet et al. 2003; Yamada et al. 2005; Jones and Baylin 2007). Thus hypomethylation predisposes to genetic damage and increases the risk of tumor development. Conversely in some tissues global hypomethylation can also inhibit tumor outgrowth (Laird et al. 1995).

In addition to global hypomethylation, it was also found that the cancer cell genome frequently contains regions with increased cytosine methylation (Baylin et al. 1986). This regional hypermethylation often affects CpG islands that are associated with promoter regions (Herman and Baylin 2003; Feinberg and Tycko 2004; Jones and Baylin 2007). Regional hypermethylation attracted attention when it was linked to transcriptional silencing of the *RB* tumor suppressor gene in patients with retinoblastoma tumors (Greger et al. 1989; Greger et al. 1994). Multiple follow up studies revealed that in cancer many tumor relevant genes, in particular tumor suppressor genes, are transcriptionally silenced by hypermethylation. Aberrant DNA methylation patterns are stable and maintained throughout cell division and therefore regional hypermethylation has been proposed to contribute to carcinogenesis (Herman and Baylin 2003). However when unbiased screens were conducted to identify hypermethylated regions in tumor DNA many of the affected genes were not directly involved in tumor pathogenesis (Suzuki et al. 2002). These observations stirred a debate whether the widespread regional hypermethylation in tumors is a consequence rather than a cause of cancer. Two recent discoveries shed more light on the mechanisms and the conceptual understanding of regional hypermethylation in tumors. Firstly, it was found that Polycomb group (PcG) repressor complexes can directly recruit *de novo* methyltransferases to target genes and promote regional methylation (Vire et al. 2006). Secondly recent studies demonstrated that hypermethylation in cancer targets developmental regulator genes that are occupied by polycomb repressor complexes in embryonic stem cells (Ohm et al. 2007; Schlesinger et al. 2007; Widschwendter et al. 2007). This is consistent with the notion that genes marked by PcG complexes during early development may be particularly susceptible to methylation mediated silencing.

Epigenetic silencing of genes in cancer poses a number of as yet unresolved questions. For example an important issue is whether *de novo* methylation of genes in cancer is causally involved in tumor development or rather represents an adaptive change reflecting the oncogenic phenotype (Bestor 2003). An early *in vitro* study using the colon carcinoma cell line HCT116 suggested a causal role of *de novo* methylation in carcinogenesis (Myohanen et al. 1998). Correlative evidence from human studies is also consistent with regional hypermethylation being causally involved in the tumorigenic process. For example hypermethylation is already detectable at the earliest stages of tumor development and some tumor characteristics correlate well with methylation mediated silencing of certain tumor relevant genes (Chan et al. 2006). However most *in vivo* experiments addressing the role of DNA methylation in cancer are loss of function studies using chemical or genetic inhibition of maintenance DNA methylation (Robert et al. 2003) and therefore do not directly address the causal role of hypermethylation in cancer. Such interventions affect global methylation as well as regional methylation complicating the interpretation of results. One gain of function study using *in vitro* overexpression of Dnmt1 in mammalian cells indeed supported the idea that hypermethylation can promote cell transformation (Wu et al. 1993) but attempts to expand this approach into an adult *in vivo* model were unsuccessful (Biniszkiewicz et al. 2002). To prove that *de novo* methylation is causally involved in tumor formation, loss of function studies need to be complemented by gain of function studies *in vivo*.

Other open questions relate to the mechanism and specificity of the *de novo* methylation process: Are altered methylation patterns in tumors the result of sporadic methylation events and selection or are certain genes directly and efficiently targeted for methylation? Also, the identity and the specificity of DNA methylating enzymes involved in gene silencing remain unresolved questions. Both *de novo* DNA methyltransferases (Dnmt) 3a and 3b are frequently expressed in cancer tissue, but their relative contribution to hypermethylation in cancer is unclear. A recent study from our lab suggested that Dnmt3b is required for tumor development (Lin et al. 2006) but target genes have not been identified. It remains to be clarified which of the known *de novo* methyltransferases is involved in tumor associated hypermethylation *in vivo* and whether these enzymes have inherent target specificity. To address these questions we generated several mouse strains that allow for conditional overexpresssion of Dnmt3a1*,* Dnmt3b1 and isoforms of Dnmt3b1 and studied the effect of these enzymes on tumorigenesis in the *Apc*$^{Min/+}$ mouse model for intestinal neoplasia. Both Dnmt3a and 3b have been shown to mediate *de novo* methylation of previously

unmethylated double stranded DNA. For example during embryogenesis expression of Dnmt3a and 3b coincides with periods of high *de novo* methylation activity and this process is impaired by genetic deletion of these enzymes (Okano et al. 1998; Okano et al. 1999). Both enzymes are therefore considered the major *de novo* DNA methyltransferases of mammalian cells (Goll and Bestor 2005).

Results

Transgenic mice with inducible Dnmt3a and Dnmt3b transgenes

We generated tetracycline inducible alleles of the DNA methyltransferases 3a1 and 3b1 and two isoforms of Dnmt3b1, Dnmt3b3 and Dnmt3b6 (Figure 1 A, B) that are produced by alternative splicing. The isoforms Dnmt3b3 and 3b6 are truncated versions of the full length protein Dnmt3b1 and lack part of motif IX of the catalytic domain (Chen et al. 2003). These isoforms are enzymatically inactive and were included in our experiment to allow for a distinction between methylation related and non methylation related effects of Dnmt3b1. The tetracycline inducible Dnmt3 transgenes were targeted to the 3' UTR of the *collagen I* gene and the tetracycline responsive transactivator (M2-rtTA) was inserted into the *ROSA26* locus as described previously (Beard et al. 2006). To analyze the effect of Dnmt3a and 3b activation on tumorigenesis, the respective transgene was crossed into the $Apc^{Min/+}$ mouse model for intestinal neoplasia, which is heterozygous for the mutant *Min* allele of the *adenomatus polyposis coli* (*Apc*) tumor suppressor gene (Su et al. 1992). Previous experiments from our lab detected increased expression of Dnmt3b in some intestinal adenomas (Figure 1C) and showed that deletion of *Dnmt3b* protects against intestinal tumor initiation (Lin et al. 2006). All Dnmt transgenic strains were first backcrossed into the C57BL/6 background and then crossed with $Apc^{Min/+}$ mice to generate the experimental genotype M2-rtTA +/-, Dnmt transgene +/-, $Apc^{Min/+}$.

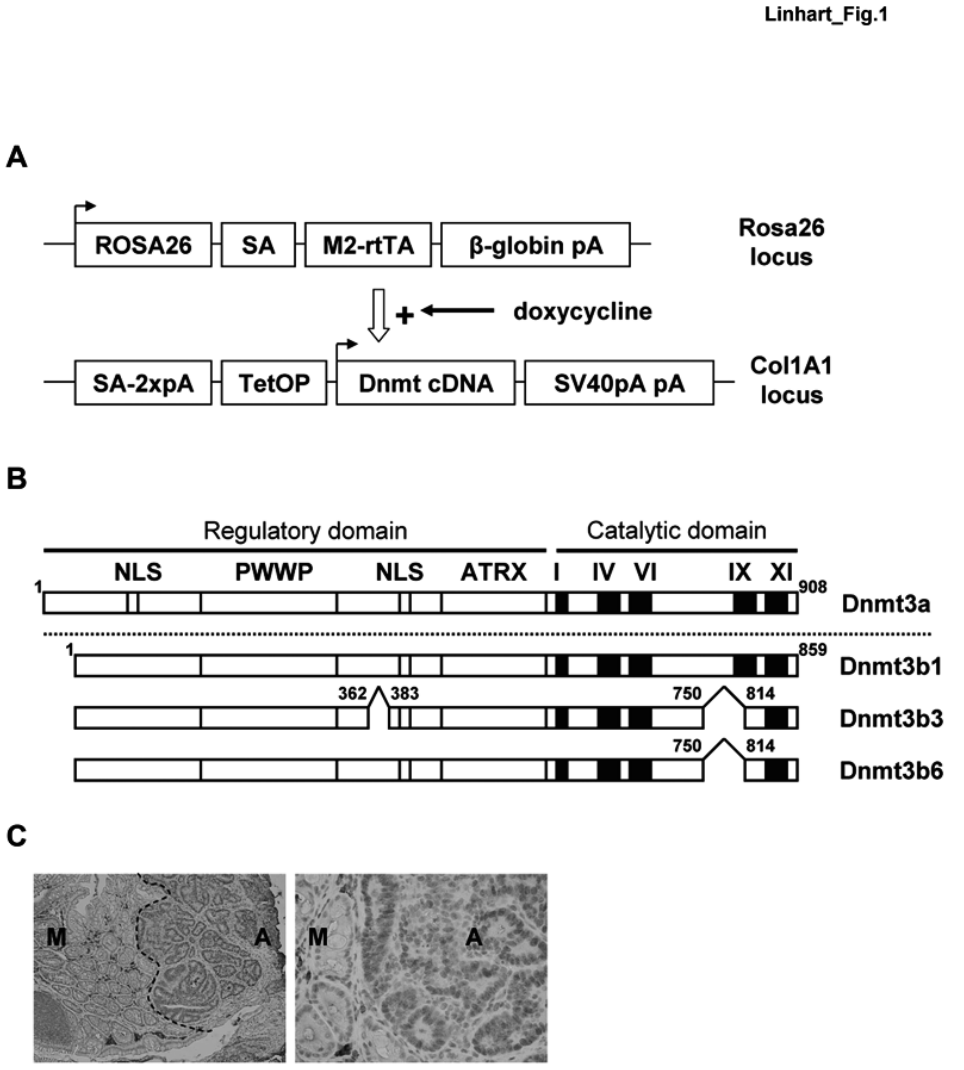


Figure 1. Transgene constructs.
A: Strategy used to generate transgenic mice with tetracyline inducible Dnmt's: M2-rtTA was targeted to the *ROSA26* locus and is under control of the endogenus *ROSA26* promotor. An expression cassette containing the respective Dnmt cDNA under control of a tetracycline responsive promotor was inserted ~500bp downstream of the 3'UTR of the collagenA1 gene. SA: splice acceptor, pA: polyadenylation signal, TetOP: tetracycline responsive operator. Arrows indicate the transcriptional start site (Beard et al. 2006). B: Diagram of the Dnmt cDNA constructs used: The position of the conserved PWWP domain, ATRX homology domain, putative NLS's, the methyltransferase motifs (I, IV, VI, IX) and sites of alternative splicing are shown. Numbers represent amino acids (Chen et al. 2003). C: Colon section of a control *Apc*$^{Min/+}$ mouse, stained with a Dnmt3b antibody (left 10x, right 40x). Regions with normal mucosa (M) showed weak expression of Dnmt3b, whereas regions with adenoma tissue (A) showed strong expression of Dnmt3b.

Inducible transgene expression

For transgene induction the experimental animals were fed doxycycline in the drinking water, starting at 4 weeks of age and were compared to the control group that received regular drinking water. The top four sections of Figure 2A show transverse sections of colon samples derived from the four DOX treated mice stained with antibodies against Dnmt3a (top left) or Dnmt3b. Each section covers areas with both normal intestinal mucosa and tumor tissue. The immunostaining demonstrated strong transgene expression in normal epithelial cells of the intestinal mucosa and neoplastic tissue in all experimental groups. The third row illustrates transgene expression in the colon mucosa at higher magnification from treated (left) and untreated (right) animals. The doxycycline treated mice showed strong transgene expression with the expected nuclear localization (Figure 2A) whereas very little or no nuclear staining was detectable in the uninduced mice. Also, Dnmt3b1 was overexpressed in spleen of doxycycline treated animals (bottom row). In general Dnmt transgene expression was low or undetectable in the non-epithelial cells of the intestinal wall. To compare transgene expression in the different anatomic regions of the intestine we harvested epithelial cells from the proximal, middle and distal small intestine and colon from induced and uninduced mice. We found that transgene expression was lower in the proximal small intestine and increased towards the distal intestine, with the highest expression observed in the colon (Figure 2B and Figure S1). Dnmt3 mediated *de novo* methylation is thought to require association with the Dnmt3L isoform (Ooi et al. 2007). To assess whether Dnmt3b1 overexpression also induces Dnmt3L, we performed quantitative PCR analyses. Figure S2 shows that Dnmt3b1 induction did not alter Dnmt3L expression.

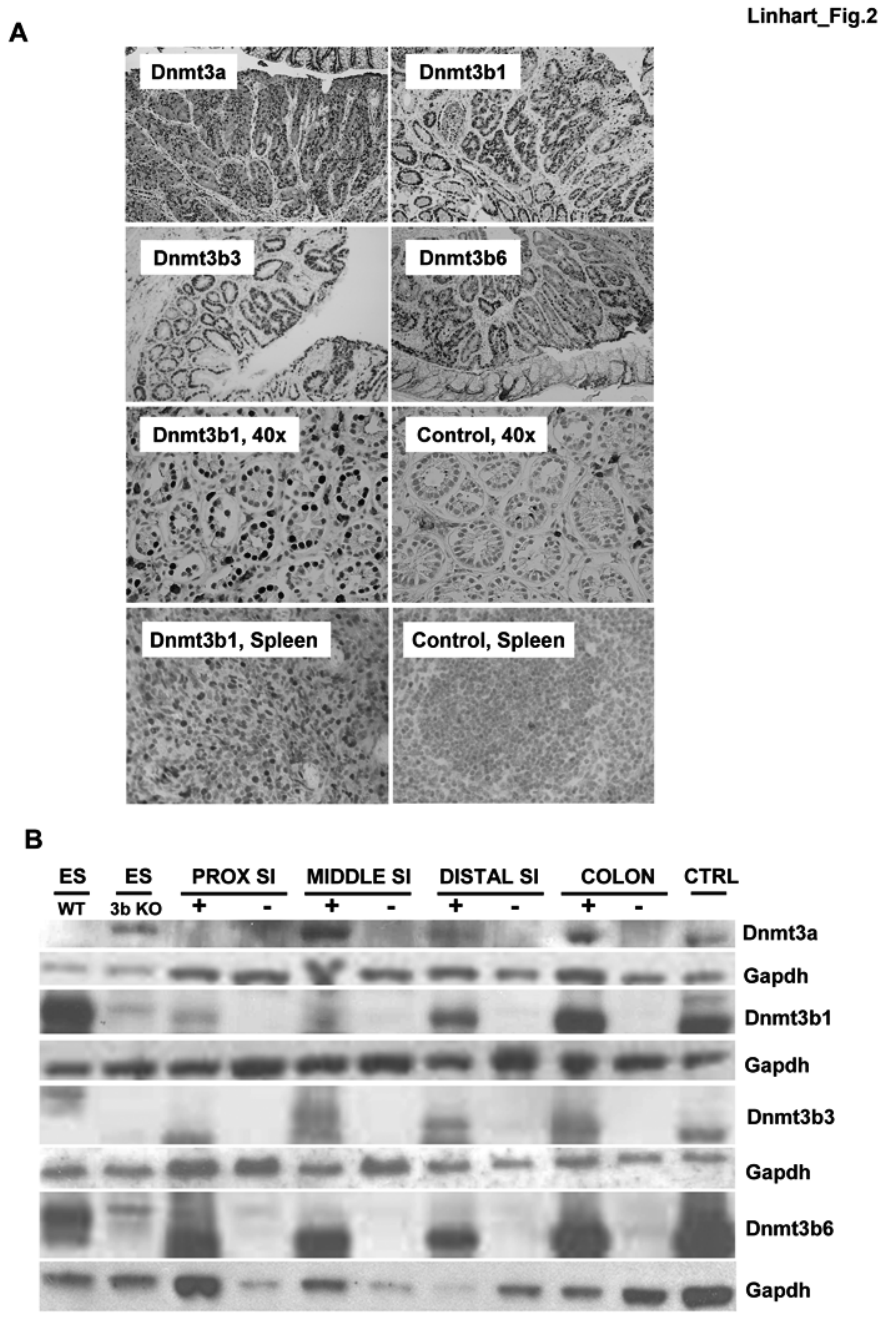


Figure 2. Transgene expression analysis.
A: Colon and spleen sections of transgenic mice induced with doxycycline and stained with a Dnmt3a (top left) or Dnmt3b antibody. All strains showed clearly visible doxycycline induced transgene expression in both normal mucosa and adenoma tissue (first and second panel, 10X). The third panel

demonstrates appropriate nuclear localization of the transgene product (Dnmt3b1 third panel left, 40X), whereas mucosa from an uninduced control mouse showed weak to no nuclear Dnmt3b staining (third panel right, 40X). The bottom panel demonstrates that the Dnmt3b1 transgene is also widely expressed in the spleen. B: Western blot demonstrating doxycycline inducible protein expression of transgenic mouse strains in different regions of the intestinal mucosa. Top panel stained with Dnmt3a antibody, other panels stained with Dnmt3b antibody, Gapdh as loading control. In each case protein from wild type ES cells (ES WT) was used as a positive control and protein from ES cells knocked out for Dnmt3b (ES 3bKO) was used as a negative control. CTRL protein was extracted from immortalized and doxycycline induced tail tip fibroblasts derived from the same respective mouse strain. All strains showed doxycycline inducible transgene expression. In general Dnmt expression was lowest in the proximal small intestine (SI) and highest in the colon. This is particularly the case with Dnmt3b1. The weak band in the ES 3bKO lane (runnning slightly above the Dnmt3b1 band) that is seen in blots with Dnmt3b antibody, is most likely nonspecific.

Dnmt3b1 activation increases the number of intestinal adenomas

When analyzing the colon of experimental mice at 5 months of age, we found a more than twofold increase in macroscopic tumors in transgenic mice expressing full length Dnmt3b1 when compared to controls (11.7 ± 1, versus 5.3 ± 0.3 colon tumors/mouse; $p < 0.0002$, Mann-Whitney U test) and this difference was statistically significant. All other transgenic strains either showed very small or no changes in colon tumor numbers when compared to controls (Figure 3A). Expression of full length Dnmt3b1 also significantly increased the number of small intestinal adenomas (39.5 ± 3.7 versus 25.3 ± 3.6 small intestinal tumors/mouse; $n = 12$ each, $p < 0.01$, Mann Whitney), with the relative increase being less pronounced than in the colon, possibly due to lower expression of the transgene (Figure 3B). Tumor formation in the intestine occurs in two stages: microscopic adenomas and macroscopic adenomas where microadenomas are considered precursor lesions to macroscopic tumors (Yamada et al. 2002; Lin et al. 2006; Yamada and Mori 2007). To evaluate whether Dnmt3b1 overexpression affects these early tumor stages, we analyzed the morphology of microadenomas in the colon of Dnmt3b1 expressing mice using horizontal en face sections of the colon mucosa. Figure 4A and B show that the average size of microadenomas was increased almost twofold in mice expressing Dnmt3b1 when compared to controls (364µm ± 38 versus 211µm ± 25; $n=58$ and $n=70$ respectively, $p< 0.0005$, Mann-Whitney). No difference was found in the ratio of microscopic to macroscopic adenomas when comparing control mice to Dnmt3b1 expressing mice (ratio microdaenomas/macroadenomas control: 3.6 ± 1.9 and Dnmt3b1: 4.8 ± 1.6, $n = 5$ each, $p = 0.75$). These findings suggest that Dnmt3b1 overexpression affects the earliest stages of tumor development.

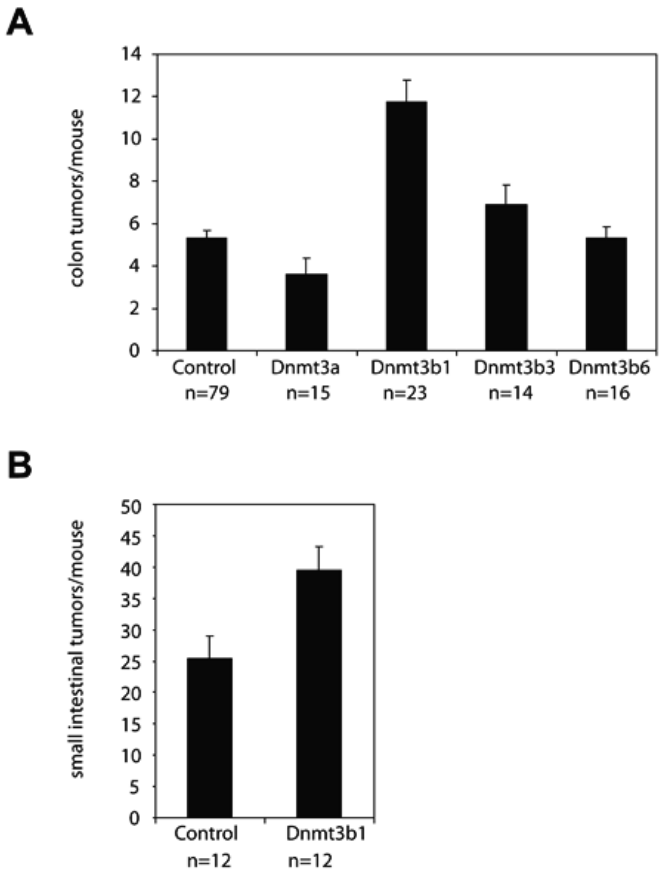


Figure 3. Dnmt3b1 overexpression increases the number of intestinal tumors.
A: Number of macroscopic colon tumors per mouse. Increased expression of Dnmt3b1 caused a 2.2 fold increase in the number of colon tumors per mouse (11.7 ± 1 versus 5.3 ± 0.3 colon tumors/mouse; $p < 0.0002$, Mann-Whitney test). Other mouse strains showed small changes (Dnmt3b3 1.3 fold increase, $p< 0.02$ and Dnmt3a 0.68 fold decrease, $p<0.01$) or no change (Dnmt3b6) in colon tumor numbers. B: Number of small intestinal adenomas per mouse. Expression of Dnmt3b1 caused a 1.6 fold increase in small intestinal adenomas (39.5 ± 3.7 versus 25.3 ± 25.3 small intestinal tumors/mouse; $p < 0.02$, Mann-Whitney test). Values are given as mean ± SE.

Dnmt3b1 promotes loss of imprinting of *Igf2*

The increased tumorigenesis by Dnmt3b1 overexpression but not by enzymatically inactive isoforms is consistent with silencing of genes involved in tumor formation by hypermethylation. To identify possible target genes we analyzed genes that are known to be silenced in cancer by *de novo* methylation. Insulin-like growth factor II (*Igf2*) has been shown to promote intestinal tumorigenesis in mouse models and increased *Igf2* expression

in humans due to loss of imprinting has been linked to an increased risk of colon cancer (Cui et al. 2003). To analyze whether overexpression of Dnmt3b1 caused loss of imprinting and increased expression of Igf2 we first compared the Igf2 expression in colon tumors from control mice with Igf2 expression in tumors derived from Dnmt3b1 overexpressing mice using quantitative PCR. Figure 4C shows that Igf2 expression was increased almost twofold in tumors from Dnmt3b1 expressing mice when compared to tumors from control mice. Bisulfite sequencing analysis of the *H19 DMR* showed that tumors from Dnmt3b1 expressing mice were hypermethylated at this locus, when compared to controls (Figure 5A). Analysis of the non tumor bearing mucosa also showed a slight increase in *H19 DMR* methylation in Dnmt3b1 expressing mice but to a lesser degree than in the macroscopic tumors (Figure 5A). Both the expression and the bisulfite sequencing data are consistent with bi-allelic expression of *Igf2* due to loss of imprinting. We used liquid chromatography coupled to a mass spectrometer (LC-MS/MS) to assess whether Dnmt3b1 overexpression altered the overall DNA methylation level. Figure S3 shows that the 5-methylcytosine content of DNA from Dnmt3b1 overexpressing mice did not differ from control samples, suggesting that global DNA methylation was not affected by Dnmt3b1 overexpression.

Methylation of the *Snurf/Snrpn* imprinting center

To evaluate, whether Dnmt3b1 expression also affects imprinted regions that do not promote cell growth and are therefore not subject to positive selection, we analyzed methylation of the differentially methylated region 1 (*DMR1*) of the *Snurf/Snrpn* imprinting center. This imprinting center is normally methylated on the suppressed maternal allele, with the umethylated paternal allele being expressed. Methylation of this imprinting center negatively controls expression of the *Snurf/Snrpn* genes, which are predominantly expressed in the developing and adult brain (Reinhart et al. 2006). Bisulfite sequencing analysis indeed showed that in contrast to the *H19 DMR*, the monoallelic methylation pattern of this region was not significantly altered in Dnmt3b1 overexpressing mice (Figure 5B).

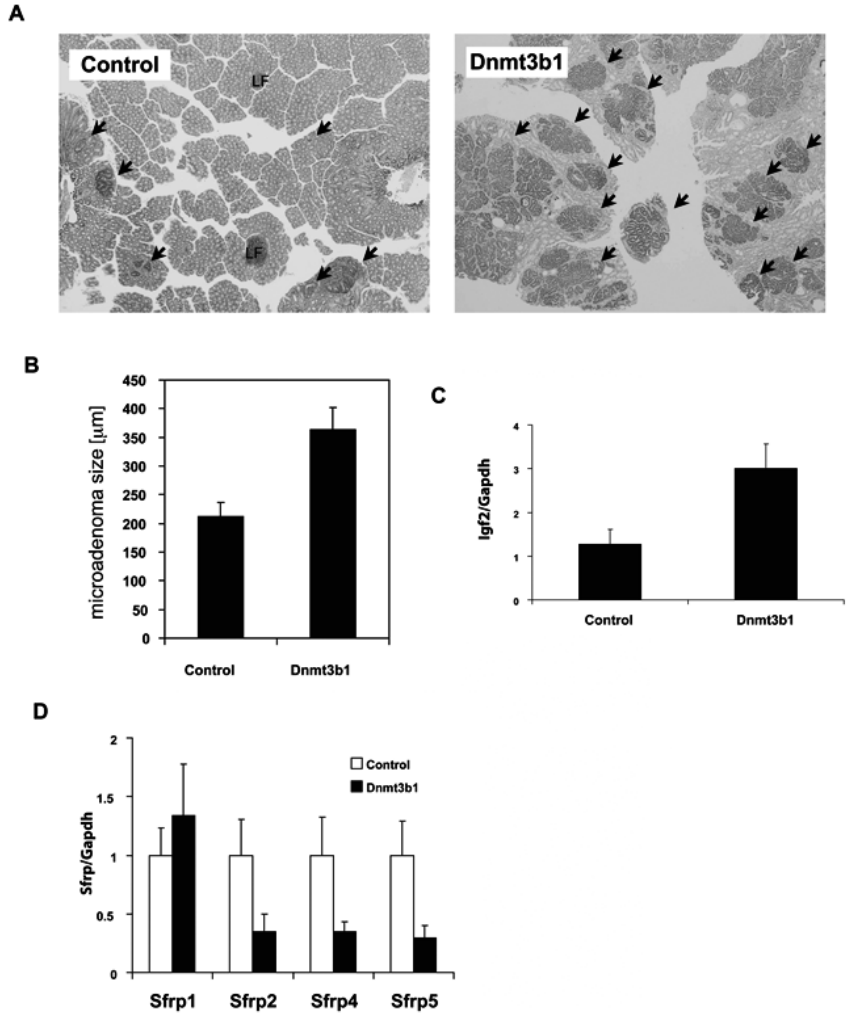


Figure 4. Dnmt3b1 overexpression increases the size of colonic microadenomas, increases expression of Igf2 and decreases expression of Sfrp2, 4 and 5 in colon tumors.
A: Horizontal *en face* sections of colon mucosa derived from a control and Dnmt3b1 expressing mouse (H/E stain, 10X). Arrows indicate microadenomas, LF: lymph follicule. The size of microadenomas in the mouse with increased Dnmt3b1 expression was much larger than in the control mouse. B: The average size of colonic microadenomas in Dnmt3b1 expressing mice was increased 1.7 fold when compared to controls (364µm ± 38 versus 211µm ± 25 colon; $p < 0.0005$). Values represent mean ± SE. C: Quantitation of Igf2 expression in colon tumors normalized to Gapdh expression using real time PCR. The relative Igf2 expression in tumors derived from Dnmt3b1 mice (2.9 ± 0.6, n=10) was significantly higher than Igf2 expression in control mice (1.3 ± 0.4, n=11), $p<0.03$ Mann Whitney U test. D: Quantitation of Sfrp expression in colon tumors normalized to Gapdh expression using real time PCR. To facilitate comparisons in one graph measurements of each Sfrp RNA were normalized to the average value of the respective control group. The expression of Sfrp2, 4 and 5 was significantly lower in tumors derived from Dnmt3b1 mice (Sfrp2 $p<0.002$, control 1 ± 0.3, n = 10 / Dnmt3b1 0.35 ± 0.2, n = 10; Sfrp4 $p<0.05$: control 1 ± 0.3, n = 9, Dnmt3b1+ 0.26 ± 0.15, n = 10; Sfrp5 $p<0.003$: control 1 ± 0.3, n=9 / Dnmt3b1+ 0.29 ± 0.1, n=10, Mann-Whitney U test). Values represent mean ± SE.

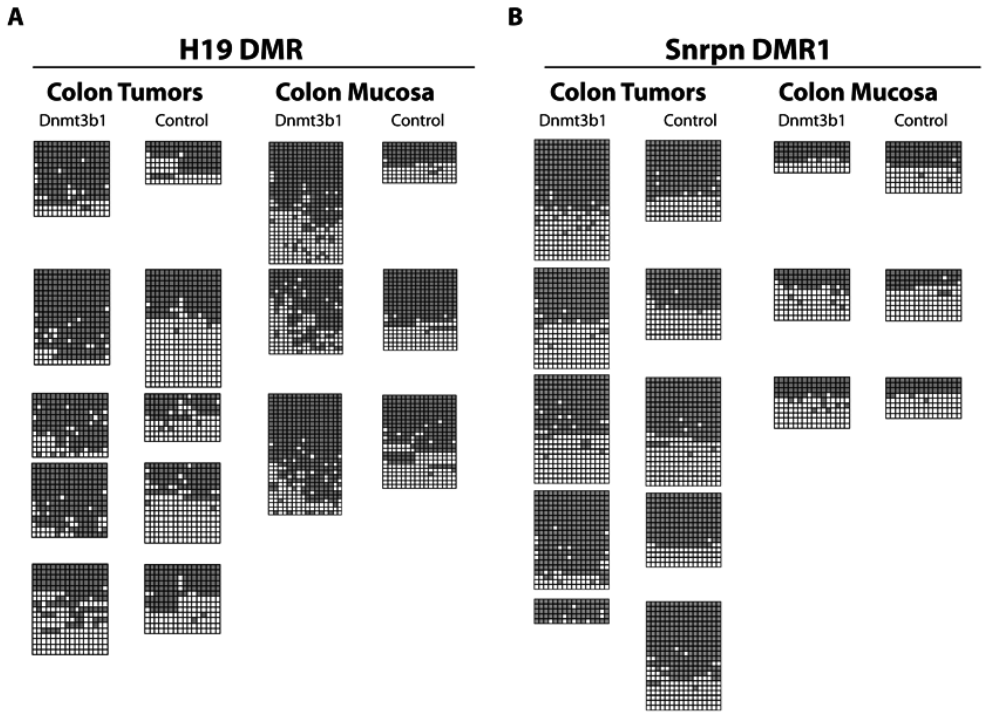


Figure 5. Dnmt3b1 overexpression causes biallelic methylation of the *H19 DMR* but does not affect methylation of the *Snrpn DMR1.*
A: Bisulfite sequencing analysis of the *H19 DMR* of tumor DNA and colon mucosa DNA derived from control (right columns) and Dnmt3b1 (left columns) expressing mice (five tumors each and three colon mucosa samples each). The region analyzed covers 16 CpG sites. Each horizontal line represents one individual sequence, each block of sequences represents data from one sample. Black boxes: Methylated CpG's, white boxes unmethylated CpG's. For each sample the biallelic or monoallelic status of the methylation pattern was assumed from the ratio of methylated and unmethylated alleles. Four out of five Dnmt3b1 expressing tumors showed clearly increased *H19 DMR* methylation, whereas all control tumors showed mostly mono allelic methylation. B: Bisulfite sequencing analysis of the *Snrpn DMR1* of tumor DNA and colon mucosa DNA derived from control (right column) and Dnmt3b1 (left column) expressing mice (five tumors each and three colon mucosa samples each). 16 CpG sites were analyzed. Only one Dnmt3b1 expressing tumor showed clear hypermethylation at this site. A second tumor was possibly hypermethylated, however the number of sequences is low. All colon mucosa samples show monoallelic methylation.

Binding, methylation and transcriptional downregulation of Sfrp family members by Dnmt3b1

Activation of the Wnt pathway is a key component of most intestinal tumors (Clevers 2006). We therefore investigated whether Dnmt3b1 expression contributed to epigenetic silencing of secreted frizzled related protein (*Sfrp*) genes which are considered Wnt pathway inhibitors (Suzuki et al. 2004). We first analyzed the expression of *Sfrp 1, 2, 4* and *5* in colon

tumors from experimental and control mice using quantitative PCR. Figure 4D shows that expression of *Sfrp 2, 4* and *5* was significantly downregulated in tumors derived from Dnmt3b1 expressing mice when compared to controls. Based on this finding we conducted bisulfite sequencing analysis of the promoter regions of *Sfrp2, 4* and *5.* Figure 6A shows that methylation of *Sfrp2* and *5* genes was significantly increased in Dnmt3b1 expressing tumors, when compared to controls. Methylation of *Sfrp4* was also increased as shown in Figure S4. Importantly, analysis of the tumor free mucosa also showed a significant increase in *Sfrp2* and *Sfrp5* methylation in samples derived from Dnmt3b1 overexpressing mice and for *Sfrp2* this finding was confirmed in *Ap*c wildtype mice (Figure 6B). This suggests that overexpression of Dnmt3b1 directly targets *Sfrp2, 4* and *5* for *de novo* methylation and silencing without clonal selection of cells carrying randomly silenced tumor suppressor genes being involved. In contrast to Dnmt3b1, Dnmt3a1 overexpression did not cause significant methylation of *Sfrp2* (Figure 6A). *Sfrp2* was not *de novo* methylated in spleen (Figure S5), suggesting tissue specificity of gene silencing.

To further evaluate the target specificity of Dnmt3b1 we analyzed the methylation pattern of three CpG islands associated with the *Cdx2* promoter region. The tumor suppressor gene *Cdx2* is transcriptionally downregulated in tumors from *Apc*$^{Min/+}$ mice and therefore a potential target for *de novo* methylation. However, in contrast to the *Sfrp* genes we found that the *Cdx2* associated CpG islands were completely unmethylated in Dnmt3b1 overexpressing tumors (Figure S6) supporting the hypothesis that Dnmt3b1 targets specific sequences. To screen for additional tumor relevant targets of Dnmt3b1 we also analyzed the methylation status of *Mlh1*, *Mgmt*, *Cdkn2b*, *Apc*, *Rb1*, *Vhlh* and *Brca1* by MALDI-TOF mass spectrometry, using the previously identified targets *Sfrp2* and *4* as positive controls. Figure 7 demonstrates that none of these additional loci was methylated by Dnmt3b1, whereas methylation of *Sfrp2* and *4* was confirmed, supporting the concept that this enzyme targets only specific loci. We also analyzed whether Dnmt3b1 was directly bound to methylated target loci using chromatin immunoprecipitation. The results (Figure S7) show that the Dnmt3b1 protein was physically associated with the DMRs of the methylated target loci *Sfrp2, 4, 5* and *H19* but not with the control loci *Cdx2* and *beta-actin* consistent with Dnmt3b1 binding to selected target genes.

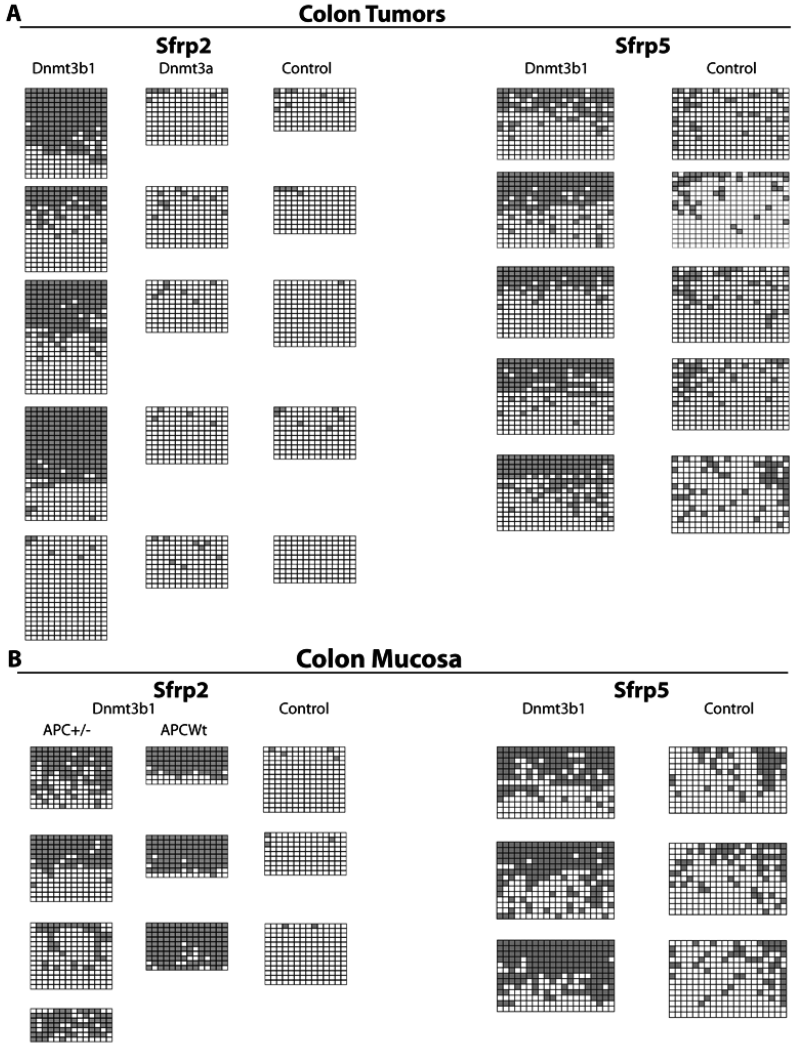


Figure 6. Dnmt3b1 causes hypermethylation of *Sfrp2* and *Sfrp5* in both tumors and normal colon epithelial cells.
A: Bisulfite sequencing analysis of the *Sfrp2* and *Sfrp5* promoter region of tumor DNA derived from control mice, Dnmt3b1 expressing mice and Dnmt3a expressing mice (*Sfrp2*). In each case five colon tumors from five different mice were analyzed. In the case of *Sfrp2* fourteen CpG sites were analyzed and in the case of *Sfrp5* twenty CpG sites were analyzed. Four out of five Dnmt3b1 expressing tumors showed clearly increased methylation at the *Sfrp2* promoter, and all five such tumors showed increased methylation at the *Sfrp5* promoter, when compared to control tumors. No significant methylation of *Sfrp2* was detected in tumors derived from Dnmt3a1 overexpressing mice B: Bisulfite sequencing analysis of the *Sfrp2* and *Sfrp5* promoter region of DNA derived from non tumor bearing colon epithelial cells (colon mucosa). *Sfrp2* methylation was analyzed in four *Apc* heterozygote mice (+/-) and in three *Apc* wildtype mice with Dnmt3b1 overexpression. Both *Apc* wildtype mice and *Apc* heterozygote mice show a clear increase in *Sfrp2* methylation following Dnmt3b1 expression when compared to controls, suggesting that Dnmt3b1 directly methylates this target in the absence of coexisting pathology. Methylation of *Sfrp5* is also increased in colon epithelial cells from Dnmt3b1 expressing mice when compared to controls (bottom right).

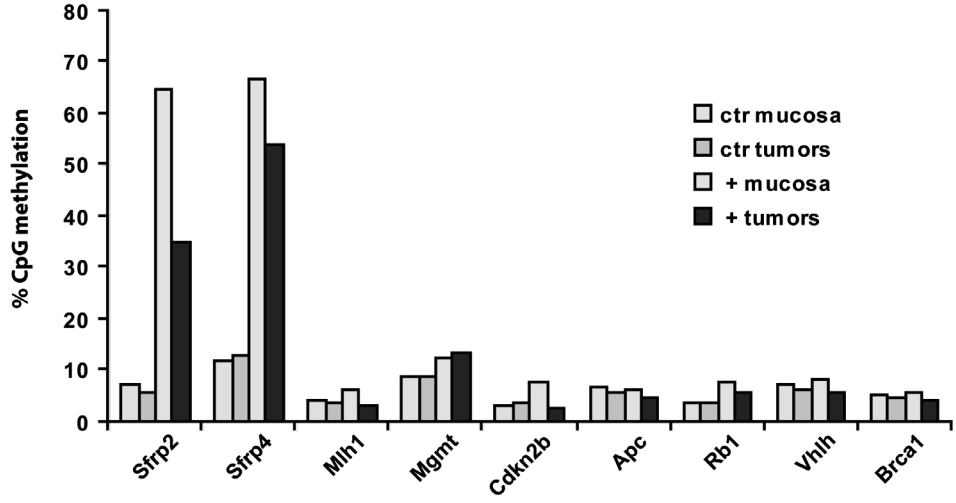


Figure 7. Dnmt3b1 does not cause hypermethylation of the tumor relevant genes *Mlh1*, *Mgmt*, *Cdkn2b*, *Apc*, *Rb1*, *Vhlh*, *Brca1*.
The methylation status of seven tumor relevant target genes (*Mlh1*, *Mgmt*, *Cdkn2b*, *Apc*, *Rb1*, *Vhlh*, *Brca1*) was analyzed by MALDI-TOF mass spectrometry, using two previously identified methylation targets (*Sfrp2* and *4*) as positive controls. We analyzed DNA from colon epithelial cells and colon tumors derived from Dnmt3b1 overexpressing mice and uninduced control mice (Dnmt3b1+ colon n = 4, control colon n = 3, Dnmt3b1+ tumors n = 5, control tumors n = 4) . The average CpG methylation rate was calculated for each amplicon (one amplicon per gene) of each experimental group and these averages are shown in the bar graph. The data demonstrate that none of these seven genes show signs of increased methylation upon overexpression of Dnmt3b1, whereas methylation of *Sfrp2* and *4* is confirmed, further suggesting that Dnmt3b1 only methylates specific genetic loci.

Apc LOH analysis

The $Apc^{Min/+}$ mouse strain contains one wildtype (+) and one mutant (*Min)* allele of the *Apc* tumor suppressor. Loss of function of *Apc* is an important component of tumor development in these mice and loss of heterozygosity (LOH) of this gene has been detected in the majority of intestinal tumors. We therefore investigated whether epigenetic inactivation of the *Apc* wildtype allele (+) might have contributed to the increased tumor load found in Dnmt3b1 expressing mice. We isolated DNA from colon tumors with Dnmt3b1 overexpression and from control tumors and analyzed the frequency of *Apc* LOH. Figure S8 shows that in both groups almost all tumors showed loss of the wildtype *Apc* allele. This indicates that epigenetic loss of *Apc* function is unlikely to contribute to the increased tumor load of Dnmt3b1 expressing mice and supports the methylation analysis of the *Apc* locus, which suggested that this gene was not methylated by Dnmt3b1 (Figure 7). Only one tumor in the Dnmt3b1 group appeared to have retained the wildtype allele. It is possible that in

this particular tumor aberrant methylation contributed to silencing of the wildtype allele and loss of *Apc* function, however the low frequency of this event cannot account for the observed increase in tumor numbers.

Discussion

Our study focused on three main goals: To evaluate a possible cause effect relationship between *de novo* methylation and tumor development, to establish an experimental system that can unmask and help identify genes susceptible to *de novo* methylation and to analyze how different *de novo* methyltransferases affect regional DNA methylation *in vivo*.

To address these questions we generated four transgenic mouse strains that allow for conditional overexpression of full length Dnmt3a1, full length Dnmt3b1 and two isoforms of Dnmt3b1 and studied the effect of overexpression on tumor development in the $Apc^{Min/+}$ model. We found that full length Dnmt3b1 increased the number of intestinal adenomas and microadenomas, whereas the truncated isoforms Dnmt3b3 and Dnmt3b6 had a small if any effect on the tumor number. Both Dnmt3b3 and 3b6 have no enzymatic activity because they lack part of the methyltransferase catalytic domain (Chen et al. 2002; Chen et al. 2003). It is therefore likely that the observed tumor promoting effect of Dnmt3b1 is mediated by DNA methylation. This distinction is important because it had been shown that Dnmt3b can suppress transcription in the absence of DNA methylating activity (Bachman et al. 2001). When evaluating possible molecular targets of Dnmt3b1 mediated methylation we followed a candidate approach. We selected genes for expression and methylation analyses that are relevant for intestinal tumor development and are known targets for *de novo* methylation in cancer.

Igf2 is an imprinted gene encoding a single chain polypetide with paracrine and autocrine activity that promotes mitosis mostly through signaling via the Igf-1 receptor (Dupont et al. 2003). Igf2 has been shown to promote tumor development in mouse models (Hassan and Howell 2000; Sakatani et al. 2005) and its increased expression in humans correlates with an increased risk for tumor development, in particular colorectal cancer (Cui et al. 2003; Woodson et al. 2004). Monoallelic expression of *Igf2* depends on the methylation status of the imprinting control region located between the *Igf2* and the *H19* gene (*H19 DMR*). The unmethylated maternal allele allows binding of the CTCF insulator protein which blocks downstream enhancer sequences from interacting with the upstream *Igf2* gene. Methylation of this sequence inhibits binding of the insulator protein and allows expression

of the respective *Igf2* allele. Thus, *de novo* methylation of the *H19 DMR* results in loss of imprinting and increased expression of *Igf2* (Bell and Felsenfeld 2000; Hark et al. 2000; Kurukuti et al. 2006). In a recent study it was demonstrated that loss of imprinting (LOI) and biallelic expression of *Igf2*, due to deletion of the *H19* DMR on the maternal allele, causes a twofold increase in intestinal tumors in *Apc*$^{Min/+}$ mice (Sakatani et al. 2005), which is similar to the relative tumor increase that we observed. This suggests that the *H19 DMR* hypermethylation and increased *Igf2* expression is partially responsible for the increased tumor load found in Dnmt3b1 expressing mice. Enhanced *Igf2* expression is also a possible explanation for the increased size of microscopic adenomas observed in these animals. Our observations provide direct *in vivo* evidence that loss of imprinting of *Igf2* can be caused by *de novo* methylation, as opposed to genetic rearrangements that cause loss of the maternal and duplication of the paternal *Igf2* allele (Haddad and Held 1997; Cooper et al. 2007). Deletion of Dnmt3b did not affect size or number of microadenomas suggesting that the phenotype of the Dnmt3b1 overexpressing mice is not complementary to the phenotype of the Dnmt3b knock-out model (Lin et al. 2006).

To evaluate whether Dnmt3b1 expression results in general loss of imprinting we analyzed the differentially methylated region 1 (*DMR1*) of the *Snurf/Snrpn* imprinting center (Reinhart et al. 2006). *Snurf/Snrpn* is a bicistronic imprinted gene encoding two polypeptides: The SmN splicing factor which is involved in RNA processing and the Snurf polypeptide of unknown function. *Snrpn* also encodes a long (~460 kb) alternatively spliced RNA transcript that contains several families of snoRNAs. A direct effect on cell proliferation has not been reported for the products of this locus, suggesting that growth mediated selection of methylation events at this imprinting center is unlikely (Rodriguez-Jato et al. 2005). In contrast to the *H19 DMR* we found that most samples from Dnmt3b1 expressing mice maintained normal imprinting patterns at this site. This suggests that the *H19 DMR* is either inherently more susceptible to *de novo* methylation by Dnmt3b1 or that cells with LOI of *Igf2* have a selective growth advantage.

In addition to *Igf2* we also analyzed the expression and methylation status of secreted frizzled related protein (*Sfrp*) genes. Five members of this protein family have been identified (Sfrp1-5). They contain regions homologous to the frizzled receptor, including a cysteine rich domain, which allows for binding and sequestering of Wnt ligands. These proteins are, therefore, considered inhibitors of the Wnt pathway (Jones and Jomary 2002). Importantly, it has been shown that the Sfrp proteins 1, 2, 4 and 5 inhibit the activity of the Wnt pathway even in the presence of downstream activating mutations, such as loss

of function of Apc (Suzuki et al. 2004). Each of these genes is associated with a CpG island that meets the formal criteria published by Takai and Jones (Takai and Jones 2002). We found that *Sfrp2, 4* and *5* are significantly downregulated in tumors derived from Dnmt3b1 mice and that the promoter regions of *Sfrp2, 4* and *5* are significantly *de novo* methylated in tumor samples from Dnmt3b1 mice when compared to controls. Importantly, increased methylation of *Sfrp2* and *5* was already detectable in the normal intestinal mucosa. Since Sfrp proteins can inhibit the Wnt pathway and methylation and downregulation of *Sfrp* genes is strongly associated with colorectal cancer in human patients (Caldwell et al. 2004; Suzuki et al. 2004), these observations suggest that epigenetic silencing of these targets contributed to the increased tumor load of Dnmt3b1 expressing mice. Our results show that *de novo* DNA methylation can induce silencing of *Sfrp* genes supporting the view that DNA methylation is a cause rather than a consequence of transcriptional silencing (Gu et al. 2006). This concept is also supported by the observation that the *Cdx2* gene remained unmethylated in Dnmt3b1 overexpressing tumors even though it is transcriptionally downregulated in *Apc*$^{Min/+}$ tumors. To further evaluate the specificity of *de novo* methylation by Dnmt3b1 we analyzed the methylation status of seven additional tumor relevant genes, selecting candidates from different functional categories, such as DNA repair (*Mlh1, Mgmt, Brca1*), Cdk inhibitor (*Cdkn2b*) and tumor suppressor genes (*Apc, Rb1, Vhlh*). None of these loci was hypermethylated upon Dnmt3b1 overexpression, supporting the hypothesis that Dnmt3b1 targets and methylates specific genetic loci.

Little is known about the relative role of different *de novo* methyltransferases in cancer. In contrast to Dnmt3b1, Dnmt3a1 overexpression did not cause any significant methylation of *Sfrp2,* which is consistent with the observation that Dnmt3a1 overexpression did not enhance tumor development. Several studies have shown different roles for Dnmt3a and Dnmt3b in development. For example Dnmt3a is required for methylation of imprinted loci during gametogenesis, whereas Dnmt3b is not (Kaneda et al. 2004). Also major satellite repeats of the pericentromeric region are preferentially methylated by Dnmt3a, whereas minor satellite repeats are methylated by Dnmt3b (Chen et al. 2003). A recent study identified a specific gene (*Fgf-1*) that is preferentially methylated by Dnmt3a and not by Dnmt3b (Oka et al. 2006). Our findings suggest that these differences may be significant for tumor development and that Dnmt3b1 is possibly a more relevant mediator of *de novo* methylation in cancer than Dnmt3a.

Our analysis of *Apc* LOH in colon tumors shows that the majority of tumors derived from Dnmt3b1 expressing mice lost the *Apc* wild type allele, which is similar to control mice.

It is, therefore, unlikely that epigenetic silencing of the *Apc* wildtype allele contributed to the increased tumor load of Dnmt3b1 overexpressing mice. Hypermethylation of the *APC* gene has been reported for selected cases of intestinal tumors in FAP patients, where loss of APC function was documented and at the same time LOH of the *APC* gene was excluded (Esteller et al. 2000; Esteller et al. 2001). However even though hypermethylation of the *APC* gene was found in these selected cases, it was not detected in unbiased screens to identify methylation targets in tumors and in our own study we also did not detect any methylation of this locus (Weber et al. 2005; Keshet et al. 2006; Widschwendter et al. 2007).

An important question is whether the epigenetic changes induced by Dnmt3b1 overexpression correspond to stochastic epimutations with subsequent selection due to proliferative advantage or whether the enzyme specifically targets some genes more efficiently than others. Our observations suggest that the aberrant DNA methylation in our model is localized and not stochastic. First, analysis of Dnmt3b1 overexpressing tissue showed no increase in global 5-methylcytosine content, when compared to control tissue, supporting the idea that this DNA methyltransferase affects regional methylation and not global methylation (Figure S3). Secondly, we found that overexpression of one *de novo* methyltransferase (Dnmt3b1) causes hypermethylation of *Sfrp2*, whereas overexpression of another *de novo* methyltransferase (Dnmt3a1) does not, suggesting differential targeting of these enzymes. Third and more importantly, methylation of affected target regions by Dnmt3b1 was not only detectable in clonal tumor tissue but also in normal colon mucosa. Because in normal mucosa selection for tumor suppressor gene silencing is unlikely, our results support the hypothesis that Dnmt3b1 can cause sequence specific *de novo* methylation of some tumor suppressor genes. This concept is further supported by the methylation screen of seven additional tumor relevant target loci, which did not show *de novo* methylation in mucosa or tumor samples from Dnmt3b1 overexpressing mice. In contrast to the normal intestinal mucosa, *Sfrp2* was not methylated in the spleen, even though the Dnmt3b1 transgene was expressed in this tissue. This suggests that the specificity of Dnmt3b1 mediated *de novo* methylation may be influenced by tissue specific chromatin states of target genes.

The results of our study raise several interesting questions with regard to the mechanisms that mediate specific targeting of Dnmt3b1 to certain loci. Is targeting specificity an inherent property of Dnmt3b1 or are additional binding partners such as Dnmt3L required for recruitment? What role does the DNA sequence play and which chromatin states of target genes are required for this process? Reports on the DNA sequence specificity of methylation

targets in cancer are conflicting. One study suggested enrichment of certain sequence motifs in tumor related methylation targets (Keshet et al. 2006) whereas another study could not detect any sequence specificity of *de novo* methylation in cancer (Weber et al. 2005). In contrast to DNA sequence specificity recent studies found good correlations between the chromatin state of genes and their methylation frequency in cancer, in particular H3K27 marks correlated with an increased frequency of DNA methylation in tumors (Schlesinger et al. 2007; Widschwendter et al. 2007). *Sfrp 2, 4* and *5* are reported targets of Polycomb complexes and are marked by H3K27 methylation in mouse and human embryonic stem cells (Boyer et al. 2006; Lee et al. 2006a). Similarly, when the expression of *Runx3* was analyzed, which is also a PcG target and in humans is frequently methylated in colon cancer (Widschwendter et al. 2007) we found reduced expression in tumors derived from Dnmt3b1-overexpressing mice (data not shown). On the other hand we could not detect any methylation of the *Cdx2* gene, which is occupied by PcG complexes in ES cells (Boyer et al. 2006; Lee et al. 2006a) and the *H19 DMR*, which was methylated in our study, reportedly does not contain any H3K27 marks (Mikkelsen et al. 2007). Clearly methylation analysis of more regions combined with histone mapping is necessary to allow a more detailed correlation of Dnmt3b1 mediated *de novo* methylation and histone modifications.

At this point it is unclear whether binding of Dnmt3b1 to specific target sites is an inherent property of the protein itself, or if additional mediators such as Dnmt3L are required for this process (Ooi et al. 2007). However, Dnmt3L expression was not increased upon overexpression of Dnmt3b1. Initial chromatin immunoprecipitation experiments suggested that Dnmt3b1 was indeed physically bound to the same loci that were found to be methylated (Figure S7) consistent with its role in *de novo* methylation of specific target genes. Our results support recent models on the contribution of epigenetic changes to tumor development: Based on the finding that epigenetic suppression of tumor relevant genes is often already detectable in pre-neoplastic tissue, it has been proposed that regional hypermethylation predisposes these tissues to later tumor development (Baylin and Ohm 2006; Feinberg et al. 2006; Jones and Baylin 2007). In our model Dnmt3b1 expression establishes a methylation pattern that is already detectable in non-tumor bearing tissues. This is consistent with the hypothesis that epigenetic rather than genetic alterations may be the very first changes in the process that converts a normal to a malignant cell (Baylin and Ohm 2006; Feinberg et al. 2006; Jones and Baylin 2007). Further analyses will also help clarifying whether inappropriate activation of Dnmt3b is the molecular basis for the CpG island methylator phenotype (CIMP) which is found in a subset of human cancers (Weisenberger et al. 2006).

Methods

DNA constructs and targeting of ES cells

Plasmids containing the coding region of the respective Dnmt's were kindly provided by Dr. En Li, Novartis Institute of Medicine, Cambridge, USA. Briefly: The respective Dnmt cDNA was cloned into the HindIII-EcoRI sites (for Dnmt3a1) or the BamhI-EcoRI sites (for Dnmt3b1 and isoforms) of the recipient plasmid pcDNA6/V5-HisA (Invitrogen), (Chen et al. 2002). In our lab these coding regions were released from pcDNA6/V5-HisA using HindIII/EcoRI (for Dnmt3a1) and BamHI/EcoRI (for Dnmt3b1 and isoforms) and inserted into the EcoRI site of the targeting vector pBS31 via blunt end ligation. All final targeting vectors were verified by sequencing, before transfection into ES cells. Targeting of transgenes to the *ColA1* locus of KH2 cells was conducted as previously described (Beard et al. 2006). Properties of the targeting vector are detailed in the methods supplement. Clones with correct integration of the targeting vector into the homing site of KH2 cells were identified by hygromycin resistance and southern blotting.

Generation of mice, genotyping, transgene induction

Transgenic ES cells were used to generate mice as as previously described (Beard et al. 2006). Mice with tet-inducible Dnmt alleles were backcrossed to the C57BL/6 strain for four generations prior to crossing with *Apc*$^{Min/+}$ mice. C57BL/6 *Apc*$^{Min/+}$ mice were obtained from The Jackson Laboratory. All final experimental mice analyzed in this study had been backcrossed for at least 6 generations to the C57BL/6 background. For genotyping DNA from tail tips was PCR amplified using primers and conditions listed in the methods supplement. For transgene induction, mice were fed 0.5 mg/ml doxycycline in the drinking water supplemented with 10 mg/ml sucrose. Control mice received regular drinking water.

Tissue harvesting, tumor analysis

All experimental mice were sacrificed at 5 months of age and macroscopic and microscopic adenomas were analyzed as previously described (Yamada et al. 2005). Counting was conducted blind with regard to genotype, p values were obtained using the Mann-Whitney U test. Colon epithelial cells were harvested from tumor free colon samples as previously described (Fujimoto et al. 2002).

Histological analysis, immunostaining, western blotting, chromatin immunoprecipitation

Paraffin sections (5 μm) were used for hematoxylin and eosin (H&E) staining and immunohistochemical analysis. Primary antibodies for immunostaining were anti-Dnmt3a (monoclonal, 1:150 dilution, Imgenex) and anti-Dnmt3b (monoclonal, 1:150 dilution, Imgenex). For western blotting we used anti-Dnmt3a (chicken polyclonal, 1:2000 dilution, Abcam), anti-Dnmt3b (monoclonal, 1:500 dilution, Imgenex) and anti-GAPDH (rabbit polyclonal, 1:2000, Abcam). Chromatin immunoprecipitation (CHIP) was conducted as previously described (Lee et al. 2006b), using the Dnmt3b antibody ab2851 (rabbit polyclonal, Abcam). Primer pairs used for quantitative PCR following CHIP are listed in the methods supplement.

cDNA and real time PCR

cDNA was generated from total RNA using oligo dT primers. For each sample a minus RT reaction was generated and pooled minus RT reactions were used as negative controls for quantitative PCR reactions (SYBR Green, Invitrogen). For quantitation standard dilution curves were included on each plate. All samples were analyzed in triplicates, β-actin was used as an endogenous control. Individual data were converted to relative values based on the standard curve and then normalized to the β-actin values of the same sample. Primers used are listed in the methods supplement.

Bisulfite sequencing, methylation screen, 5-methylcytosine quantitation and *Apc* LOH analysis

For bisulfite sequencing analysis DNA was mutagenized using the Epitect kit (Qiagen). Target regions were PCR amplified, subcloned and minipreps of individual colonies were sequenced. Only sequences with more than 95% non CpG C conversion were used for analysis. Primers and PCR conditions for individual loci are listed in the methods supplement. Methylation screening using MALDI-TOF MS was conducted as previosuly described (Ehrich et al. 2005). Primer pairs used for this analysis are listed in the methods supplement. Quantitative analysis of 5-methylcytosine content of DNA was measured by LC-MS/MS as described previosuly (Crain 1990). 2'-deoxycytidine (dC), and 5-methyl-2'-deoxycytidine (5mdC) were monitored in the analysis and 2'-deoxycytidine ($^{15}N_3$) was used as an internal standard. LOH of the *Apc* gene was analyzed by PCR as described previously (Yamada et al. 2002).

Acknowledgements

We would like to thank Jessica Dausman, Ruth Flannery and Dongdong Fu for help with maintenance of the mouse colony and histological analysis. We are also very grateful for the help of Tom DiCesare in the preparation of Figures. Heinz Linhart is supported by a postdoctoral fellowship from the Fritz-Thyssen Stiftung. This work was supported by a research grant from Philip Morris International and National Institutes of Health grant NIH CA087869 to R.J.

References

Bachman, K.E., M.R. Rountree, and S.B. Baylin. 2001. Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin. *J Biol Chem* 276: 32282-7.

Baylin, S.B., J.W. Hoppener, A. de Bustros, P.H. Steenbergh, C.J. Lips, and B.D. Nelkin. 1986. DNA methylation patterns of the calcitonin gene in human lung cancers and lymphomas. *Cancer Res* 46: 2917-22.

Baylin, S.B. and J.E. Ohm. 2006. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* 6: 107-16.

Beard, C., K. Hochedlinger, K. Plath, A. Wutz, and R. Jaenisch. 2006. Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. *Genesis* 44: 23-8.

Bell, A.C. and G. Felsenfeld. 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* 405: 482-5.

Bestor, T.H. 2003. Unanswered questions about the role of promoter methylation in carcinogenesis. *Ann N Y Acad Sci* 983: 22-7.

Biniszkiewicz, D., J. Gribnau, B. Ramsahoye, F. Gaudet, K. Eggan, D. Humpherys, M.A. Mastrangelo, Z. Jun, J. Walter, and R. Jaenisch. 2002. Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality. *Mol Cell Biol* 22: 2124-35.

Boyer, L.A., K. Plath, J. Zeitlinger, T. Brambrink, L.A. Medeiros, T.I. Lee, S.S. Levine, M. Wernig, A. Tajonar, M.K. Ray, G.W. Bell, A.P. Otte, M. Vidal, D.K. Gifford, R.A. Young, and R. Jaenisch. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 441: 349-53.

Caldwell, G.M., C. Jones, K. Gensberg, S. Jan, R.G. Hardy, P. Byrd, S. Chughtai, Y. Wallis, G.M. Matthews, and D.G. Morton. 2004. The Wnt antagonist sFRP1 in colorectal tumorigenesis. *Cancer Res* 64: 883-8.

Chan, T.L., S.T. Yuen, C.K. Kong, Y.W. Chan, A.S. Chan, W.F. Ng, W.Y. Tsui, M.W. Lo, W.Y. Tam, V.S. Li, and S.Y. Leung. 2006. Heritable germline epimutation of MSH2 in a family with hereditary nonpolyposis colorectal cancer. *Nat Genet* 38: 1178-83.

Chen, T., Y. Ueda, J.E. Dodge, Z. Wang, and E. Li. 2003. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Mol Cell Biol* 23: 5594-605.

Chen, T., Y. Ueda, S. Xie, and E. Li. 2002. A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. *J Biol Chem* 277: 38746-54.

Clevers, H. 2006. Wnt/beta-catenin signaling in development and disease. *Cell* 127: 469-80.

Cooper, W.N., R. Curley, F. Macdonald, and E.R. Maher. 2007. Mitotic recombination and uniparental disomy in Beckwith-Wiedemann syndrome. *Genomics* 89: 613-7.

Crain, P.F. 1990. Analysis of 5-methylcytosine in DNA by isotope dilution gas chromatography-mass spectrometry. *Methods Enzymol* 193: 857-65.

Cui, H., M. Cruz-Correa, F.M. Giardiello, D.F. Hutcheon, D.R. Kafonek, S. Brandenburg, Y. Wu, X. He, N.R. Powe, and A.P. Feinberg. 2003. Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. *Science* 299: 1753-5.

Dupont, J., A. Pierre, P. Froment, and C. Moreau. 2003. The insulin-like growth factor axis in cell cycle progression. *Horm Metab Res* 35: 740-50.

Eden, A., F. Gaudet, A. Waghmare, and R. Jaenisch. 2003. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 300: 455.

Ehrich, M., M.R. Nelson, P. Stanssens, M. Zabeau, T. Liloglou, G. Xinarianos, C.R. Cantor, J.K. Field, and D. van den Boom. 2005. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc Natl Acad Sci U S A* 102: 15785-90.

Esteller, M., M.F. Fraga, M. Guo, J. Garcia-Foncillas, I. Hedenfalk, A.K. Godwin, J. Trojan, C. Vaurs-Barriere, Y.J. Bignon, S. Ramus, J. Benitez, T. Caldes, Y. Akiyama, Y. Yuasa, V. Launonen, M.J. Canal, R. Rodriguez, G. Capella, M.A. Peinado, A. Borg, L.A. Aaltonen, B.A. Ponder, S.B. Baylin, and J.G. Herman. 2001. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. *Hum Mol Genet* 10: 3001-7.

Esteller, M., A. Sparks, M. Toyota, M. Sanchez-Cespedes, G. Capella, M.A. Peinado, S. Gonzalez, G. Tarafa, D. Sidransky, S.J. Meltzer, S.B. Baylin, and J.G. Herman. 2000. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. *Cancer Res* 60: 4366-71.

Feinberg, A.P., R. Ohlsson, and S. Henikoff. 2006. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 7: 21-33.

Feinberg, A.P. and B. Tycko. 2004. The history of cancer epigenetics. *Nat Rev Cancer* 4: 143-53.

Feinberg, A.P. and B. Vogelstein. 1983. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 301: 89-92.

Fujimoto, K., R.D. Beauchamp, and R.H. Whitehead. 2002. Identification and isolation of candidate human colonic clonogenic cells based on cell surface integrin expression. *Gastroenterology* 123: 1941-8.

Gaudet, F., J.G. Hodgson, A. Eden, L. Jackson-Grusby, J. Dausman, J.W. Gray, H. Leonhardt, and R. Jaenisch. 2003. Induction of tumors in mice by genomic hypomethylation. *Science* 300: 489-92.

Goll, M.G. and T.H. Bestor. 2005. Eukaryotic cytosine methyltransferases. *Annu Rev Biochem* 74: 481-514.

Greger, V., N. Debus, D. Lohmann, W. Hopping, E. Passarge, and B. Horsthemke. 1994. Frequency and parental origin of hypermethylated RB1 alleles in retinoblastoma. *Hum Genet* 94: 491-6.

Greger, V., E. Passarge, W. Hopping, E. Messmer, and B. Horsthemke. 1989. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum Genet* 83: 155-8.

Gu, P., D. Le Menuet, A.C. Chung, and A.J. Cooney. 2006. Differential recruitment of methylated CpG binding domains by the orphan receptor GCNF initiates the repression and silencing of Oct4 expression. *Mol Cell Biol* 26: 9471-83.

Haddad, R. and W.A. Held. 1997. Genomic imprinting and Igf2 influence liver tumorigenesis and loss of heterozygosity in SV40 T antigen transgenic mice. *Cancer Res* 57: 4615-23.

Hark, A.T., C.J. Schoenherr, D.J. Katz, R.S. Ingram, J.M. Levorse, and S.M. Tilghman. 2000. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* 405: 486-9.

Hassan, A.B. and J.A. Howell. 2000. Insulin-like growth factor II supply modifies growth of intestinal adenoma in Apc(Min/+) mice. *Cancer Res* 60: 1070-6.

Herman, J.G. and S.B. Baylin. 2003. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 349: 2042-54.

Jones, P.A. and S.B. Baylin. 2007. The epigenomics of cancer. *Cell* 128: 683-92.

Jones, S.E. and C. Jomary. 2002. Secreted Frizzled-related proteins: searching for relationships and patterns. *Bioessays* 24: 811-20.

Kaneda, M., M. Okano, K. Hata, T. Sado, N. Tsujimoto, E. Li, and H. Sasaki. 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 429: 900-3.

Keshet, I., Y. Schlesinger, S. Farkash, E. Rand, M. Hecht, E. Segal, E. Pikarski, R.A. Young, A. Niveleau, H. Cedar, and I. Simon. 2006. Evidence for an instructive mechanism of de novo methylation in cancer cells. *Nat Genet* 38: 149-53.

Kurukuti, S., V.K. Tiwari, G. Tavoosidana, E. Pugacheva, A. Murrell, Z. Zhao, V. Lobanenkov, W. Reik, and R. Ohlsson. 2006. CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2. *Proc Natl Acad Sci U S A* 103: 10684-9.

Laird, P.W., L. Jackson-Grusby, A. Fazeli, S.L. Dickinson, W.E. Jung, E. Li, R.A. Weinberg, and R. Jaenisch. 1995. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell* 81: 197-205.

Lee, T.I., R.G. Jenner, L.A. Boyer, M.G. Guenther, S.S. Levine, R.M. Kumar, B. Chevalier, S.E. Johnstone, M.F. Cole, K. Isono, H. Koseki, T. Fuchikami, K. Abe, H.L. Murray, J.P. Zucker, B. Yuan, G.W. Bell, E. Herbolsheimer, N.M. Hannett, K. Sun, D.T. Odom, A.P. Otte, T.L. Volkert, D.P. Bartel, D.A. Melton, D.K. Gifford, R. Jaenisch, and R.A. Young. 2006a. Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* 125: 301-13.

Lee, T.I., S.E. Johnstone, and R.A. Young. 2006b. Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nat Protoc* 1: 729-48.

Lin, H., Y. Yamada, S. Nguyen, H. Linhart, L. Jackson-Grusby, A. Meissner, K. Meletis, G. Lo, and R. Jaenisch. 2006. Suppression of intestinal neoplasia by deletion of Dnmt3b. *Mol Cell Biol* 26: 2976-83.

Mikkelsen, T.S., M. Ku, D.B. Jaffe, B. Issac, E. Lieberman, G. Giannoukos, P. Alvarez, W. Brockman, T.K. Kim, R.P. Koche, W. Lee, E. Mendenhall, A. O'Donovan, A. Presser, C. Russ, X. Xie, A. Meissner, M. Wernig, R. Jaenisch, C. Nusbaum, E.S. Lander, and B.E. Bernstein. 2007. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* 448: 553-60.

Myohanen, S.K., S.B. Baylin, and J.G. Herman. 1998. Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia. *Cancer Res* 58: 591-3.

Ohm, J.E., K.M. McGarvey, X. Yu, L. Cheng, K.E. Schuebel, L. Cope, H.P. Mohammad, W. Chen, V.C. Daniel, W. Yu, D.M. Berman, T. Jenuwein, K. Pruitt, S.J. Sharkis, D.N. Watkins, J.G. Herman, and S.B. Baylin. 2007. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet* 39: 237-42.

Oka, M., N. Rodic, J. Graddy, L.J. Chang, and N. Terada. 2006. CpG sites preferentially methylated by Dnmt3a in vivo. *J Biol Chem* 281: 9901-8.

Okano, M., D.W. Bell, D.A. Haber, and E. Li. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99: 247-57.

Okano, M., S. Xie, and E. Li. 1998. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* 19: 219-20.

Ooi, S.K., C. Qiu, E. Bernstein, K. Li, D. Jia, Z. Yang, H. Erdjument-Bromage, P. Tempst, S.P. Lin, C.D. Allis, X. Cheng, and T.H. Bestor. 2007. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature* 448: 714-7.

Reinhart, B., A. Paoloni-Giacobino, and J.R. Chaillet. 2006. Specific differentially methylated domain sequences direct the maintenance of methylation at imprinted genes. *Mol Cell Biol* 26: 8347-56.

Robert, M.F., S. Morin, N. Beaulieu, F. Gauthier, I.C. Chute, A. Barsalou, and A.R. MacLeod. 2003. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat Genet* 33: 61-5.

Rodriguez-Jato, S., R.D. Nicholls, D.J. Driscoll, and T.P. Yang. 2005. Characterization of cis- and trans-acting elements in the imprinted human SNURF-SNRPN locus. *Nucleic Acids Res* 33: 4740-53.

Sakatani, T., A. Kaneda, C.A. Iacobuzio-Donahue, M.G. Carter, S. de Boom Witzel, H. Okano, M.S. Ko, R. Ohlsson, D.L. Longo, and A.P. Feinberg. 2005. Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice. *Science* 307: 1976-8.

Schlesinger, Y., R. Straussman, I. Keshet, S. Farkash, M. Hecht, J. Zimmerman, E. Eden, Z. Yakhini, E. Ben-Shushan, B.E. Reubinoff, Y. Bergman, I. Simon, and H. Cedar. 2007. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet* 39: 232-6.

Su, L.K., K.W. Kinzler, B. Vogelstein, A.C. Preisinger, A.R. Moser, C. Luongo, K.A. Gould, and W.F. Dove. 1992. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* 256: 668-70.

Suzuki, H., E. Gabrielson, W. Chen, R. Anbazhagan, M. van Engeland, M.P. Weijenberg, J.G. Herman, and S.B. Baylin. 2002. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet* 31: 141-9.

Suzuki, H., D.N. Watkins, K.W. Jair, K.E. Schuebel, S.D. Markowitz, W.D. Chen, T.P. Pretlow, B. Yang, Y. Akiyama, M. Van Engeland, M. Toyota, T. Tokino, Y. Hinoda, K. Imai, J.G. Herman, and S.B. Baylin. 2004. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 36: 417-22.

Takai, D. and P.A. Jones. 2002. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci U S A* 99: 3740-5.

Vire, E., C. Brenner, R. Deplus, L. Blanchon, M. Fraga, C. Didelot, L. Morey, A. Van Eynde, D. Bernard, J.M. Vanderwinden, M. Bollen, M. Esteller, L. Di Croce, Y. de Launoit, and F. Fuks. 2006. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 439: 871-4.

Walsh, C.P., J.R. Chaillet, and T.H. Bestor. 1998. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nat Genet* 20: 116-7.

Weber, M., J.J. Davies, D. Wittig, E.J. Oakeley, M. Haase, W.L. Lam, and D. Schubeler. 2005. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet* 37: 853-62.

Weisenberger, D.J., K.D. Siegmund, M. Campan, J. Young, T.I. Long, M.A. Faasse, G.H. Kang, M. Widschwendter, D. Weener, D. Buchanan, H. Koh, L. Simms, M. Barker, B. Leggett, J. Levine, M. Kim, A.J. French, S.N. Thibodeau, J. Jass, R. Haile, and P.W. Laird. 2006. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 38: 787-93.

Widschwendter, M., H. Fiegl, D. Egle, E. Mueller-Holzner, G. Spizzo, C. Marth, D.J. Weisenberger, M. Campan, J. Young, I. Jacobs, and P.W. Laird. 2007. Epigenetic stem cell signature in cancer. *Nat Genet* 39: 157-8.

Woodson, K., A. Flood, L. Green, J.A. Tangrea, J. Hanson, B. Cash, A. Schatzkin, and P. Schoenfeld. 2004. Loss of insulin-like growth factor-II imprinting and the presence of screen-detected colorectal adenomas in women. *J Natl Cancer Inst* 96: 407-10.

Wu, J., J.P. Issa, J. Herman, D.E. Bassett, Jr., B.D. Nelkin, and S.B. Baylin. 1993. Expression of an exogenous eukaryotic DNA methyltransferase gene induces transformation of NIH 3T3 cells. *Proc Natl Acad Sci U S A* 90: 8891-5.

Yamada, Y., K. Hata, Y. Hirose, A. Hara, S. Sugie, T. Kuno, N. Yoshimi, T. Tanaka, and H. Mori. 2002. Microadenomatous lesions involving loss of Apc heterozygosity in the colon of adult Apc(Min/+) mice. *Cancer Res* 62: 6367-70.

Yamada, Y., L. Jackson-Grusby, H. Linhart, A. Meissner, A. Eden, H. Lin, and R. Jaenisch. 2005. Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. *Proc Natl Acad Sci U S A* 102: 13580-5.

Yamada, Y. and H. Mori. 2007. Multistep carcinogenesis of the colon in Apc(Min/+) mouse. *Cancer Sci* 98: 6-10.

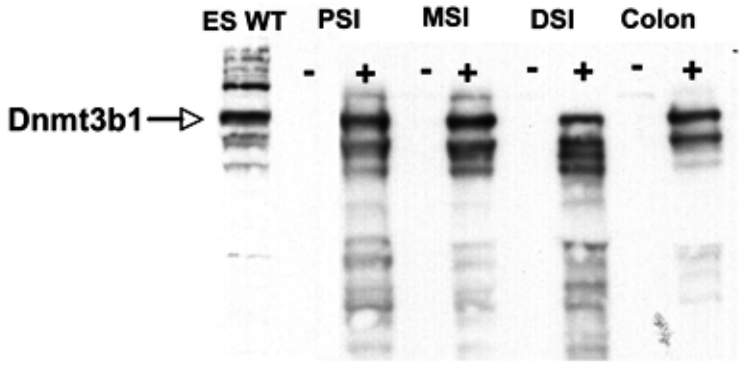


Figure S1. Doxycycline dependent Dnmt3b1 transgene expression in different regions of the mouse intestine.
Western Blot of intestinal epithelial cells from proximal small intestine (PSI), middle small intestine (MSI), distal small intestine (DSI) and colon. – indicates samples from a transgenic mouse that received no doxycycline and + indicates samples from a transgenic mouse that received doxycycline in the dirnkineg water, wildtype ES cells (ES WT) were used as a positive control. The figure demonstrates doxycyline dependent expression of the Dnmt3b1 transgene in all regions of the intestine.

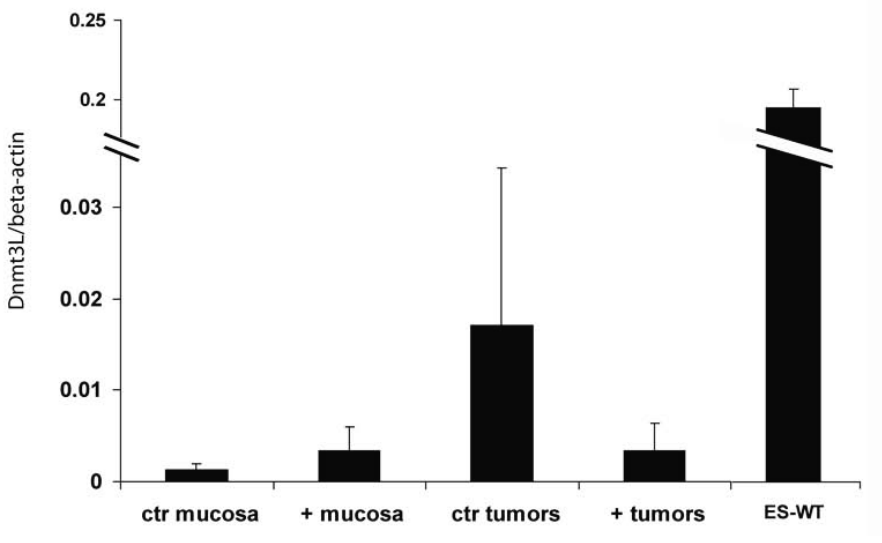


Figure S2. Dnmt3b1 overexpression does not cause increased expression of Dnmt3L.
Dnmt3L expression was measured by quantititave PCR in colon epithelial cells (n = 3) and colon tumors (n = 4) derived from control mice (ctr) or Dnmt3b1 overexpressing mice (+). Beta-actin was used as reference and cDNA from wildtype ES cells (ES-WT) was included as a positive control. The data demonstrate that the tissues from Dnmt3b1 overexpressing mice did not show a consistent increase in Dnmt3L expression when compared to controls.

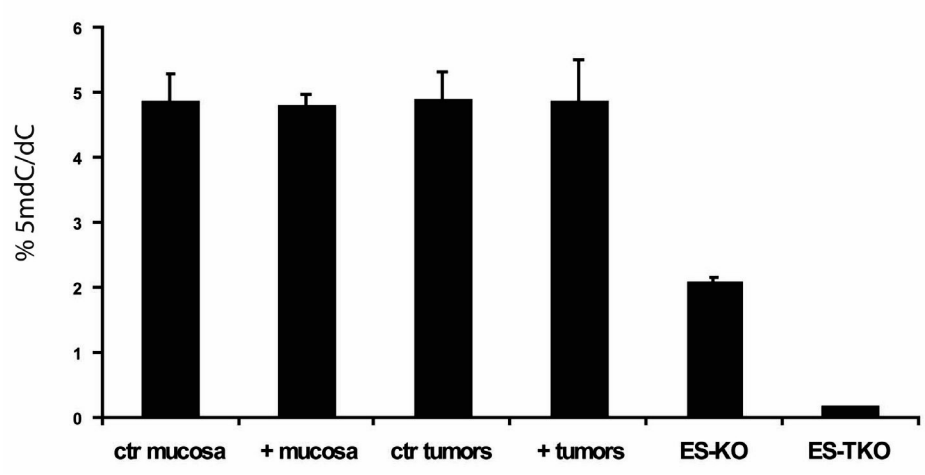


Figure S3. Dnmt3b1 overexpression does not global increase in DNA cytosine methylation.
Global DNA 5-methylcytosine content was measured by mass spectrometry (LC-MS/MS) in colon epithelial cells and colon tumors derived from either control mice (ctr) or Dnmt3b1 overexpressing mice(=), n = 3 each. As controls we included DNA from Dnmt1 knock-out ES cells (ES-KO) and from combined Dnmt3a and 3b knock-out ES cells with additional RNAi-induced knockdown of Dnmt1 (ES-TKO). These data demonstrate that Dnmt3b1 overexpression did not cause a measurable increase in global DNA cytosine methylation in colon epithelial cells or colon tumors.

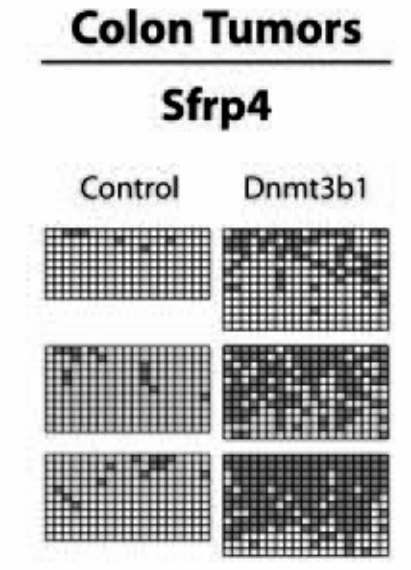


Figure S4. Dnmt3b1 overexpression caused hypermethylation of *Sfrp4*.
Bisulfite sequencing analysis of the Sfrp4 promoter region of tumor DNA dereived from control mice and Dnmt3b1 overexpressing mice. In each case three colon tumors from three different mice were analyzed. In total 19 CpG sites were studied. All Dnmt3b1 overexpressing tumors showed increased methylation of the *Sfrp4* promoter region when compared to control tumors. This suggests that *Sfrp4*, in addition to *Sfrp2* and *5*, also represents a target of Dnmt3b1.

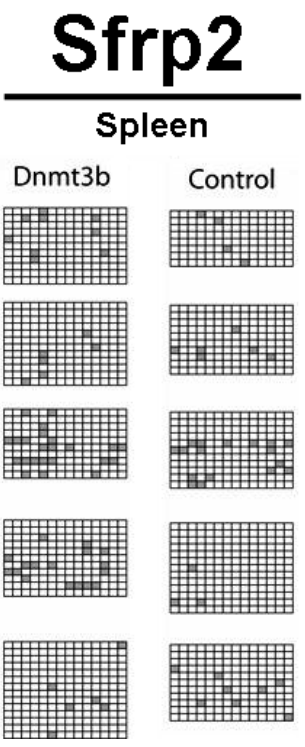


Figure S5. Dnmt3b1 overexpression does not cause hypermthethyaltion of *Sfrp2* in the spleen. Bisulfite sequencing analyis of *Sfrp2* promoter region of spleen DNA derived from control mice and Dnmt3b1 overexpressing mice. In each case samples from five different mice were analyzed with the analysis covering a total of 14 CpG sites. The promoter region of *Sfrp2* remained mostly unmethylated in both control and Dnmt3b1 overexpressing mice. This is in contrast to intestinal epithelial cells where *Sfrp2* was strongly methyalted by Dnmt3b1, suggesting that the specificity of Dnmt3b1 mediated methylation may be influenced by the tissue specific chromatin state of a target gene.

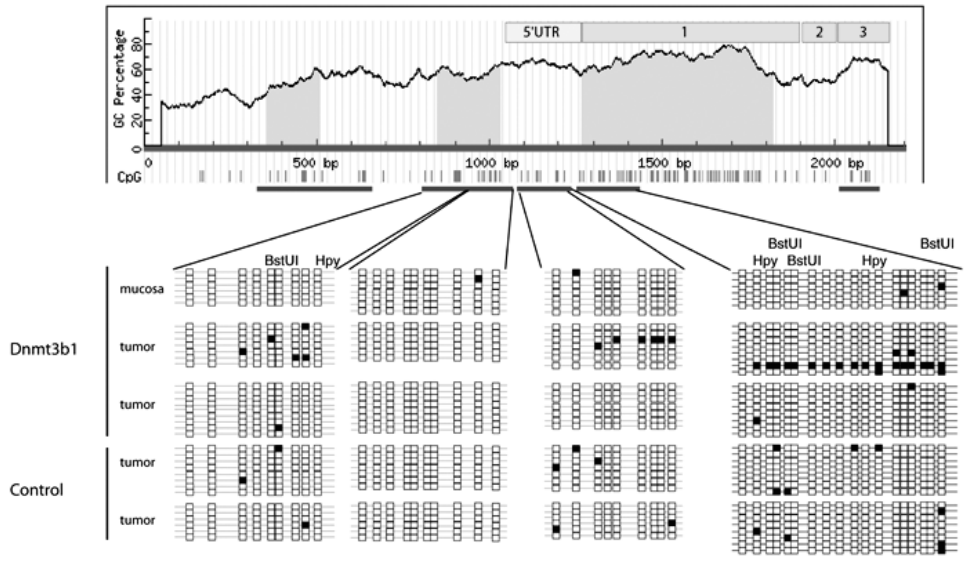


Figure S6. Dnmt3b does not methylate *Cdx2*.
Bisulfite sequencing analyis of *Cdx2* promoter region of colon tumor DNA derived from control mice and Dnmt3b1 overexpressing mice. One colon mucosa sample from Dnmt3b1 overexpressing mouse was also included in the analysis. *Cdx2* was chosen for analysis because it functions as a tumor suppresor in the intestinal mucosa and is transcriptionally silenced in adenomas of APC Min mice. It has been proposed that DNA methylation is the consequence rather than the cause of transcriptional silencing. According to this hypothesis the promoter region of the *Cdx2* gene should be susceptible to DNA methylation in adenomas where this gene is transcriptionally silent. In total four CpG islands that are associated with the *Cdx2* promoter region were studied. Black circles represent methylated CpG's and open circles represent unmethylated CpG sites. The figure demonstrates that Dnmt3b1 overexpression did not cause methylation of this target. This is in contrast to the Sfrp genes studied, which were methylated even though they were transcriptionally active. This suggests that transcriptional silencing is not a prerequisite for methylation, nor does it neccesarily promote methylation.

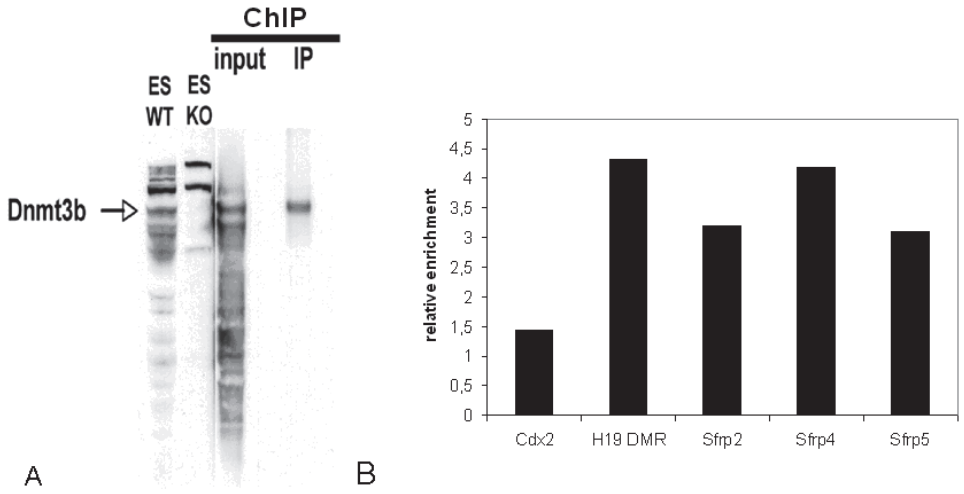


Figure S7. Dnmt3b1 binds to methylated target sequences.
To evaluate direct binding of Dnmt3b1 to methylated target regions in intestinal epithelial cells of Dnmt3b1 overexpressing mice we conducted chromatin immunoprecipitation with a Dnmt3b antibody followed by quantitative PCR. A: Western Blot stained with Dnmt3b antibody of wildtype ES cells (ES WT), Dnmt3b knock-out ES cells (ES KO), whole cell extract from intestinal epithelial cells (input) and Dnmt3b immunoprecipitated intestinal whole cell extract (IP). The figure demonstrates that Dnmt3b protein was succesfully immunoprecipitated. B: Quantitative PCR of two control regions (*beta-actin* and *Cdx2*) and four methylated regions (*H19 DMR, Sfrp2, Sfrp4, Sfrp5*) using extract from intestinal epithelial cells (input) and Dnmt3b immunoprecipitated extract (output) from a Dnmt3b1 overexpressing mouse. The data show that methylated target sequences were enriched three to four fold by Dnmt3b immunoprecipitation, when compared to the two control regions, suggesting that Dnmt3b1 directly binds to these methylated regions.

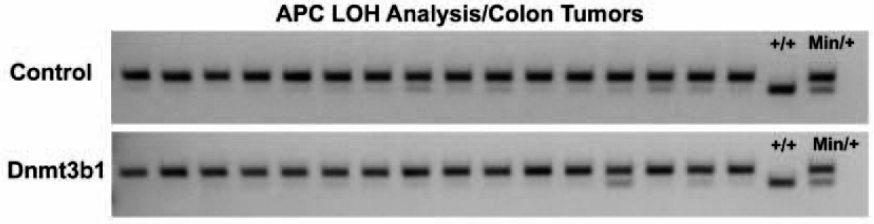


Figure S8. Dnmt3b1 overepression does not affect the frequency of *Apc* LOH in colon tumors.
Apc LOH analysis of 16 tumors derived from control mice (top panel) and of 16 tumors derived from Dnmt3b1 expressing mice (bottom panel). The *Apc* LOH analysis was conducted as previously described (1). In each panel the upper band represents the mutant Min allele and the bottom band represents the wildtype (+) allele. Almost all tumors showed loss of the wildtype band, suggesting LOH. Only one of the tumors derived from Dnmt3b1 expressing mice appeared to have retained the wildtype allele.

1. Yamada, Y., K. Hata, Y. Hirose, A. Hara, S. Sugie, T. Kuno, N. Yoshimi, T. Tanaka, and H. Mori. 2002. Microadenomatous lesions involving loss of Apc heterozygosity in the colon of adult Apc(Min/+) mice. *Cancer Res* 62: 6367-70.

CHAPTER 3

GENES METHYLATED BY DNA METHYLTRANSFERASE 3B ARE SIMILAR IN MOUSE INTESTINE AND HUMAN COLON CANCER

Eveline J. Steine[1, †], Mathias Ehrich[2, †], George W. Bell[1], Arjun Raj[3], Seshamma Reddy[1], Alexander van Oudenaarden[3], Rudolf Jaenisch[1]* and Heinz G. Linhart[1,4,5]*

1 Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Nine Cambridge Center, Cambridge, MA 02142, USA

2 Sequenom, Inc., 3595 John Hopkins Court, San Diego, CA 92121, USA

3 Department of Physics, Department of Biology, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

4 Department of Gastroenterology and Hepatology, University Hospital Freiburg, Hugstetter Str. 49, 79106 Freiburg, Germany

5 Departments of Translational Oncology and Epigenetics, DKFZ/NCT, INF 581, 69120 Heidelberg, Germany

† These authors contributed equally

* To whom correspondence should be addressed.

Published in *Journal of Clinical Investigation* 2011; 121(5):1748-52

Abstract

We demonstrate that transgenic expression of DNA methyltransferase 3b (Dnmt3b) in the mouse colon initiates *de novo* DNA methylation of genes that are similar to genes that become methylated in human colon cancer. This is consistent with the notion that aberrant methylation in cancer may be due to targeting of specific sequences by Dnmt3b rather than due to random methylation followed by clonal selection. We also show that Dnmt3b induced aberrant DNA methylation is maintained in regenerating tissue even in the absence of continuous Dnmt3b expression. This supports the concept that transient stressors can cause permanent epigenetic changes in somatic stem cells that accumulate over the lifetime of an organism in analogy to DNA mutations.

Introduction

Regional hypermethylation of DNA is frequently found in human cancer, causes transcriptional silencing of affected genes and promotes tumor formation (1, 2). An unresolved question is whether aberrant DNA methylation of cancer is caused by stochastic DNA methylation followed by clonal selection or whether specific sequences are targeted by the *de novo* methyltransferases (3). Furthermore it is unknown whether "cancer specific" DNA methylation only occurs in neoplastic cells or whether it can also occur in normal cells. Another important question is whether aberrations in DNA methylation are maintained in the absence of the initiating cause.

Abundant evidence indicates that Dnmt3b is involved in *de novo* methylation of mammalian cells during development, is frequently activated in human tumors and promotes tumor development in $Apc^{min/+}$ mice (4, 5). To address whether Dnmt3b targets specific sequences for *de novo* methylation we used transgenic mice that allow tetracycline inducible expression of the DNA methyltransferase 3b in wild-type mice and in $Apc^{min/+}$ mice, a model of intestinal tumorigenesis.

Results and discussion

To determine whether aberrant *de novo* methylation is a stochastic or a targeted process we compared DNA methylation of colon tumors, representing a mono-/oligoclonal cell population with normal colon epithelial cells of $Apc^{min/+}$ mice that had been induced to express the Dnmt3b transgene by exposure to doxycycline (dox). We analyzed DNA methylation of 23 candidate genes that are either frequently methylated in human colon cancer or represent classical tumor suppressor genes (6). Three imprinted loci were included as control regions and embryonic stem (ES) cells deficient for Dnmt1 (ES c/c), ES cells deficient for Dnmt1, Dnmt3a and Dnmt3b (ES tko) and wild-type ES cells (ES wt) were included as control samples. For methylation analysis we used gene specific amplification of bisulfite treated DNA followed by reverse transcription and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis (7). As demonstrated in Figure 1A, an almost identical methylation pattern was seen in all tumor samples and non-tumor cell samples from Dnmt3b-induced mice. A significant increase in DNA methylation (defined as methylation increase ≥ 0.1 and $p \leq 0.05$, t-test) was detected in 11/26 candidate regions (including imprinted loci) in both tumor samples and non-tumor samples and pairwise analysis of

methylation data confirmed a strong correlation of *de novo* methylation patterns amongst all Dnmt3b expressing samples (correlation between induced sample pairs 0.76-0.97, mean r = 0.90; Figure 1B). The observation that clonal and non-clonal cell populations showed the same *de novo* methylation pattern across multiple samples strongly suggests that *de novo* methylation by Dnmt3b is not random but targets specific genetic loci. The data shown in Figure 1A also support the hypothesis, that *de novo* methylation of targets such as *Abpa2* (also known as *Mint2*), *Hic1*, *Gata4*, *Gata5*, *Cacna1g*, *Neurog1* that are considered to be hallmarks of methylated genes in cancer (6), can occur prior to tumor formation in normal cells (also see Supplemental Table 1).

To further compare DNA methylation in the mouse model with human colon cancer we analyzed additional candidate genes that were previously reported as methylated or unmethylated in human colon cancer (6). To confirm that *de novo* methylation of cancer specific targets can occur in normal cells the expanded candidate gene analysis was conducted using *Apc* wild-type mice as opposed to the previously studied mutant $Apc^{min/+}$ mice. Indeed, the Dnmt3b induced DNA methylation pattern in *Apc* wild-type mice fully reproduced the DNA methylation pattern previously detected in $Apc^{min/+}$ mice (Figure 1C). This shows that the *de novo* methylation patterns obtained in Figure 1A do not require the presence of a mutant *Apc* allele. Further, the expanded candidate gene analysis (Figure 1C) demonstrates that human cancer related methylation targets were consistently methylated in mice with Dnmt3b expression: The target list of Figure 1C includes 31 genes that are frequently methylated in human colon cancer (6). 26 of these 31 human cancer methylation targets were also significantly methylated when Dnmt3b was induced in the colonic mucosa of wild-type mice (absolute increase in DNA methylation ≥ 0.1 and $p \leq 0.05$, t-test), (Supplemental Table 1). Conversely 8/8 candidate genes reportedly unmethylated in human colon cancer also remained unmethylated in the Dnmt3b mouse model (*de novo* methylation ≤ 0.1 and $p \geq 0.05$, Supplemental Table 1).

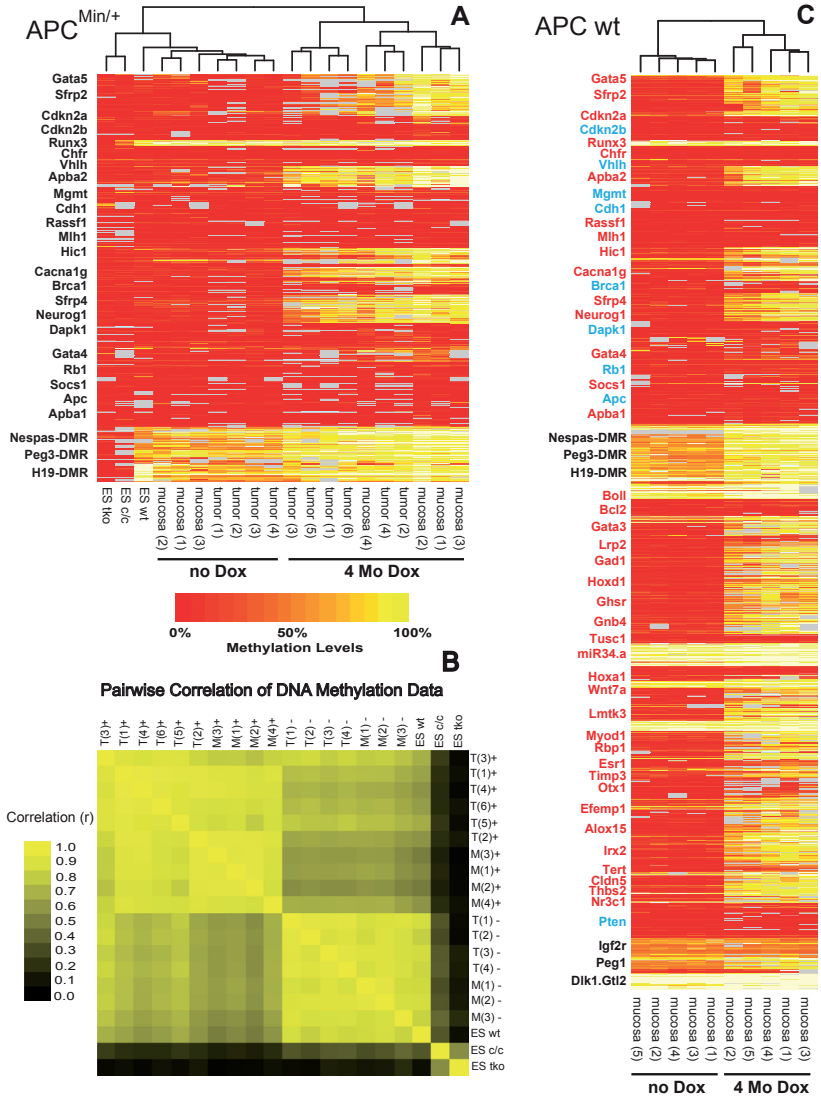


Figure 1. DNA methylation analysis of colon tumors and colon epithelial cells +/- Dnmt3b transgene expression.

4 Mo dox = Dnmt3b induction, no dox = no Dnmt3b induction. ES wt = wild-type ES cells , ES c/c = ES cells deficient for Dnmt1, ES tko = ES cells deficient for Dnmt1, 3a and 3b, grey color = no data. Heat map data were generated by MassARRAY analysis and subjected to sample based unsupervised hierarchical clustering. (A): Tumors and mucosa samples with Dnmt3b induction showed reproducible *de novo* DNA methylation of specific genomic loci. Imprinted regions (*Nespas*, *Peg3* and *H19 DMR*) were partially methylated, ES tko and ES c/c cells showed loss of DNA methylation. (B): Pairwise correlation of methylation data, confirmed similar *de novo* methylation in all samples with Dnmt3b induction (r = 0.76-0.97, mean r = 0.90). (C): Dnmt3b mediated DNA methylation in colon mucosa of *Apc* wild-type mice closely resembles *de novo* methylation reported for human colon cancer (Supplemental Table 1), suggesting that Dnmt3b can induce cancer specific *de novo* methylation in normal cells (red = methylated genes, blue = unmethylated genes, black = control/imprinted regions).

To complement this candidate gene approach and to further explore the similarities between *de novo* methylation in our transgenic model and aberrant DNA methylation of colon cancer we next conducted genome wide analysis of Dnmt3b mediated *de novo* methylation using reduced representation bisulfite sequencing (RRBS) (8). Using three colon mucosa samples with three months of Dnmt3b induction and three samples from age matched control mice, we scored Dnmt3b mediated *de novo* methylation in 13361 promoter regions. In analogy to the previous experiments (Figure 1) we observed almost identical *de novo* methylation patterns amongst all three induced samples (pairwise correlation between induced sample pairs: $r > 0.95$, Supplemental Figure 2). This further supports the concept that Dnmt3b directly targets certain promoter regions. Interestingly, a discrete subset of genes (11.7 %, 1564/13361) showed a strong methylation gain upon Dnmt3b expression (absolute methylation gain > 0.5, FDR p value < 0.01, 1 = complete methylation), whereas the majority of promoter regions remained unmethylated in both test and control DNA (Figure 2A, B). This suggests that a subgroup of genes in the genome is inherently "methylation sensitive". Importantly, in agreement with the results of the candidate gene analysis, we again found a strong concordance when comparing the murine Dnmt3b mediated *de novo* methylation data with published data from human colon cancer (6): For the human cancer/mouse comparison we selected human genes from the study of Widschwendter et al. (6) that were classified as either cancer related methylation targets (absolute methylation gain > 0.1 in tumors, PMR gain in tumors > 10) or classified as "non targets" (tumor methylation gain < 0.1, genes with baseline methylation in human control tissue > 100% (PMR < 100) were excluded). Mouse RRBS data were available for 44 human colon cancer methylation targets and for 71 non targets (115 total). As shown in Supplemental Tables 2 and 3, 84% (37/44) of genes methylated in human colon cancer were also methylated by Dnmt3b (methylation gain > 0.1 and FDR $p < 0.01$) and conversely 92% (65/71) of genes not methylated in human colon cancer were also not methylated by Dnmt3b (methylation gain < 0.1). This supports the concept that Dnmt3b targets the very same set of genes that are frequently methylated in human cancer. In addition, the similarity between murine and human data suggests conservation of DNA methylation-sensitivity across species.

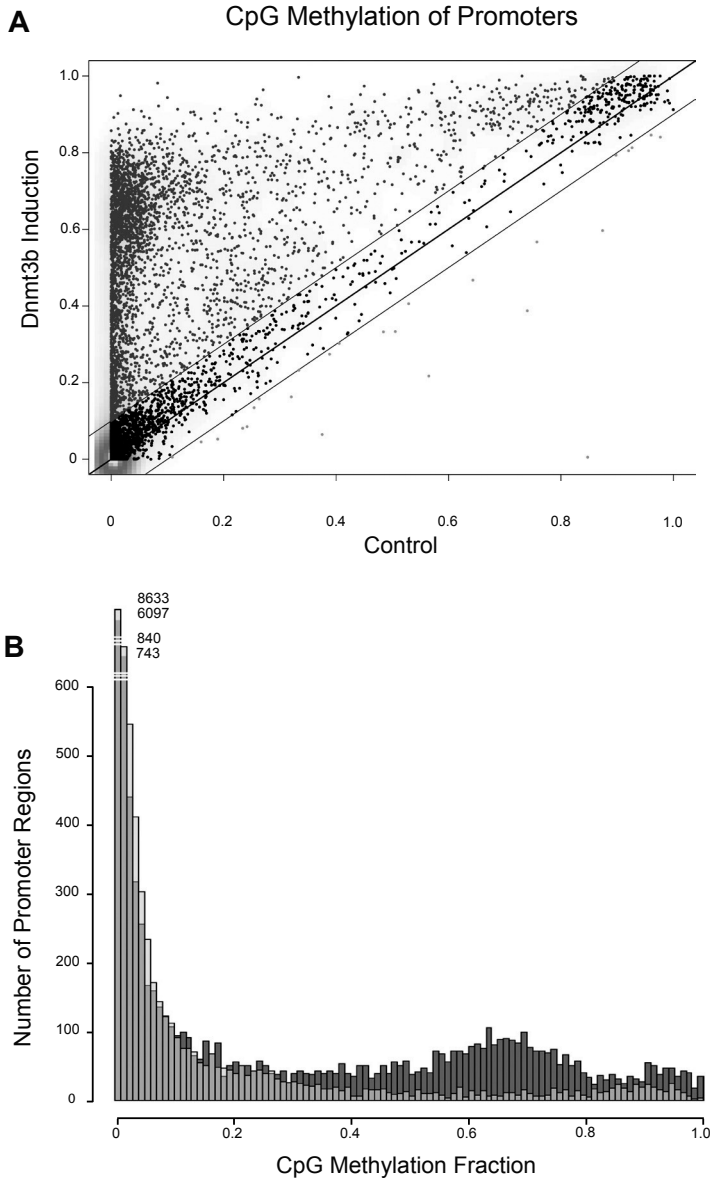


Figure 2. Reduced representation bisulfite sequencing (RRBS) analysis of colon epithelial cells +/- Dnmt3b transgene expression *in vivo*.

RRBS data from colon epithelial cells +/- Dnmt3b transgene expression (n = 3 each) were used to calculate *de novo* methylation in 13361 annotated promoter regions. (A) The scatter plot demonstrates Dnmt3b mediated hypermethylation of a distinct subpopulation of promoter regions (11.7% of promoters showed a methylation gain > 0.5 in Dnmt3b expressing tissue, lines parallel to the diagonal indicate the position of 0.1 methylation gain or loss). (B) The histogram further illustrates the appearance of a discrete subpopulation of hypermethylated promoters in Dnmt3b expressing samples (blue), whereas the control samples (brown) contained only few promoters regions with a methylation score > 0.5.

Previous studies had shown a strong correlation between genes methylated in human cancer and genes targeted by the polycomb repressive complex 2 (PRC2) in embryonic stem cells (6, 9). We therefore used published data on mouse ES cells (10) to compare the PRC2 occupancy of genes targeted by Dnmt3b (methylation gain > 0.5) with the PRC2 occupancy of genes not targeted by Dnmt3b (methylation gain < 0.1). In analogy to previous reports we defined genes associated with either Suz12, Eed or H3K27 methylation in mouse embryonic stem cells as PRC2 targets. Interestingly the ES cell PRC2 occupancy was 71 % (721/1015) for genes targeted by Dnmt3b and only 13% (716/5564) for genes not targeted by Dnmt3b (Supplemental Tables 4 and 5). This strong enrichment of PRC2 targets (in ES cells) amongst genes methylated by Dnmt3b is in good agreement with the reported enrichment of PRC2 targets in genes methylated in human cancer (6) and further supports the concept that Dnmt3b targets the same genes that are methylated in cancer. Taken together, our results indicate that colon epithelial cell methylation in the Dnmt3b model predicts DNA methylation of human colon cancer with high confidence.

After having established that Dnmt3b can induce a cancer specific signature of *de novo* methylation in healthy tissue we next analyzed the stability of such alterations. Such information would help resolve the question whether altered DNA methylation is a potential initiating cause of disease rather than only an effect of disease. For this we induced *de novo* methylation in the colon of transgenic mice by adding dox to the drinking water for five months and analyzed maintenance of these methylation marks after four months of dox withdrawal and silencing of transgene expression. The epithelial cell layer of the colon fully renews every five to seven days (11) and is therefore ideal to test maintenance of epimutations *in vivo*. As demonstrated in Figure 3A, Dnmt3b expression in transgenic mice was rapidly activated by dox in the drinking water and quickly returned to baseline within one week of dox withdrawal. Importantly, as shown in Figure 3B, *de novo* methylation of individual genes was almost identical in mice on doxycyline (On) when compared to mice that were maintained in the absence of dox for four months (On/Off). This supports the hypothesis that aberrant *de novo* DNA methylation is maintained in regenerating healthy tissue even in the absence of the initiating cause. To test whether this was possibly caused by *de novo* methylation of the intestinal stem cell compartment we used RNA FISH analysis to measure Dnmt3b transgene expression in colonic epithelial cells that express the intestinal stem cell marker Lgr5 (11, 12), (Supplemental Figure 1): Indeed, Dnmt3b expression in Lgr5 positive cells of a dox induced transgenic mouse was approximately two fold higher than in a littermate control (control: 0.0037 ± 0.00063 Dnmt3b particles/μm^3, Dnmt3b

overexpressor: 0.0076 ± 0.00124 Dnmt3b particles/μm^3, $p \leq 0.001$), supporting the concept that transgene mediated *de novo* methylation of intestinal stem cells contributed to the irreversible epigenetic alteration of the colonic mucosa.

Several previous studies have shown a strong correlation between cell proliferation and the acquisition of aberrant *de novo* DNA methylation (8, 13), suggesting that infrequently dividing cells, such as somatic stem cells, are possibly less sensitive to Dnmt3b mediated *de novo* methylation than more rapidly dividing cells. To address this question we analyzed Dnmt3b mediated *de novo* methylation of proliferating and growth arrested (mitomycin C treated or irradiated) mouse embryonic fibroblasts after 14 days of dox induced transgene expression. The data in Figure 3C failed to show a significant difference between *de novo* methylation of actively dividing cells and growth arrested cells ($p = 0.23$ for mitomycin C treated and $p = 0.41$ for gamma-irradiated cells, when comparing with proliferating cells, paired t-test). This further supports the concept that Dnmt3b activation in the slow cycling stem cell compartment can contribute to permanent epigenetic alterations in regenerating tissues.

In summary, our results show that Dnmt3b induction in the mouse colon generates *de novo* methylation that closely resembles aberrant DNA methylation of human colon cancer. We demonstrate that “cancer specific” DNA methylation can be a targeted process and may not be the result of stochastic methylation followed by clonal selection in the incipient tumor cell population. Importantly, our results show that *de novo* methylation can occur in normal cells, suggesting that regional hypermethylation may contribute to the earliest stages of tumor formation. Finally, we demonstrate that transient induction of aberrant DNA methylation, most likely in intestinal stem cells, causes permanent epigenetic alterations of the intestinal mucosa. This supports the concept that transient stressors can cause permanent epigenetic changes in somatic stem cells that accumulate over the lifetime of an organism in analogy to DNA mutations.

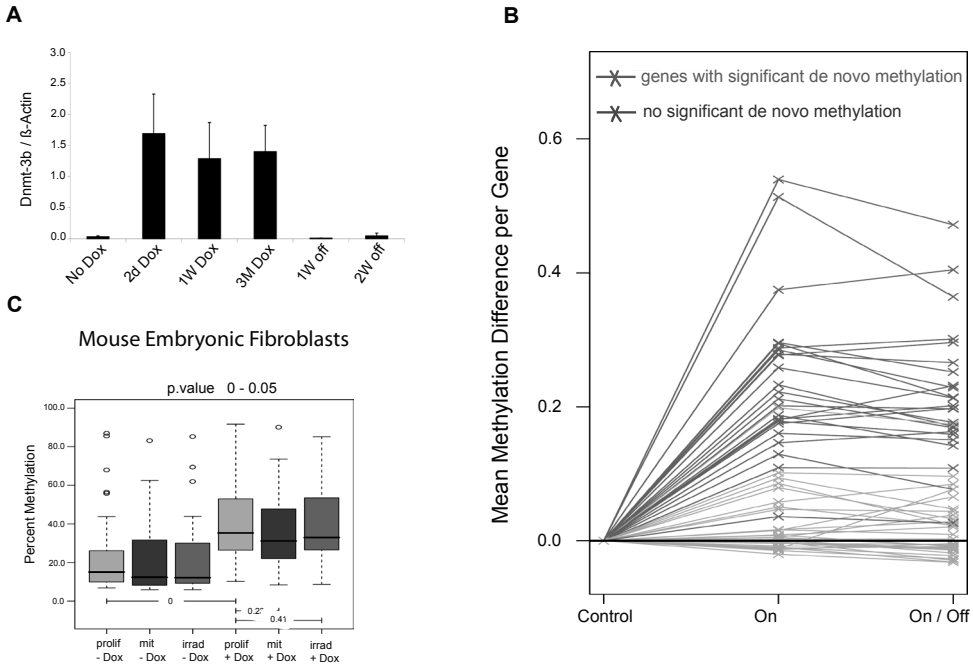


Figure 3. Transient expression of Dnmt3b *in vivo* causes permanent epigenetic alterations of colonic mucosa.

(A) Dnmt3b transgene expression in colon epithelial cells *in vivo* was activated within 2 days of dox administration and returned to baseline within 1 week of dox withdrawal (quantitative PCR analysis, mean +/- stdev, n = 2 each, off = dox withdrawal). (B) Dnmt3b was induced in transgenic mice for five months (On, n = 6) and a subset was followed up for an additional period of four months after dox withdrawal (On/Off, n = 6, control mice = age matched to On/Off group, n = 4). Almost all genes that undergo significant *de novo* methylation ($p < 0.01$, t-test) were equally methylated in the "On" group and "On/Off" group (C) Dnmt3b mediated *de novo* methylation was analyzed in dividing (prolif) and non-dividing mouse embryonic fibroblasts (mitomycin C treated = mit or γ-irradiated = irrad) after 14 days of dox induced Dnmt3b transgene expression. The box plot shows combined methylation data of genes with significant *de novo* methylation in proliferating cells ($p < 0.05$, n = 25, paired t-test), line = median, boundaries = first and third quartile, whiskers extend to 1.5 x the interquartile range and dots are outliers from that range. Significant Dnmt3b mediated *de novo* methylation was detectable in both proliferating and growth arrested cells, suggesting that cell division is not required for this process.

Methods

Transgenic mice

All mice used in this study and the genotyping protocol were described previously (5). Transgene induction was achieved by administering doxycycline (dox) in the drinking water (0.5g/liter). All animal studies were reviewed and approved by the Comittee on Animal Care at MIT, under protocol number 1107-088-10 (Department of Comparative Medicine, MIT, 77 Mass. Ave, Cambridge, MA 02139).

Tissue harvesting

Colon tumors from *Apc*$^{min/+}$ mice were harvested under the dissecting microscope. Colon epithelial cells from tumor free colon samples of *Apc*$^{min/+}$ mice and *Apc* wild-type mice were harvested as described previously (14).

Mouse embryonic fibroblasts

Double homozygous embryos (Dnmt3b transgene +/+ and Rosa-rtTA +/+) were harvested 12.5 days post conception. Growth arrest of cells was achieved by irradiation with 24 Gy or treatment with Mitomycin C respectively and verified using BrdU staining. After 14 days of dox treatment cells were harvested for DNA methylation analysis. For details see supplemental methods.

Lgr5 and Dnmt3b mRNA FISH

Frozen sections of colon samples were hybridized with labeled probes targeting mouse Lgr5 and Dnmt3b mRNA (12). Individual mRNA molecules were located using the semi-automated method described in (12). Lgr5 positive cells were manually identified to calculate Dmnt3b mRNA density in intestinal stem cells. For details and probe sequences see supplemental methods.

cDNA and quantitative PCR

cDNA production and quantitative PCR was conducted as described previously (5). For details and primer sequences see supplemental methods.

DNA methylation analysis (MassARRAY platform)

Genomic DNA was sodium bisulfite converted and PCR amplified. When feasible, amplicons were designed to cover CpG islands in the same region as the 5`UTR. MassCLEAVE biochemistry, Mass spectra acquisition and methylation ratio analysis was performed as previously described (7). A list of primers is provided in the Supplemental Methods.

DNA methylation analysis (reduced representation bisulfite sequencing)

RRBS methylation analysis was conducted as described previously (8). Briefly, mouse genomic DNA was digested with MspI and resulting fragments between 40-220 bp were subjected to sequencing (Illumina genome Analyzer II).(8) Sequence reads that mapped to a promoter region (defined as 2 kb up- and downstream of the transcription start site)

were then used to calculate promoter methylation. Raw data and processed RRBS data were deposited at the GEO database, accession number GSE26758, link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hrepveuqoqcgqdu&acc=GSE26758. For further details see supplemental methods.

Statistics

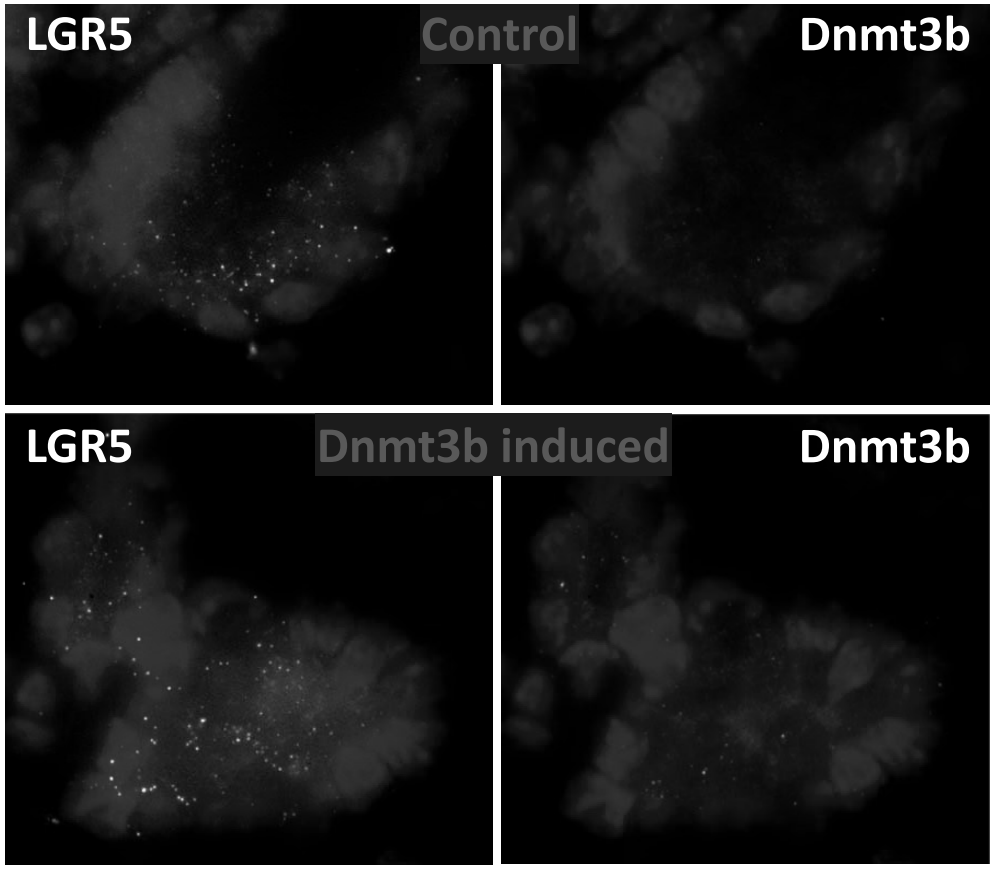
All correlations were performed using the Pearson correlation. Significant difference in methylation fraction for the RRBS data was assayed with the moderated t-test (two-tailed), corrected for False Discovery Rate, with significance defined as FDR < 0.01. Significant difference for colon MassARRAY methylation data was assayed by t-test (two-tailed) and for cell culture MassARRAY data by paired t-test (two-tailed). For RNA FISH we obtained errors by bootstrap resampling and for p-values we computed the fraction of events that the null hypothesis (no difference) would be satisfied given a random resampling of the dataset. In all cases p values < 0.05 were considered significant.

Acknowledgements

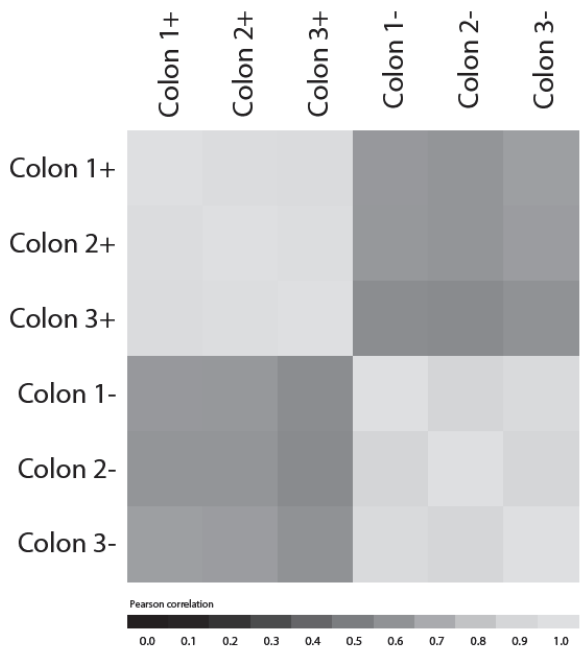
We thank Jessica Dausman, Ruth Flannery and Qing Gao for help with maintenance of the mouse colony and Tom DiCesare for support in preparation of figures. Eveline Steine is supported by Pr. Bernhard Cultuurfonds, Banning-de Jong Fonds, Heinz Linhart is supported by a fellowship from the Fritz-Thyssen Stiftung. Research described in this article was supported by grants to RJ by Philip Morris International and by NIH grant RO1-CA087869. A.R. was supported by a Burroughs-Wellcome Fund Career Award at the Scientific Interface. A.R. was supported by a Burroughs-Wellcome Fund Career Award at the Scientific Interface, A.v.O. is supported by a National Institutes of Health (NIH) Director's Pioneer award and an award from the National Cancer Institute (U54CA143874).

References

1. Jones, P.A., and Baylin, S.B. 2007. The epigenomics of cancer. *Cell* 128:683-692.
2. Feinberg, A.P. 2007. Phenotypic plasticity and the epigenetics of human disease. *Nature* 447:433-440.
3. Opavsky, R., Wang, S.H., Trikha, P., Raval, A., Huang, Y., Wu, Y.Z., Rodriguez, B., Keller, B., Liyanarachchi, S., Wei, G., et al. 2007. CpG island methylation in a mouse model of lymphoma is driven by the genetic configuration of tumor cells. *PLoS Genet* 3:1757-1769.
4. Nosho, K., Shima, K., Irahara, N., Kure, S., Baba, Y., Kirkner, G.J., Chen, L., Gokhale, S., Hazra, A., Spiegelman, D., et al. 2009. DNMT3B expression might contribute to CpG island methylator phenotype in colorectal cancer. *Clin Cancer Res* 15:3663-3671.
5. Linhart, H.G., Lin, H., Yamada, Y., Moran, E., Steine, E.J., Gokhale, S., Lo, G., Cantu, E., Ehrich, M., He, T., et al. 2007. Dnmt3b promotes tumorigenesis *in vivo* by gene-specific *de novo* methylation and transcriptional silencing. *Genes Dev* 21:3110-3122.
6. Widschwendter, M., Fiegl, H., Egle, D., Mueller-Holzner, E., Spizzo, G., Marth, C., Weisenberger, D.J., Campan, M., Young, J., Jacobs, I., et al. 2007. Epigenetic stem cell signature in cancer. *Nat Genet* 39:157-158.
7. Ehrich, M., Nelson, M.R., Stanssens, P., Zabeau, M., Liloglou, T., Xinarianos, G., Cantor, C.R., Field, J.K., and van den Boom, D. 2005. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc Natl Acad Sci U S A* 102:15785-15790.
8. Meissner, A., Mikkelsen, T.S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B.E., Nusbaum, C., Jaffe, D.B., et al. 2008. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 454:766-770.
9. Schlesinger, Y., Straussman, R., Keshet, I., Farkash, S., Hecht, M., Zimmerman, J., Eden, E., Yakhini, Z., Ben-Shushan, E., Reubinoff, B.E., et al. 2007. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for *de novo* methylation in cancer. *Nat Genet* 39:232-236.
10. Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., et al. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 441:349-353.
11. Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., et al. 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449:1003-1007.
12. Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A., and Tyagi, S. 2008. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat Methods* 5:877-879.
13. Velicescu, M., Weisenberger, D.J., Gonzales, F.A., Tsai, Y.C., Nguyen, C.T., and Jones, P.A. 2002. Cell division is required for *de novo* methylation of CpG islands in bladder cancer cells. *Cancer Res* 62:2378-2384.
14. Fujimoto, K., Beauchamp, R.D., and Whitehead, R.H. 2002. Identification and isolation of candidate human colonic clonogenic cells based on cell surface integrin expression. *Gastroenterology* 123:1941-1948.



Supplemental Figure 1. Illustration of RNA FISH analysis.
Illustration of RNA FISH analysis of Lgr5 (left panel) and Dmnt3b expression (right panel) in colon crypts isolated from a wild-type (top row) and Dmnt3b induced (bottom row) mouse. Particulate signals represent individual LGR5 (left) or Dmnt3b (right) molecules. Nuclei stained with DAPI (purple). Lgr5 was used to identify crypt stem cells and Dmnt3b expression was quantified in Lgr5 positive cells. Dnmt3b expression in crypt stem cells of the dox induced transgenic mouse was on average ~ 2 fold higher than in the littermate control mouse.



Supplemental Figure 2. **Methylation fraction of methylation fractions: pairwise correlations.**
Pairwise correlation of promoter methylation in colon epithelial cells +/-Dnmt3b transgene expression (+ = Dnmt3b transgene induction, - = no Dnmt3b induction). Promoter methylation fractions were calculated using reduced representation bisulfite sequencing data. The strong correlation of de novo methylation amongst samples with Dnmt3b induction supports the concept that Dnmt3b directly targets certain promoter regions.

	mouse data		
	Δ meth.	p value	human (references)
Alox15	0.40	< 0.001	6
Apba2	0.58	< 0.001	2
Boll	0.18	< 0.001	3
Cacna1g	0.42	< 0.001	1
Cdkn2a	0.32	< 0.001	5
Efemp1	0.35	< 0.001	3
Gad1	0.50	< 0.001	1
Gata3	0.31	< 0.001	1
Gata4	0.12	< 0.001	1, 2
Gata5	0.51	< 0.001	1
Gnb4	0.47	< 0.001	3
H19.DMR	0.24	< 0.001	9
Hic1	0.39	< 0.001	1
Hoxa1	0.15	< 0.001	1
Hoxd1	0.48	< 0.001	3
Lrp2	0.50	< 0.001	4
Myod1	0.39	< 0.001	1, 4
Neurog1	0.50	< 0.001	1
Nr3c1	0.32	< 0.001	1, 2
Otx1	0.19	< 0.001	2
Rbp1	0.69	< 0.001	1
Sfrp2	0.48	< 0.001	1
Sfrp4	0.47	< 0.001	1
Tert	0.21	< 0.001	1, 4
Thbs2	0.49	< 0.001	4
Timp3	0.31	< 0.001	1, 4

	mouse data		
	Δ meth.	p value	human (references)*
Apc	0.00	0.622	1
Bcl2	0.03	< 0.001	1, 2
Brca1	0.00	0.739	7
Cdh1	0.01	0.020	1
Cdkn2b	0.02	< 0.001	1
Chfr	0.00	0.505	1
Dapk1	0.03	0.006	1
Mgmt	0.03	< 0.001	1, 8
Mlh1	0.01	< 0.001	1
Pten	0.00	0.801	1
Rb1	0.03	< 0.001	1
Socs1	0.01	0.127	1
Vhlh	0.01	0.173	1

Supplemental Table 1. **Comparison of DNA methylation in colon epithelial cells of Dnmt3b overexpressing mice with DNA methylation of human colon cancer.**
The mouse data show the absolute increase in DNA methylation caused by Dnmt3b overexpression (from 0 to 1) and the corresponding p value (comparison no dox versus plus dox, n = 5 each, t-test). Genes with an absolute increase ≥ 0.1 and p ≤ 0.05 were defined as methylated and are colored in red, unmethylated genes are colored in blue. The column with human data shows the respective literature reference, red fields indicate genes that were reported as methylated in human colon cancer and blue fields indicate genes that were reported as unmethylated. In the case of *Mgmt*, labeled yellow, one reference indicates methylation and one reference indicates no methylation. To facilitate the comparison, genes with more than one promoter in human or mouse were not included in the analysis.

References Supplemental Table 1:

1. Widschwendter, M., Fiegl, H., Egle, D., Mueller-Holzner, E., Spizzo, G., Marth, C., Weisenberger, D. J., Campan, M., Young, J., Jacobs, I., and Laird, P. W. (2007) *Nat Genet* **39**, 157-158
2. Irizarry, R. A., Ladd-Acosta, C., Wen, B., Wu, Z., Montano, C., Onyango, P., Cui, H., Gabo, K., Rongione, M., Webster, M., Ji, H., Potash, J. B., Sabunciyan, S., and Feinberg, A. P. (2009) *Nat Genet* **41**, 178-186
3. Schuebel, K. E., Chen, W., Cope, L., Glockner, S. C., Suzuki, H., Yi, J. M., Chan, T. A., Van Neste, L., Van Criekinge, W., van den Bosch, S., van Engeland, M., Ting, A. H., Jair, K., Yu, W., Toyota, M., Imai, K., Ahuja, N., Herman, J. G., and Baylin, S. B. (2007) *PLoS Genet* **3**, 1709-1723
4. Shen, L., Toyota, M., Kondo, Y., Lin, E., Zhang, L., Guo, Y., Hernandez, N. S., Chen, X., Ahmed, S., Konishi, K., Hamilton, S. R., and Issa, J. P. (2007) *Proc Natl Acad Sci U S A* **104**, 18654-18659
5. Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J. G., Baylin, S. B., and Issa, J. P. (1999) *Proc Natl Acad Sci U S A* **96**, 8681-8686
6. Zuo, X., Shen, L., Issa, J. P., Moy, O., Morris, J. S., Lippman, S. M., and Shureiqi, I. (2008) *FASEB J* **22**, 1981-1992
7. Xu, X. L., Yu, J., Zhang, H. Y., Sun, M. H., Gu, J., Du, X., Shi, D. R., Wang, P., Yang, Z. H., and Zhu, J. D. (2004) *World J Gastroenterol* **10**, 3441-3454
8. Kondo, Y., Shen, L., Cheng, A. S., Ahmed, S., Boumber, Y., Charo, C., Yamochi, T., Urano, T., Furukawa, K., Kwabi-Addo, B., Gold, D. L., Sekido, Y., Huang, T. H., and Issa, J. P. (2008) *Nat Genet* **40**, 741-750

Gene Symbol	Dnmt3b	Colon Cancer (1)	Gene Symbol	Dnmt3b	Colon Cancer (1)
Gata5	0.69	479	HoxA1	0.00	52
Sfrp5	0.75	443	Chfr	0.02	51
Igf2	0.56	366	Sez6l	0.67	50
Twist1	0.66	284	Mt3	0.08	49
Ebf3	0.51	273	Timp3	0.61	49
Hic1	0.61	266	Neurog1	0.61	44
Sfrp2	0.65	179	Rbp1	0.89	44
Sfrp1	0.60	148	Cdkn1c	0.29	43
Neurod2	0.70	147	Epm2aip1	0.01	42
Gata4	0.45	94	Crabp1	0.63	38
Nr3c1	0.16	93	Bdnf	0.66	36
Gata3	0.22	89	Cdh13	0.53	36
Tert	0.34	89	Socs1	0.17	30
Itga4	0.46	84	Gabra2	0.43	29
Kl	0.67	84	Esr2	0.23	24
Cacna1g	0.51	79	Pgr	0.59	23
Sfrp4	0.60	70	Cyp27B1	0.45	23
Bcl2	0.01	64	Mlh1	0.01	23
Tmeff2	0.67	60	Mgmt	0.19	19
MyoD1	0.50	57	Drd2	0.61	14
Gad1	0.63	54	Gstp1	0.01	13
Gdnf	0.55	52	Thbs1	0.26	10

Supplemental Table 2. **The table shows a comparison of DNA methylation data from colon epithelial cells of Dnmt3b expressing mice as measured by RRBS with published DNA methylation data of human colon cancer (1).**

The mouse RRBS data are shown in the column "Dnmt3b" with numbers indicating the absolute methylation gain induced by Dnmt3b expression (0= no methylation, 1= complete methylation). Human colon cancer data are shown in the column "colon cancer" with numbers indicating percent of methylated reference (PMR, 0= no methylation, 100 = methylation equals methylated reference). The supplemental table S1 of Widschwendter et al. (1) lists 77 genes with a significant methylation increase in human colon cancer. For comparison with Dnmt3b data human genes with <0.1 absolute gain in human colon cancer (PMR gain in tumors < 10) or baseline methylation > 100% (PMR>100) were excluded. Based on these criteria 62 genes were eligible for the Dnmt3b/colon cancer comparison. Dnmt3b RRBS data were available for 44/62 eligible human genes. 37/44 (84%) of these genes were methylated both by Dnmt3b and in human colon cancer (red color), only 7/44 (16 %) were methylated in colon cancer and not methylated by Dnmt3b model.

References Supplemental Table 2:

1. Widschwendter, M., Fiegl, H., Egle, D., Mueller-Holzner, E., Spizzo, G., Marth, C., Weisenberger, D.J., Campan, M., Young, J., Jacobs, I., et al. 2007. Epigenetic stem cell signature in cancer. *Nat Genet* 39:157-158.

Gene Symbol	Dnmt3b	Colon Cancer (1)	Gene Symbol	Dnmt3b	Colon Cancer (1)
Smad3	0.001	1.970	Prkar1a	0.003	0.000
Apc	0.003	1.850	Uqcrh	0.000	0.000
Jup	0.000	0.970	Cdk2ap1	0.002	0.000
Rpa3	0.000	0.530	Axin1	0.000	0.000
Grin2b	0.610	0.490	Rb1	0.010	0.000
Smad6	0.050	0.340	Tgfbr2	0.002	0.000
Rpa2	0.000	0.320	App	0.001	0.000
Stk11	0.013	0.080	Smad2	0.002	0.000
Xpa	0.018	0.020	Faf1	0.001	0.000
Atm	0.009	0.020	Tnfrsf10b	0.023	0.000
Ercc4	0.001	0.010	Smad9	0.170	0.000
Ctnnb1	0.043	0.010	Xpc	0.000	0.000
Ercc2	0.002	0.000	Rad23A	0.000	0.000
Msh2	0.005	0.000	Xab2	0.010	0.000
Dph1	0.066	0.000	Atr	0.001	-0.010
Stat1	0.003	0.000	Pttg1	0.016	-0.010
Ctsd	0.002	0.000	Ercc6	0.002	-0.020
Cxadr	0.001	0.000	Hsd17b4	0.005	-0.030
Pparg	0.000	0.000	Mbd2	0.000	-0.040
Clic4	0.005	0.000	Vdr	0.002	-0.050
Ncl	0.000	0.000	Ercc5	0.000	-0.070
Ung	0.002	0.000	Ldlr	0.000	-0.090
Mbd4	0.050	0.000	Ccdn1	0.010	-1.030
Ogg1	0.003	0.000	Psen1	0.000	-0.230
Apex1	0.011	0.000	Psat1	0.030	-0.270
Xrcc1	0.060	0.000	Cdkn2b	0.079	-1.130
Parp1	0.005	0.000	Dapk1	0.048	-1.400
Parp2	0.005	0.000	Cdh1	0.004	-1.990
Ercc8	0.003	0.000	Msh4	0.110	-2.340
Ddb1	0.013	0.000	Erbb2	0.005	-3.310
Brca2	0.011	0.000	Ptgs2	0.440	-3.510
Pold1	0.020	0.000	Onecut2	0.620	-4.490
Pten	0.000	0.000	Dnajc13	0.001	-5.870
Arpc1b	0.001	0.000	Mthfr	0.267	-6.490
Vhl	0.001	0.000	Sash1	0.001	-10.040
Tgfvbr1	0.002	0.000			

Supplemental Table 3. **Genes not methylated in human colon cancer are also largely not methylated by Dnmt3b.**
The table shows a comparison of DNA methylation data from colon epithelial cells of Dnmt3b expressing mice as measured by RRBS with published DNA methylation data of human colon cancer (1). The mouse RRBS data are shown in the column "Dnmt3b" with numbers indicating the absolute methylation gain induced by Dnmt3b expression (0= no methylation, 1= complete methylation). Human colon cancer data are shown in the column "colon cancer" with numbers indicating percent of methylated reference (PMR, 0= no methylation, 100 = methylation equals methylated reference). The supplemental table S1 of Widschwendter et al. (1) lists 100 genes with no significant methylation increase in human colon cancer. For comparison with Dnmt3b data human genes with baseline

methylation > 100% (PMR>100) were excluded. Based on these criteria 94 genes were eligible for the Dnmt3b/colon cancer comparison. Dnmt3b RRBS data were available for 71/94 eligible human genes. 65/71 (92 %) of these genes were not methylated in colon cancer and also not methylated by Dnmt3b (blue color). Only 6/71 (8 %) genes were methylated by Dnmt3b (red) and not methylated in colon cancer.

Reference Supplemental Table 3:

1. Widschwendter, M., Fiegl, H., Egle, D., Mueller-Holzner, E., Spizzo, G., Marth, C., Weisenberger, D.J., Campan, M., Young, J., Jacobs, I., et al. 2007. Epigenetic stem cell signature in cancer. *Nat Genet* 39:157-158.

ADDENDUM CHAPTER 3

LOCI METHYLATED BY ECTOPIC EXPRESSION OF DNMT3B ARE ENRICHED FOR H3K27ME3 AND VARY BETWEEN TISSUES

Eveline J. Steine, M. Inmaculada Barrasa, Dongdong Fu, Menno P. Creygthon, Heinz G. Linhart and Rudolf Jaenisch

Summary

In previous studies (described in Chapter 3) we observed that ectopic expression of Dnmt3b in the mouse colon induced a specific DNA methylation pattern, and that genes that became methylated are also methylated in human colon cancer. Using the same mouse model, here we describe that loci methylated upon ectopic Dnmt3b expression differ across small intestine and liver. Most loci that became methylated were associated with trimethylated lysine 27 of histone 3 (H3K27me3, a histone mark associated with gene silencing). Most genes with CpG promoters were found to be already silenced prior DNA methylation. In addition, we found that a small subset of the genes which carried H3K27me3 prior DNA methylation loses H3K27me3 when those genes become DNA methylated. The biological relevance of this latter finding still needs to be resolved. The present study indicates that it is warranted to perform additional and more detailed studies on the mechanism and consequences of Dnmt3b-mediated DNA methylation.

Introduction

Silencing of tumor suppressor genes by promoter methylation is frequently observed in cancer [1,2]. In agreement with this, we have demonstrated that over-expression of the *de novo* methyltransferase Dnmt3b in the *Apc*$^{min/+}$ mouse model of colon cancer resulted in an increased number and size of intestinal adenomas [3] (Chapter 2) . When we analyzed the DNA methylation pattern in tumors from these mice, we found that DNA methylation induced by Dnmt3b occurred at specific loci, rather than in a stochastic manner. Interestingly, when we examined DNA methylation in the phenotypically normal mucosa of the *Apc*$^{min/+}$ mouse, we found that DNA methylation occurred at the same loci in both normal and tumor tissue. The DNA methylation pattern found is not simply attributable to the genetic background, as we observed that the same loci are methylated in cells of the intestinal mucosa when we induced Dnmt3b ectopically in a wild type *Apc* mouse. The human homologs of these loci are also hypermethylated in human colon cancer, indicating that the methylation sensitive loci in the intestine are conserved across species [4] (Chapter 3). Conversely, ectopic Dnmt3b expression in the spleen does not induce methylation at the colon epithelial specific methylation target *Sfrp2* [3] (Chapter 2). Taken together, DNA methylation patterns are a consequence of targeting of specific loci, which are conserved across species. The unmethylated *Sfrp2* promoter in the spleen indicates that DNA methylation patterns might be tissue specific, at least for a number of genes. Possibly, the chromatin state of a given cell type might dictate which loci can be *de novo* methylated.

Promoter DNA methylation usually correlates with gene repression, and this also correlates for some histone modifications, such as trimethylation of lysine 27 of histone 3 (H3K27me3). This histone modification is catalyzed by polycomb repressive complex 2 (PRC2), consisting of the core components Eed, Ezh2, Suz12 [5]. Previous studies have shown that genes methylated in human colon cancer are marked by PRC2 proteins in human ES cells [6-8]. These reports suggested that genes that carry PRC2 complex proteins in ES cells are more susceptible to DNA methylation in cancer. Moreover, in our previous study, we observed that DNA hypermethylated loci in the colon are enriched for PRC2 complex targets in mouse ES cells [4] (Chapter 3). It should be noted, however, that not all DNA methylated loci carried a PRC2 component in ES cells, suggesting that there could be other components involved.

The presence of an unmethylated *Sfrp2* promoter in the spleen suggested that there are tissue specific differences in DNA methylation [3] (Chapter 2). As every tissue has a distinct chromatin landscape, it is conceivable that Dnmt3b-catalyzed DNA methylation occurs at loci marked by specific modified histones. For example, the histone modification H3K27me3 is established and maintained by Ezh2. Vire (2006) showed that EZH2 binds DNMT3b and is essential for the DNMT3b-mediated DNA methylation of EZH2 targets [9]. In addition, for prostate cancer cells, Gal-Yam et al. (2008) observed that loci that are H3K27me3 targets in normal cells lose this mark and become CpG methylated upon transformation [10]. Preliminary ChIP-on-Chip experiments carried out in our laboratory (unpublished) suggested that H3K27me3 marks in small intestinal epithelial cells are lost globally upon increased Dnmt3b expression. Consequently, we asked whether the loci that become methylated upon ectopic expression of Dnmt3b are marked by H3K27me3 prior to DNA methylation, and whether the Dnmt3b methylated targets lose H3K27me3 upon DNA methylation. Additionally, since DNA methylation in the promoter region is associated with gene silencing, we also investigated the expression changes upon Dnmt3b methylation to analyze which genes change expression upon DNA methylation. Finally, the observation that Dnmt3b overexpression could induce a colon cancer-associated DNA methylation pattern on normal colon tissue (Chapter 3) raised the question whether ectopic Dnmt3b expression might induce a cancer specific methylation pattern depending on the tissue in which it is expressed.

To address these questions, we analyzed DNA methylation patterns and histone modifications on a global level using reduced representation bisulfite sequencing (RRBS) and ChIP-Seq. Since ChIP-Seq experiments require a large amount of material, we decided to use mouse small intestinal epithelial cells and liver, tissues from which vast amounts of material can be harvested. In addition, it has been shown that transgene expression induced by doxycycline is efficient in these mouse tissues [11]. Finally, and most importantly, these tissues are relevant in carcinogenesis, since it is known that aberrant Dnmt3b expression can be found in cancer of these tissues in human [12-14].

Results

CpG islands are important regulators of gene expression and are known to be dynamically regulated during cancer. Therefore, we were interested to study the regulation of these loci in our mouse model with ectopic Dnmt3b expression. We used reduced representation bisulfite sequencing (RRBS) to analyze the CpG islands that become methylated upon induction of Dnmt3b [15]. After confirming ectopic Dnmt3b expression in the hepatocytes and the small intestinal epithelial cells by QRT-PCR and immunohistochemistry (Figure 3.1), we subjected the samples to RRBS analysis, which yielded more than 66 million high quality reads. Previously, it has been reported that high density CpG areas in the genome are mainly unmethylated, while low density CpG areas are highly methylated [15,16].

To validate whether our data are consistent with these observations, we divided our sequences in high density CpG areas > 10 CpGs per 1 kb region and low CpG density areas with <10 CpGs per 1 kb region. We were able to confirm that the high density CpG areas were mainly unmethylated and low CpG density areas were highly methylated (data not shown). For subsequent analysis, we grouped the promoters in previously defined high density promoters (HCP) and low density promoters (LCP) [17]. Most promoters are classified as HCP, which regulate genes that are important for embryonic development and ubiquitously expressed housekeeping genes. LCP mainly regulate tissue specific genes. Unclassified promoters are referred to as intermediate CpG promoters (ICP). We observed that DNA methylation level did not change in most promoters (Figure 3.2). A quarter of the HCP promoters increased in methylation by more than 10%; however, the highest average methylation change occurred in the LCP group. When we analyzed the liver and the intestine side by side, we observed that twice as many promoters became methylated in the liver than in the intestine. Interestingly, while most promoters methylated in the intestine were common with the promoters methylated in the liver, a substantial number of promoters were specifically methylated in the intestine.

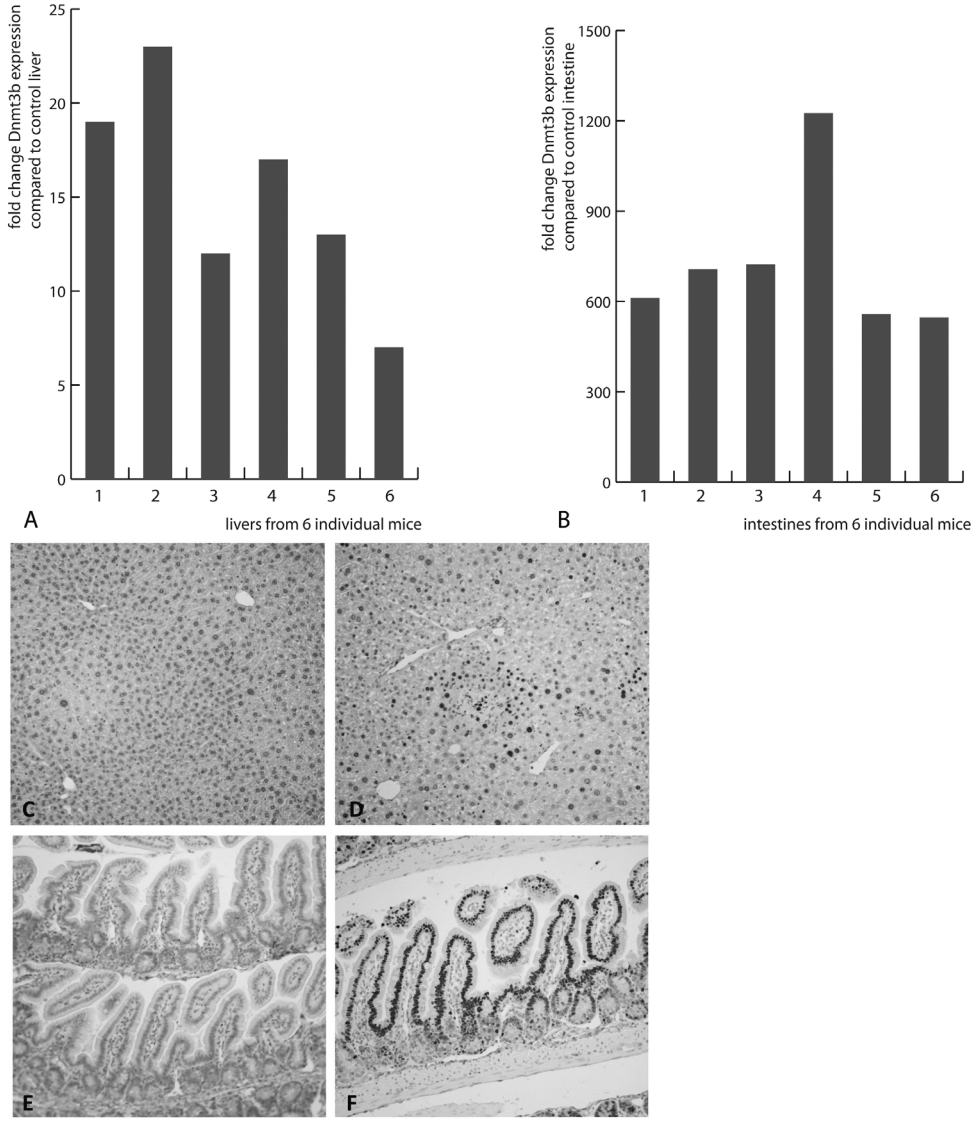


Figure 3.1. **Transgene expression analysis.**
RT-PCR data showing the fold change of Dnmt3b expression in liver (A) and small intestinal epithelial cells (B) of 6 mice induced for Dnmt3b expression, compared to control mice. Dnmt3b expression analyzed by IHC in liver (C and D) and small intestinal epithelial cells (E and F) from induced mouse # 6 (D and F) and control mouse (C and E).

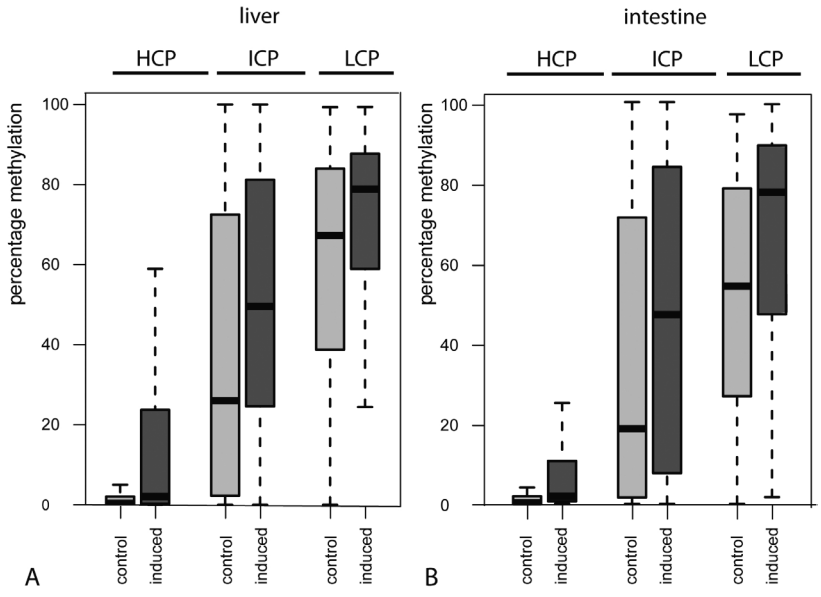


Figure 3.2. **Average percentage of cytosine methylation in promoters.**
Average percentage of cytosine methylation in the promoters in liver (A) and in small epithelial intestinal cells (B). The average methylation level of the CpGs in each promoter is shown on the y-axis. Dark gray bars denote Dnmt3b induced mice and light gray bars denote control mice. Promoters are grouped in high density CpG promoters (HCP), low density CpG promoters (LCP) and intermediate CpG promoters (ICP). Box represents interquartile range; line within box represents median; whiskers represent 1.5× interquartile range.

Since we hypothesized that the chromatin landscape has a guiding role in DNA methylation we set out to find chromatin marks, which correlate with sites that are DNA methylated upon ectopic Dnmt3b expression. By ChIP-Seq, we investigated the localization of H3K27me3 and H3K4me3 genome wide in the liver and intestine. In this analysis, we also incorporated an H3K4me3 liver ChIP-Seq dataset previously performed in our lab (unpublished results). In liver, we observed that promoters that become DNA methylated upon Dnmt3b expression were H3K27me3 targets in the non-induced sample. In liver, we also observed that DNA methylation increased upon ectopic Dnmt3b expression at bivalent promoters; these loci are trimethylated at lysine 4 and lysine 27 of histone 3 and their corresponding genes are poised [18] (Figure 3.3). Additionally, both in the liver and intestine, we observed a reduced number of H3K27me3 peaks in the more DNA methylated Dnmt3b induced sample compared to the less DNA methylated control sample (Figure 3.4). Further research is needed to confirm whether H3K27me3 is lost from the promoters that are DNA methylated upon ectopic Dnmt3b expression.

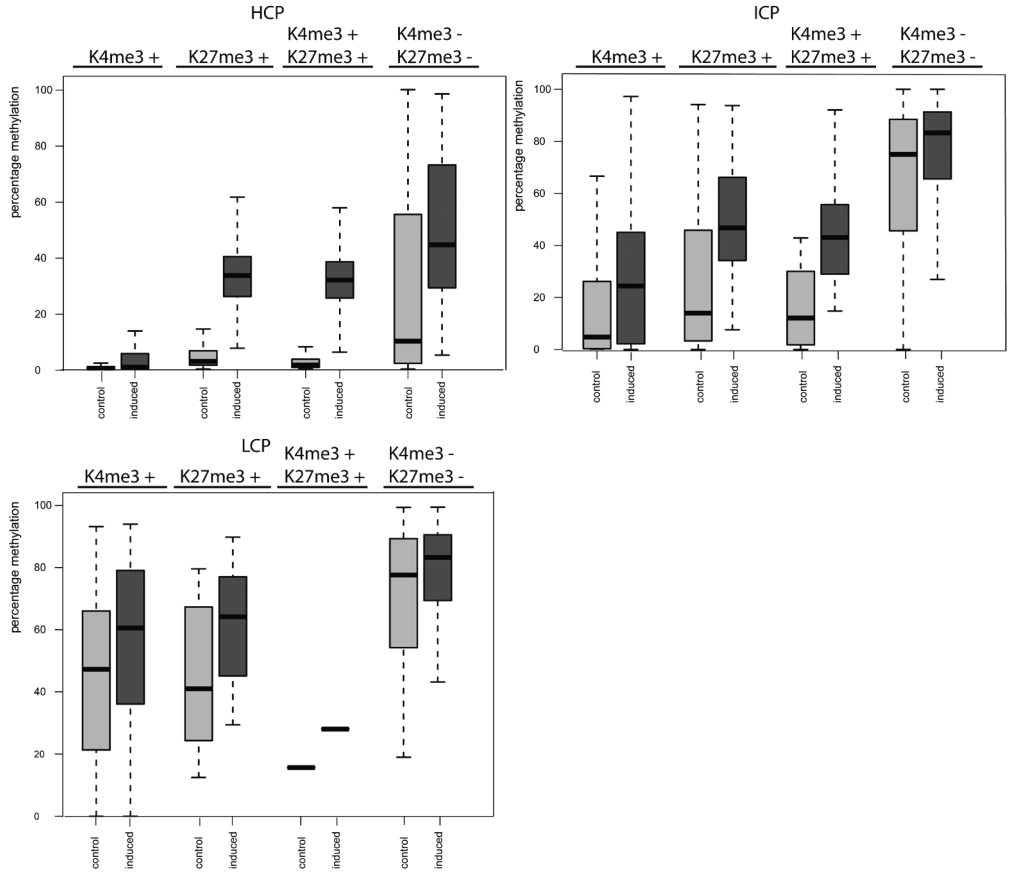


Figure 3.3. Average percentage of cytosine methylation in the promoters overlapping with H3K4me3 targets, H3K27me3 targets, or both targets, and for promoters not overlapping with any of these modifications, prior to ectopic Dnmt3b expression.
The HCP (A), ICP (B), LCP (C) promoters of the liver of the Dnmt3b induced mice are shown in dark gray and control mice in light gray. Promoters with H3K27me3 become methylated upon Dnmt3b induction.

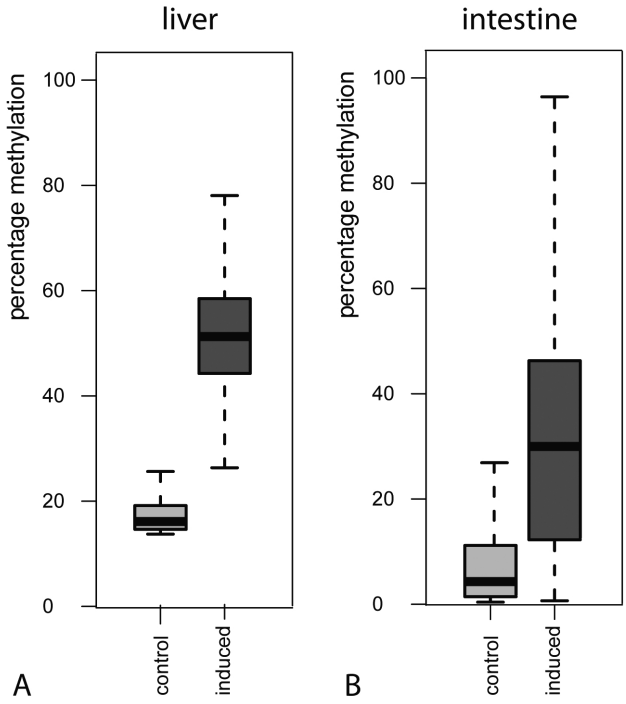


Figure 3.4. Average percentage of cytosine methylation in the promoters overlapping with H3K27me3 peaks that are only present in the control samples, but not in the Dnmt3b induced sample.
Dnmt3b induced (dark gray) and control sample (light gray). In both the liver (A) and intestine (B), the promoter methylation level of genes that lose the H3K27me3 upon ectopic Dnmt3b expression is increased.

Since DNA hypermethylation of gene promoters is associated with gene silencing, we decided to investigate the expression levels of genes that became DNA methylated upon ectopic Dnmt3b expression. In the intestine we did not found an expression change upon DNA methylation (Figure 3.5). For genes that became methylated, the expression levels were low prior and after DNA methylation. Interestingly, this was also the case for Dnmt3b mediated DNA methylated genes marked only with H3K4me3, a histone modification, which is generally associated with active genes.

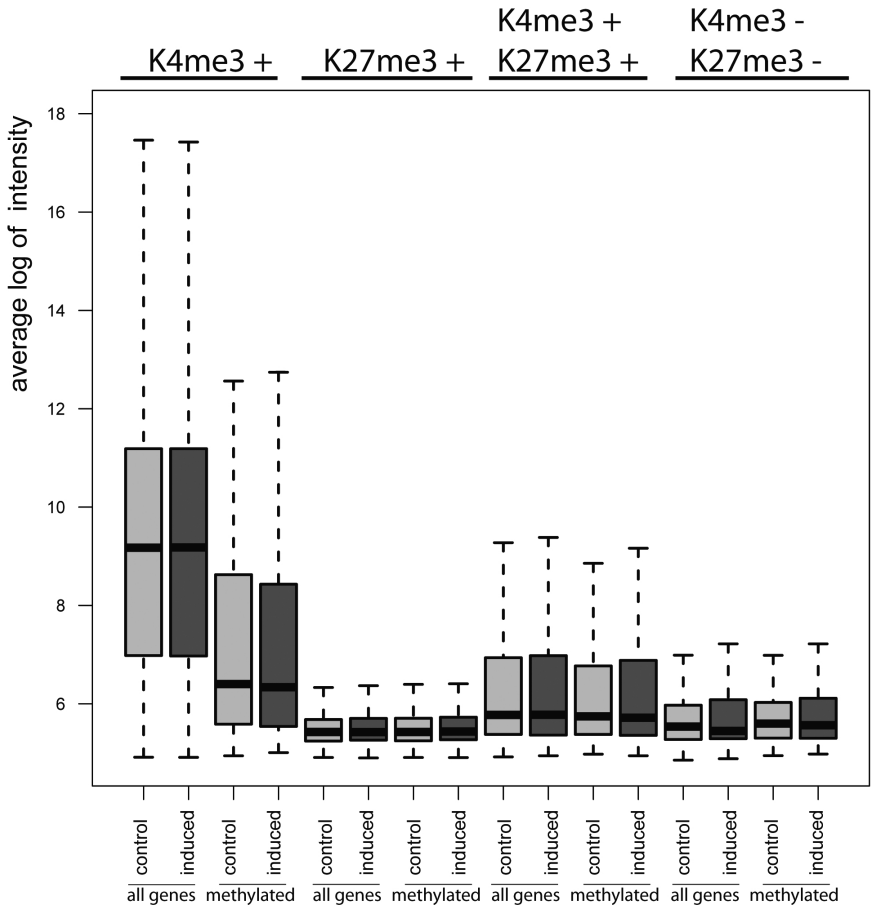


Figure 3.5. No change in gene expression upon DNA methylation in the intestine.
Expression data were analyzed for genes with HCP promoters that get methylated upon ectopic Dnmt3b expression (methylated) and for all genes (all genes) in the liver. Dnmt3b induced samples are depicted as dark gray bars, control samples are depicted as light gray bars. Genes that are methylated upon ectopic Dnmt3b expression tend to be lowly expressed genes. Notably most H3K4me3 targets are active genes, but H3K4me3 targets that acquire DNA methylation are expressed at lower levels than genes for which DNA methylation is not changed upon Dnmt3b expression. LCP and ICP promoters show similar results as HCP promoters (data not shown).

Discussion

Here, we found that the LCP promoters had the highest average DNA methylation change upon Dnmt3b induction in small intestinal epithelial cells and hepatocytes. Most promoters that became DNA methylated upon Dnmt3b induction were classified as HCP, the largest promoter group. Further, we observed Dnmt3b induced DNA methylation varies per tissue. The present data suggest that the Dnmt3b induced DNA methylation is not specifically directed to either HCP or LCP promoters.

Next, we investigated the correlation of chromatin marks and Dnmt3b mediated DNA methylation. We observed that most loci that are methylated upon ectopic Dnmt3b expression carried the gene silencing histone modification H3K27me3 prior DNA methylation. Interestingly, upon DNA methylation the number of H3K27me3 targets was reduced; however, further research is needed to study whether the loss of H3K27me3 and increase of DNA methylation occurs at the same locus. Conversely, some loci that were methylated upon Dnmt3b expression carried the gene activating histone modification H3K4me3. Other loci that were methylated upon ectopic Dnmt3b expression did not carry any of the above histone modifications. Possibly another chromatin mark recruits Dnmt3b to these loci. For example, DNA methylation occurs on loci marked with H3K9me2 [19]. Additionally, we cannot exclude that other components such as nucleosomes might direct Dnmt3b mediated DNA methylation, as previously suggested [20]. Moreover, it has recently been shown that chromatin regions associated with DNA hypermethylation in colon cancer associates with nuclear lamina. How this mechanism exactly works remains to be elucidated, but if this is studied, this mechanistic aspect could potentially shed light on regulation of Dnmt3b mediated DNA methylation [21].

Ultimately, promoter DNA methylation is correlated with gene silencing. Interestingly, when we analyzed the gene expression changes upon DNA methylation we saw that most genes were not or minimally expressed prior DNA methylation, which is consistent with observations reported previously. For example, Keshet (2006) reported that genes that become methylated in cancer are usually already silenced before they become methylated [22]. In addition, Choi et al. (2011) observed that when *Dnmt3b* was overexpressed in HEK 293T cells, only 10% of genes that became DNA methylated were changed in their expression levels [23]. Other interesting questions could address which genes become DNA methylated in different tissues. Are there commonalities between these genes? Are these genes relevant to the respective tissues, and possibly involved in tumorigenesis?

In conclusion, the loci DNA methylated upon ectopic Dnmt3b expression differ per tissue, and further research should address possible functional implications of specific methylation of these loci.

Acknowledgements

We thank R. Flannery, and K. Ganz for maintenance of the mice; Mike lodato and the Young lab for critical discussions. We thank D. Cook, A. Leshinski, and C. Whittaker for running the Solexa pipeline; and J. Kwon, J. Love, and S. Gupta for performing microarray hybridization.

Materials & methods

Transgenic mice

All transgenic mice and genotyping protocols used in this study were described previously [3]. Briefly, the doxycycline inducible *Dnmt3b* transgenic mice are generated from ES cells where the doxycycline inducible transgene was targeted to the collagen A1 locus (*ColA1*) and the reverse transactivator M2-rtTA was targeted to the *Rosa26* locus (R26-rtTA). The sample mice were heterozygous for both alleles; the control mice were heterozygous for R26-rtTA and wild type for the *ColA1* locus. All animal studies were reviewed and approved by the Committee on Animal Care (protocol 1110-096-13, Department of Comparative Medicine, MIT).

Treatment of mice

All mice were fed 2mg/ml doxycycline in drinking water supplemented with 10mg/ml sucrose for 11 weeks, for transgene induction or to control for side effects of the transactivator M2-rtTA. When harvesting the tissues, livers and intestines of six mice were pooled for RRBS, microarray and ChipSeq experiments. Performing these experiments in triplicate on three individual mouse samples would likely yield data reflecting differences at the level of biological random fluctuations, 'noise', between individual mice, Therefore, we pooled samples of several mice and executed our ChIP experiments on these samples.

Tissue harvesting

Mice were sacrificed and perfused with PBS to wash out the blood, which could cloud the analysis. For RNA samples the tissue was immediately dissolved in trizol and snap frozen, for DNA methylation analysis, the liver was homogenized and washed with ice cold PBS. Equal aliquots of 6 livers were pooled and snap frozen for DNA. Small intestinal samples were harvested as previously described [24]. Samples for RNA, protein and DNA were frozen separately. For IHC, samples were fixed in 10% buffered formaline for 24h at 4°C.

RNA isolation

Total RNA was isolated using trizol reagent (Invitrogen) according to manufacturer's protocol. After DNAse treatment (R1028, Zymo), cDNA was synthesized by Superscript III first strand synthesis supermix (Invitrogen). Quantitative PCR analysis was performed in triplicate at an ABI Prism7900 (Applied biosystem) using Sybr green master (Roche). For *Dnmt3b* quantification we used primers Dnmt3b forward: GTTCGAGCTGGCAAGACCTT and Dnmt3b reverse: TGGTCCTCCAGTGACTCTCCA and for *Gapdh* we used primers Gapdh forward: TTCACCACCATGGAGAAGGC and Gapdh backward: CCCTTTTGGCTCCACCCT. All samples were analyzed in triplicate, and *Gapdh* was used as an endogenous control. The *Dnmt3b* expression was converted to the fold change of Dnmt3b expression compared to the control samples.

ChIP

Cells were chemically cross-linked by 1.1% formaldehyde solution (1 mM EDTA, 0.5 mM EGTA, 50 mM Hepes-KOH, and 100 mM NaCl) for 15 minutes at RT. Samples were washed twice with PBS, and snap frozen. 10^8 cells were resuspended in lysis buffer 1 (50 mM Hepes-KOH, 140 mM EDTA, 10% glycerol, 0.5% Igepal and 0.25% triton), rocked at rotating platform for 10 min at 4 °C, and spun down at 2500 rpm for 5 min at 4 °C. Next, cells were lysed in lysis buffer 2 (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10mM Tris), rocked at rotating platform for 10 min at 4 °C, and spun down at 2500 rpm at 4 °C. Cells were resuspended in 3 ml sonication buffer (20 mM TrisHCl, 150 mM NaCl, 2 mM EDTA and 0.1% SDS, 1% triton). Samples were sonicated for 30 sec ON, 60 sec OFF at 27 Watt for 12 cycles by using Misonix sonicator 3000 at 4 °C. Samples were cleared by 14000 rpm, 10 minute spin at 4 °C. As input 50 ul of the lysate was stored; at 4 °C overnight, the rest of the lysate was incubated on a rocking platform with 100 ul magnetic dynal beads, which were preincubated with 10 mg antibody (H3K727me3: ab6002). Beads were then first washed once with the sonication

buffer, secondly with wash buffer 1 (20 mM Tris, 500 mM NaCl, 2 mM EDTA, 0.1 % SDS, 1% Triton), thirdly with wash buffer 2 (10 mM Tris, 250 mM LiCl, 1 mM EDTA, 1% Igepal), and last with Tris-EDTA buffer supplemented with 50mM NaCl. Next, bound protein complexes were eluted from the beads in elution buffer (50 Tris, 10 EDTA, 1% SDS), by heating the beads in elution buffer at 65 °C for 20 minutes and vortexing the beads every 2-3 minutes. After transferring the elution buffer to a new tube, the protein-chromatin complexes were reversed-cross-linked at 65 °C overnight. The immunoprecipitated DNA was diluted with TE buffer (1:1) and RNAse treated (0.2 ug/ul) for 2 hours at 37°C followed by treatment with proteinase K (0.2 ug/ul) supplemented with CaCl for 2 hours at 55°C. DNA was twice phenol-chloroform extracted using phaselock tubes. Libraries for the Genome analyzer II genome sequencer were prepared with the Illumina ChIP-Seq prep kit according to manufacturers protocol. 150-250 bp fragment was gel selected and run on the analyzer. H3K4me3 ChIP was preformed at wild type mice, according to the same protocol using H3K4me3 antibody (Upstate 07-473).

IHC

After antibody retrieval by boiling the sections (5 um) in EDTA (zymed), immunostaining was performed with envision™ + System–HRP (DAB) (Dakocytomation K4010). Peroxidase activity was quenched with peroxidase block for 5 min at RT, sections were incubated with antibody Dnmt3b (1:3000, Imgenex 184A) for 30 minutes at RT, followed by 30 minute incubation with labeled polymer for 30 minutes at RT, and 5 minute incubation with DAB as chromogen. Hematoxylin was used as counter staining.

Reduced representation bisulfite sequencing (RRBS)

The library was constructed with the DNA sample kit (Illumina, IP-102-1001). Five microgram Msp1 (50U) digested genomic DNA was run on a 3% nusive gel to isolate small and a large size fragment bands, 40-120 and 120-220 bp, respectively. Phenol:chloroform extracted DNA was end-repaired and adenylated and ligated to the 3'T-overhang methylated-cytosine-containing adapters. Subsequently DNA was bisulfite treated with Epitect kit (Qiagen) with two extra bisulfite conversion cycles. Bisulfite-treated DNA was PCR amplified for 24 cycles. The small and large fragment libraries were gel purified and size selected (120-220 and 220-310 bp, respectively.) on a 3% nusive gel. Libraries were sequenced on the Genome Analyzer II (Illumina).

Analysis ChIP-Seq

We used the promoter classification described in Mikkelsen (2007)[17]. High density CpG promoters (HCPs) contain a 500-bp interval within -0.5 kb to +2 kb of the transcription start site with a (G+C)-fraction greater or equal to 0.55 and a CpG observed to expected ratio (O/E) greater or equal to 0.6. LCPs contain no 500-bp interval with CpG observed to expected ratio greater or equal to 0.4. Promoters not meeting either of the criteria above were classified as a set of intermediate CpG content promoters (ICP). Mikkelsen et al. (2007) had classified promoters using mm8 coordinates; we converted these coordinates to the mm9 release using liftover. Of the 17762 promoters classified we recovered mm9 coordinates for 17745. Liftover changed the length of 35 promoters, of those five were longer than 2.5 kb (2790 bps was the longest promoter kept) and 10 were smaller than 2490 pb, the smallest promoter being 2388 bps.

We performed H3k27me3 ChIP-Seq two different times in duplicate each time. We analyzed the two data sets separately. We combined the reads from the two replicas and if the number of reads was very different between IP and whole cell extract we selected a subset of reads from the sample with the highest number of reads to have a comparable number of reads to input to MACS. We mapped the reads with Bowtie, allowing mapping to only one place on the genome, and used MACS 1.4.1 to call regions bound. We combined the peaks found in both experiments by keeping only peaks present in both experiments, using bed tool “intersectBed” with default settings. Box represents interquartile range; line within box represents median; whiskers represent 1.5× interquartile range.

Analysis reduced representation bisulfite sequencing

A bisulfite-aware short-read aligner was used to align reads to the appropriate RRBS library, allowing up to 2 mismatches. The RRBS library was generated by *in silico* MspI digestion, fractionation, and conversion. Mapped reads were assigned to promoters using “intersectBed” from Bed tools. Promoters were defined as in Mikkelsen 2007 except that coordinates were lifted over from mm8 to mm9 [17]. A CpG was defined as scorable if it was assayed by at least 5 reads, and a promoter with least 5 scorable CpGs was itself defined as scorable. In each sample, the methylation fraction of a promoter was calculated as the mean of scorable CpGs.

References

1 Jones, P. A. & Baylin, S. B. The epigenomics of cancer. *Cell* **128**, 683-692, doi:10.1016/j.cell.2007.01.029 (2007).
2 Feinberg, A. P. Phenotypic plasticity and the epigenetics of human disease. *Nature* **447**, 433-440, doi:10.1038/nature05919 (2007).
3 Linhart, H. G. *et al.* Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. *Genes & development* **21**, 3110-3122, doi:10.1101/gad.1594007 (2007).
4 Steine, E. J. *et al.* Genes methylated by DNA methyltransferase 3b are similar in mouse intestine and human colon cancer. *The Journal of clinical investigation* **121**, 1748-1752, doi:10.1172/JCI43169 (2011).
5 Margueron, R. & Reinberg, D. The Polycomb complex PRC2 and its mark in life. *Nature* **469**, 343-349, doi:10.1038/nature09784 (2011).
6 Ohm, J. E. *et al.* A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nature genetics* **39**, 237-242, doi:10.1038/ng1972 (2007).
7 Schlesinger, Y. *et al.* Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nature genetics* **39**, 232-236, doi:10.1038/ng1950 (2007).
8 Widschwendter, M. *et al.* Epigenetic stem cell signature in cancer. *Nature genetics* **39**, 157-158, doi:10.1038/ng1941 (2007).
9 Viré, E. *et al.* The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439**, 871-874, doi:10.1038/nature04431 (2006).
10 Gal-Yam, E. N. *et al.* Frequent switching of Polycomb repressive marks and DNA hypermethylation in the PC3 prostate cancer cell line. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 12979-12984, doi:10.1073/pnas.0806437105 (2008).
11 Beard, C., Hochedlinger, K., Plath, K., Wutz, A. & Jaenisch, R. Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. *Genesis (New York, NY : 2000)* **44**, 23-28, doi:10.1002/gene.20180 (2006).
12 Miyake, T., Endo, K., Honjo, S., Hirooka, Y. & Ikeguchi, M. Expression of DNA Methyltransferase (DNMT) 1, 3a and 3b Proteins in Human Hepatocellular Carcinoma. *Yonago Acta medica*, 1-7 (2010).
13 Robertson, K. D. *et al.* The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic acids research* **27**, 2291-2298 (1999).
14 Xie, S. *et al.* Cloning, expression and chromosome locations of the human DNMT3 gene family. *Gene* **236**, 87-95 (1999).
15 Meissner, A. *et al.* Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* **454**, 766-770, doi:10.1038/nature07107 (2008).
16 Weber, M. *et al.* Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature genetics* **39**, 457-466, doi:10.1038/ng1990 (2007).
17 Mikkelsen, T. S. *et al.* Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**, 553-560, doi:10.1038/nature06008 (2007).
18 Bernstein, B. E. *et al.* A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. *Cell* **125**, 315-326, doi:10.1016/j.cell.2006.02.041 (2006).
19 Lehnertz, B. *et al.* Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Current biology : CB* **13**, 1192-1200 (2003).
20 Jeong, S. *et al.* Selective anchoring of DNA methyltransferases 3A and 3B to nucleosomes containing methylated DNA. *Molecular and cellular biology* **29**, 5366-5376, doi:10.1128/MCB.00484-09 (2009).

21 Berman, B. P. *et al.* Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina–associated domains. *Nature genetics*, doi:10.1038/ng.969 (2011).
22 Keshet, I. *et al.* Evidence for an instructive mechanism of de novo methylation in cancer cells. *Nature genetics* **38**, 149-153, doi:10.1038/ng1719 (2006).
23 Choi, S. H. *et al.* Identification of preferential target sites for human DNA methyltransferases. *Nucleic acids research* **39**, 104-118, doi:10.1093/nar/gkq774 (2011).
24 Fujimoto, K., Beauchamp, R. D. & Whitehead, R. H. Identification and isolation of candidate human colonic clonogenic cells based on cell surface integrin expression. *YGAST* **123**, 1941-1948, doi:10.1053/gast.2002.37065 (2002).

CHAPTER 4

DELETION OF THE *DE NOVO* DNA METHYLTRANSFERASE *DNMT3A* PROMOTES LUNG TUMOR PROGRESSION

Qing Gao[a], Eveline J Steine[a], M. Inmaculada Barrasa[a], Dirk Hockemeyer[a], Mathias Pawlak[a], Dongdong Fu[a], Seshamma Reddy[a,c], George W Bell[a], & Rudolf Jaenisch[a,b,1]

[a] Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA

[b] Department of Biology, Massachusetts Institute of Technology, 31 Ames Street, Cambridge, MA 02139, USA

[c] Current address: OralCDx Laboratories inc, 2 Executive Blvd., Suite 102, Suffern, NY 10901

[1] To whom correspondence should be addressed.

Published in *Proceedings of the National Academy of Sciences* 2011; 108(44);18061-18066

Abstract

Alterations in DNA methylation have been associated with genome-wide hypomethylation and regional *de novo* methylation in numerous cancers. *De novo* methylation is mediated by the *de novo* methyltransferases Dnmt3a and 3b but only Dnmt3b has been implicated in promoting cancer by silencing of tumor suppressor genes. In this study we have analyzed the role of Dnmt3a in lung cancer using a conditional mouse tumor model. We show that Dnmt3a deficiency significantly promotes tumor growth and progression but not initiation. Changes in gene expression show that Dnmt3a deficiency affects key steps in cancer progression, such as angiogenesis, cell adhesion and cell motion, consistent with accelerated and more malignant growth. Our results suggest that *Dnmt3a* may act like a tumor suppressor gene in lung tumor progression and may be a critical determinant of lung cancer malignancy.

Introduction

Changes in the DNA methylation status are among the most common molecular alterations in cancer (1, 2). Genome-wide hypomethylation and promoter hypermethylation are hallmarks of a great variety of cancers contributing to tumorigenesis. Global hypomethylation can result in increased genomic instability, reactivation of silenced parasitic genes and loss of imprinting (3, 4) whereas hypermethylation of CpG islands in gene promoters can silence tumor suppressor genes and affect critical cellular processes, such as cell-cycle control, apoptosis, DNA repair, cell-cell interaction and angiogenesis (5).

The de novo methyltransferases Dnmt 3a and 3b are highly expressed during early embryonic development and down-regulated in most differentiated somatic cells (6). In human cancers, however, Dnmt3b is frequently overexpressed (7, 8), consistent with the notion that inappropriate expression of Dnmt3b may silence tumor suppressor genes and thus contribute to tumorigenesis. Based upon tissue-specific gene deletion and induction, Dnmt3b has been identified in mouse models to carry out *de novo* methylation of genes that are typically silenced in human colon cancer and to promote intestinal tumor formation (9-11). In contrast to Dnmt3b, the role of Dnmt3a in cancer is far less understood. DNMT3A was found to be overexpressed in some human cancers (12) and in a mouse xenograft model, the knockdown of *Dnmt3a* was shown to suppress melanoma growth and metastasis (13). In contrast, induction of Dnmt3a in $Apc^{min/+}$ mice had no effect on intestinal tumor formation (9). The role of DNMT3A in human cancer was highlighted by reports of *DNMT3A* mutations in approximately 20% of patients with acute myeloid leukemia (AML)(14, 15). The occurrence of these mutations correlated with reduced enzymatic activity and genomic regions with decreased methylation. *DNMT3A* mutations were also identified in 8% of patients with myelodysplastic syndrome (16). In all these reports the *DNMT3A* mutations correlated with poor prognosis.

Lung cancer is the leading cause of cancer death in the US (17) and can be divided into four types: adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell lung carcinoma with adenocarcinoma being the most common type. Both genetic and epigenetic factor have been implicated in lung cancer. The mutation of *K-ras* is one of the most common genetic lesions and can be found in a large fraction of lung cancers. Promoter hypermethylation is perhaps the best characterized epigenetic aberration and can be used as a screening marker for early detection, prevention and prognosis (18, 19).

In this study we have established an experimental system to investigate the role of Dnmt3a in lung adenocarcinoma. We show that deletion of *Dnmt3a* in mutant *K-ras* induced lung tumors significantly promotes tumor progression suggesting that this gene functions like a tumor suppressor.

Results

Dnmt3a deletion accelerates tumor growth

In order to test the effect of *Dnmt3a* deletion on lung cancer, we generated mice carrying a conditional *K-ras*$^{LSL-G12D}$ allele (20) and 2Lox alleles of *Dnmt3a* (21) (*K-ras*$^{LSL-G12D}$ / *Dnmt3a*$^{2Lox/2Lox}$, Figure 1A). Oncogene activation and *Dnmt3a* deletion were induced by intratracheal infusion with adenoviral Cre recombinase (Ad-Cre) (22).

The lungs of infected animals were removed and prepared for histology at weeks 8, 16 and 24 after Ad-Cre administration. No significant differences in tumor number and size were seen in lungs of animals at week 8. In contrast, Dnmt3a deficient [knockout (KO)] and wild type (WT) mice showed a dramatic difference at weeks 16 and 24 after infection. While most tumors in lungs of Dnmt3a WT animals were small (up to 0.2 cm in diameter), lungs of Dnmt3a deficient mice were characterized by a significant increase in the number of large tumors (Figure 1B). This was confirmed on histological sections of the lungs (Figure 1C). As summarized in Figure 1D, the average size of Dnmt3a deficient tumors was about 4 times larger at week 16 and 6 times larger at week 24 as compared to WT tumors. Similarly, the fraction of the total lung area occupied by Dnmt3a deficient tumors was about 4 times larger than that occupied by WT tumors at weeks 16 and 24 (Figure 1E). However, the total number of tumors (adenoma and adenocarcinoma, AD) and atypical adenomatous hyperplasia (AH, a pre-tumor lesion) did not vary significantly between Dnmt3a deficient and control animals at weeks 8 and 16 (Figure 1F, Table S1 and S2). These results suggest that Dnmt3a deficiency does not affect the initiation of K-ras induced lung tumors but significantly promotes tumor growth.

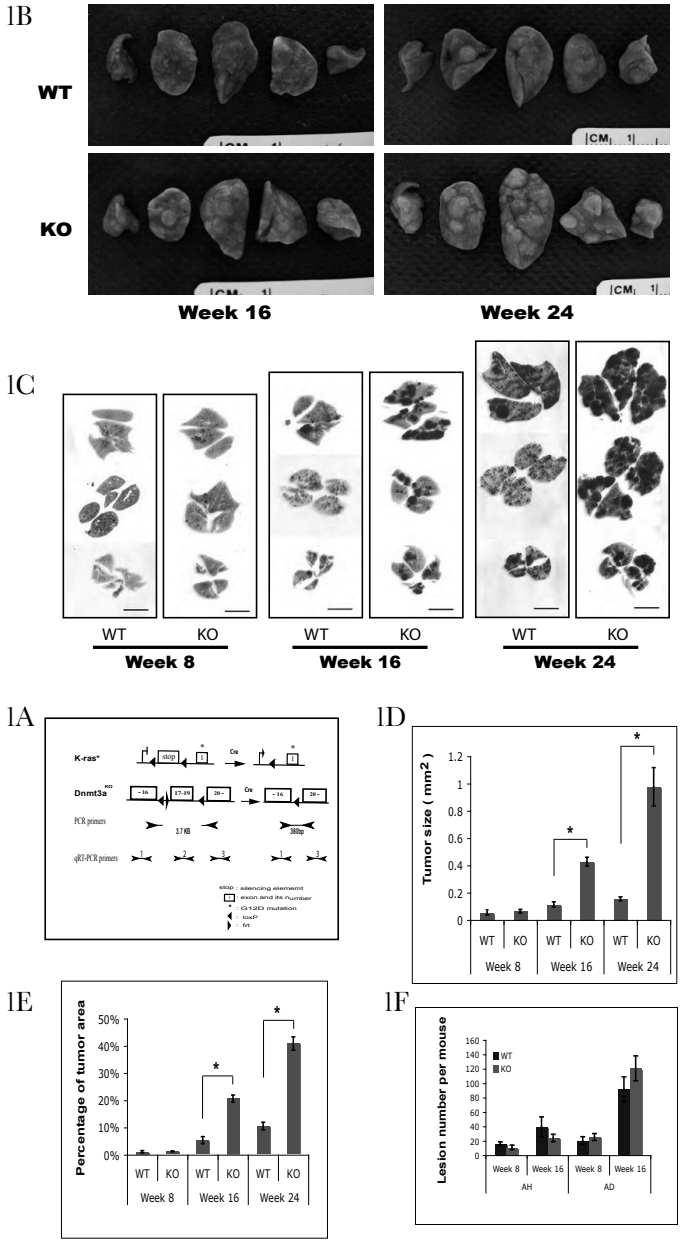


Figure 1. Dnmt3a deficiency accelerates lung tumor growth in K-ras^{G12D} mouse.
(*A*) Schematic representation of the engineered locus in *K-ras* conditional knock-in and *Dnmt3a* conditional deletion mice before and after Cre-mediated recombination [modified from references (20) and (21)]. Positions of the PCR primers to detect recombination and the positions of 3 pairs of qRT-PCR primers to detect Dnmt3a mRNA expression are also indicated. stop : siliencing element; 1 : exon and its number; *: G12D mutation; ◀: loxP site; ▶: frt site; ➤ and ➤: PCR primers; > and < : qRT-PCR primers. (*B*) Tumors in lungs of Dnmt3a deficient mice were significantly larger than those in Dnmt3a WT mice at weeks 16 and 24 after Ad-Cre infection. Scale bar, 5mm. (*C*) Representative

H&E stained sections of lungs in Dnmt3a deficient and WT mice at weeks 8, 16 and 24 after Ad-cre infection. The tumors are seen as blue areas because of crowded nuclei stained blue by hemotoxylin. Dnmt3a deficient mice had an increased number of large tumors. Scale bar, 6mm. (*D,E*) Comparison of tumor size (area) and fraction of lung area occupied by tumors. Both tumor size and tumor area fraction were significantly larger in Dnmt3a deficiency mice than in WT mice at weeks 16 and 24. ★: comparisons that are significantly different. (Tumor size: week 16, $p < 0.0001$; week 24, $p = 0.0012$. Tumor area fraction: weeks 16 and 24, $p < 0.0001$). (*F*) Comparison of number of tumor (adenoma and andenocarcinoma, AD) and atypical adenomatous hyperplasia (AH) at weeks 8 and 16. No singnificant difference was detected. n=4 for both Dnmt3a KO and WT at weeks 8 and 24; n=5 for both KO and WT mouse at week 16. Error bar, SEM.

Deletion of *Dnmt3a* in tumors

To verify *Dnmt3a* deletion we used PCR with primers flanking the Lox-P sites to detect recombination (Figure 1A). Figure 2A shows that all tumors tested had evidence for *Dnmt3a* deletion. This was confirmed by qRT-PCR using primers located 5' to the deletion (primer pair 1), within the deletion (primer pair 2) and 3' to the deletion (primer pair 3). Figure 2B demonstrates that primer pairs 1 and 3 detected a relatively high level of mRNA whereas primer pair 2 generated a >10-fold lower signal in Dnmt3a deficient tumors. This is consistent with efficient Cre mediated deletion of exons 17 to 19 which encode essential residues of the catalytic center of Dnmt3a and with the production of a shortened Dnmt3a mRNA from the deletion allele. The low level of RNA detected in Dnmt3a deficient tumors by primer pair 2 is likely due to the presence of some stromal cells in the tumor samples.

To confirm that the deletion affects Dnmt3a protein expression we performed immunohistochemical analyses on lung sections of Dnmt3a deficient and WT mice using an antibody that recognizes the amino-terminal region of the Dnmt3a protein. Figure 2C showed strong nuclear as well as weak cytoplasmic staining in Dnmt3a WT tumors. In contrast, Dnmt3a deficient tumors lacked nuclear staining and revealed only weak cytoplasmic immunoreactivity (Figure 2D). This difference in staining pattern suggests that, while *Dnmt3a* mutant cells may produce low levels of truncated Dnmt3a protein, they do not express functional protein that can localize to the nucleus and thus would be unable to methylate genomic DNA.

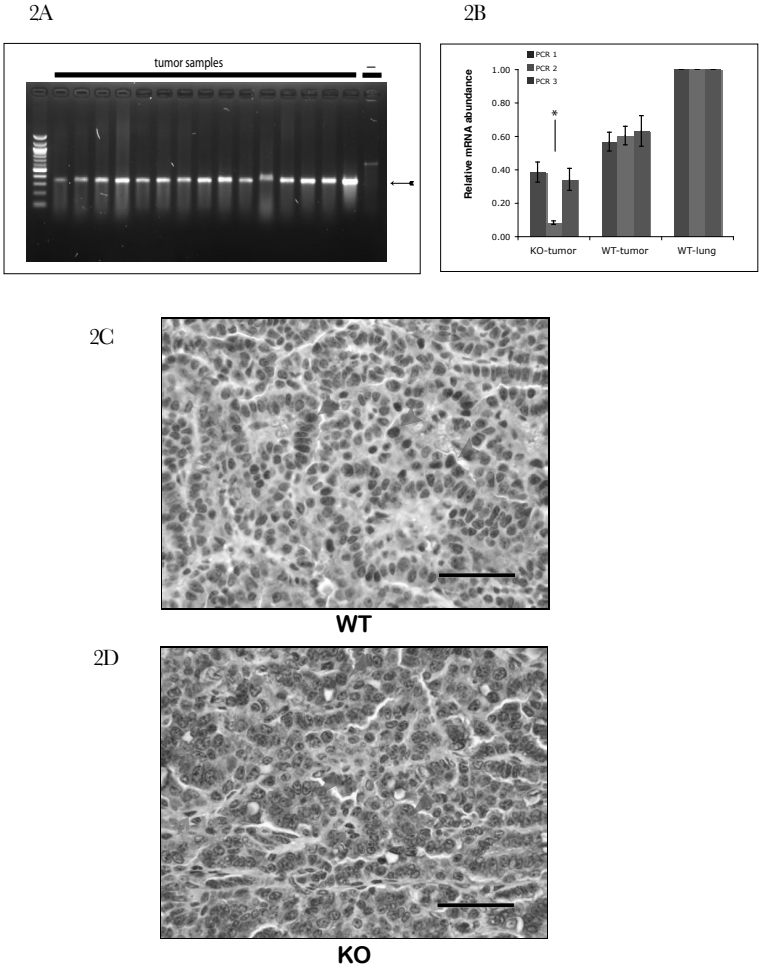


Figure 2. Deletion and expression of Dnmt3a.
(*A*) The targeted segment of *Dnmt3a* is deleted in Dnmt3a deficient tumors. The expected 380bp recombination bands (arrowhead) were detected in all the tumors tested (40 tumors from 9 Dnmt3a KO mice). - : negative control. (*B*) Relative Dnmta3a mRNA abundance by quantitative RT-PCR. Primer pairs (PP) 1 to 3 are located to the 5' side, within, and to the 3' side of the targeted deletion respectively (see Figure 1A). Primer pair 2, which detects the segment removed in the deleted mRNA, gave a significantly lower signal (indicated by *) in Dnmt3a deficient tumors than those in normal lungs and WT tumors ($p < 0.0001$), indicating efficient deletion of the targeted segment. Primers pairs 1 and 3 detected a relatively high level of Dnmt3a mRNA in Dnmt3a deficient tumors, suggesting production of a shortened Dnmt3a mRNA. Tumor number, KO: n=19; WT, n=20. Error bar, SEM. (*C, D*) Immunohistochemical staining for Dnmt 3a protein with an Ab recognizing the amino-terminal region of Dnmt3a. In WT tumors (*C*), positive cells showed strong nuclear staining (indicated by arrowheads) with weak cytoplasmatic signals, whereas in Dnmt3a KO tumor cells (*D*), no typical nuclear signal was detected with only occasional weak cytoplasmatic staining (indicated by arrowheads). Strong nuclear signals were observed in 78 out of 170 WT tumors (6 mice) and no typical nuclear staining was detected in 160 Dnmt3a KO tumors (6 mice). Also note that some stromal cells in Dnmt3a deficient tumor tissue display nuclear signals indicated by arrows (*D*). Scale bar, 50μm.

***Dnmt3a* deletion facilitates tumor progression and shortens life span**

To assess tumor progression and malignancy we classified the tumors into three grades according to tumor differentiation [modified from reference (23)]. Grade 1 tumors were well differentiated and the tumor cells resembled type 2 pneumocytes with small round nuclei and granular cytoplasm. In contrast, grade 3 tumors represented poorly differentiated tumors with marked cellular and nuclear pleomorphism, high nucleus to cytoplasm ratio, prominent nucleoli and numerous mitotic figures. Grade 2 tumors were moderately differentiated, with a degree of differentiation between grade 1 and 3. As summarized in Figure 3A, a significantly higher fraction of tumors in Dnmt3a deficient animals at weeks 16 and 24 after infection was of grade 2 and 3 as compared to tumors in Dnmt3a WT mice (also see Table S2 and S3). .

We also evaluated the tumor growth pattern. Two types of tumor growth, a solid and a papillary growth patterns, can be distinguished in mouse lung tumor, with the papillary growth pattern being considered as characteristic for advanced tumors (24, 25). In solid tumors, tumor cells proliferate in a nest- or sheet-like fashion, whereas in papillary tumors, the cells grow around a fibrovascular core (Figure 3B). Compared with solid growth pattern, the papillary structure enables tumor cells to access blood circulation more efficiently, which is critical for tumor growth and progression. Figure 3B shows that Dnmt3a deficient mice had a significantly higher percentage of tumors with papillary structure (papillary tumors) at weeks 16 and 24 after infection than control mice (also see Table S2 and S3). Finally, we observed tumor invasion in four Dnmt3a deficient tumors (Figure 3C) but no invasion was detected in WT tumors.

Consistent with the high tumor load and with the more malignant tumor phenotype, lifespan of Dnmt3a deficient mice was significantly shorter than that of Dnmt3a heterozygous or WT mice (Figure 3D). No significant difference was observed between Dnmt3a heterozygous and WT mice. Autopsies of about 70 animals revealed lung tumors as the most likely cause of death.

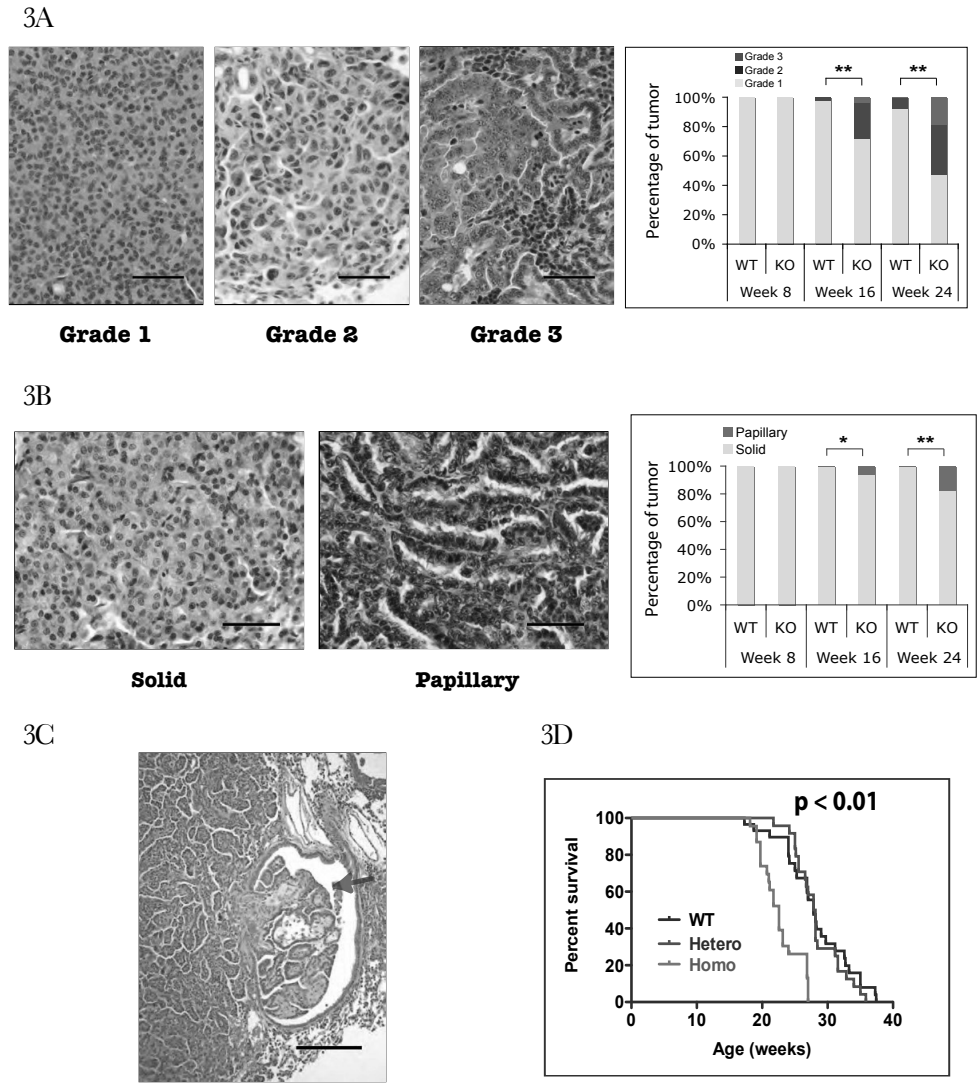


Figure 3. Dnmt3a deficiency leads to more advanced tumors.
(*A*) Dnmt3a deficient mice had more grade 2 and grade 3 tumors than WT mice. Photomicrographs show the morphology of tumors of the three histological grades (arrowhead : a mitotic figure). Bar graph demonstrates that Dnmt3a deficient mice had significantly higher percentage of grade 2 + 3 tumors at weeks 16 and 24 (week 16: $p = 0.0019$; week 24, $p < 0.0001$) and grade 3 tumors at week 24 ($p=0.0034$). (*B*) Dnmt3a deficient mice had more papillary tumors than WT mice. Photomicrographs show morphology of solid and papillary growth pattern. Bar graph demonstrates that Dnmt3a deficient mice had significantly higher percentage of papillary tumors at weeks 16 and 24 (week 16, $p = 0.026$; week 24, $p = 0.0062$). n=4 for both Dnmt3a KO and WT at week 8 and 24; n=5 for both KO and WT at week 16. (*C*) Representative section showing invasion into a bronchiole (arrow) in a Dnmt3a deficient tumor. (*D*) Dnmt3a deficient (homozygous KO) mice have significantly shorter lifespan than Dnmt3a heterozygous and WT mice ($p < 0.0001$). No significant difference was detected between Dnmt3a heterozygous and WT mice ($p=0.63$). WT: Dnmt3a wild type, n=29; Homo: Dnmt3a homozygous KO, n=23; Hetero: Dnmt3a heterozygous, n=24. ✶: comparisons that are significantly different (p values, see above). Photomicrographs, H&E staining. Scale bar, 50μm (*A*, *B*), 200μm (*C*).

Dnmt3a deficient tumors have a high proliferation index

To investigate the possible basis of the accelerated growth of Dnmt3a deficient tumors, we examined the cell proliferation and apoptosis indexes by immunostaining for proliferation marker Ki67 and apoptosis marker cleaved caspase-3. Figure 4A and 4B show that significantly more cells per unit area in Dnmt3a deficient tumors were positive for Ki67 than in WT tumors, indicating that mutant tumors were characterized by a higher proliferation index. In contrast, cells positive for cleaved caspase-3 were rare and no significant difference was detected between Dnmt3a deficient and WT tumors (Figure 4C). These results suggest that the increased growth of Dnmt3a deficient tumors is due to increased proliferation rather than decreased apoptosis.

Gene expression and DNA methylation in Dnmt3a deficient tumors

To gain insights into overall gene expression changes we performed transcriptional profiling analyses using whole mouse genome microarrays. A comparison between 12 pairs of Dnmt3a deficient and WT tumors revealed that mRNA abundance of about 1970 genes was significantly different between Dnmt3a deficient and WT tumors (FDR < 0.05) with approximately 1020 genes expressed at a higher and 940 genes expressed at a lower level in Dnmt3a deficient tumors (Figure 5A). Gene ontology enrichment analysis (26) revealed that the top three biological processes affected most significantly by these differentially expressed genes were blood vessel formation, cell adhesion and regulation of cell motion (Figure 5B). These processes are key steps in cancer progression(27), consistent with the morphological findings that Dnmt3a deficient tumors appear to be larger and more advanced. Also, no significant difference (using a raw p-value threshold of 0.05) of the mRNA abundance was detected in Dnmt1 (p=0.46) or Dnmt3b (p=0.34) between Dnmt3a deficient and WT tumors.

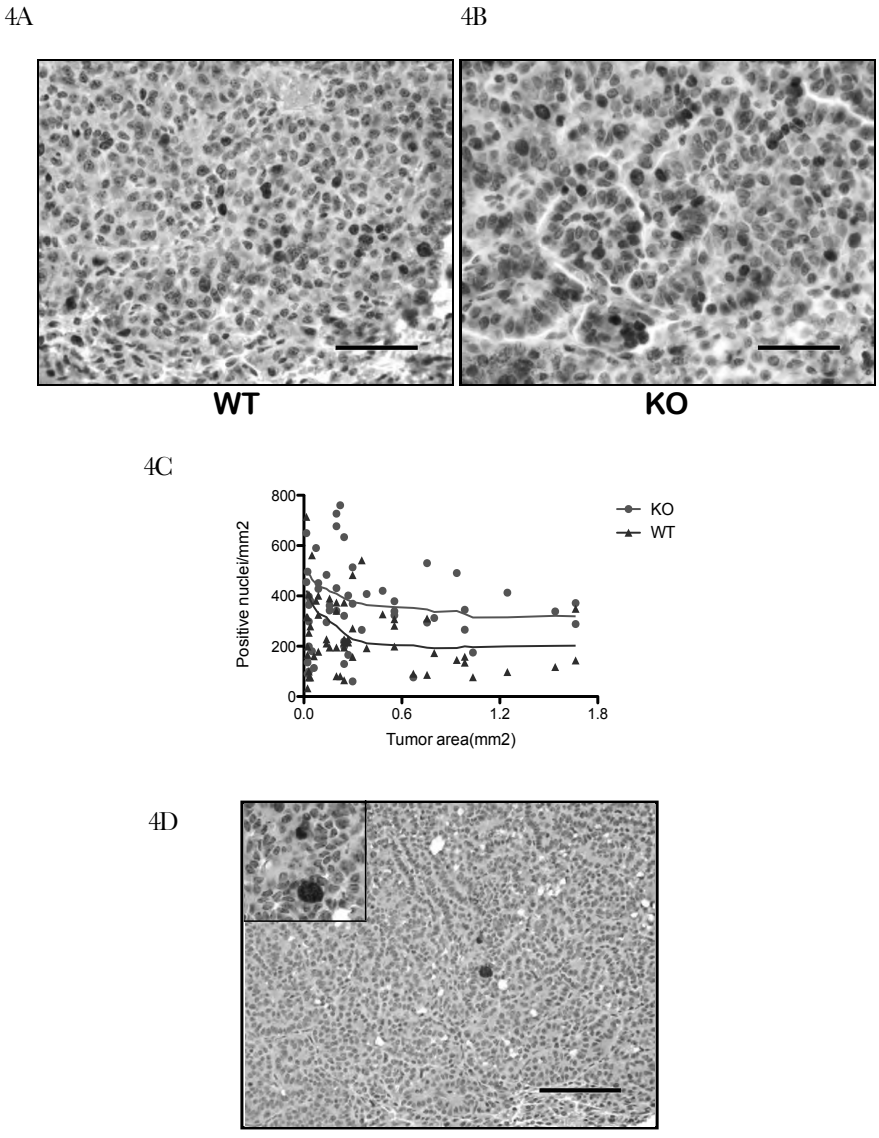
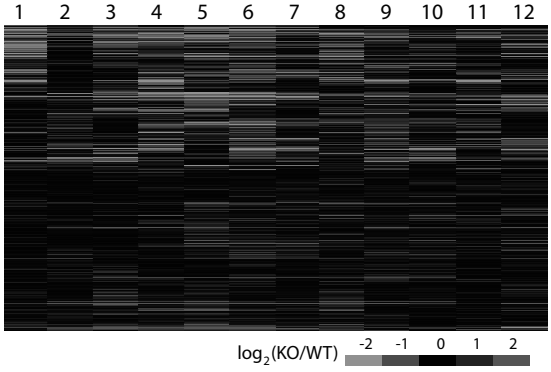


Figure 4. Dnmt3a deficient tumors have a higher proliferation index.
(*A, B*) Representative photomicrographs of immunohistochemical staining for proliferation marker Ki-67 in Dnmt3a WT (*A*) and KO mice (*B*). The positive signal localizes to the nuclei. (*C*) Plotting of proliferation index (positive nuclei/mm^2) against tumor size (mm^2). Dnmt3a deficient tumors had significantly higher proliferation index compared with WT tumors [p = 0.0006, proliferation index was also influenced by tumor size (p = 0.032)]. (*D*) A representative photomicrograph of immunohistochemical staining for apoptosis marker cleaved caspase-3, showing that positive cells are rare in both Dnmt3a deficient or WT tumors. The insert shows the details of two positive cells with cytoplasmatic staining. Scale bar, 50μm (*A, B*) and 200μm (*D*).

To examine whether Dnmt3a deficiency resulted in genomic methylation changes we performed an unbiased methylome analysis. Methylated DNA isolated from three Dnmt3a deficient and two WT tumors was immunoprecipitated and subjected to high throughput sequencing (MeDIP-Seq), producing a total of 101 million reads. Due to the CpG density dependency of MeDIP, we only analyzed relative methylation levels comparing the two exact same regions in Dnmt3a deficient and WT tumors. To identify differentially methylated regions (DMRs) we mapped the reads and scanned the genome in 1 kb windows with 100 bp overlap (see Materials and Methods). We observed that 99.6% of the DMRs were less methylated in the Dnmt3a deficient compared to the WT tumors (Table S4). Based on their overall distribution on the genome, a higher than expected percentage of less methylated regions (LMRs) overlapped gene bodies, particularly exons (Table S4). We further tested all genes and promoter regions for differential methylation (see methods) and observed that genes that were less expressed in Dnmt3a deficient tumors tended to have lower methylation levels in gene bodies (Figure S1A). In contrast, gene expression changes did not correlate with promoter methylation (Figure S1B). These results are consistent with a previous report showing a positive relation between gene body methylation and gene expression in Dnmt3a deficient neural cultures, suggesting that Dnmt3a mediated non-promoter methylation facilitates gene expression (28).

A previous study found methylation changes of repetitive sequences in established Dnmt3a mutant ES cells (29). We therefore performed Southern blot-based methylation assays to compare the DNA methylation status of minor satellite repeats and detected no obvious differences between Dnmt3a deficient and WT tumors (Figure S2). This suggests that Dnmt3a dependent methylation changes of these sequences become manifest only after extensive cell passage and cell proliferation.

5A



5B

Cluster 1. Enrichment Score: 3.50

Process	GO id	Count	PValue	Benjamini
blood vessel morphogenesis	GO:0048514	34	1.91E-04	0.114
blood vessel development	GO:0001568	39	2.74E-04	0.117
angiogenesis	GO:0001525	25	4.23E-04	0.126
vasculature development	GO:0001944	39	4.47E-04	0.122

Cluster 2. Enrichment Score: 3.01

Process	GO id	Count	PValue	Benjamini
cell adhesion	GO:0007155	75	1.58E-04	0.154
biological adhesion	GO:0022610	75	1.68E-04	0.125
cell-cell adhesion	GO:0016337	30	0.035	0.672

Cluster 3. Enrichment Score: 2.67

Process	Term	Count	PValue	Benjamini
regulation of cell motion	GO:0051270	22	2.94E-04	0.111
regulation of cell migration	GO:0030334	17	0.005	0.319
regulation of locomotion	GO:0040012	19	0.006	0.328

Cluster 4. Enrichment Score: 2.62

Process	GO id	Count	PValue	Benjamini
cellular macromolecule catabolic process	GO:0044265	77	7.07E-04	0.149
modification-dependent macromolecule catabolic process	GO:0043632	65	0.001	0.168
modification-dependent protein catabolic process	GO:0019941	65	0.001	0.168

Cluster 5. Enrichment Score: 2.41

Process	GO id	Count	PValue	Benjamini
regulation of body fluid levels	GO:0050878	19	5.34E-04	0.123
coagulation	GO:0050817	16	8.09E-04	0.149
blood coagulation	GO:0007596	16	8.09E-04	0.149

Cluster 6. Enrichment Score: 2.17

Process	GO id	Count	PValue	Benjamini
negative regulation of molecular function	GO:0044092	30	2.24E-06	0.007
negative regulation of catalytic activity	GO:0043086	23	4.20E-05	0.065
negative regulation of kinase activity	GO:0033673	14	3.04E-04	0.102

Figure 5. Gene expression in Dnmt3a deficient and WT tumor.
(*A*) Global expression profiling using whole mouse genome microarrays arrays. Heat map showing the top 1967 differentially expressed genes between Dnmt3a deficient and WT tumors (FDR<0.05). Columns represent 12 matched KO-WT tumor pairs and rows differentially expressed genes. (*B*) Gene enrichment analysis of the differentially expressed genes (FDR<0.05) by Functional Annotation Tool in DAVID using default thresholds. Shown are the top 6 clusters in biological process. Only the top 3 annotation terms are shown in Cluster 4 to 6.

Discussion

The role of DNA methylation in the *Apc*$^{min/+}$ model of intestinal tumors has been well established with Dnmt3b deletion protecting against tumorigenesis and over-expression increasing tumor load by *de novo* methylation and silencing of tumor suppressor genes (9, 10). In contrast, no role of Dnmt3a in *Apc*$^{min/+}$ tumor formation has been established. Here we find that deletion of *Dnmt3a* promotes tumor growth and progression but not tumor initiation suggesting that this gene, counter to expectation, acts like a tumor suppressor rather than like a cancer-promoting gene as has been shown for Dnmt3b. Expression of genes involved in processes such as angiogenesis, cell adhesion and cell motion were significantly altered in Dnmt3a deficient tumors, consistent with an effect on tumor growth and progression.

The methylation status of DNA may affect cancer by several mechanisms. (i) Global hypomethylation may increase genomic instability (3, 4). (ii) Hypermethylation of promoters can mediate tumor suppressor gene silencing (5, 9). However, the mechanism of how Dnmt3a affects gene expression and tumor formation is unclear. The genome of embryonic stem cells, in contrast to that of somatic cells, has methylated cytosine residues at non-CpG contexts, which has been suggested to be due to the activity of Dnmt3a (30). More recently, whole-genome profiles of DNA methylation at single base pair resolution of ES cells detected non-CpG methylation in gene bodies, which was positively correlated with gene expression (31). Another study demonstrated that Dnmt3a–dependent gene body methylation correlated with expression of genes involved in neural differentiation (28). Consistent with this observation we found that gene bodies were less methylated in Dnmt3a deficient tumors than in WT tumors, which also correlated with lower gene expression. Given that tumors in the mutant K-ras lung cancer model may arise from stem cells giving rise to more differentiated cells in the tumor (32), it is possible that Dnmt3a-dependent gene body methylation may be important for expression of genes that promote differentiation in a fraction of the tumor cells. Though mechanistic insights are lacking, our observation that Dnmt3a deficient mice harbored more poorly differentiated and more advanced tumors is consistent with the notion that Dnmt3a deficiency interferes with the differentiation process in tumor cells promoting the formation of less mature and more malignant tumors.

The majority of *DNMT3a* mutations found in myeloid neoplasm patients are missense mutations and some of the mutations have been shown or predicted to result in reduced translation (14-16). However, because nearly all patients are heterozygous for the mutant allele it is not clear whether the *DNMT3a* mutations have dominant negative effects or cause hemizygous insufficiency. In this context our data using a *Dnmt3a* null allele are of interest as they argue against the possibility that hemizygous insufficiency affects lung tumors.

Based on the well-established role of *de novo* DNA methylation mediated gene silencing in cancer, inhibitors of methyltransferases are being actively investigated (33) and two drugs, vidaza and decitabine, have been approved by the FDA for treatment of myelodysplastic syndrome (34, 35). Our data raise the possibility that such treatments, in addition to activating silenced tumor suppressor genes, may have the unintended consequence of inhibiting DNMT3A and thus affect its proposed tumor suppressor function. Therefore, it will be of great importance to elucidate the molecular mechanisms of how this gene affects cancer progression. The availability of a genetically defined experimental system will greatly facilitate these efforts.

Materials and methods

Animal models

K-ras$^{LSL\text{-}G12D}$ mice, which were kindly provided by Dr. Jacks (20) ,were crossed with the Dnmt3a conditional KO mice (21) to generate mice with the following genotypes: *K-ras*$^{LSL\text{-}G12D/WT}$*/Dnmt3a*$^{2lox/2lox}$*, K-ras*$^{LSL\text{-}G12D/WT}$*/Dnmt3a*$^{WT/WT}$ *and K-ras*$^{LSL\text{-}G12D/WT}$*/Dnmt3a*$^{2lox/WT}$. After Ad-Cre–mediated recombination, mice were generated with lung cells that carried the K-ras mutation and were homozygous (KO), heterozygous and WT at the *Dnmt3a* locus.

Ad-Cre (Gene Transfer Vector Core, University of Iowa) was delivered via intratracheal approach (22). Briefly, 8 to 12 weeks old mice were anesthetized by intra-peritoneal injection of avertin (~0.4mg/g body weight) and suspended on a bar of a mouse platform via their front teeth. A Fiber-lite Illuminator was used to illuminate the opening of the trachea and an IV catheter (22 gauge) was inserted into the trachea until the top of the catheter was near the mouse's front teeth, 60ul Ad-Cre ($5X10^7$ PFU in MEM containing 10mM $CaCl_2$) was directly delivered into the catheter. In some of the microarray studies we also used intranasal infection approach. The procedure was similar to the intratracheal approach except that the

virus was directly pipetted over the opening of one nostril of the mouse instead of delivering Ad-Cre via a catheter into the trachea.

Animal care was in accordance with institutional guidance and all animal studies were reviewed and approved by the Committee on Animal Care, Department of Comparative Medicine, MIT.

Mouse examination, tissue preparation and histological examination

For histological comparison, the mice were euthanized at weeks 8, 16 and 24 after Ad-Cre infection (number of mice analyzed: week 8, Dnmt3a WT=4, KO=4, 3 pairs were littermates; week 16, WT=5, KO=5, 4 littermate pairs; week 24, WT=4, KO=4, 3 littermates pairs). During dissection, each organ was carefully examined with particular attention to mediastinal structures, especially lymph nodes. Any abnormal tissue was sampled for histological examination. The lungs were fixed by infusion of 10% formalin. After infiltrated by paraffin, the left lobe was trisected and the other lobes were bisected, then they were embedded in paraffin, sectioned (4μm) and stained by hematoxylin and eosin (H&E). Thus, each mouse's lungs were represented by 11 sections that were used for histopatholological comparisons. In the survival study, mice were euthanized at end stage (mouse number: Dnmt3a WT=29, homozygous=23, heterozygous=24). Before tissue fixation, representative lung tumors and normal tissue were flash-frozen in liquid nitrogen and stored at -80°C for genomic DNA and RNA extraction. Tumor image was captured and analyzed by Pixel Link Capture SE (Pixel Link).

Immunohistochemistry

Immunohistochemistry was performed using Vectastain Elite ABC kit (Vector) following manufacturer's instruction, with primary antibodies: anti-Dnmt3a (sc-20703, 1:200 dilution, Santa Cruz), anti-Ki-67 (Clone TEC-3, 1:50 dilution, DakoCytomation), anti-Cleaved Caspase-3 (#9661, 1:1000 dilution. Cell Signaling). Dnmt3a staining was performed on 160 tumors in 6 Dnmt3a deficient mice and 170 tumors in 6 WT mice. Ki-67 and cleaved caspase-3 staining were performed on 57 Dnmt 3a deficient tumors from 2 mice and 69 WT tumors from 2 mice.

PCR and RT-PCR

We used regular PCR to detect Cre-medicated recombination in tumors of *Dnmt3a* KO mice. The sequences of the PCR primes were (see also Figure 1A): sense: 5'ggcttttcctcagacagtgg3'; antisense: 5' tcaatcatcacggggttaga3'. PCR program was 95˚C 2min, 95˚C 30sec-60˚C 30sec-72˚C 45sec, 30cycles; 72˚C 6min. Forty tumors with tumor diameter from 0.1cm to 0.4cm were tested from 9 *Dnmt3a* KO mice. Either genomic DNA (for big tumors) or tumor lyses (for small tumors) was used as template. Genomic DNA was isolated with AllPrep DNA/RNA mini kit (Qiagen). Lyses solution: proteinase K 0.5 mg/ml, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl2, 0.1 mg/ml gelation, 0.45% Nonidet P40, 0.45% Tween20.

The Dnmt 3a mRNA abundance was analyzed in 19 tumors in 4 KO mice and 20 tumors in 6 WT mice by qRT-PCR. Three pairs of qRT-PCR primers were designed to detect Dnmt3a mRNA using primers located 5' to the deletion (primer pair 1), within the deletion (primer pair 2) and 3' to the deletion (primer pair 3). The primer sequences are, primer pair 1: 5'ggagccaccagaagaagaga3', 5'gctctttctgggtttcttgg3'; primer pair 2: 5'cctgcaatgacctctccatt3', 5'cggccagtaccctcataaag3'; primer pair 3: 5'gaacgagaggacggagaaaa3', 5'tcctcctctgtggtttgctt3'. Total RNA was isolated with AllPrep DNA/RNA mini kit (Qiagen). First strand cDNA was synthesized using Superscript III First-Strand Synthesis Supermix (Invitrogen). Real time PCR was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems) using SYBR Green Master (Roche), following manufacturers' protocols. The expression data was analyzed by comparative C_T method.

Microarray

Total RNA was isolated from 12 matched Dnmt3a KO ~WT tumor pairs from eight pairs of Dnmt3a KO ~ WT mice. Each pair of mice shared at least one parent. These 12 pairs of tumors included six pairs of large tumors (> 0.4cm in diameter) and six pairs of small tumors (< 0.25 cm in diameter). The hybridization was performed on Agilent Whole Mouse Genome 4 x 44K Microarrays (Two-channel) by Whitehead Genome Technology Core.

The two-color microarray raw data were normalized across channels by loess (locally weighted scatterplot smoothing, using spot quality weights) and between arrays by quantile normalization of average intensities ("Aquantile") using Bioconductor. Following summarization of replicate probes by median, differential expression was assayed by moderated t-test and corrected for false discovery rate, as implemented by the limma package in Bioconductor.

For gene enrichment analysis, we used DAVID (the Database for Annotation, Visualization and Integrated Discovery)(26) to analyze the differentially expressed genes list between Dnmt 3a deficient and WT tumors (FDR <0.05).

DNA methylation assays

MeDIP-seq was performed on three Dnmt3a deficient and two WT tumors using the Magmedip kit (Diagenode) according to manufacturers' protocol. Libraries were sequenced on the genome analyzer II (Illumina). Data Analysis was similar to the one described by Bock et al. (36).

Details of MeDIP-Seq and Southern blot analysis were provided in *SI Materials and Methods.*

Statistical analysis

We used Prism 5 (GraphPad Software) to perform statistical analysis, with two-tailed Student's t Test for the comparison of tumor number, size, grade, growth pattern, invasion and fraction of tumor area; Kaplan-Meier survival analysis for comparison of lifespan; two-way ANOVA for comparison of proliferation and apoptosis indexes. Unless indicated otherwise, 0.05 was used as the p-value threshold for statistical significance.

Acknowledgements

We thank J Dausman, R Flannery, and K Ganz for maintenance of the mouse colony and F. Soldner, L Medeiros, G. Welstead, S. Sarkar, D. Faddah, Y. Buganim for helpful discussions and critical comments on the manuscript. We thank T. Jacks (MIT) for providing K-ras mutant mice, A. Cheung and A Dooley for technical assistance on intro-tracheal virus delivery. We also thank B. Yuan, R. Bronson and D. Crowley for advice in statistics or histopathology; D Cook, A Leshinski, and C Whittaker for running the Solexa pipeline; J Kwon, J Love and S Gupta for performing microarray hybridization. This work was supported by grants from Philip Morris International and from the NIH (RO1-CA087869).

References

1. Feinberg AP (2007) Phenotypic plasticity and the epigenetics of human disease. *Nature* 447(7143):433-440.
2. Jones PA & Baylin SB (2007) The epigenomics of cancer. *Cell* 128(4):683-692.
3. Gaudet F*, et al.* (2003) Induction of tumors in mice by genomic hypomethylation. *Science* 300(5618):489-492.
4. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, & Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. *Nature* 395(6697):89-93.
5. Sharma S, Kelly TK, & Jones PA (2010) Epigenetics in cancer. *Carcinogenesis* 31(1):27-36.
6. Okano M, Xie S, & Li E (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* 19(3):219-220.
7. Mizuno S*, et al.* (2001) Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood* 97(5):1172-1179.
8. Nosho K*, et al.* (2009) DNMT3B expression might contribute to CpG island methylator phenotype in colorectal cancer. *Clin Cancer Res* 15(11):3663-3671.
9. Linhart HG*, et al.* (2007) Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. *Genes Dev* 21(23):3110-3122.
10. Lin H*, et al.* (2006) Suppression of intestinal neoplasia by deletion of Dnmt3b. *Mol Cell Biol* 26(8):2976-2983.
11. Steine EJ*, et al.* (2011) Genes methylated by DNA methyltransferase 3b are similar in mouse intestine and human colon cancer. *J. Clin. Invest.* :doi:10.1172/JCI43169.
12. Robertson KD*, et al.* (1999) The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res* 27(11):2291-2298.
13. Deng T*, et al.* (2009) An essential role for DNA methyltransferase 3a in melanoma tumorigenesis. *Biochem Biophys Res Commun* 387(3):611-616.
14. Ley TJ*, et al.* (2010) DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* 363(25):2424-2433.
15. Yan XJ*, et al.* (2011) Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet* 43(4):309-315.
16. Walter MJ*, et al.* (2011) Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. *Leukemia* .
17. Jemal A, Siegel R, Xu J, & Ward E (2010) Cancer statistics, 2010. *CA Cancer J Clin* 60(5):277-300.
18. Brock MV*, et al.* (2008) DNA methylation markers and early recurrence in stage I lung cancer. *N Engl J Med* 358(11):1118-1128.
19. Belinsky SA (2004) Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer* 4(9):707-717.
20. Jackson EL*, et al.* (2001) Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev* 15(24):3243-3248.
21. Nguyen S, Meletis K, Fu D, Jhaveri S, & Jaenisch R (2007) Ablation of de novo DNA methyltransferase Dnmt3a in the nervous system leads to neuromuscular defects and shortened lifespan. *Dev Dyn* 236(6):1663-1676.
22. DuPage M, Dooley AL, & Jacks T (2009) Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protoc* 4(7):1064-1072.
23. Jackson EL*, et al.* (2005) The differential effects of mutant p53 alleles on advanced murine lung cancer. *Cancer Res* 65(22):10280-10288.
24. Nikitin AY*, et al.* (2004) Classification of proliferative pulmonary lesions of the mouse: recommendations of the mouse models of human cancers consortium. *Cancer Res* 64(7):2307-2316.
25. Renne R*, et al.* (2009) Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. *Toxicol Pathol* 37(7 Suppl):5S-73S.

26. Huang da W, Sherman BT, & Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44-57.
27. Talmadge JE & Fidler IJ (2010) AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res* 70(14):5649-5669.
28. Wu H*, et al.* (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. *Science* 329(5990):444-448.
29. Liang G*, et al.* (2002) Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Mol Cell Biol* 22(2):480-491.
30. Ramsahoye BH*, et al.* (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc Natl Acad Sci U S A* 97(10):5237-5242.
31. Lister R*, et al.* (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462(7271):315-322.
32. Kim CF*, et al.* (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 121(6):823-835.
33. Belinsky SA*, et al.* (2011) Combination therapy with vidaza and entinostat suppresses tumor growth and reprograms the epigenome in an orthotopic lung cancer model. *Cancer Res* 71(2):454-462.
34. Taby R & Issa JP (2010) Cancer epigenetics. *CA Cancer J Clin* 60(6):376-392.
35. Rodriguez-Paredes M & Esteller M (2011) Cancer epigenetics reaches mainstream oncology. *Nat Med* 17(3):330-339.
36. Bock C*, et al.* (2010) Quantitative comparison of genome-wide DNA methylation mapping technologies. *Nat Biotechnol* 28(10):1106-1114.

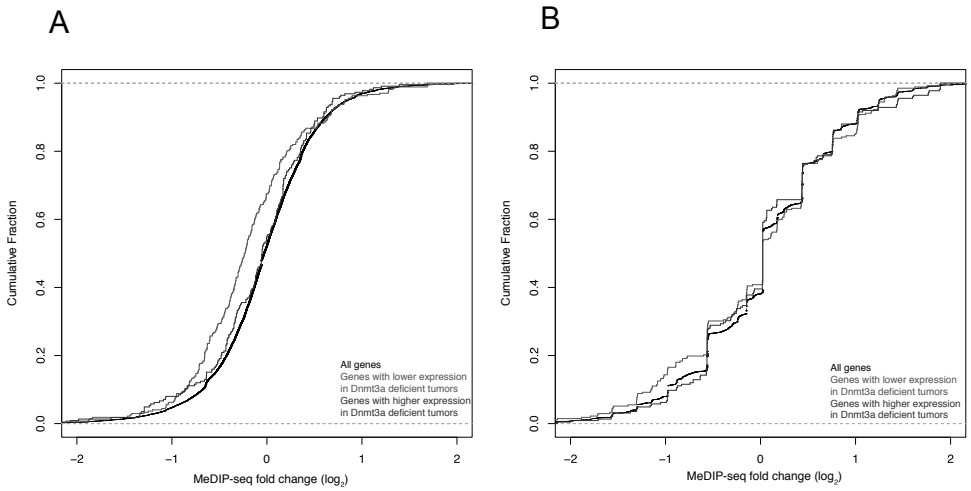


Figure S1. Genes with lower expression in the Dnmt3a-deficient tumors are less methylated in the gene body.

Shown are the cumulative distributions of methylation changes for different subsets of genes. Each line shows the percentage of genes (y axes) that have a log2 fold change (FC) (Dnmt3a-deficient/WT) of methylation lower than or equal to the value shown on the x axes. (A) Genes with lower expression (log2FC ≤ −0.7; red) in the Dnmt3a-deficient tumors are significantly less methylated in gene bodies (P = 1.54 °— 10−9, ks-test); genes with higher expression (log2FC ≥ 0.7; blue) show no significant difference in methylation (P = 0.32, ks-test); all genes on the array are shown in black. (B) Changes in gene expression are not associated with changes in methylation of promoter regions (1 kb upstream of TSS). The figure is otherwise as in A.

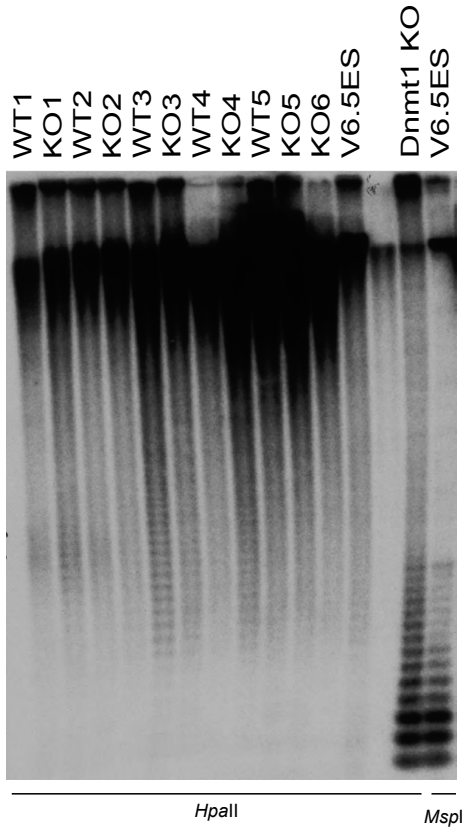


Figure S2. Dnmt3a-KO and WT tumors show no obvious difference in the methylation status of minor satellite repeats.
Southern blot analysis: genomic DNA from six Dnmt3a-KO and five WT tumors (KO1-6, WT1-5) were digested with HpaII and probed for minor satellite repeats (pMR150). Genomic DNA from Dnmt1-KO ES cells digested with HpaII and that from V6.5 ES cells digested with MspI were used as control.

Mouse	AH	AD
WT		
1	13	34
2	23	23
3	13	9
4	16	16
Subtotal	65	82
KO		
1	9	16
2	6	27
3	20	38
4	9	21
Subtotal	44	102

All tumors at week 8 are grade 1 and solid tumors. AD, adenoma/adenocarcinoma; AH, atypical adenomatous hyperplasia.

Table S1. Comparison of lesion number of Dnmt3a WT and KO mice at week 8 after Ad-Cre infection.

			Grade			Growth pattern	
Mouse	AH	AD	1	2	3	Solid	Papillary
WT							
1	46	58	57	1	0	58	0
2	21	118	112	6	0	116	2
3	91	137	135	2	0	137	0
4	25	99	97	2	0	99	0
5	15	46	46	0	0	46	0
Subtotal	198	458	447	11	0	456	2
KO							
1	33	118	61	40	17	111	7
2	19	170	138	29	3	156	14
3	38	142	111	29	2	141	1
4	10	106	86	19	1	101	5
5	20	68	44	24	0	67	1
Subtotal	120	604	440	141	23	576	28

AD, adenoma/adenocarcinoma; AH, atypical adenomatous hyperplasia.

Table S2. Comparison of lesion number of Dnmt3a WT and KO mice at week 16 after Ad-Cre infection.

		Grade			Growth Pattern	
Mouse	AD	1	2*	3*	Solid	Papillary*
WT						
1	299	279	18	2	298	1
2	239	225	12	2	237	2
3	266	244	19	3	264	2
4	182	165	17	0	181	1
Subtotal	986	913	66	7	980	6
KO						
1	219	113	84	22	182	37
2	150	58	58	34	106	44
3	140	61	41	38	117	23
4	176	97	53	26	160	16
Subtotal	685	329	236	120	565	120

AD, adenoma/adenocarcinoma.
*Statistically signi fi cant: grade 2 + 3, $P < 0.0001$; grade 3, $P = 0.0003$; papillary, $P = 0.0043$.

Table S3. Comparison of tumor number of Dnmt3a WT and KO mice at week 24 after Ad-Cre infection.

Region	Percentage of total regions tested	Percentage ofl ess methylated regions (4,382) in Dnmt3a KO tumors	Percentage of more methylated regions (19) in Dnmt3a KO tumors
Gene body	46.6	65.5	31.6
Intron	28.7	33.3	31.6
Coding exon	16.4	29.4	0.0
5 UTR	0.4	0.8	0.0
3 UTR	1.1	2.0	0.0
Promoter	1.2	1.8	5.3
Intergenic region	52.2	32.7	63.2

Table S4. Distribution of DMRs.

ADDENDUM CHAPTER 4

FUNCTION OF DNMT3A IN INTESTINAL CANCER

Eveline J. Steine & Rudolf Jaenisch

Summary

Dnmt3a acts as a tumor suppressor in lung cancer development; however, it is unknown if this is a general function of Dnmt3a in all tumor types. In this study, we have deleted the *Dnmt3a* gene in the intestinal stem cells of $Apc^{min/+}$ mice, which develop intestinal adenomas. The generation of this compound mouse model would allow us to examine the function of Dnmt3a in intestinal tumor progression, and to determine whether Dnmt3a suppresses tumorigenesis in cancer types other than lung cancer.

Introduction

Dnmt3a deletion in a lung cancer mouse model (*K-ras*$^{lox\text{-}stop\text{-}lox\text{-}G12D}$) results in bigger and more invasive tumors compared to *Dnmt3a* wild type tumors, suggesting that Dnmt3a suppresses lung cancer development [1]. Furthermore, when Dnmt3a was ectopically expressed in the intestine of *Apc*$^{min/+}$ mice no significant effect on intestinal tumor formation was observed, indicating that Dnmt3a does not promote tumorigenesis in the intestine [2]. In contrast, ectopic expression of Dnmt3b in *Apc*$^{min/+}$ mice promotes intestinal tumorigenesis [2]. These data suggest that Dnmt3a and Dnmt3b have distinct roles, where Dnmt3a functions as a tumor suppressor in lung cancer and Dnmt3b promotes tumorigenesis in the intestine. To further investigate whether Dnmt3a might suppress tumor development also for other types of cancer, we aim to assay the effects of Dnmt3a deficiency on progression of the intestinal tumors of *Apc*$^{min/+}$ mice.

Experimental design

To study the role of Dnmt3a in intestinal tumor progression, we used the *Apc*$^{min/+}$ mouse model (Chapter 2). When intestinal cells of these mice spontaneously lose their wild type *Apc* allele, they develop microadenomas which may grow out to adenomas [3]. The intestinal adenomas in *Apc*$^{min/+}$ mice arise from intestinal stem cells [4], which are marked by leucine-rich-repeat-containing G protein-coupled receptor 5 (Lrg5) [5]. In order to study the function of Dnmt3a in intestinal tumorigenesis, *Dnmt3a* will be deleted in the intestinal stem cells by Cre-recombinase expressed under control of the *Lrg5* promoter (*Lrg5-EGFP-IRES-creER*^{T2}) [5]. *Dnmt3a* deletion should occur before or at the same time the cell loses the wild type *Apc* allele and develops a microadenoma [6]. To assay the Cre-recombinase efficiency in our mice, we will utilize the Cre-activatable Rosa26-lacZ reporter [7]. LacZ is expressed from this allele when the stop-cassette in front of the *LacZ* gene is deleted by Cre-recombinase. The tumors of *Apc*$^{min/+}$, *Dnmt3a 2lox/2lox, Lrg5-EGFP-IRES-creER*^{T2}*, Rosa26R LacZ* mice will be analyzed and compared to the tumors of *Apc*$^{min/+}$*, Lrg5-EGFP-IRES-creER*^{T2}*, Rosa26R LacZ* mice (Figure 4.1).

Dnmt3a deficiency in lung tumors of the *K-ras*$^{lox\text{-}stop\text{-}lox\text{-}G12D}$ mice reduced survival of these mice, as compared to *K-ras*$^{lox\text{-}stop\text{-}lox\text{-}G12D}$ mice carrying wild type *Dnmt3a* alleles [1]. We hypothesized that Dnmt3a deletion would have a similar effect on *Apc*$^{min/+}$ mice, Consequently, the life span of *Dnmt3a* deficient *Apc*$^{min/+}$ mice might be considerably

shortened, as compared to the life span of *Apc$^{min/+}$* mice. *Apc$^{min/+}$* mice typically die at an age of 4-5 months due to tumor development [6]. In order to compare the tumors of the Dnmt3a deficient and Dnmt3a wild type mice, both on the *Apc$^{min/+}$* background and of the same age, the colon will be dissected and analyzed at 3 months. If Dnmt3a deficient mice have a more severe tumor phenotype (such as larger tumors or more invasive tumors), we will perform additional experiments to examine the role of Dnmt3a in intestinal cancer in more detail.

Progress

We have bred the mice to obtain *Apc$^{min/+}$, Dnmt3a 2lox/2lox, Lrg5-EGFP-IRES-creER^{T2}, Rosa26R LacZ* mice for this tumor study. In order to delete *Dnmt3a* prior the loss of the wild type *Apc* allele, we decided to treat two-week-old mice with tamoxifen, which is significantly earlier than the *Lgr5-EGFP-IRES-creER^{T2}* mice were treated in previous studies. In published protocols, 6-8 week old *Lrg5-EGFP-IRES-CreER* mice were injected intraperitoneally (IP) with 100 ul of 20 mg/ml tamoxifen for 7 consecutive days, which is close to the maximum dose for adult mice (personal communication Pekka Katajisto and Omer Yilmaz, laboratory of David Sabatini). However, this protocol is not tolerated by two-week-old mice. Therefore, we are currently determining the optimal treatment with tamoxifen to induce *Dnmt3a* deletion in younger mice. We adapted the protocol to subcutaneous injections, since the tamoxifen will be absorbed in the body more slowly. Further, we are injecting the mice every other day for a total of 4 injections. Additionally, we are testing whether 50 ul of a 40 mg/ml solution is better tolerated than 100 ul of a 20 mg/ml solution to improve survival. Since tamoxifen treatment dehydrates mice, the mice are supplemented with IP saline injections every day during the treatment period. We will assay the Cre-recombinase-inducing efficiency of the different injection conditions using the LacZ reporter, to determine optimal tamoxifen treatment for deletion of the gene. Once a protocol for tamoxifen administration in two-week-old mice is established, we will begin our analysis of the function of Dnmt3a in intestinal tumors.

We hypothesize that the Dnmt3a deficient *Apc$^{min/+}$* mice will develop a more severe phenotype than *Apc$^{min/+}$* mice with wild type Dnmt3a expression, resulting in a shorter survival time. This phenotype may include more tumors, larger tumors and more invasive tumors. If this is observed, the tumors will be dissected to analyze the gene expression and DNA methylation changes compared to tumors from *Dnmt3a* wild type mice. The results of this analysis should shed more light on the role of Dnmt3a in tumorigenesis.

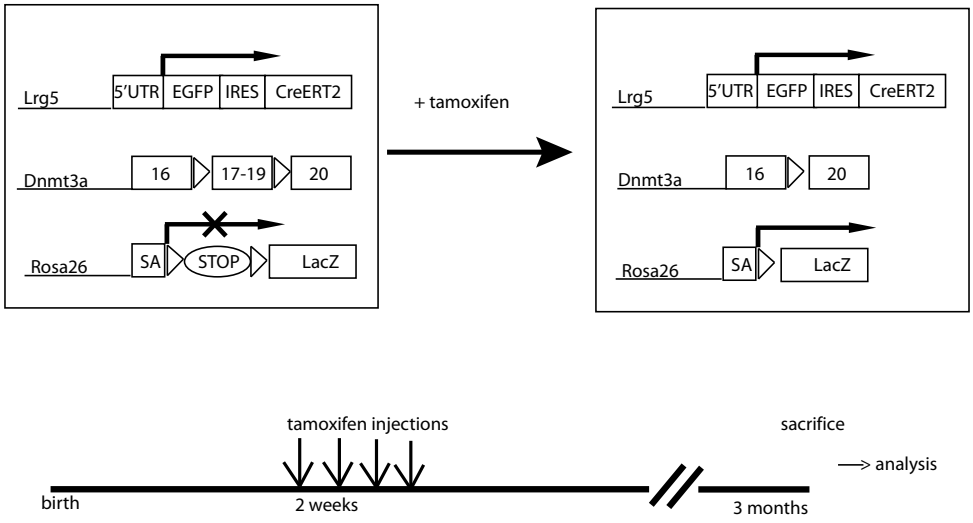


Figure 4.1. Schematic representation of genetic strategy (top) and experimental outline (bottom). Schematic representation of the genetic strategy to delete *Dnmt3a* and the reporter gene LacZ in the intestinal stem cell. The tamoxifen dependent Cre-recombinase is expressed under control of the *Lrg5* promoter. Exon 17-19 of *Dnmt3a* and the stop cassette in front of LacZ reporter gene under Rosa26 control are excised upon tamoxifen treatment.
Experimental outline. Tamoxifen (arrow) will be given subcutaneously every other day for 4 times in total to two-week-old mice. Animals will be sacrificed and analyzed at 3 months of age.

Acknowledgements

We thank R. Flannery, and K. Ganz for maintenance of the mouse facility. We thank David Sabatini for providing the *Lrg5-EGFP-IRES-CreER* mouse and Pekka Katajisto, Omer Yilmaz and Alejo Efeyan for helpful discussions.

References

1 Gao, Q. *et al.* Deletion of the de novo DNA methyltransferase Dnmt3a promotes lung tumor progression. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 18061-18066, doi:10.1073/pnas.1114946108 (2011).

2 Linhart, H. G. *et al.* Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. *Genes & development* **21**, 3110-3122, doi:10.1101/gad.1594007 (2007).

3 Moser, A. R., Pitot, H. C. & Dove, W. F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science (New York, NY)* **247**, 322-324 (1990).

4 Barker, N. *et al.* Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* **457**, 608-611, doi:10.1038/nature07602 (2009).

5 Barker, N. *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**, 1003-1007, doi:10.1038/nature06196 (2007).

6 Moser, A. R. *et al.* ApcMin, a mutation in the murine Apc gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 8977-8981 (1993).

7 Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nature genetics* **21**, 70-71, doi:10.1038/5007 (1999).

CHAPTER 5

GENERAL DISCUSSION

5. General discussion

5.1 Overview of our findings

The expression and activities of maintenance and *de novo* DNA methyltransferases are frequently deregulated in cancer cells. As a consequence, the epigenome of cancer cells is often globally hypomethylated while specific promoter regions are hypermethylated. Previous studies have shown that lack of maintenance methyltransferase Dnmt1 both suppresses and promotes tumorigenesis, as lack of this enzymatic activity, hence a reduced level of DNA methylation, correlates with higher expression of tumor suppressor genes, but also with increased genome instability and possibly with a higher expression of oncogenes in specific tissues [1-4]. As described in the present thesis, we investigated the function of *de novo* methyltransferases Dnmt3a and Dnmt3b in tumorigenesis. To do this, we performed loss- and gain-of-function studies for these *de novo* methyltransferases using lung and colon cancer mouse models. $Apc^{min/+}$ mice ectopically expressing Dnmt3b developed more and larger intestinal tumors, whereas ectopic expression of Dnmt3a did not promote tumorigenesis, in this mouse model [5]. These results indicate that only Dnmt3b promotes intestinal tumorigenesis. $K\text{-}ras^{lox\text{-}stop\text{-}lox\text{-}G12D}$ mice deficient for Dnmt3a in the lung developed larger and more invasive lung tumors than tumors found in Dnmt3b deficient or wild type Dnmt mice [6]. These results suggest that Dnmt3a suppresses lung tumorigenesis. In conclusion, *de novo* methyltransferases have distinct roles in these tumor types; Dnmt3b promotes intestinal tumorigenesis and Dnmt3a suppresses lung tumorigenesis.

To investigate whether ectopic Dnmt3b expression promotes intestinal tumor formation as a result of clonal outgrowth of cells with randomly methylated loci, or might be due to methylation of specific genes, we analyzed the DNA methylation of a few loci [5]. We observed that the same loci are methylated in the intestinal tumor as in the intestinal mucosa, when Dnmt3b was ectopically expressed, indicating that the Dnmt3b mediated DNA methylation is loci specific. By analyzing more loci in tumors and mucosa of $Apc^{min/+}$ and *Apc* wild type mice, we showed that Dnmt3b methylation is specific to certain loci and that the genetic background of the mice does not influence DNA methylation [7]. Further, we found that upon ectopic expression of Dnmt3b the same loci in the mouse intestine become methylated as in human colon cancer. These results indicate that DNA methylation sensitive loci are conserved across species and that our mouse model provides a great tool to study Dnmt3b mediated methylation [7].

To investigate the mechanism that regulates the target specificity of Dnmt3b, we analyzed DNA methylation data and H3K27me3 and H3K4me3 ChIP-seq data in liver and small intestinal epithelial cells. We observed that most loci that are DNA methylated upon ectopic Dnmt3b expression already had H3K27me3 modification prior to Dnmt3b mediated DNA methylation, suggesting that H3K27me3 might attract Dnmt3b to its targets. However, not all loci had H3K27me3 before DNA methylation, thus additional histone modifications or factors may direct Dnmt3b to specific loci.

Since it is known that genes are silenced when their promoter is methylated, we examined the gene expression profiles of liver and small intestinal epithelial cells before and after ectopic Dnmt3b expression. We observed that most loci methylated upon ectopic Dnmt3b expression were already expressed at low levels preceding DNA methylation. This could potentially be explained by the fact that most loci carried H3K27me3, a histone modification associated with gene silencing.

Additionally, it remains to be determined whether the genes, that are silenced upon ectopic Dnmt3b expression have some common characteristics; i.e. to answer the question if these genes belong to the same functional group. Further research will be needed to resolve these questions.

5.2 Specificity of *de novo* DNA methylation by Dnmt3b

One of the challenges in this evolving field concerns the question how Dnmt3b's loci specificity is regulated. In order to unravel the mechanism regulating Dnmt3b loci specificity, it would be relevant to study components and aspects related to chromatin, such as histone modifications, positioning of nucleosomes, and interaction with the nuclear lamina. It is known that DNA methylation is influenced by histone modifications, as H3K27me3, H3K9me2, H3K9me3 and unmethylated H3K4 have been shown to positively correlate with DNA methylation [8-13]. Indeed, we also observed a positive correlation between H3K27me3 and DNA methylation in our analysis. It would be possible to generate genome maps of different histone modifications in samples that are induced for ectopic Dnmt3b expression, compared to control samples. These maps could then be analyzed in combination with our Reduced representation bisulfite sequencing (RRBS) data, which would yield information about whether Dnmt3b-mediated DNA methylation correlates with any specific type of histone modification. The limitation of our obtained RRBS data is that yields only information of 5% of the total cpG, which included 90% of the CpG islands. As an alternative for using the RRBS data, ectopic Dnmt3b expressing and control samples could be analyzed by genome

wide bisulfite sequencing to obtain information about the DNA methylation covering the entire genome. These genome wide maps might provide better insight into whether histone modifications direct Dnmt3b to the loci it methylates. Further, it has been shown that Dnmt3a and Dnmt3b preferentially bind to nucleosomes which are associated with methylated CpG islands and DNA repetitive elements [14]. Thus, one could assess whether and how aspects of nucleosome dynamics influence Dnmt3b methylation. In addition, specific regions of chromatin are organized by the nuclear lamina, which is a fibrillar structure composed of lamins and lamin-associated proteins, located near the inner nuclear membrane and the peripheral chromatin. It has recently been reported that, in colon cancer cells, DNA hypermethylation occurs in long stretches of hypomethylated regions which are associated with the nuclear lamina [15]. From this, it can be suggested that the nuclear lamina might be involved in Dnmt3b targeting. To investigate this, maps of nuclear lamina interactions with the chromatin could be generated with DamID (DNA adenine methyltransferase identification) to Lamin B [16], and overlapped with DNA methylation data. Taken together, investigation of all these chromatin-related components and dynamic aspects may potentially clarify the mechanism, which directs Dnmt3b to specific loci.

The mechanism of Dnmt3b loci specificity could also be addressed by investigating whether Dnmt3b has interaction partners that guide Dnmt3b to its DNA methylation targets. For example, a yeast two-hybrid assay could be performed to screen for possible interaction partners of Dnmt3b. However, this method usually yields many false positives. In an alternative approach, interacting proteins could be identified by immunoprecipitating Dnmt3b and potential interacting proteins followed by mass spectrometry analysis. In order to immunoprecipitate Dnmt3b it is crucial to generate a good Dnmt3b antibody, as it is not commercially available. Alternatively, one could tag Dnmt3b and immunoprecipitate Dnmt3b by using an antibody against its tag. Once potential interacting proteins are identified, further studies should verify the putative binding partners and investigate whether they determine which loci Dnmt3b methylates. Additionally, RRBS data or preferentially genome wide bisulfite sequencing data could be examined for enrichment for DNA methylated sequence motifs. The obtained motifs could be compared to DNA binding motifs of transcription factors, and thus it could suggest whether Dnmt3b is directed by certain transcription factors. Last, it has been reported for plants that siRNAs guide DNA methyltransferases to DNA [17]. Such a mechanism might also implicate small RNAs in guiding Dnmts to specific loci in mammalian cells. One could perform a RIP-Seq (RNA immunoprecipitation-sequencing) to investigate whether small RNAs interact with Dnmt3b. If an interaction is detected, one

could analyze whether the complementary sequences of the respective small RNAs are methylated in the genome. These experiments should shed more light on the mechanism that directs Dnmt3b to the specific loci.

5.3 More on the role of Dnmts in cancer

In our studies, Dnmt3a was found to suppress lung tumorigenesis [6]. However, whether Dnmt3a plays a tumor suppressive role also in other types of cancer remains to be determined. A recent report that 20% of the acute monocytic leukemia (AML) patients harbor a mutation in the *DNMT3A* gene suggests that Dnmt3a may act as a tumor suppressor also in other cancers [18,19]. On the other hand, in many human cancer types increased DNMT3A expression has been detected [20-24]. It is however unclear whether the increased DNMT3A protein levels have a function in human tumorigenesis. While we demonstrated that ectopic Dnmt3a expression in the *Apc$^{min/+}$* mouse does not promote intestinal tumorigenesis [5], Samuel et al. (2011) reported that ectopic Dnmt3a expression promotes intestinal tumorigenesis [25]. The different transgenic systems used in the studies may explain the contradicting results. In our study, we induced Dnmt3a in the mice at 4-6 weeks of age by using the tet(O) system, with the rtTA in the Rosa26 locus [26]. Using this system Dnmt3a is ectopically expressed in most tissues at 4-6 weeks, but prior to induction Dnmt3a is normally expressed. In the Samuel et al. (2011) study, *Dnmt3a* was knocked-in the *A33* locus [25]. A33 glycoprotein is expressed in the ICM of the blastocyst and in intestinal epithelium. Consequently, Dnmt3a is ectopic expressed earlier in their study than in ours. Of note, *Dnmt3b* knocked-in the *A33* locus was embryonic lethal [25]. To further elucidate the function of Dnmt3a in intestinal cancer development, we are currently investigating the role of *Dnmt3a* deletion in the intestine of *Apc$^{min/+}$* mice.

Our analysis of the DNA methylation and gene expression patterns in lung tumors did not reveal the mechanism for Dnmt3a tumor suppression [6]. To unravel the mechanism of the suppressive function of Dnmt3a, one could analyze the lung tumors using genome wide bisulfite sequencing in order to find aberrantly methylated loci in Dnmt3a deficient tumors. These loci may identify a tumor suppressor gene or a group of genes, which would help to explain how Dnmt3a suppresses lung tumorigenesis. Additionally, if Dnmt3a suppresses intestinal tumorigenesis, one could also examine these tumors using whole genome bisulfite sequencing. Identification of changes in DNA methylation in the same regions in the intestinal and lung cancer tumors might give new insights into the role of Dnmt3a in tumorigenesis.

5.4 DNA methylation as target to treat cancer

Since DNA methylation is reversible, there is a huge opportunity to improve cancer treatment by targeting this type of epigenetic modification or the proteins catalyzing DNA methylation. We observed that Dnmt3b expression promotes intestinal cancer progression and that ectopic Dnmt3b expression induces methylation of the same genes that are known to be methylated in human intestinal tumors [5,7]. Vidaza and Decitabine are demethylating drugs currently used to treat patients suffering from blood disorders [27,28]. However, these drugs are rather inefficient in penetrating solid tumors and cause significant hematological and gastrointestinal side effects. In addition, one potential problem with these compounds is that demethylating drugs act genome wide, and might cause genome instability or imbalanced gene expression. The observation that Dnmt3a suppresses lung cancer development suggests that global demethylation could lead to tumor progression rather than inhibition [6]. Consequently, it may be better to develop drugs that selectively target DNMT3b and DNMT1 rather than global DNA methylation. Since Dnmt1 has been shown to promote and inhibit tumor formation [1,3,4], drugs against DNMT1 should specifically target tumor tissue, since global demethylation in other tissues may favor tumor progression. Therefore, DNMT3b promises to be the best target for cancer treatment, since Dnmt3b promotes intestinal cancer tumorigenesis and is aberrantly expressed in many types of cancer cells, but Dnmt3b has no significant role in somatic tissues [20-24,29]. Further, no tumors were observed when Dnmt3b was deleted in the intestine or in the lungs of mice [5,6], suggesting that loss of Dnmt3b does not lead to other complications. Taken together, it appears a matter of urgency to promote further research, which aims to elucidate the role of DNA methylation and DNA methyltransferases in tumorigenesis, as it has a promising perspective to improve cancer treatment.

References

1 Eden, A., Gaudet, F., Waghmare, A. & Jaenisch, R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science (New York, NY)* **300**, 455, doi:10.1126/science.1083557 (2003).

2 Howard, G., Eiges, R., Gaudet, F., Jaenisch, R. & Eden, A. Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. *Oncogene* **27**, 404-408, doi:10.1038/sj.onc.1210631 (2008).

3 Gaudet, F. *et al.* Induction of tumors in mice by genomic hypomethylation. *Science (New York, NY)* **300**, 489-492, doi:10.1126/science.1083558 (2003).

4 Yamada, Y. *et al.* Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 13580-13585, doi:10.1073/pnas.0506612102 (2005).

5 Linhart, H. G. *et al.* Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. *Genes & development* **21**, 3110-3122, doi:10.1101/gad.1594007 (2007).

6 Gao, Q. *et al.* Deletion of the de novo DNA methyltransferase Dnmt3a promotes lung tumor progression. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 18061-18066, doi:10.1073/pnas.1114946108 (2011).

7 Steine, E. J. *et al.* Genes methylated by DNA methyltransferase 3b are similar in mouse intestine and human colon cancer. *The Journal of clinical investigation* **121**, 1748-1752, doi:10.1172/JCI43169 (2011).

8 Edwards, J. R. *et al.* Chromatin and sequence features that define the fine and gross structure of genomic methylation patterns. *Genome research* **20**, 972-980, doi:10.1101/gr.101535.109 (2010).

9 Meissner, A. *et al.* Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* **454**, 766-770, doi:10.1038/nature07107 (2008).

10 Weber, M. *et al.* Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature genetics* **39**, 457-466, doi:10.1038/ng1990 (2007).

11 Wu, S. C. & Zhang, Y. Active DNA demethylation: many roads lead to Rome. *Nature reviews Molecular cell biology* **11**, 607-620, doi:10.1038/nrm2950 (2010).

12 Delaval, K. *et al.* Differential histone modifications mark mouse imprinting control regions during spermatogenesis. *The EMBO Journal* **26**, 720-729, doi:10.1038/sj.emboj.7601513 (2007).

13 Viré, E. *et al.* The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439**, 871-874, doi:10.1038/nature04431 (2006).

14 Jeong, S. *et al.* Selective anchoring of DNA methyltransferases 3A and 3B to nucleosomes containing methylated DNA. *Molecular and cellular biology* **29**, 5366-5376, doi:10.1128/MCB.00484-09 (2009).

15 Berman, B. P. *et al.* Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina–associated domains. *Nature genetics*, doi:10.1038/ng.969 (2011).

16 Guelen, L. *et al.* Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions. *Nature* **453**, 948-951, doi:10.1038/nature06947 (2008).

17 Chinnusamy, V. & Zhu, J.-K. RNA-directed DNA methylation and demethylation in plants. *Science in China. Series C, Life sciences / Chinese Academy of Sciences* **52**, 331-343, doi:10.1007/s11427-009-0052-1 (2009).

18 Ley, T. J. *et al.* DNMT3A mutations in acute myeloid leukemia. *The New England journal of medicine* **363**, 2424-2433, doi:10.1056/NEJMoa1005143 (2010).

19 Yan, X.-J. *et al.* Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nature genetics* **43**, 309-315, doi:10.1038/ng.788 (2011).

20 Mizuno, S. *et al.* Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood* **97**, 1172-1179 (2001).
21 Robertson, K. D. *et al.* The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic acids research* **27**, 2291-2298 (1999).
22 Xie, S. *et al.* Cloning, expression and chromosome locations of the human DNMT3 gene family. *Gene* **236**, 87-95 (1999).
23 Xiong, Y. *et al.* Opposite alterations of DNA methyltransferase gene expression in endometrioid and serous endometrial cancers. *Gynecologic Oncology* **96**, 601-609, doi:10.1016/j.ygyno.2004.11.047 (2005).
24 Miyake, T., Endo, K., Honjo, S., Hirooka, Y. & Ikeguchi, M. Expression of DNA Methyltransferase (DNMT) 1, 3a and 3b Proteins in Human Hepatocellular Carcinoma. *Yonago Acta medica*, 1-7 (2010).
25 Samuel, M. S. *et al.* Elevated Dnmt3a Activity Promotes Polyposis in ApcMin Mice by Relaxing Extracellular Restraints on Wnt Signaling. *YGAST* **137**, 902-913.e911, doi:10.1053/j.gastro.2009.05.042 (2011).
26 Lin, H. *et al.* Suppression of intestinal neoplasia by deletion of Dnmt3b. *Molecular and cellular biology* **26**, 2976-2983, doi:10.1128/MCB.26.8.2976-2983.2006 (2006).
27 Gore, S. D., Jones, C. & Kirkpatrick, P. in *Nature Reviews Drug Discovery* Vol. 5 891-892 (2006).
28 Kaminskas, E. *et al.* Approval summary: azacitidine for treatment of myelodysplastic syndrome subtypes. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 3604-3608, doi:10.1158/1078-0432.CCR-04-2135 (2005).
29 Jin, F. *et al.* Up-regulation of DNA methyltransferase 3B expression in endometrial cancers. *Gynecologic Oncology* **96**, 531-538, doi:10.1016/j.ygyno.2004.10.039 (2005).

SUMMARY

The human body contains many different cell types with different characteristics, but all cells contain the same genetic code so called DNA. Gene expression is regulated by the mode of packaging of the DNA, which differs between different cell types and ensures expression of tissue specific genes. Certain parts of the DNA in a cell are condensed and inaccessible for the transcription machinery, whereas other parts of the DNA are accessible for the transcription machinery, resulting in gene expression. The packaging of the DNA is controlled by epigenetic modifications, which are heritable through cell divisions, but do not alter the base sequence of the DNA. These modifications ensure that a daughter cell receives developmental information from its mother cell, and it is known that these modifications are important for normal development. In turn, dysregulation of these epigenetic additions may lead to all kind of problems for cells and tissues, including uncontrolled cell divisions and cancer. Several modifications of DNA have been well described, but additional knowledge is necessary to understand how they contribute to health and disease; this thesis focuses on *de novo* methyltransferases Dnmt3a and Dnmt3b and their role in cancer development.

DNA methylation describes the addition of a methyl (CH3) group to the DNA, which in most cases renders DNA inaccessible for gene expression. DNA methyltransferases (Dnmts) are responsible for DNA methylation. Dnmts can be subdivided in 1) maintenance DNA methyltransferase that copies the DNA methylation pattern of the mother DNA strand to the daughter DNA strand, and 2) *de novo* methyltransferases that add a methyl group to DNA that has not been methylated before.

In the experiments described in this thesis. we showed that ectopic expression of Dnmt3b causes more and larger intestinal tumors in a mouse model for colon cancer, and that increased Dnmt3b expression is a cause for tumor development, rather than a consequence. We further demonstrated that Dnmt3b methylated specific DNA loci and that this methylation pattern is established before the tumor develops. Thus, upon ectopic Dnmt3b expression, DNA methylation is induced at specific loci rather than at random loci. We subsequently extended our analysis in order to study the DNA methylation of more genes. We demonstrated that mouse homologs of genes that are found to be methylated in human colon cancer are also methylated in our mouse model. We further observed that genes once methylated remain methylated even when Dnmt3b induction has been terminated. These data indicate that epimutations may play a similar role in cancer development as mutations of the DNA.

We next investigated the role of Dnmt3a in lung cancer, by deletion of Dnmt3a in a lung cancer mouse model. We observed that lung tumors deficient for *Dnmt3a* were larger and more invasive than the lung tumors from mice wild type for *Dnmt3a*. Our data indicate that *de novo* methyltransferase Dnmt3a acts as a tumor suppressor in lung cancer development. Currently, we are investigating whether Dnmt3a has a similar role in intestinal tumorigenesis.

Since DNA methylation is a reversible modification, it is important to investigate the role of DNA methylation in cancer in more detail. The goal is try to develop novel tools and compounds that can be used to for diagnosis and treatment of cancer.

SAMENVATTING

Het menselijk lichaam is opgebouwd uit veel verschillende celtypes met verschillende eigenschappen, maar alle cellen bevatten dezelfde genetische code in de vorm van DNA. Genexpressie is gereguleerd door de uiteenlopende manier van het verpakken van het DNA in verschillende celtypes. Dit verzekert de expressie van weefsel-specifieke genen. Bepaalde delen van het DNA in een cel zijn gecondenseerd en niet toegankelijk voor het transcriptie-apparaat, terwijl andere delen van het DNA wel toegankelijk zijn voor het transcriptie-apparaat, wat resulteert in genexpressie. De verpakking van het DNA is gereguleerd door epigenetische modificaties, die overgeërfd worden door verschillende celdelingen, maar niet gebaseerd zijn op een verandering van de volgorde van de baseparen in het DNA. Deze modificaties zorgen ervoor dat de dochtercel de ontwikkelingsinformatie van haar moedercel ontvangt. Aangezien dit belangrijk is voor normale ontwikkeling, kan misregulatie van deze epigenetische toevoegingen leiden tot allerlei verschillende problemen voor de cellen en weefsels, inclusief ongecontroleerde celdeling en kanker. Verscheidene modificaties van het DNA zijn goed beschreven, maar meer kennis is noodzakelijk om te begrijpen hoe deze modificaties bijdragen aan gezondheid en ziekte. Dit proefschrift is gericht op de *de novo* methyltransferases Dnmt3a en Dnmt3b en hun rol in kanker-ontwikkeling.

DNA methylatie beschrijft de toevoeging van een methylgroep aan het DNA, dat in de meeste gevalllen het DNA ontoegankelijk maakt voor genexpressie. DNA methyltransferases (Dnmts) zijn verantwoordelijk voor DNA methylatie. Dnmts kunnen worden onderverdeeld in 1) onderhouds DNA methyltransferase, dat het DNA methylatiepatroon van de moeder DNA keten op de dochter DNA keten kopieert, en 2) de *de novo* methyltransferases, die een methylgroep toevoegen aan DNA wat nog niet gemethyleerd was.

In de experimenten beschreven in dit proefschrift hebben we laten zien dat verhoogde expressie van Dnmt3b in een muismodel voor dikke darmkanker meer en grotere darmtumoren veroorzaakt, en dat de verhoogde Dnmt3b expressie eerder een oorzaak dan een gevolg van kankerontwikkeling is. Bovendien konden we aantonen dat Dnmt3b specifieke DNA loci methyleert en dat dit methylatiepatroon tot stand wordt gebracht voordat een tumor zich ontwikkelt. Dus, als Dnmt3b verhoogd aanwezig is, wordt DNA methylatie geïnitieerd op specifieke loci in plaats van random loci. Vervolgens hebben we onze analyse uitgebreid, zodat we de DNA methylatie van meer genen konden bestuderen. We lieten zien dat de muis-homologen van de genen die in dikke darmkanker van mensen gemethyleerd zijn, ook gemethyleerd zijn in ons muismodel. We observeerden verder dat

als de genen éénmaal gemethyleerd zijn, ze gemethyleerd blijven zelfs als de verhoogde Dnmt3b expressie wordt afgebroken. Deze data wijzen er op dat epimutaties (epigenetische mutaties) een belangrijke rol in kankerontwikkeling kunnen spelen, vergelijkbaar met het effect van DNA mutaties.

Vervolgens onderzochten we de rol van Dnmt3a in longkanker door middel van het verwijderen van Dnmt3a in een longkanker muismodel. We vonden dat longtumoren deficiënt voor Dnmt3a groter en invasiever waren dan de longtumoren van muizen met wild type Dnmt3a. Onze data duiden aan dat *de novo* methyltransferase Dnmt3a als een tumor suppressor functioneert in longkanker ontwikkeling. Op het moment van het schrijven van dit proefschrift, betrof lopend onderzoek de vraag of Dnmt3a een vergelijkbare rol heeft bij dikke darmkankerontwikkeling.

Aangezien DNA methylatie een reversibele modificatie is, is het van belang dat we de rol van DNA methylatie in kanker in meer detail onderzoeken. Het doel is om te proberen nieuwe methoden en medicijnen te ontwikkelen, die gebruikt kunnen worden om kanker te diagnosticeren en te behandelen.

ABBREVIATIONS

AS: Angelman syndrome
BWS: Beckwith-Wiedemann syndrome
DPC: days post coitum
Dnmt: DNA methyltransferases (Dnmts)
ES: embryonic stem cell
GK repeat: glycine-lysine repeat
H3K4: histone 3 lysine 4
H3K4me3: H3K4 trimethylation
H3K9: histone 3 lysine 9
H3K9me3: histone 3 lysine 9 trimethylation
H3K27: histone 3 lysine 27
H3K27me3: histone 3 lysine 27 trimethylation
H3K36: histone 3 lysine 36
H3K36me3: H3K36 trimethylation
HAT: histone acetylation transferase
HCP: high density CpG promoters
IAP: intracisternal A particle
ICP: intermediate CpG promoter
ICR: imprinted control region
KO: knockout
LCP: low density CpG promoters
LOH: loss of heterozygosity
LOI: loss of imprinting
NLS: nuclear localization signal
PBD: PCNA binding domain (PCNA: proliferating cell nuclear antigen)
PWS: Prader-Willi syndrome
PWWP: proline-tryptophan-tryptophan-proline
SAM: S-adenosyl-methionine
SAH: S-adenosyl homocysteine
TRF target replication foci
TSS: transcription start site

PH.D. PORTFOLIO

Summary of PhD training and teaching activities

Name PhD Student:	Eveline J. Steine
Erasmus MC department:	Reproduction and Development
Outside Erasmus:	Rudolf Jaenisch lab, Whitehead Institute for Biomedical Research, Cambridge, MA, USA
Research school:	Medical Genetics Centre South-West Netherlands (MGC)
PhD period:	March 2008-June 2012
Promotors:	prof.dr. J.Anton Grootegoed, Prof.dr. Joost Gribnau, Prof.dr. Rudolf Jaenisch
Supervisor:	Prof.dr. Rudolf Jaenisch

General academic skills

2011	Manuscript writing, MIT
2008-2012	Laboratory animal science, MIT/Whitehead Institute for Biomedical Research

Research skills

2011	Analysis of next generation sequencing experiments with Galaxy, Whitehead Institute
2011	Juggling with genome coordinates, Whitehead Institute
2011	Analysis of Chip-Seq experiments, Whitehead Institute
2011	Introduction in R graphics, Whitehead Institute
2010	Introduction in Unix, Whitehead Institute, Whitehead Institute
2010	Gene list enrichment analysis, Whitehead Institute
2010	Mapping next generation sequence reads, Whitehead Institute
2010	Introduction in R, Whitehead Institute
2009	Introduction in Perl, Whitehead Institute
2009	Analysis of microarray data, Whitehead Institute
2008	Statistics for biologists, Whitehead Institute

Presentations

2011	Epigenetic Landscape in Development and Disease, Kobe, Japan
	Cancer center meeting, MIT & Cancer Center & Whitehead Institute
	Poster session, Cancer center meeting, MIT/Whitehead Institute
	Whitehead Institute Scientific Retreat, Waterville valley, NH, USA
2010	Whitehead Institute scientific retreat, Waterville valley, NH, USA
	Poster session, Cancer center meeting, MIT/Whitehead Institute
2009	Whitehead Institute scientific retreat, Waterville valley, NH, USA
	Cancer center meeting, MIT & Cancer Center & Whitehead Institute
	Poster session, Cancer center meeting, MIT/Whitehead Institute
2008	Whitehead Institute scientific retreat, Waterville valley, NH, USA
	Poster session, Cancer center meeting, MIT/Whitehead Institute

Fellowships

2011	CDB travel fellowship, RIKEN Center for Developmental Biology, Kobe, Japan
2010	International Talents in Life Science & Health Programme, the Netherlands
2008	PhD fellowship Banning-de Jong Fonds - Prins Bernhard Cultuurfonds, the Netherlands

International conferences

2011	Epigenetic Landscape in Development and Disease, Kobe, Japan
2008	ISCCR, Philadelphia, PA, USA

Seminars and workshops

2008-2012	Weekly Biology colloquium, Biology department, MIT
2008-2012	Weekly Cancer Center meeting, MIT & Koch Institute & Whitehead Institute
2008-2012	Weekly scientific seminar series 'forum', Whitehead Institute for Biomedical Research

Didactic skills, lecturing and teaching activities

2010-2012	High school teachers program, Whitehead Institute for Biomedical Research
2011-2012	Spring Lecture Series for High School Students, Whitehead Institute for Biomedical Research
2011	Massachusetts Biotechnology Job Shadow Day, Whitehead Institute for Biomedical Research
2009-2010	Organiser & host of Whitehead scientific weekly seminar series.

CURRICULUM VITAE

Eveline Josephine Steine

Day of Birth: November 26, 1980 • Place of Birth: Rotterdam

Email: evelinesteine@gmail.com

EDUCATION

Ph.D. candidate in Biology; Epigenetics, Cancer and Stem Cells MIT & Whitehead Institute for Biomedical Research, and Erasmus MC – University Medical Center Expected graduation date:June 27, 2012	2008 – present Cambridge, MA, USA Rotterdam, NL
Master of Science (M.Sc) Biomedical Science, cum laude Universiteit van Amsterdam	2008 Amsterdam, NL
Bachelor of Science (B.Sc) Biomedical Science, cum laude Universiteit van Amsterdam	2005 Amsterdam, NL
VWO Gymnasium Coornhert Gymnasium, Gouda, NL	1995-1999 Gouda, NL
VWO Gymnasium Emmaus College, Rotterdam, NL	1993-1995 Rotterdam, NL

WORK EXPERIENCE

Ph.D student in the group of Professor Rudolf Jaenisch Massachusetts Institute of Technology & Whitehead Institute for Biomedical Research	2008 – present Cambridge, MA
Master research internship in the group of Professor Rudolf Jaenisch Massachusetts Institute of Technology & Whitehead Institute for Biomedical Research	2007 Cambridge, MA
Master research internship in the group of Professor Hans Clevers Hubrecht Institute for Developmental Biology	2006 – 2007 Utrecht, NL
Research Internship – genetic metabolic diseases Amsterdam Medical Center	2005 – 2006 Amsterdam, NL
Bachelors thesis research Swammerdam Institute for Life Sciences	2005 Amsterdam, NL

LIST OF PUBLICATIONS

1. Steine, EJ., Ehrich, M., Bell, GW., Raj, A., Reddy, S., van Oudenaarden, A., Jaenisch, R. & Linhart, HG. (2011) *Genes targeted by Dnmt3b in the mouse intestinal mucosa correspond to genes methylated in human colon cancer.* J Clin Invest 121(5):1748-52

2. Carey, Bw., Markoulaki, S., Hanna, J., Faddah, DA., Buganim, Y., Kim, Jp., Ganz, K., Steine, EJ., Cassady, JP., Creyghton, M., Welstead, GG., Gao, Q., & Jaenisch, R. (2011) *Reprogramming factor stoichiometry influences the epigenetic state and biological properties of induced pluripotent stem cells.* Cell Stem Cell 9(6):588-598

3. Gao, Q., Steine, EJ., Barrasa, MI., Hockemeyer, D., Pawlak, M., Fu, D., Reddy, S., Bell, GW. & Jaenisch, R. (2011) *Deletion of Dnmt3a promotes lung tumor progression* PNAS 108(44);18061-18066

4. Kim, JP., Lengner, CL., Kirak, O., Hanna, J., Cassady, JP., Lodato, MA., Wu. S., Faddah, DA., Steine, EJ., Gao, G., Fu, D., Dawlaty, M. & Jaenisch, R. (2011) *Reprogramming of Postnatal Neurons into Induced Pluripotent Stem Cells by Defined Factors.* Stem cells 29(6):992-1000

5. Creyghton, M., Cheng, A., Welstead, GG., Kooistra, T., Carey. B., Sharp, P., Steine, EJ., Hanna, J., Lodato, M., Frampton, G., Boyer, L., Young, R. & Jaenisch, R. (2010) *Histone H3K27ac separates active from poised enhancers and predicts developmental state*. PNAS 107(50):21931-6

6. DeKelver, RC., Choi, VM., Moehle, EA., Paschon, DE., Hockemeyer, D., Meijsing, SH., Sancak, Y., Cui, X., Steine, EJ., Miller, JC., Tam, P., Bartsevich, V., Meng, X., Rupniewski, I., Gopalan, SM., Sun, HC., Pitz, KJ., Rock JM., Zhang, L., Davis, GD., Rebar, EJ., Cheeseman, IM., Yamamoto, KR., Sabatini, DM., Jaenisch, R., Gregory PD., & Urnov, FD. (2010) *Functional Genomics, Proteomics, and Regulatory DNA Analysis in Isogenic Settings Using Zinc Finger Nuclease-Driven Transgenesis Into a Safe Harbor Locus in the Human Genome.* Genome Res 20(8):1133-42

7. Linhart, HG., Troen, A., Bell, GW., Cantu, E., Chao WH., Moran, E., Steine, EJ., He, T., & Jaenisch, R. (2009) *Folate deficiency in wildtype and Ung -/- mice induces DNA uracil misincorporation and genomic hypomethylation but does not increase DNA point mutations.* Gastroenterology 136(1):227-23

8. Wernig, M., Lengner, CJ., Hanna, J., Lodato, MA., Steine, E., Foreman, R., Staerk, J., Markoulaki, S. & Jaenisch, R. (2008) *A Drug-inducible transgenic system for direct reprogramming of multiple somatic cell types.* Nat Biotechnol 26(8):916-24

9. Hanna, J., Markoulaki, S., Schorderet, P., Beard, C., Wernig, M., Creyghton, MP., Steine, EJ., Carey, BW., Cassady, JP., Lengner, CJ., Dausman, JA. & Jaenisch, R. (2008) *Direct Reprogramming of Terminally Differentiated Mature B Lymphocytes to Pluripotency.* Cell 133(2):250-64

10. Linhart, HG, Lin, H., Yamada, Y., Moran, E., Steine, EJ., Gokhale, S., Lo, G., Cantu, E., Ehrich, M., He, T., Meissner, A. & Jaenisch, R. (2007) *Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing*. Genes Dev 21:3110-3122

ACKNOWLEDGEMENTS

Finally, I can thank you all! Without you, this 'boekje' would not have existed!

First for all, thank you Rudolf for giving me the opportunity to do my Ph.D. research in your lab. Thank you for being such a great advisor and for giving me so much freedom. I admire you and all your achievements. It was great to be in your lab during the exciting years after the discovery of the factor-mediated nuclear reprogramming! It was intense, it was fun!

Thank you Anton and Joost for being my promoters in Holland, for taking all the time in the world when I would visit Erasmus MC, for being engaging from such a distance and for all the feedback you gave me.

I would like to thank my committee Peter Verrijzer, Dies Meijer and Frank Grosveld for accurately reading my manuscript.

The former and current members of the Jaenisch lab, who made the lab an amazing environment to do my Ph.D., thank you for all your helpful discussions and suggestions, for your great insights and for "being there all the time!" I thank specifically Heinz for teaching me all the ins and outs of DNA methylation, Ruthie for all the tail appointments, Jessie for all the fun times in the mouse house, Dongdong for helping me with all the mouse work upstairs, Carrie for all grammar-related issues and John for being there. Thanks to all my bay mates, Jacob, Bryce, Caroline, Judith and Yarden. It was loud, it was festive, it was cozy, and "it didn't make sense!"

Furthermore, I would like to extend my gratitude to the supportive WI & MIT facilities and my collaborators. In particular, Inma, it was always good to get the email: "can you make me an espresso?"

Special thanks to all my buddies, who kept me sane by keeping me moving. Gymbuddies, you felt like my family. MIT Women's Volleyball Club, it was great to spend my Sunday nights with you. Everybody on the slopes and all the heartwarming visits you paid me when I could not move after the GSC ski trip. Rogier, KC, the Harvard cyclist club riders and all the other

cyclists with whom I spent so many hours on the bike, I loved it! “Movements in time” every Friday and Sunday – I was like a little child, so happy to go to rehearsal!

To all my friends in the greater Boston area, thank you! I had so much fun with you during our coffees, our nice dinners, our amazing weekend trips, at the crazy house parties, while going out, on the beach, all other events we have shared and especially at Halloween. In particular I would like to thank Judith for the fascinating places we saw in the States; the German crowd Ulrike, Doro, Katrin and Fiona (sorry you belong to the German crowd!); the Dutchies Elke, Marti, Marthe, Nadia, Karin en Bjorn, Carlijn en Derek; my “sister” Grace and all my roommates and Jaime “that’s why we are roommates”.

Pap, Mam, Thijs, Martijn, Pim & Renée. Bedankt voor alles, zelfs met het water er tussen, waren jullie er altijd. Ik hoefde mijn bureau la maar open te schuiven en daar waren jullie op de foto. Hoe trots jullie op mij zijn op het “buisjes vullen en buisjes legen” is onbeschrijflijk. Bedankt voor alles, voor de leuke doosjes, de relaxte vakanties, voor het broeder spelen. Nooit hebben jullie iets gezegd dat ik zo ver weg was, totdat ik vertelde ik mijn boekje ging schrijven en eerste reactie was “waar ga je dan een baan zoeken?”.

Bedankt!

Eveline (Eve)