Functional Analysis of DNA Hypomethylation in Development and Disease

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an der Fakultät für Biologie

Ludwig-Maximilians-Universität München

Vorgelegt von
François Gaudet
aus Montreal, Kanada

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i. Abstract

The genome of mammals harbors chemical modifications at some cytosine residues in the form of a methyl group. These modified residues, termed 5'-methylcytosines, have been discovered more than 50 years ago (Hotchkiss 1948) and have since been shown to play important roles in the regulation of gene expression and in the execution of developmental programs. Patterns of cytosine methylation (also referred to as *DNA methylation*) are carefully set and preserved during cellular expansion and global methylation levels are well regulated throughout development. Changes in methylation patterns and levels have been associated with disease progression and death (Li et al. 1992; Okano et al. 1999; Ehrlich 2002). Specifically, elevated levels of global genomic methylation have been shown to play a role in the inactivation of tumor suppressor genes in many types of cancer (Ehrlich 2002). In contrast, reduced levels of methylation have been observed in a wide variety of tumors and complete demethylation in vivo causes embryonic death (Li et al. 1992; Ehrlich 2002).

In an effort to study the effect of changed methylation levels in vivo and its effect on disease progression, we developed a genetic approach to study the effect of hypomethylation during embryogenesis and adulthood. DNA methyltransferase 1 (Dnmt1) is the major methyltransferase in mammals and genetic inactivation of the *Dnmt1* gene causes demethylation that results in cell death in tissue culture and

embryonic lethality of homozygous mutant mice at E8.5 (Li et al. 1992). In a first step, the 5' end of the *Dnmt1* gene was characterized to determine the structure of a new oocyte-specific isoform found in oocytes and early embryos. Upon elucidation of the structure of this isoform, assays were developed to test its function in vivo. Loss of this oocyte-specific isoform protein resulted in hypomethylation of an IAP reporter element suggesting a role for this protein in early development. In contrast, the somatic Dnmt1 isoform, which is present in all somatic cells, was important for maintaining this IAP element methylated following implantation of the embryo and throughout adulthood. Reduced levels of Dnmt1 in adults caused global hypomethylation and resulted in the development of thymic lymphomas which displayed a duplication of chromosome 15 (trisomic 15). The *c-myc* oncogene, which resides on chromosome 15, was overexpressed, and a gene expression array analysis revealed that another oncogene, *Notch-1*, was also overexpressed in all tumors. Cooperation between those oncogenes has been previously shown to induce thymic lymphomas. Analysis of the *Notch-1* locus demonstrated the presence of IAP insertions upstream of the oncogenic cytoplasmic domain capable of activating transcription of truncated oncogenic *Notch-1*. IAP elements were shown to be activated by hypomethylation albeit not as much as traditional mutagenic retroviruses. These results thus show that hypomethylation may induce tumorigenesis in this model following two mechanisms. First by inducing chromosome instability and second by creating insertional mutagenesis of defective retroviral elements such as IAPs. These results demonstrate for the first time that hypomethylation can directly induce tumorigenesis in mice and induce chromosome instability.

Chapter 1

Introduction

1.1. A fifth DNA base

Developmental stages in mammals are established by genetic and epigenetic programs. Epigenetic information is defined as those marks which are inherited through mitosis or meiosis but are independent of the DNA sequence. They constitute signals that are interpreted to regulate gene expression and cellular differentiation. The most common of these marks is DNA methylation, which consists in the addition of a methyl group to the 5′ carbon of cytosine residues (Figure 1). Cytosine-5 methylation is present in vertebrates, vascular plants and other eukaryotes and prokaryotes.

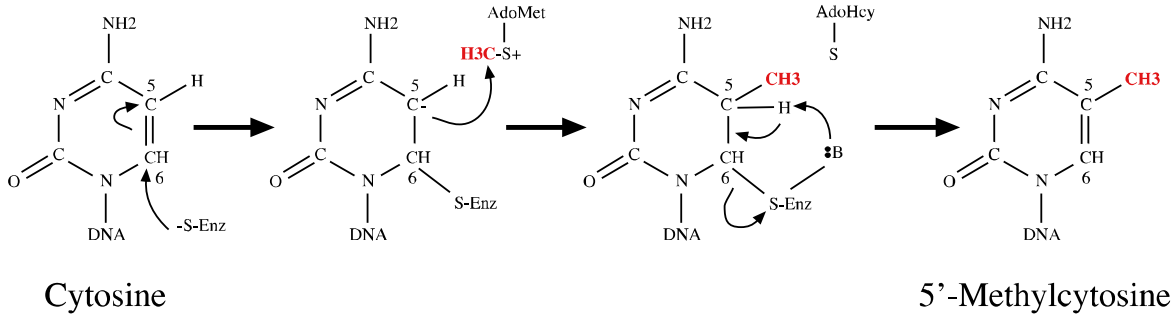

Figure 1. The methylation reaction. The enzyme first binds covalently to the 6′ carbon of the cytosine ring via a cysteine thiol group on the enzyme. The binding causes an electron transfer from the C-5/C-6 double bond to the 5′ carbon. The energetic doublet on the 5′ carbon then attacks the carbon from the methionine moiety of S-adenosyl L-methionine, resulting in the transfer of a methyl group from SAM to the 5′ carbon of the cytosine and the release of S-adenosyl-L-homocysteine. The release of the enzyme is achieved by the attack and capture of a proton on C-6 by a base, recreating the C-5/C-6 double bond and resulting in 5-methyl cytosine (Gerlt and Gassman, 1993).

The biological significance of this epigenetic modification was established in 1992 when the gene responsible for maintaining cytosine methylation was inactivated in mice,

resulting in genomic demethylation and death of the embryo at day E10.5 (Li,,et al. 1992). Subsequent knockout of methyltransferase family members in mice have resulted in embryonic lethal phenotypes and early post-natal death, consistent with a crucial function for cytosine methylation in developmental regulation (Okano et al. 1999).

DNA methylation has been shown to play an important role in the suppression of many genes including tissue-specific genes, imprinted genes and genes of the inactive X chromosome (Keshet et al. 1986; Becker et al. 1987; Li, Beard et al. 1993; Panning and Jaenisch 1996). In addition, methylation can silence endogenous proviruses including SINES, LINES, IAPs and other proviruses so as to prevent insertional mutagenesis (Jaenisch et al. 1985; Walsh et al. 1998). It has also been shown that methylation provides additional stability to the genome, perhaps by providing a more tightly packed chromatin that is less prone to recombination events (Colot et al. 1996; Chen et al. 1998; (Hashimshony et al. 2003).

Cytosine methylation normally occurs in the context of the palindromic 5′-CG-3′ (CpG) dinucleotide (Holliday and Pugh 1975; Bird 1978). About 70% of CpGs in mammalian genomes are methylated (Ehrlich et al. 1982), most of which at repetitive elements scattered throughout the genome (Yoder et al. 1997; Baylin and Bestor 2002). An interesting characteristic of CpGs is that they are greatly under-represented in the genome (Sved and Bird 1990). The higher spontaneous deamination rate of 5-methylcytosine compared to cytosine might account for this observation. In addition, 5-methylcytosine deaminates to thymine causing a C⇐T mutation whereas deamination of

cytosine creates a uracil residue that is efficiently removed by the DNA repair machinery thus making the former a more likely mutation site. CpGs are mutation hotspots that contribute to 30% of all point mutations in the germline (Cooper and Youssoufian 1988; Jones et al. 1992; Laird and Jaenisch 1994). Surely, since DNA methylation poses such a substantial mutagenic burden on the genome, it must contribute a strong selective advantage, perhaps by presenting an efficient means of regulating transcription of complex genomes.

Several models have been proposed to explain the evolutionary advantage of methylation, of which two hypotheses stand out. One hypothesis suggests that DNA methylation works as a silencer of background "transcriptional noise" (Bird 1995). This hypothesis is supported by the fact that 1) it is restricted to large genome organisms but practically absent from species with smaller genomes such as *Drosophila melanogaster* and *Caenorhabtidis elegans*, 2) it is largely confined to intergenic, non-coding and repetitive regions and 3) it is absent from the promoter regions of active genes. The other hypothesis argues that DNA methylation functions mainly as a defense system by silencing parasitic elements throughout the genome (Yoder et al. 1997). It has been shown that methylation silences expression of retroviral elements in somatic cells (Jaenisch et al. 1985; Walsh et al. 1998). However, this hypothesis fails to explain why undifferentiated cells of the early embryo are hypomethylated and retroviral elements such as IAPs are expressed in them (Kuff and Lueders 1988; Poznanski and Calarco 1991), leaving a possibility for these elements to become active and cause insertional mutagenesis. Therefore if DNA methylation constituted a defense system uniquely in

somatic cells, it would not be subject to evolutionary selection because mutations occurring late in development would be confined to a few subset of clonal cells, making it unlikely to operate as an efficient defense mechanism.

In any case, changes in patterns of methylation have been associated with disease progression such as ICF syndrome and cancer. Importantly, DNA methylation is a stable modification that is *reversible*, making it ideal for regulation of developmental programs. Understanding the mechanisms that regulate it will provide a unique opportunity for therapy and intervention.

1.2. Enzymes of DNA methylation

DNA methylation patterns vary significantly throughout development. These changes involve both methylation as well as demethylation activities. While it is still unclear which components play an active role in genomic demethylation, cytosine methylation is established and maintained by DNA methyltransferases whose expression is tightly regulated during development. These enzymes catalyze the transfer of a methyl moiety from S-adenosyl-L-methionine to the 5' carbon of a cytosine pyrimidine ring (Wu and Santi 1987). There are two distinct DNA methyltransferase activities: de novo methylation and maintenance methylation (Figure 2).

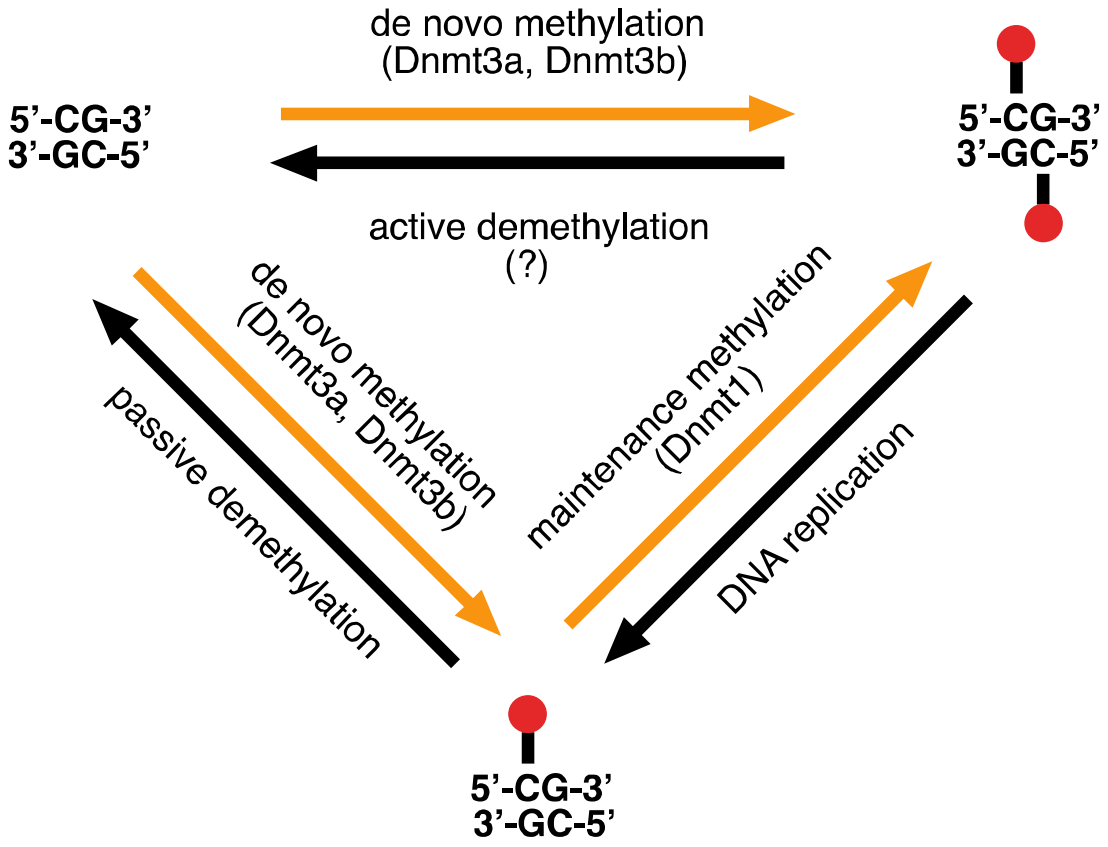

Figure 2. Changes of methylation patterns during development. The levels of methylation are relatively high in both mature gametes. After fertilization, a global wave of passive demethylation takes place until implantation of the embryo after which a wave of remethylation occurs and somatic cells become highly methylated by EXXX. In contrast, primordial germ cells remain demethylated until the beginning of gametogenesis when they differentiate and become methylated. Recent findings argued that primordial germ cells are methylated after implantation and soon followed by a wave of demethylation (REF) but these results have yet to be replicated.

1.2.1. Methylation of unmethylated DNA

The methylation of previously unmethylated DNA, referred to as de novo methylation, is carried by two enzymes in mammals: Dnmt3a and Dnmt3b (Figure 2). These proteins contain well conserved motifs in the catalytic domain that are present in all DNA methyltransferases. Inactivation of *Dnmt3a* and *Dnmt3b* by gene targeting in ES cells abolishes the ability of these cells to de novo methylate foreign DNA (Okano et

al. 1999). Both proteins are essential for normal development as *Dnmt3a* knockout mice become runted after birth and die after 4 weeks of age and *Dnmt3b* knockout induces various embryonic defects and causes death before E15.5. Animals homozygous mutant for both *Dnmt3a* and *Dnmt3b* die before E11.5, a phenotype that is more severe than any individual phenotype, suggesting an overlapping function for these proteins in embryogenesis (Okano et al. 1999). Double mutant embryos are also unable to de novo methylate genomic DNA following implantation of the embryo like wild-type embryos do. Both de novo methyltransferases are expressed at various levels in adult somatic tissues suggesting that they may have distinct roles at that stage.

De novo methylation may play an important function in organizing and compartmentalizing the genome during somatic differentiation so that genes are expressed in the proper sequence and cell types. In humans, cells from ICF syndrome patients were shown to contain mutations in DNMT3B, and DNMT3B mutant cells were found to contain chromosomal abnormalities. Additionally, several groups have reported overexpression of Dnmt3a and Dnmt3b in various tumors (Robertson et al. 1999; Girault et al. 2003). Tissue-specific inactivation and overexpression of these enzymes will be necessary to elucidate their function in mature organs, tissues and in tumorigenesis.

1.2.2. Maintenance of DNA methylation patterns

Following replication when the newly synthesized DNA strand is unmethylated, an enzyme binds to hemimethylated sites and catalyzes the transfer of a methyl group on the daughter strand to restore the palindromic methyl CpG configuration (Figure 2). This activity is catalyzed by Dnmt1, the first mammalian methyltransferase characterized more than 10 years ago and has been referred to as *maintenance methylation*. This methylation activity ensures that established patterns of methylation are preserved over many cell generations and provides a mean for heritable transcriptional control during development. The somatic form of Dnmt1 contains 1581 amino acid residues, consisting of a regulatory N-terminus of ~1000 amino acids that is linked by a segment of 12 alternating glycyl and lysyl residues to a C-terminus catalytic domain of ~ 500 amino acids that is closely related to prokaryotic cytosine methylases (Bestor et al. 1988). The regulatory domain contains a cysteine-rich region capable of binding zinc ions (Bestor 1992), a proliferating cell nuclear antigen (PCNA) binding domain (Chuang et al. 1997), several nuclear localization sequences (Cardoso and Leonhardt 1999), a polybromo homology domain (Liu et al. 1998) and a targeting sequence controlling subnuclear localization (Leonhardt et al. 1992) whereas the catalytic domain contains the conserved methyltransferase motifs. Several interactions have been described between DNMT1 and different chromatin-associated proteins and cell cycle regulators such as pRb, HDACs, MeCP2, SUV39H1 and HP1-beta, and PCNA (Chuang et al. 1997; Robertson et al. 2000; Rountree et al. 2000; Fuks et al. 2003; Kimura and Shiota 2003). Homozygous gene inactivation of *Dnmt1* results in demethylation in the embryos and

death by E9.5. Inactivation of *Dnmt1* in T cells in vivo causes a depletion of this cell type in the animal (Lee et al. 2001) and inactivation in fibroblasts caused cellular death after 2 weeks in vitro (Jackson-Grusby et al. 2001) suggesting that maintenance methylation is necessary for cellular differentiation and expansion of differentiated cells. Dnmt1 was shown to possess a maintenance activity in vivo in concert with Dnmt3a but no de novo activity (Lyko et al. 1999). Interestingly, Dnmt1 null ES cells grow normally in the absence of Dnmt1 and primordial germ cells do not contain Dnmt1, suggesting that this protein is not always necessary for cell division and survival (Li et al. 1992).

Dnmt1 is expressed during much of embryonic development and in the adult and exists as two isoforms in vivo. A shorter isoform is present in oocytes and early embryos and a longer isoform is expressed in postimplantation embryos and in somatic cells. The levels of the shorter isoform are very high in the oocyte and the fertilized egg and progressively decrease together with global methylation until embryonic implantation. The expression of the protein goes up again soon after implantation of the embryo together with the wave of de novo methylation, and remain present at various levels in adult tissues. In addition, Dnmt1 overexpression has often been linked to tumorigenesis (Robertson et al. 1999).

1.2.3. Are there additional mammalian DNA methyl-transferases?

A gene containing all the highly conserved catalytic domain methyltransferase motifs was identified and was termed Dnmt2 (Okano et al. 1998; Van den Wyngaert et al. 1998; Yoder and Bestor 1998). However, no methyltransferase activity was found for this protein when it was overexpressed from a baculovirus expression vector. In addition, *Dnmt2* knockout in ES cells did not affect their de novo or maintenance methyltransferase activities (Okano et al. 1998) and genetic inactivation of *Dnmt2* in mice did not result in any detectable phenotype. The function of Dnmt2 remains to be determined. A protein called Dnmt3L was isolated and was found to play a role in the setting of imprints in oocytes. This protein does not contain the conserved methyltransferase motifs and has no known enzymatic activity. The genome of primordial germ cells becomes methylated as the gametes differentiate. In addition, genomic imprints are known to be set in the gametes, but the factors necessary to conduct these functions remain to be identified (Margot et al. 2003; Bourc'his et al. 2001).

1.2.4. Genomic demethylation

Passive demethylation of the DNA can occur when methyl groups fail to be added onto the new DNA strand following DNA replication. This can happen when proper cellular maintenance methyltransferase activities are impaired such as when 5-

azacytidine is present (Jones 1984) or when a loxed (Cre/ lox) *Dnmt1* gene is looped out and inactivated in tissue culture (Jackson-Grusby et al. 2001). Such demethylation was shown to cause the activation of retroelements and developmentally regulated genes in the cultured cells (Jackson-Grusby et al. 2001). In addition, passive demethylation is thought to occur in early embryogenesis when the levels of Dnmt1 decrease and become mostly localized in the cytoplasm (Carlson et al. 1992; Rougier et al. 1998; Cardoso and Leonhardt 1999). Active demethylation (independent of DNA replication) has been shown following induction of the vitellogenin gene in chick liver (Wilks et al. 1984) or of the globin gene in erythroleukemia cells (Razin et al. 1984) and has also been observed in transiently transfected myoblasts (Paroush et al. 1990), in postmeiotic spermatocytes (Trasler, Hake et al. 1990) and in preimplantation mouse embryos (Kafri et al. 1993). Recently, active demethylation has been shown to occur on the paternal genome soon after fertilization (Mayer et al. 2000; Oswald et al. 2000) by an unknown mechanism. It has been suggested that active demethylation is at least partly mediated by an RNA component and that demethylation occurs by the removal of DNA nucleotides after their conversion to RNase-sensitive molecules (Weiss et al. 1996). Surely, additional components remain to be identified. More recently, active demethylation of the Il-2 promoter was shown in T cells following their activation (Bruniquel and Schwartz 2003). The mechanism of demethylation in this case is unknown and research is intensifying to characterize components responsible for these activities.

1.3. Dynamics of DNA methylation changes during development

Overall degrees of genomic methylation vary substantially during development and methylation level changes are well coordinated with developmental stages. In early embryogenesis, the levels of genomic methylation progressively decrease until implantation, and shortly thereafter are increased until the DNA is highly methylated in somatic cells. In contrast to embryogenesis, the levels of methylation in somatic lineages remain fairly stable throughout development. The significance of these global changes of methylation during embryogenesis are unknown but may serve as a way to reprogram the genome to suppress early developmental gene expression after implantation and to make accessible the loci needed for subsequent lineage-specific differences (Figure 3).

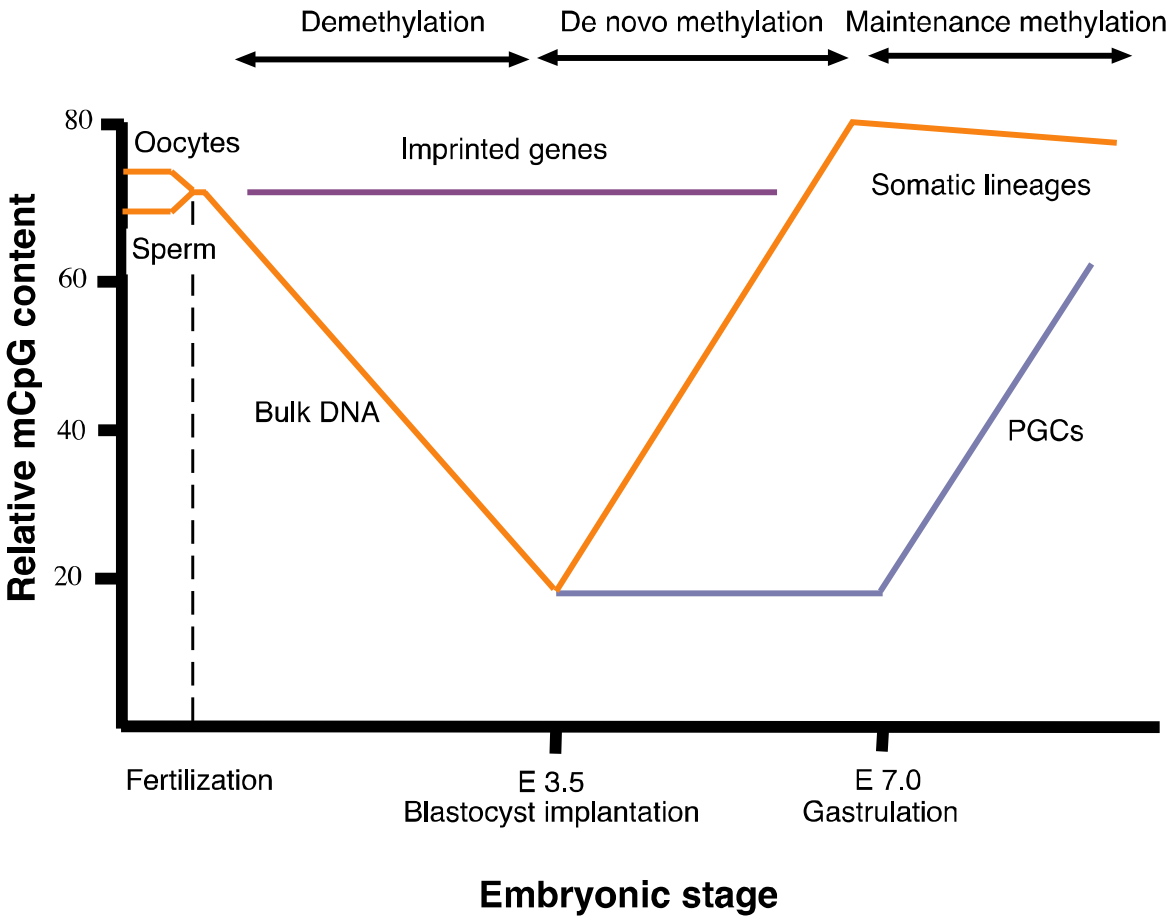

Figure 3. Regulation of DNA methylation by DNA methyltransferases. The major de novo methyltransferases Dnmt3a and Dnmt3b can use unmethylated CpG as substrates to create a double-stranded methylated CpG dinucleotide. This pattern is lost following DNA methylation as the newly synthesized strand is unmethylated. This hemimethylated CpG becomes a substrate for the maintenance methyltransferase Dnmt1 soon after replication of the DNA strand and restores the fully methylated configuration.

1.3.1. Changes of DNA methylation levels during gameto-genesis

As gametogenesis begins, the demethylated primordial germ cells undergo a wave of methylation that coincide with cellular differentiation. During this process, imprinted patterns are established in both parental gametes. During gametogenesis, the genome becomes methylated and imprinted gene methylation is set. An enzyme that bears resemblance to Dnmt3 (termed

Dnmt3L for Dnmt3-*like*) is expressed at that time and is responsible for setting the imprints in oocytes (Howell et al. 2001). Imprinted genes are genes whose allelic expression is determined by the parental origin (Bartolomei and Tilghman 1997; Jaenisch 1997). Dnmt3L lacks the highly conserved methyltransferase motifs and has no enzymatic activity. Dnmt1 is present at high levels in mature oocytes and is sequestered in the cytoplasm and it is not clear whether it has a function in setting the imprints. Dnmt3L is also expressed in the male gamete and *Dnmt3L* homozygous knockout is incompatible with spermatogenesis (Howell et al. 2001). In contrast, the mature sperm is devoid of Dnmt1 but although Dnmt1 is found in the nucleus of leptotene to pachytene stage spermatocytes (Mertineit et al. 1998). A few loci have been described that escape this global wave of reprogramming. For example, the methylation status of the mouse A^{iapy} allele can be inherited in the next generation. This event, termed transgenerational inheritance of epigenetic states, has been observed in mice at a variety of loci and circumstantial evidence suggests that this process may also occur in humans (Morgan et al. 1999; Greally 2002).

1.3.2. Changes of DNA methylation levels during embryo-genesis

After fertilization, the paternal genome soon becomes actively demethylated (Mayer et al. 2000; Oswald et al. 2000) followed by passive demethylation of both the maternal and paternal genomes (Monk et al. 1987; Kafri et al. 1992; Rougier et al. 1998). This demethylation occurs in the bulk DNA which consists of repetitive elements and most gene sequences. This period is associated with a progressive loss of Dnmt1 and by its localization in the cytoplasm the vast majority of the time. The embryo becomes

further demethylated until the postimplantation stage when a wave of remethylation occurs as a result of the expression of the de novo methyltransferases Dnmt3a and Dnmt3b and the maintenance methyltransferase Dnmt1. The levels of methylation remain high in somatic cells except in the germ cell lineage where the degree of methylation stays low until gametogenesis.

In contrast to bulk DNA, the methylation of imprinted genes and CpG islands are affected quite differently. The methylation of imprinted genes is established during gametogenesis and remain resistant to demethylation or de novo methylation in embryogenesis and in adulthood. The only exception is the differentiating germ cells which have the ability to reprogram imprinted genes (Tucker et al. 1996). CpG islands are unmethylated in normal cells except on the inactive X chromosome of female cells (Bird 1987). They reside in the proximity of promoters or first exons, are usually 1-2 k long, and are associated with housekeeping genes (Bird 1987). These islands are estimated at 45 000 copies, are associated with 60% of genes (Gonzalgo and Jones 1997) and contain the predicted frequency of CpGs of 12.5%, representing 15% of all CpG sites. They have an open chromatin structure that is deficient in the linker histone H1 and contain nucleosomes enriched in acetylated forms of histones H3 and H4 that are associated with active chromatin. It has been suggested that transcriptional silencing may be necessary for DNA methylation to occur and that genes containing CpG islands are not silenced in post-implantation embryogenesis when the embryo becomes methylated, making them resistant to DNA methylation (Bird 2002). CpG islands can be classified in three categories.

The CpG islands associated with housekeeping genes on chromosome X are methylated upon inactivation of the chromosome and those patterns of methylation are stably inherited after each cell division. The CpG islands associated with tumor suppressor genes are unmethylated in normal tissues but often become methylated as tumors develop (Laird and Jaenisch 1994; Baylin et al. 1998) and may play a role in tumorigenesis (Myohanen et al. 1998). All other CpG islands are unmethylated during development. This resistance to methylation was shown to be dependent on an intact Sp1 binding site within CpG islands (Brandeis et al. 1994). In addition to housekeeping genes which contain CpG islands, about 50% of tissue-specific genes also contain CpG islands (Jones, P.A. 1999).

1.4. Interpretation of DNA methylation signals

The most common effect of DNA methylation is gene silencing. This process represents a unique mechanism of gene regulation because it is both reversible and stable. Patterns of methylation can be modulated through development resulting in activation or inactivation of proper developmental programs. The stability of this modification makes it quite energy-efficient as they can be inherited though mitotic cell division. In fact, cell culture experiments have shown that the stability of the

modification approaches the genetic point mutation frequency of DNA (Harris 1982; Holliday 1990). The silencing of gene expression by DNA methylation is achieved by a number of proteins including methyl-binding proteins and chromatin remodeling proteins that can read the methylation status and alter gene transcription.

1.4.1. Numerous genes and elements are regulated by DNA methylation

The expression of many genes and DNA elements is modulated by DNA methylation. For example, imprinted genes possess DNA methylation marks that allow allele-specific expression (Bartolomei and Tilghman 1997; Jaenisch 1997). These marks are set during gametogenesis and are maintained throughout life. Failure to properly maintain methylation patterns at imprinted gene loci results in either biallelic expression or complete loss of expression (Li et al. 1993; Tucker et al. 1996; Caspary et al. 1998). The expression of the *Xist* gene, which is involved in chromosome X inactivation, also correlates with DNA methylation. Demethylated *Dnmt1* mutant embryos activate *Xist* which induces inactivation of the active X chromosome, resulting in silencing of both X chromosomes (Panning and Jaenisch 1996). Proper regulation of X inactivation is very important to compensate for the different dosage of X-linked genes between males and females and is achieved by the random inactivation of an X chromosome in females. In addition to these genes, the expression of many tissue-specific genes correlates with the methylation status of their promoters regions (Eden and Cedar 1994). Methylation generally correlates with gene silencing whereas demethylation is associated with

transcriptional activation. Finally, endogenous retroviruses and other transposable elements are also silenced by methylation (Jähner 1984). These sequences are normally methylated and transcriptionally silent. Hypomethylation induced by hypomethylating drugs or by genetic inactivation of *Dnmt1* results in the activation of endogenous viruses in vitro and in vivo (Jaenisch et al. 1985; Walsh et al. 1998).

While it is widely known that methylation can affect gene expression, the mechanisms by which methylation exerts its effect on transcriptional activity has become more clear only recently. Methylation signals can be interpreted through several mechanisms to inhibit specific genes or genomic regions so as to signal specific cellular or developmental programs.

1.4.2. DNA methylation can prevent the binding of transcription factors to their recognition sequence

Methylation can physically prevent transcription factors from binding to their recognition sequences (Watt and Molloy 1988) although this type of regulation is rather uncommon in vivo (Takizawa et al. 2001). For example, the glial fibrillary acidic protein (*GFAP*) gene is activated during astrocyte differentiation by the demethylation of a CpG dinucleotide located in a STAT3 (signal transducer and activator of transcription 3) – binding element (Takizawa et al. 2001). While the end result of methylation is generally transcriptional silencing, methylation of repressor protein-binding elements in the imprinted insulin-like growth factor 2 (*Igf2*) gene can increase its expression (Eden et al.

2001; Murrell et al. 2001). In addition, methylation of a CTCF binding site on the paternal allele of *Igf2* prevents the binding of the repressor protein and allows the *H19* enhancer to promote *Igf2* transcription. In contrast, hypomethylation of the CTCF binding site on the maternal allele allows binding of the repressor to this site and isolates the *H19* enhancer, resulting in *Igf2* silencing (Bell and Felsenfeld 2000; Hark et al. 2000). In any case, methylation of such recognition sites does not necessarily prevent target proteins from binding to the DNA (Brandeis et al. 1994; Macleod D et al. 1994).

1.4.3. Transcriptional repressors can bind to methylated DNA and induce chromatin remodeling

Methylation signals can be interpreted directly by methyl-CpG-binding proteins (MeCPs) that can alter gene transcription. There are 5 known MeCPs in mammals (Nan et al. 1993; Cross et al. 1997; Hendrich and Bird 1998; Prokhortchouk et al. 2001). Four of these proteins, MeCP2, MBD1, MBD2 and MBD4 bind methyl-CpGs through a conserved protein motif called the methyl-CpG binding domain (MBD, (Nan et al. 1996)). Another MeCP, Kaiso, differs from the other 4 by binding to methyl-CpGs through a zinc finger binding domain (Prokhortchouk et al. 2001). MBD3 also contains the MBD but does not have the ability to bind to methylated DNA (Hendrich and Bird 1998). All MeCPs except MBD4 have been shown to act as transcriptional repressors in vitro (Hendrich and Bird 1998). In contrast, MBD4 has been implicated in DNA repair (Bellacosa et al. 1999; Hendrich et al. 1999) and may act to minimize mutations at 5-methylcytosine. The repression activity of MeCPs is largely achieved by their interaction

with histone deacetylase complexes (Prokhortchouk et al. 2001) that help condense the chromatin into higher order structures that are transcriptionally silent. For example, MeCP2 forms a complex with histone deacetylases and the co-factor protein Sin3a to repress transcription after binding methyl-CpG (Nan, X., et al, Nature, 393, p.386, 1998; Jones, P.L. et al, Nature Genet., 19, p.187, 1998). Another complex made of the methyl-CpG-binding protein MBD2 and the multisubunit NuRD (previously known as MeCP1) can repress transcription in a similar fashion. The NuRD complex contains the ATP-dependent chromatin remodeling protein Mi-2 and histone deacetylases (Wade et al. 1999; Zhang, Ng et al. 1999). MBD3 was also shown to be a structural component of the NuRD complex (Zhang et al. 1999). In addition to suppress transcription of methylated promoters, this complex can also remodel methylated chromatin (Ng et al. 1999; Feng and Zhang 2001). These two methyl-CpG-binding complexes provide a link between DNA methylation-mediated transcriptional repression, histone deacetylation and chromatin remodeling.

1.4.4. Packaging of transcriptionally silent methylated chromatin

The significance of the global waves of demethylation and remethylation is not known. However, recent developments suggest that demethylation in cleavage embryos could serve to decondense the chromatin to facilitate the expression of genes necessary for early development. In addition, the opening of the chromatin allows for repackaging into a “non-pluripotent conformation” that may act as a silencing mechanism for early

development and pluripotent genes. The wave of remethylation that follows implantation is coordinated with chromatin condensation and somatic differentiation. DNA methylation at this stage may act as a mark to initiate chromatin assembly and help to maintain this structure during mitotic inheritance (Hashimshony et al. 2003).

DNA methylation patterns are read by methyl-binding transcriptional repressors that interact with histone deacetylase complexes that help remodel the chromatin into transcriptionally silent units. The regulation of chromatin packaging involves a number of histones that can be selectively modified at their amino terminus. These reversible reactions involve many histone modification enzymes and are very complex. For example, acetylation of core histones can occur on lysine residues number 9, 14, 18 and 23 of H3, lysines 5, 8, 12 and 16 of H4 and lysines of H2A and H2B. The status of acetylation of histones has been shown to set the level of transcriptional activity of the chromatin (Strahl, B.D. and Allis C.D. 2000) and knockout of the *Hdac1* gene, which encodes a histone deacetylase (HDAC), result in embryonic death at day E9.5 (Lagger, G. et al. 2002). Interestingly, it has been shown that Dnmt1 and Dnmt3a can interact with HDACs and repress transcription (Burgers, W.A. et al. 2002).

In addition to acetylation, *methylation* can occur on lysine residues 4, 9 27 and 79 and arginines 2, 17 and 26 of H3 and lysine 20 and arginine 3 of H4. However, less is known of the function of histone methylation but the evidence suggests that it may influence transcriptional activity. Genetic knockout of the histone methyltransferases G9a and Suv39h1/ Suv39h2 results in embryonic lethality at day E9.5 and E14.5 respectively (Peters et al. 2001). *G9a* $^{-/-}$ mutant embryos have a loss of H3-K9 methylation

in euchromatin (decondensed, transcriptionally active regions) whereas Suv39h1/ Suv39h2 double mutant embryos have a loss of H3-K9 methylation in heterochromatin (contains repetitive elements and other protein-coding genes and is generally transcriptionally silent) (Peters et al. 2001). H3-K9 methylation has been associated with transcriptional silencing whereas H3-K4 methylation (as achieved by H3K4 histone methyltransferases such as MLL and SET7) has been correlated with active gene expression (Jenuwein and Allis 2001; Zhang and Reinberg 2001). The identification of several H3-K9 methyltransferases in mammals indicate that specific histone methyltransferases may be targeted to different regions in the genome.

All modifications of DNA and histones require that the chromatin be accessible. ATP-dependent chromatin remodeling proteins with DNA helicase activities are present in mammalian cells and are necessary to give accessibility of DNA to DNA methyltransferases (Gibbons et al. 2000). These enzymes use the energy from the hydrolysis of ATP to induce twists into the DNA that results in the formation of nucleosomes (Peterson 2002). For example, genetic knockout of the lymphoid-specific helicase (*Lsh*), a member of the SNF2/ helicase family, results in global demethylation of genomic DNA at embryonic day E13.5 and post-natal death (Dennis et al. 2001). Other studies have shown that chromatin-remodeling proteins play a very important role in mammalian development as demonstrated by several gene knockouts that result in lethal phenotypes at various stages of development (Li 2002). The specific mechanisms that regulate the interaction between chromatin remodeling, histone modification and DNA methylation remain to be elucidated.

Histone modifications may act to modify the structure and chemical reactivity of histones making them amenable for interactions with regulatory proteins such as HP1 which binds to H3K9, (Lachner et al. 2001), the Polycomb repressor which binds to H3K27, (Cao et al. 2002) as well as transcriptional regulators which binds bromodomains of acetylated histones, (Jacobson et al. 2000; Owen et al. 2000).

Although evidence is lacking in mammals, studies to date showed a link between histone methylation and DNA methylation in the filamentous fungus *Neurospora crassa* and *Arabidopsis thaliana* (Tamaru and Selker 2001; Jackson et al. 2002). Specifically, functional mutations of histone methyltransferases resulted in loss of DNA methylation. In addition, Suv39h-mediated methylation of H3K9 directs DNA methylation to major satellite repeats at pericentric heterochromatin in mammals (Lehnertz et al. 2003). It is still unclear how histone methylation may regulate DNA methylation. Interestingly, reduction of cytosine methylation leads to an increase of H3K9 and H3K14 acetylation and H3K4 methylation while causing a decrease in H3K9 methylation in mammals (Nguyen et al. 2001; Bachman et al. 2003). The potential cross-regulation and inter-dependence between DNA methylation and histone methylation remains to be clarified.

1.4.5. Is there a histone code?

There is considerable evidence supporting the *histone code* hypothesis. The identification of proteins capable of writing a potential *code* on histone tails and factors

capable of reading this code and influence gene transcription strongly support this idea. This hypothesis has generated considerable interest and effort is on-going to decipher the *code* so that histone tails can be read with reasonable predictability outcomes in any region of the genome. The complete elucidation of the *code* will, however, take significantly more understanding of the complex dynamics of histone modification in gene regulation. Such an understanding will be important to develop treatment for diseases stemming from defects in histone modifications and of transcriptional regulator proteins interacting with these factors.

1.5. Methylation and disease

A number of disorders have been associated with methylation-related abnormalities. Such diseases can be classified into methylation pathways deficiencies such as methyltransferase mutations or into deficiencies related to target genes of methylation such as imprinted genes and tumor suppressor genes. Methylation aberrations have also been observed in a wide variety of cancer types and may play a role in disease progression through various mechanisms.

1.5.1. Deficiencies of methylation pathways

A number of diseases are caused by genetic mutations in enzymes that play key roles in methylation pathways. These include ICF, Rett and fragile X syndrome. The ICF syndrome (immunodeficiency, centromeric region instability, and facial anomalies syndrome) is a rare autosomal recessive disease. In addition to the many developmental defects and mental retardation, an interesting characteristic is the presence of multibranched, deleted or duplicated chromosome arms, and centromeric breakage. These regions are normally heavily methylated in somatic cells but are hypomethylated in ICF cells suggesting that methylation is important for centromeric structure and stability. It has been shown that ICF syndrome is linked to a null mutation in the DNMT3B gene which may be important to maintain methylation at centromeres.

The Rett syndrome (RTT) is an X-linked dominant disorder and is a very common cause of mental retardation in females (1/ 10 000). A large proportion of Rett patients are heterozygous for mutations in the MECP2 gene, which encodes a methyl CpG-binding protein that is X-linked (Guy et al. 2001). Females develop normally until 6 to 18 months of age but then lose speech, voluntary movements and hand skills (Guy et al. 2001). Knockout of MeCP2 in post-natal brains results in a phenotype similar to that of Rett syndrome (Chen et al. 1998; Chen et al. 2001; Guy et al. 2001). However, the mechanism responsible for this phenotype is unknown.

The Fragile X syndrome is the most common form of inherited mental retardation (1/ 5000 in males) after Down syndrome and affects mostly males (Kooy et al. 2000). The syndrome is defined by cognitive impairment, enlarged testes and behavioral hyperactivity. The gene associated with the syndrome, FMR1 (Fragile X Mental Retardation-1), contains a CGG repeat in the 5' untranslated region (UTR) that is greatly amplified in the syndrome (more than 200 repeats compared to 5-50 in normal patients) (Verkerk et al. 1991). Expansion of the repeat causes methylation (Pieretti et al. 1991) and deacetylation (Coffee et al. 1999) of the repeat and surrounding sequences, including the FMR1 promoter, resulting in transcriptional suppression of the FMR1 gene. So the effect is indirect in this case. Knockout of *Fmr1* in mice results in mild, but consistent abnormalities, analogous to the clinical and pathological symptoms observed in human patients (Kooy 2003). The product of the FMR1 gene is a ubiquitously expressed RNA-binding protein that may be involved in selective RNA trafficking between the cytoplasm and the nucleus. The connection between the function of the FMR1 protein and the syndrome is unclear and *Fmr1* mouse models will be very useful in that respect (Kooy 2003).

1.5.2. Imprinted genes disorders

Imprinted genes have been implicated in several diseases. The Beckwith-Wiedemann syndrome (BWS) is a clinical condition associated with somatic fetal overgrowth and variable predisposition to cancer. The most common molecular abnormality in this syndrome is biallelic expression of the imprinted gene insulin

growth factor-2 (Igf2 – an important fetal growth factor) which occurs in 80 % of all patients without cytogenetic abnormalities (Weksberg et al. 1993). Overexpression of Igf2 in transgenic mice confers most of the phenotype of BWS suggesting a direct role for elevated Igf2 levels in BWS. Experiments suggest that mutations in some of the imprinted genes surrounding Igf2 may act as transcriptional activators of this gene in BWS (Reik and Maher 1997).

The Prader-Willi syndrome (PWS) is another condition which is linked to abnormal imprinted gene expression. It is a developmental and behavioral disease linked to de novo intrachromosome deletions within an imprinted domain that causes altered expression of multiple contiguous imprinted genes. The end result is generally a loss of expression of paternally inherited alleles. It is characterized by hypotonia, respiratory distress, hyperphagia, small hands and feet and mental retardation, temper tantrums, and obsessive-compulsive mannerisms (Nicholls and Knepper 2001).

A closely linked disorder, the Angelman syndrome (AS), also results in intrachromosomal deletions within the same region linked to PWS but is in contrast associated with losses of maternally inherited alleles. Evidence indicates that the disorder results from disrupted expression of the maternal UBE3A brain-specific expression. (Nicholls and Knepper 2001). The syndrome is characterized by developmental delay, severe mental retardation with a lack of speech, movement ataxia, hyperactivity, seizures, aggressive behavior and excessive inappropriate laughter (Jiang

et al. 1999). While PWS and AS result most frequently from LOH of the functional allele, a number of other cases appear entirely epigenetic.

1.5.3. Methylation and cancer

Methylation has been postulated to play a role in cancer for many decades. DNA methylation patterns are significantly altered in many tumor types and include both hypermethylation and hypomethylation. The former is usually localized to specific regions surrounding gene promoters while the latter is genome-wide. Promoter hypermethylation is the most well categorized epigenetic change to occur in tumors. It is found in virtually every type of human neoplasm and is associated with inappropriate gene silencing. A large number of tumor-suppressor genes have been shown to be silenced by hypermethylation in cancer and include *RB* (Greger et al. 1989), *VHL* (Herman et al. 1994) and *p16ink4a* (Gonzalez-Zulueta et al. 1995; Merlo et al. 1995) as well as DNA repair genes such as *MLH1* (Baylin et al. 2001). Interestingly, promoter silencing by hypermethylation is at least as common as the disruption of classic tumor-suppressor genes in human cancer by mutation. Another effect of hypermethylation besides gene silencing is the higher frequency of point mutations that results from increased deamination of 5'-methylcytosine to uracil, resulting in a C⇐T mutation after the subsequent round of DNA replication (Jones et al. 1992; Laird and Jaenisch 1994). Indeed, CpG to TpG mutations account for many acquired somatic mutations that lead to cancer. For example, the p53 gene displays such mutations in 50% of all inactivating

mutations in colon cancer and in 25% of cancers in general despite having only 4% of methyl CpG in its sequence (Rideout et al. 1990).

The evidence linking hypomethylation to tumorigenesis also includes a considerable body of literature. A large variety of different tumor types in mammals have been shown to be hypomethylated, in many cases in conjunction with local hypermethylation although the evidence that the two mechanisms are independent of each other is lacking (Ehrlich 2002). Hypomethylation is as prevalent as hypermethylation in cancers but its potential role in tumorigenesis has not been well studied. It is generally observed at repeated sequences such as LINES and other retrotransposons, centromeric repeats and to a lesser extent to specific genes (Ehrlich 2002). It has been suggested that reduction in S-adenosyl-L-methionine metabolism may induce hypomethylation and play an important role in carcinogenesis (Chiang et al. 1996). For example, reduced supply of methionine, folate and choline in rats, all precursors of S-adenosyl-L-methionine, leads to genomic hypomethylation, overexpression of *c-H-ras, c-jun* and *c-myc* and results in liver tumors (Simile et al. 1994). Similarly, methyl donor deficiencies is correlated with liver and colon tumors in humans (Giovannucci et al. 1993). These experiments suggest a link between dietary factors and cancer and argue that hypomethylation may play a role in tumorigenesis. It has been suggested that hypomethylation represents an early stage in the development of some tumors (Goelz et al. 1985). Surprisingly, hypomethylation reduces tumor number in APC^{min} mice suggesting a protective role against tumor formation (Laird et al. 1995). While hypermethylation generally silences gene expression, it is unclear what the result

of hypomethylation might be in cancer development. Demethylation of genes silenced by methylation has not been frequently reported and evidence is lacking to support this mechanism. Many experiments have suggested that hypomethylation promotes genomic instability. It has been shown that hypomethylation in ES cells causes an increase in mutation rate and LOH by mitotic recombination (Chen et al. 1998). In a sterile hybrid obtained from two species of kangaroos, global hypomethylation was linked to chromosomal rearrangements including telomere elongation and various translocations (O'Neill et al. 2001). In addition, in the fungus *Neurospora crassa*, knockout of the main DNA methyltransferase (dim-2) resulted in chromosome instability (Foss et al. 1995). The association of hypomethylation with histone acetylation favors an open chromatin conformation which might render the genome more prone to recombination events than tightly packed DNA. To date, no clear evidence exists to suggest that hypomethylation leads to genomic instability in vivo.

1.5.4. Altered hypomethylation in cancer: cause or consequence?

Despite all the evidence linking changes of methylation patterns and tumorigenesis, it is unknown whether methylation plays a causal role in cancer development. Hypomethylating agents have been shown to induce tumorigenesis in rodents but most of these compounds are mutagenic, making it difficult to attribute a phenotype to the hypomethylated state or to mutations induced by the drug itself. To establish a causal relationship between hypomethylation and cancer, a genetic approach

resulting in global hypomethylation in vivo must be developed. So far, null mutations for all methyltransferases have resulted in death during embryogenesis or soon after birth, making it difficult to engineer an experimental model of hypomethylation. A T cell-specific knockout of *Dnmt1* was studied in mice and caused death of all mature T cells (Lee et al. 2001). In addition, knockout of *Dnmt1* in mouse embryonic fibroblasts also caused cell death (Jackson-Grusby et al. 2001). These results are consistent with methylation being required for somatic differentiation and begs for a new strategy for the design of an animal model of hypomethylation.

1.6. Study of the role of hypomethylation in development and cancer

The body of this thesis project aimed at developing a genetic system to study the effect of hypomethylation in development and cancer. A strategy was designed to generate a hypomorphic allele of the maintenance methyltransferase Dnmt1 to be used in a new experimental model.

The Dnmt1 gene can produce two different protein isoforms. The shorter isoform is present in oocytes and early embryos whereas the longer isoform is present in postimplantation embryos and in adult tissues. While it has been demonstrated that the longer isoform is required for embryonic development and survival (Li et al. 1992), the exact structure and function of the shorter isoform is unknown. Therefore, structural and functional analyses of this protein was undertaken. Results showed that this shorter

isoform was generated from an ATG translational start site in exon 4 of the *Dnmt1* gene and was capable of remethylating *Dnmt1* null ES cells (Chapter 2).

Experiments were designed to specifically address the effect of reduced levels of the *Dnmt1* gene products in vivo. Mice containing a weak *Dnmt1* allele were engineered. The lower Dnmt1 levels in the animals were found to cause global genomic hypomethylation. The effect of hypomethylation on development and cancer was studied using two approaches.

In a first approach, we studied the effect of reduced levels of both Dnmt1 isoform on genomic methylation during embryogenesis by using a methylation-sensitive reporter allele (Chapter 3). These results showed that the shorter maternal Dnmt1 isoform was important to maintain the methylation of this reporter allele in cleavage embryos. In contrast, the longer Dnmt1 isoform was important in maintaining methylation patterns in postimplantation embryogenesis. Furthermore, changes in methylation patterns resulting from lower levels of either isoform were stably inherited in the adult. Thus, interference with methylation pathways during gestation such as with dietary factors may result in permanent changes that may affect gene expression (Wolff et al. 1998).

In the second approach, the effect of hypomethylation on disease was investigated by aging Dnmt1 hypomorphic mice and monitoring their health for symptoms manifestation (Chapter 4). After a few months of age, the vast majority of

these hypomethylated mice developed aggressive thymic lymphomas, demonstrating that hypomethylation can cause cancer. The mechanism of tumor formation was further investigated and whole chromosome 15 duplications were implicated as one of the steps leading to tumorigenesis. The oncogene *c-myc*, which is present on chromosome 15 was activated in these tumors. In contrast, in tumors where chromosome 15 was not duplicated, c-myc was not overexpressed suggesting that c-myc overexpression was caused by chromosome 15 duplication. Additional analyses of these tumors using gene expression arrays showed that another oncogene, *Notch-1*, was also overexpressed in all tumors. The mechanism of *Notch-1* activation was found to be an IAP insertion near the transmembrane region of the *Notch-1* gene. Insertion of these IAP caused the expression of truncated transcripts of Notch-1 that are known to carry oncogenic properties. Consistent with these findings, c-myc and Notch-1 have been shown to collaborate to induce the formation of thymic tumors in vivo (Pear et al. 1996). Thus, hypomethylation can induce tumorigenesis by at least two mechanisms: insertional mutagenesis of weak retroviral elements and whole chromosome instability. The effect of hypomethylation on chromosome stability was also shown in a different study in which the hypomorphic *Dnmt1* allele was introduced in a tumor prone mouse strain (Annex). In this case, hypomethylation accelerated tumor formation and whole chromosome loss was shown to contribute to the disease phenotype.

The work presented here further emphasizes the importance of epigenetic changes in development and disease, in particular since patterns of methylation can be

modulated by environmental factors. The nature of DNA methylation as a reversible modification also makes it an interesting approach for developing new therapies.

1.7 References

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Chapter 2

A Short DNA Methyltransferase Isoform Restores Methylation In Vivo

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A Short DNA Methyltransferase Isoform Restores Methylation *In Vivo**

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Two murine DNA methyltransferase isoforms (MTases) have been observed, a longer form in somatic and embryonic stem (ES) cells and a shorter form in oocytes and preimplantation embryos. While the longer MTase is associated with maintenance methyltransferase activity in replicating cells, little is known about the shorter form. We present genetic and biochemical evidence that both isoforms are expressed from the same *Dnmt1* gene by using different translation initiation sites in exons 1 and 4. We further demonstrate that the shorter isoform can functionally rescue Dnmt1 null ES cells that have a hypomethylated genome. These rescued ES cells differentiate *in vivo* into a variety of cell types, unlike the Dnmt1 null ES cells that die upon induction of differentiation. These results show that the shorter isoform can substitute for the longer maintenance MTase in ES and differentiated cells. Our data further indicate that the shorter MTase isoform found in oocytes is fully functional *in vivo* and may play an active role in the regulation of DNA methylation and the establishment of imprinting patterns.

DNA (cytosine-5)-methylation is essential for normal mammalian development as Dnmt1 null mouse embryos fail to maintain cytosine methylation and die at mid-gestation (1). *Dnmt1* encodes the enzyme DNA (cytosine-5)-methyltransferase (MTase)[1], which methylates hemi-methylated DNA to maintain methylation patterns following DNA replication (2, 3). Cytosine methylation has been suggested to affect chromatin structure and gene transcription (4, 5) and plays an important role in genomic imprinting (6), X chromosome inactivation (7), and development (1). DNA methylation patterns are inherited from both parents and are mostly erased during preimplantation development, when the genome undergoes global demethylation. After implantation, somatic cells are remethylated, whereas primordial germ cells remain hypomethylated until gametogenesis when their genome becomes remethylated again (8).

Recently, three additional MTase genes were cloned (*Dnmt2* (9–11) and *Dnmt3α* and *β* (12)). To date, the Dnmt1 MTase is the only mammalian enzyme that has been shown to have a DNA methyltransferase activity and a function *in vivo*. This MTase is believed to maintain methylation patterns in somatic and ES cells. Interestingly, a shorter MTase form with an apparent molecular mass of about 170 kDa was found in differentiating female germ cells (oocytes) and in preimplantation embryos, which correlates with the occurrence of an oocyte-specific, alternatively spliced *Dnmt1* mRNA (13, 14). To study possible mechanisms regulating DNA methylation during early development, it is necessary to determine which isoforms are expressed and to test their activity *in vivo*.

The present work established that the *Dnmt1* gene contains at least four in-frame ATGs which could potentially be used to generate multiple MTase isoforms. We show that the longer isoform present in ES cells and somatic tissues starts at ATG3 in exon one, and the shorter isoform present in oocytes and preimplantation embryos starts at ATG4 in exon four. This shorter isoform is fully active *in vivo* and can restore wild-type DNA methylation levels and the capacity to differentiate Dnmt1 null ES cells. The fact that the shorter, oocyte-specific isoform can substitute for the somatic form suggests that it plays an active role in the regulation of DNA methylation during early development.

EXPERIMENTAL PROCEDURES

Vectors and DNA Sequencing—Site-directed mutations were introduced in the *Dnmt1* minigene using the previously described plasmid pMT50 (15). The ATG mutant constructs pMTΔ1,2 (mutated ATG1,2), pMTΔ3 (mutated ATG3), and pMTΔ1,2,3 (mutated ATG1,2,3) were generated using standard PCR-mediated site-directed mutagenesis with mutant primers overlapping ATG1,2 or ATG3: Δ1,2-upper primer, 5′-GCCTCCGTTGCGCGCtTGCGCACTCCCTTCGGGCATAGCtTGGTCTTCCCCCACTCT-3′; Δ3-upper primer, 5′-CCTGCAAGtTGCCAGCGCGAACAGC-3′; Δ1,2-lower primer, 5′-AGAGTGGGGGAAGACCAaGCTATGCCCGAAGGGAGTGCGCAaGCGCGCAACGGAGGC-3′; and Δ3-lower primer, 5′-GCTGTTCGCGCTGGCAaCTTGCAGG-3′. The respective mutations are indicated in lower case letters. For subcloning of mutated PCR fragments, two flanking primers were chosen that are located at suitable, nearby restriction sites (the *Bst*XI-upper primer, 5′-GAGTTCCAGAAATATGGATCATGGAC-3′ (located 859-bp upstream of ATG1), and the *Eag*I-lower primer, 5′-CAGCGGCCGtAGGGCCACCCGGAGAT-3′ (located 139-bp downstream of ATG3)). The *Eag*I-lower primer contains a mutation in the *Not*I site which results in an *Eag*I site (mutation shown in lowercase). The PCR fragment containing the mutated ATG1,2 was used to perform a second round of mutagenesis, thereby generating an ATG1,2,3 mutant PCR product. The final mutated PCR products (1210 bp) were cut with *Bst*XI/*Eag*I and cloned into the *Bst*XI/*Not*I site of pMT50–7/*Eag*I, resulting in pMT50–7/*Eag*I/Δ1,2, pMT50–7/*Eag*I/Δ3, and pMT50–7/*Eag*I/Δ1,2,3. The pMT50/7 construct was generated by religating the 7-kb DNA fragment from a *Not*I-cut pMT50. The pMTΔ4 (mutated ATG4) construct was made using the same strategy. The Δ4-upper primer, 5′-GGAGAGCAGAAtTGGCAGACTCAA-3′, and Δ4-lower primer, 5′-TT-

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[1] The abbreviations used are: MTase, DNA (cytosine-5-)-methyltransferase; Ab, antibody; ES cells, embryonic stem cells; kb, kilobases; LIF, leukemia inhibitory factor; MEL, murine erythroleukemia; MBP, maltose-binding protein; bp, base pair(s); PCR, polymerase chain reaction.

GAGTCTGCCAaTTCTGCTCTCC-3′, were used to mutate ATG4. Fragments were amplified for subcloning with the *Eco*RI-upper primer, 5′-GCGCAGCTAGGCATCTTAGTTATG-3′ (located 414-bp upstream of ATG4), and the *Eco*RI-lower primer, 5′-CGGGAATTCGGACGTCGAA-GACTCCTGTTGTC-3′ (located 349-bp downstream of ATG4). The final mutant PCR product (820 bp) was digested with *Eco*RI and ligated into the 13.1-kb fragment of *Eco*RI-cut pMT50-*Eag*I (same as pMT50 except *Not*I site downstream of ATG3 was mutated to an *Eag*I site), resulting in pMT50-*Eag*I/13.1-kb-Δ4. This construct was cut with *Aat*II, and the 11-kb fragment was ligated to the 7.1-kb fragment of *Aat*II-cut pMT50, resulting in pMTΔ4. The presence of site-directed mutations was confirmed by DNA sequencing.

Cell Culture and Transfections—Wild-type J1 and Dnmt1$^{s/s}$ mutant ES cells were cultured as described (1, 16). Transfections were done using the cationic liposome reagent DOTAP (Boehringer Mannheim). Plasmid DNA (7.5 μg each) was linearized by cleavage at a *Not*I site to favor random integration. DNA was mixed with 45 μl of DOTAP for 15 min at room temperature in 20 mM Hepes, pH 7.4, and added to 4×10^6 ES cells in 1 ml of Dulbecco's modified Eagle's medium for 2 h at 37 °C. The pPgk-Puro plasmid (17) (0.5 μg) was used in all transfections as a selection marker (puromycin). ES cells were plated on irradiated puro-resistant murine fetal fibroblasts, and puromycin was added at 2 μg/ml 24 h after transfection. Clones were picked and expanded 11–14 days after the addition of puromycin. Genomic DNA was made and analyzed as described (17). Genomic DNA (10 μg each) was digested with the restriction enzyme *Hpa*II and loaded on agarose gel, electrophoresed, and blotted onto Zetabind nylon membranes using standard procedures (18) An [α-^{32}P]dCTP-labeled minor satellite centromeric repeat probe was used for detection (pMR150; Ref. 19; gift from V. Chapman, Roswell Park Memorial Institute, Buffalo, NY). Teratomas were generated by resuspending 10^7 ES cells (grown in the presence of 1000 units/ml LIF and without embryonic fibroblasts) in 1 ml of phosphate-buffered saline. ES cells were injected subcutaneously into the flanks of 2-month old 129/sv male mice (300 μl of phosphate-buffered saline/ES cells per injection). Teratomas were excised 3 weeks after injections for Western blot and histological analysis.

Generation of MTase Antibodies and Western blots—Peptides were synthesized with an additional cysteine residue on the N-terminal end as follows: ATG2-Ab, CCFRLRRPLPIGFRAREK; ATG3-Ab, CDHVR-RRLKDLERDGLTEKE; and ATG4-Ab, CRSPRSRPKPRGPRRSK. Peptides were coupled to KLH (keyhole limpet hemocyanin) via the cysteine residue and injected into chickens (ATG2-Ab and ATG3-Ab) or New Zealand White rabbits (ATG4-Ab) using standard protocols (20). For ATG2-Ab and ATG3-Ab, antibodies were purified from egg yolk using the Eggstract purification system (Promega). The C-terminal antibody was raised against the catalytic domain (amino acids 1088 to 1619) fused to maltose-binding protein (MBP) and expressed in *Escherichia coli*. Recombinant protein was purified over an amylose-agarose affinity column and eluted with maltose. Purified protein was injected into chickens using standard protocols (20), and antibodies were purified from yolk as described above. To remove unspecific antibodies binding MBP epitopes, the mix was passed over a column carrying the immobilized catalytic MBP domain.

Antibodies were stored at 4 °C with sodium azide. Antibody specificity was tested against recombinant MTases made from baculoviruses (21) (see Fig. 2, *left panels*). ES cells were harvested in 10 volumes of sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris, pH 6.8, 0.01% bromphenol blue), sonicated, boiled for 5 min, and loaded on an 8% SDS-PAGE as described (22). Two-cell embryos, eight-cell embryos, and blastocysts were harvested in the same buffer. Coomassie staining and Western blot analyses were performed as described previously (23). All antibodies were diluted 1:3000 and detected using the chemiluminescence method (ECL, Amersham Pharmacia Biotech).

Purification of MTase from ES cells and N-terminal Sequencing—J1 embryonic stem cells were grown under standard conditions in the presence of 1000 units/ml LIF (1, 16). 1×10^{10} cells were harvested by scraping, and nuclear extracts were prepared as described (24). Dialyzed extract was loaded onto a Q Sepharose fast flow ion exchange column (Amersham Pharmacia Biotech) and eluted with a salt gradient from 20 to 1000 mM NaCl (peak at 280 mM). MTase protein was monitored by Western blot analysis using the ATG4-Ab in all chromatography steps. The pooled fractions were diluted to 150 mM NaCl, loaded onto a heparin column, and eluted with a 150–2000 mM NaCl gradient (peak at 550 mM NaCl). Pooled fractions were diluted to 100 mM NaCl and loaded onto a High Q column and eluted with a 100–1000 mM NaCl gradient (peak at 280 mM). Positive fractions were concentrated by loading onto a High Q column and eluted with 1000 mM NaCl. Fractions were stored on ice until N-terminal sequencing. For sequencing, purified MTase was electrophoresed using standard protein sequencing conditions on SDS-PAGE, and the gel was transferred to polyvinylidene difluoride membrane. After staining with Coomassie Blue, the protein band was excised and subjected to N-terminal sequencing on a 477A protein sequencer (Applied Biosystems) with phenylthiohydantoin-derivative analysis.

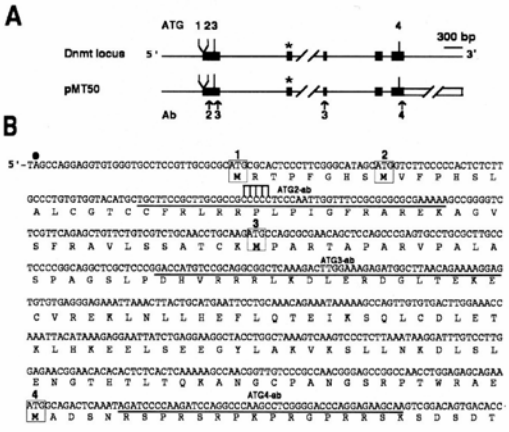

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FIG. 1. ***Dnmt1* gene has four potential translation initiation sites.** *A*, schematic diagram of the Dnmt1 locus and the Dnmt1 minigene construct pMT50. All of the sequence is genomic except for the *open box* which denotes cDNA sequence. The *filled boxes* represent exonic sequence, and the * identifies the testis-specific exon. The location of ATG 1, ATG2, ATG3, and ATG4, and the peptide sequences recognized by antibodies ATG2-Ab, ATG3-Ab, and ATG4-Ab are also shown. An intron of about 11 kb is present between the testis-specific exon and exon 2 and is represented by a broken line (/ /; H. Leonhardt, unpublished data). The pMT50 construct contains a 4.5-kb deletion in this 11-kb intron (segment between *Eco*RI and *Nae*I site is deleted, see Ref. 15). *B*, sequence of the 5′-end of the Dnmt1 gene. All four in-frame ATGs are *boxed*, and the stop codon is marked above with a *filled circle*. The five cytosine residues which are *highlighted* by five *vertical bars* mark the region where the sequence discrepancy was found. The 5′-end of exon 1 has been mapped by Rnase-H analysis and includes ATG1 (25). The peptides used for generation of the MTase antibodies are *underlined* and identified. *C*, comparison of the sequence surrounding the four ATGs with the Kozak consensus sequence. The most critical nucleotides for translation initiation are in *bold* (26). The homology to the Kozak consensus (which is 10 bases) is shown as the total number of matching bases as well as the number of bases matching the two most important bases which are *bold* (two bases). The letter "*R*" designates a purine residue (A or G).

RESULTS

The Mouse Dnmt1 Gene Codes for at Least Two MTase Isoforms—Recent reports of additional 5′-cDNA sequences of the mouse *Dnmt1* gene differed by one frameshift, which alters the number of possible upstream ATGs (15, 25). Because this discrepancy has potential consequences for the structure of the N-terminal, regulatory domain, we sequenced the corresponding genomic DNA of the mouse *Dnmt1* gene. To rule out strain-specific differences and PCR artifacts, we used genomic DNA from three different sources, the MEL cell line and tissues from two mouse strains (129/sv and BALB/c). Our data unequivocally show that the 5′-end of the *Dnmt1* gene contains three additional in-frame ATGs in all mouse DNAs tested (see ATG1–3 in Fig. 1) and thus confirm our previous observation (15). As shown in Fig. 1*C*, ATG2, ATG3, and ATG4 have all the

GAGTCTGCCAaTTCTGCTCTCC-3′, were used to mutate ATG4. Fragments were amplified for subcloning with the *Eco*RI-upper primer, 5′-GCGCAGCTAGGCATCTTAGTTATG-3′ (located 414-bp upstream of ATG4), and the *Eco*RI-lower primer, 5′-CGGGAATTCGGACGTCGAA-GACTCCTGTTGTC-3′ (located 349-bp downstream of ATG4). The final mutant PCR product (820 bp) was digested with *Eco*RI and ligated into the 13.1-kb fragment of *Eco*RI-cut pMT50-*Eag*I (same as pMT50 except *Not*I site downstream of ATG3 was mutated to an *Eag*I site), resulting in pMT50-*Eag*I/13.1-kb-Δ4. This construct was cut with *Aat*II, and the 11-kb fragment was ligated to the 7.1-kb fragment of *Aat*II-cut pMT50, resulting in pMTΔ4. The presence of site-directed mutations was confirmed by DNA sequencing.

Cell Culture and Transfections—Wild-type J1 and Dnmt1$^{s/s}$ mutant ES cells were cultured as described (1, 16). Transfections were done using the cationic liposome reagent DOTAP (Boehringer Mannheim). Plasmid DNA (7.5 μg each) was linearized by cleavage at a *Not*I site to favor random integration. DNA was mixed with 45 μl of DOTAP for 15 min at room temperature in 20 mM Hepes, pH 7.4, and added to 4×10^6 ES cells in 1 ml of Dulbecco's modified Eagle's medium for 2 h at 37 °C. The pPgk-Puro plasmid (17) (0.5 μg) was used in all transfections as a selection marker (puromycin). ES cells were plated on irradiated puro-resistant murine fetal fibroblasts, and puromycin was added at 2 μg/ml 24 h after transfection. Clones were picked and expanded 11–14 days after the addition of puromycin. Genomic DNA was made and analyzed as described (17). Genomic DNA (10 μg each) was digested with the restriction enzyme *Hpa*II and loaded on agarose gel, electrophoresed, and blotted onto Zetabind nylon membranes using standard procedures (18) An [α-^{32}P]dCTP-labeled minor satellite centromeric repeat probe was used for detection (pMR150; Ref. 19; gift from V. Chapman, Roswell Park Memorial Institute, Buffalo, NY). Teratomas were generated by resuspending 10^7 ES cells (grown in the presence of 1000 units/ml LIF and without embryonic fibroblasts) in 1 ml of phosphate-buffered saline. ES cells were injected subcutaneously into the flanks of 2-month old 129/sv male mice (300 μl of phosphate-buffered saline/ES cells per injection). Teratomas were excised 3 weeks after injections for Western blot and histological analysis.

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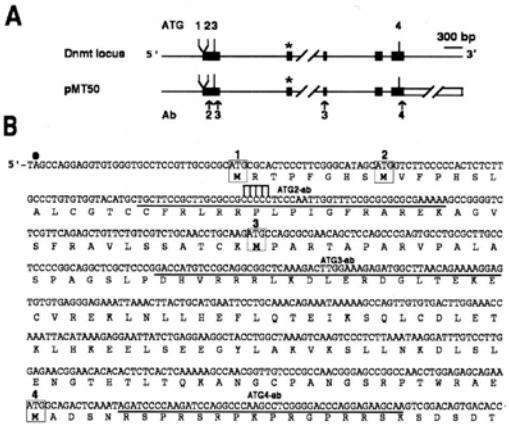

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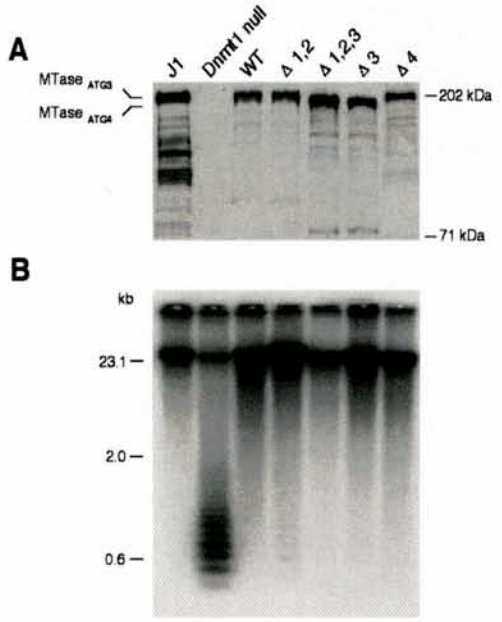

FIG. 3. **The shorter MTase isoform functionally rescues hypomethylated MTase null ES cells.** *A*, expression of MTase from various rescued Dnmt1 null ES cells. Shown is a Western blot analysis of protein extracts from wild-type J1 ES cells (*lane 1*), Dnmt1 null ES cells (*lane 2*), and Dnmt1 null ES cells rescued with: the wild-type minigene pMT50 (*lane 3*), the pMTΔ1,2 minigene (*lane 4*), the pMTΔ1,2,3 minigene (*lane 5*), the pMTΔ3 minigene (*lane 6*), and the pMTΔ4 minigene (*lane 7*). ATG4-Ab was used as a probe. Coomassie staining showed that all lanes were equally loaded except the *Dnmt1 null lane*, which was relatively underloaded (data not shown). *B*, global genomic remethylation by the shorter MTase isoform is observed. Southern analysis of genomic DNA cut with *Hpa*II and probed with a radioactive centromeric minor satellite repeat DNA fragment. All clones shown are presented in the same order as in part A above. Digestion of these clones with the methylation-insensitive restriction enzyme *Msp*I resulted in < 1-kb bands only (data not shown).

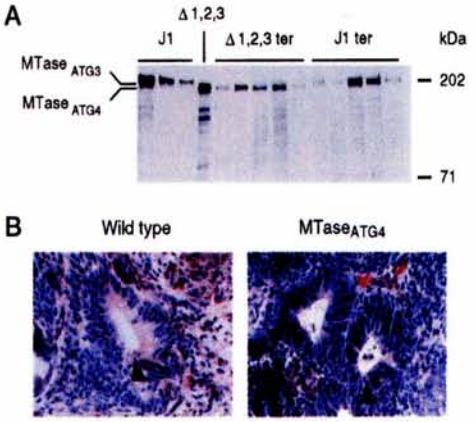

FIG. 4. **Both the longer and shorter MTase isoforms can maintain various differentiation programs *in vivo*.** *A*, Western blot analysis of protein extracts from teratomas derived from wild-type J1 ES cells (*J1 ter*, five different teratomas) or a pMTΔ1,2,3-rescued ES cell clone (*Δ1,2,3 ter*, five different teratomas). Protein extracts from the undifferentiated J1 ES cells (*J1*, three different protein amounts) and pMTΔ1,2,3-rescued undifferentiated ES cells (Δ1,2,3) are also shown. Coomassie staining showed at most a 2-fold difference in protein content between the lanes (data not shown). *B*, histological analysis of wild-type J1-derived (*left panel*) and $MTase_{ATG4}$-rescued (*right panel*) teratomas. Proteins are stained in *red* and DNA in *blue* (hematoxylin/eosin staining). *Arrows* point to well differentiated mucus-secreting glands reminiscent of colonic (endodermal) mucosa.

trast, mutation of ATG1,2 and ATG3 together or ATG3 alone resulted in the expression of the shorter MTase isoform. This protein was recognized by the ATG4-Ab but not the ATG2-Ab or ATG3-Ab (data not shown). It is unlikely that a start codon downstream of ATG4 was used because the next in-frame ATG and CTG are 266 and 97 amino acids downstream of ATG4 and would be expected to produce a noticeably shorter protein which would not be recognized by the ATG4-Ab. The ES cell clones shown in Fig. 3*A* were selected for high MTase expression levels. However, when looking at a large number of ES cell clones, we observed that Dnmt1 protein levels generated from constructs carrying deletions of ATG1,2 or ATG4 (generating $MTase_{ATG3}$) were on average comparable with those observed from the wild-type minigene, whereas protein levels obtained with deletion of ATG3 or ATG1,2 and ATG3 (generating $MTase_{ATG4}$) were higher than with the wild-type minigene (data not shown).

In summary, our data indicate that ATG3 is necessary and sufficient for expression of the longer MTase isoform in ES cells, whereas ATG1 and ATG2 seem not to be used in these cells. In addition, ATG4 is necessary for the expression of the shorter isoform. We demonstrated that two translational start sites are functional in ES cells; however, when both are present, ATG3 is dominant over ATG4 and only the longer isoform, $MTase_{ATG3}$, is expressed.

The Shorter MTase Isoform Restores Methylation in Dnmt1 Null ES Cells—To assess the function of these two MTase isoforms in ES cells, we isolated genomic DNA from Dnmt1 null ES cells rescued with the wild-type minigene or with the minigene containing mutated ATG1,2 and ATG3. The DNA was digested with the methyl-sensitive restriction enzyme *Hpa*II, separated on agarose gel, blotted, and probed with a radioactive centromeric repeat DNA fragment. Fig. 3*B* shows that both the cells containing $MTase_{ATG3}$ or $MTase_{ATG4}$ were able to restore methylation near wild-type levels, indicating that both enzyme isoforms are functional in ES cells.

The Shorter MTase Isoform Can Substitute for the Longer Form in Differentiated Tissues—Dnmt1 null ES cells die upon induction of differentiation and cannot form teratomas (15). To test whether $MTase_{ATG4}$ can rescue the differentiation capacity of these cells, we injected $MTase_{ATG4}$-transfected ES cells in the flank of 129/sv male mice to generate teratomas. We found that the $MTase_{ATG4}$-rescued ES cells produced teratomas (11/11) at the same efficiency as ES cells rescued with the wild-type minigene (6/6) or wild-type J1 ES cells (11/11). A control experiment using Dnmt1 null ES cells did not (0/5) generate any teratomas as shown previously. The teratomas were excised and tested for the presence of MTase by Western blot analysis. As expected, $MTase_{ATG4}$ teratomas contained only $MTase_{ATG4}$, whereas the teratomas derived from ES cells rescued with the wild-type minigene or the teratomas derived from wild-type J1 ES cells contained only $MTase_{ATG3}$ (Fig. 4*A* and data not shown). Histological analysis showed that both wild-type and $MTase_{ATG4}$ teratomas contained endothelial cells, secretory endothelial cells, cartilage, smooth muscle, skeletal muscle, and mature neural cells with no obvious differences. For example, glandular structures containing secretory endothelial cells are evident in stained sections of both wild-type J1 and $MTase_{ATG4}$ teratomas (Fig. 4*B*). These results show that the short isoform found in preimplantation embryos can substitute for the longer isoform in differentiated, somatic tissue. It is interesting to notice that previous attempts to express $MTase_{ATG4}$ with a heterologous promoter in ES cells failed (15), suggesting

that the MTase promoter of the minigene construct and correct transcriptional regulation is required for stable expression and cell viability.

DISCUSSION

The goal of this study was to identify and characterize possible alternative translation products of the *Dnmt1* gene. Sequencing of the 5′-end of the mouse *Dnmt1* gene indicated the presence of four potential in-frame ATGs and suggested that different translational start sites could be used *in vivo* (Fig. 1*B*). In addition to the somatic MTase form (about 190 kDa), an isoform with an apparent molecular mass of about 170 kDa has been observed in preimplantation embryos and was speculatively attributed to post-translational modification (13). We have generated specific antibodies that can distinguish three different MTase forms and show that a shorter MTase form starting at ATG4 is expressed in preimplantation embryos (see Fig. 2). These results are consistent with an earlier observation that the expression of the open reading frame starting at ATG4 in COS cells also resulted in a protein of about 170 kDa (28). With this set of antibodies, we could also show that the slower migrating MTase form found in ES cells and differentiated cells is most likely generated by translation starting at ATG3 (see Fig. 2). This conclusion was directly confirmed by protein sequencing of MTase purified from ES cells (see Table I). The same form ($MTase_{ATG3}$) has been identified by peptide mapping in transformed MEL cells (27). We failed to detect any MTase in cell extracts from ES cells, kidney, brain, and thymus with ATG2-Ab, which recognize a peptide sequence between ATG2 and ATG3. These results suggest that ATG1 and ATG2 are not used *in vivo*, but we cannot rule out that these start sites are used at a lower rate or might play a role at other developmental stages.

To directly test the role and function of these alternative MTase forms *in vivo*, we systematically mutagenized all four ATGs in a *Dnmt1* minigene construct and transfected MTase null ES cells. Even though ATG2, ATG3, and ATG4 fulfill the criteria of a good translation initiation site, only one of them, ATG3, is preferentially used *in vivo*. The shorter MTase form starting at ATG4 is only expressed in the absence of ATG3. These results fit well with the recent observation that, in oocytes, transcription starts at an alternative upstream promoter and generates a transcript lacking ATG1, ATG2, and ATG3 (14).

Interestingly, the shorter MTase form is present in growing oocytes and preimplantation embryos, a developmental stage when dramatic changes in the methylation pattern including imprinting occur (14, 29). Our genetic experiments, however, show that this shorter form has properties that are very similar if not identical to the longer, somatic MTase form. In null mutant ES cells as well as in teratomas, the shorter form can substitute for the longer form, $MTase_{ATG3}$, and can cause remethylation and normal differentiation (see Figs. 3 and 4). It will be interesting to investigate whether the different isoforms of Dnmt1 have unique functions in establishing and maintaining methylation patterns during early development. We are currently generating mice which can express either $MTase_{ATG3}$ or $MTase_{ATG4}$ but not both.

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Chapter 3

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The methylation of intracisternal A-type particle (IAP) sequences is maintained during mouse embryogenesis. Methylation suppresses IAP expression and the potential for mutagenesis by retrotransposition, but it is not clear how methylation of these elements is maintained during the embryonic stages when the bulk of the genome is being demethylated. It has been suggested that the high levels of DNA methyltransferase-1 (Dnmt1) present during cleavage could be important for keeping IAPs methylated. To test this hypothesis, we combined mutant alleles of *Dnmt1* with an *agouti* allele (A^{iapy}), which provided a coat color readout for the methylation status of the IAP insertion in the *agouti* locus. We found that reduction in Dnmt1 levels directly impacted methylation at this locus, leading to stable transcriptional activation of the *agouti* gene in the adult. Specifically, the short maternal Dnmt1 protein was important in maintaining methylation at the A^{iapy} locus in cleavage embryos, whereas the longer Dnmt1 isoform found in somatic cells was important in maintaining IAP methylation during the postimplantation stage. These results underscore the importance of maintaining proper maintenance of methylation patterns during gestation and suggest that interference with this process may stably affect gene expression patterns in the adult and may have profound phenotypic consequences.

Epigenetic marks such as genomic methylation have been shown to regulate gene expression and development (16, 21). Interference with proper establishment of methylation can result in a runted phenotype, tumorigenesis (7), and death (13, 22, 30). DNA methylation patterns established in the germ line during gametogenesis are largely erased in early embryogenesis and are reset after implantation. Patterns of methylation are then stably maintained through somatic cell divisions (21).

The various methyltransferases that regulate genomic methylation follow distinct expression patterns that are well coordinated in gametogenesis and embryogenesis (21). The establishment of new genomic methylation patterns in the differentiating gametes involves the de novo methyltransferases Dnmt3a and Dnmt3b and the Dnmt3-like (Dnmt3L) protein in the oocyte (1, 12). Dnmt3a and Dnmt3L are also expressed during male gametogenesis and are necessary for the completion of spermatogenesis (1, 12). After fertilization, the highly methylated paternal genome undergoes active demethylation in the male pronucleus (26, 31), whereas the maternal genome is only passively demethylated during the subsequent cleavage divisions (14, 17, 35). At the blastocyst stage, the bulk of the genome is hypomethylated except for alleles of imprinted genes and some repetitive elements which remain methylated (19, 34). High levels of a short form of Dnmt1 (Dnmt1o) are produced during oogenesis and are present in the cleavage embryo (27) but disappear after implantation. While this maternal store of enzyme may maintain the methylation of repetitive elements such as intracisternal A-type particles (IAPs), it is not clear why the bulk of the genome becomes demethylated. Dnmt1 is also expressed at high levels in spermatogonia and in leptotene spermatocytes but is not detectable in the nucleus of pachytene spermatocytes (27). This is consistent with a role for Dnmt1 in setting or maintaining paternal methylation patterns during the early stages of spermatogenesis. The somatic form of Dnmt1 mRNA (Dnmt1s) is transcribed postzygotically and, in concert with Dnmt3a and -b, has been shown to be responsible for the wave of global de novo methylation after implantation (22, 25, 30). All evidence suggests that the primary function of the longer somatic Dnmt1 isoform is to maintain global methylation after implantation of the embryo as the genome becomes remethylated by Dnmt3a and Dnmt3b. However, some de novo methylation activity has been observed in $Dnmt3a^{-/-}/3b^{-/-}$ double knockout embryonic stem cells, suggesting that other methyltransferases may play a role in remethylation (24). In addition, it has been shown that methylation is important for maintaining the stability of the genome (3, 6, 7).

The genome of mice contains multiple copies of retrotransposable IAP elements, and it is well established that transposition of these elements can cause insertional inactivation as well as the ectopic activation of genes (10, 40). Also, it has been demonstrated that methylation of IAPs is an important silencing mechanism that suppresses activation and transposition of the elements (15, 45). The insertion of an IAP element into the *agouti* gene (designated as the A^{vy} or A^{iapy} allele) has been shown to lead to ubiquitous ectopic activation of the gene. Hypomethylation of a cryptic promoter within the long terminal repeat (LTR) of the IAP drives constitutive ectopic expression of the *agouti* gene, leading to yellow coat color, obesity, and a high incidence of tumors (5, 28). In contrast, methylation of the IAP LTR silences the cryptic promoter and allows normal tissue-specific and regulated *agouti* expression, resulting in a "pseudoagouti" phenotype. The degree of yellow

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contribution to the fur provides an easy readout of the level of IAP methylation in the animal. The methylation state of an *agouti* IAP can be maternally transmitted to the next generation (a process termed transgenerational inheritance of epigenetic states) (29). For example, yellow A^{vy} mothers are more likely to have yellow pups than pseudoagouti mothers. Insertion of an IAP element into another gene which regulates embryonic axis formation (axin) leads to maternal as well as paternal transmission of the methylation state of this IAP and an associated kinked-tail phenotype (33). Thus, changes in methylation patterns imposed on the genome during embryogenesis can be not only maintained in the adult but also transmitted to the next generation. It has been shown that maternal diet can affect the methylation state of the IAP element and can cause a shift in coat color distribution in progeny from A^{vy} mothers (4, 44). This is an interesting result that emphasizes that environmental stimuli such as diet can profoundly affect the methylation state of the genome and result in gene expression states and phenotypic changes that are stable throughout life (16).

It has been established that methylation can suppress the expression of IAP elements (41), but the role of the specific Dnmt1 isoforms in this suppression has not yet been addressed. We tested the hypothesis that Dnmt1 plays a crucial role in the establishment and successful somatic propagation of these epigenetic states during the earliest stages of embryogenesis. For this, we examined the effect of *Dnmt1* mutant alleles that generate lower Dnmt1 levels in the embryo on the expression of A^{iapy} in the adult. Our results suggest a role for the shorter oocyte-specific Dnmt1 isoform in maintaining IAP methylation in the cleavage-stage embryo, whereas the longer isoform found in the male gametes and somatic cells is important only after implantation. Our results further suggest that interference with the methylation machinery in early embryogenesis results in stable changes of IAP methylation and altered gene expression in the adult.

MATERIALS AND METHODS

Genotypes and breeding. Four mutant alleles of *Dnmt1* were used: the null allele (c allele [20]), the hypomorphic *chip* allele (39), and the 2lox and 1lox alleles (15). The Msx2Cre transgene contains the Cre recombinase cDNA under the control of the Msx2 regulatory sequences (37) and was always transmitted through the female germ line. The A^{iapy} allele contains an IAP insertion upstream of the *agouti* gene (28) and was always transmitted through the male germ line from pseudoagouti or near-pseudoagouti males.

Phenotype classification and statistics. The overall degree of yellow fur contribution in A^{iapy} mice was determined visually. In all cases, the examiner was blind to the genotype. The mice were classified in four different groups: 0 to 10% yellow contribution (all or almost all brown), 15 to 50% contribution, 55 to 85% contribution, and 90 to 100% contribution (all or almost all yellow). The *P* values were obtained using the Mann-Whitney rank sum test.

DNA preparation and methylation assay. Tissues were digested in lysis buffer (100 mM Tris HCl [pH 8.5], 5 mM EDTA, 0.2% sodium dodecyl sulfate [SDS], 200 mM NaCl, plus 100 μg of proteinase K/ml) overnight (o/n) at 55°C, phenol-chloroform extracted, and precipitated with an equal volume of isopropanol. A 10-μg aliquot of DNA was digested with the stated restriction endonuclease for 12 to 16 h. The methyl-sensitive restriction enzyme *Hpa*II is blocked by methylation of its recognition sequence. The enzyme only cuts when the site is demethylated, yielding smaller DNA products (see Fig. 3A). The products were resolved on an agarose gel, transferred to nylon membranes (Genescreen), and hybridized in Church buffer (0.5 M $NaPO_4$ [pH 7.5], 7% SDS, 2 mM EDTA) with a radioactively labeled probe at 65°C o/n. Probe 1 was generated by PCR amplification from mouse genomic DNA using the following primers (5′ to 3′): F, GCTTCTCAGGATGGATGTCA; R, GCCCCAGTTTCATCACTTGT, yielding a 736-bp fragment. The probe was synthesized with random hexamers. Final wash was done with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS at 65°C.

Northern analysis. RNA was isolated using TRIzol (Gibco), separated on a formaldehyde-agarose gel, and transferred to a nylon membrane (Genescreen). Hybridization was carried out at 65°C o/n in Church buffer. The agouti probe (500 bp) was generated by PCR using the primers (5′ to 3′) F3 (CCACCCTA GTGAGCTTCCTG) and R2 (GCCCACAAGTCACAACCAC) and reverse-transcribed liver RNA from yellow mice. The agouti probe was radioactively labeled and synthesized with random hexamers.

Western analysis. Female mice were hormone primed with 5 IU of pregnant mare serum gonadotropin (Calbiochem) intraperitoneally followed 48 h later by 5 IU of recombinant human chorionic gonadotropin (Calbiochem) intraperitoneally. Eggs were harvested from the oviducts 24 h after human chorionic gonadotropin treatment. Ten eggs of each genotype were lysed in sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris [pH 6.8], 0.01% bromophenol blue), boiled, and loaded on an SDS–8% polyacrylamide electrophoresis gel. The gel was blotted on a nitrocellulose membrane (catalog no. RPN2020D; Amersham Pharmacia Biotech). A chicken C-terminal Dnmt1 antibody was used at 1/1,000 dilution (8), and detection was performed with the chemiluminescence method (ECL; Amersham Pharmacia Biotech).

RESULTS

To investigate the effect of reduced Dnmt1 levels in embryos on the methylation of paternally transmitted A^{iapy} alleles, we determined by visual examination the coat color distribution in offspring from $Dnmt1^{+/+}$; A^{iapy}/a and $Dnmt1^{+/-}$; A^{iapy}/a males bred to wild-type $Dnmt1^{+/+}$; a/a females. A mutation in the *agouti* locus results in no detectable *agouti* expression and a recessive black hair phenotype. Expression of *agouti* from the A^{iapy} allele is dominant and gives coat colors ranging from brown (agouti) to yellow, depending on the level of *agouti* expression. To exclude transgenerational inheritance at the *agouti* locus in our experiments, we transmitted the A^{iapy} allele through the paternal germ line and always used males that were pseudoagouti or with a very high pseudoagouti contribution. Coat colors were broken down into four categories: agouti (0 to 10% yellow coat color), mottled (15 to 50% and 55 to 85% yellow coat color), and yellow (90 to 100% yellow coat color) (Fig. 1 and 2). The distribution of coat color in the progeny from $Dnmt1^{+/+}$ fathers was 35% agouti and 13% yellow (the remainder being mottled) (Fig. 2A). The distribution of coat color in progeny from $Dnmt1^{+/-}$ fathers containing 50% less Dnmt1 than wild type (20) showed that 27% of the progeny was agouti and 12% was yellow (Fig. 2B). Thus, reduction of Dnmt1 levels by half in the male germ line did not significantly ($P = 0.8$) affect the overall coat color distribution in the offspring. However, when the coat color of the offspring was separated by genotype, we noted that 20% of the $Dnmt1^{+/-}$ animals were agouti and 19% were yellow (average yellow color = 50 to 55%) (Fig. 2D) compared to $Dnmt1^{+/+}$ animals, which were 34% agouti and 8% yellow, a difference that was significant (average yellow color = 35 to 40%; $P = 0.02$) (Fig. 2C). Specifically, the $Dnmt1^{+/-}$ progeny were about half as likely to be agouti and twice as likely to be yellow compared to $Dnmt1^{+/+}$ siblings. The coat color of $Dnmt1^{+/+}$ progeny from $Dnmt1^{+/-}$ fathers (Fig. 2C) showed a similar distribution as progeny from $Dnmt1^{+/+}$ fathers (Fig. 2A), suggesting that reducing the levels of Dnmt1 in the male differentiating gametes did not affect coat color distribution in the progeny. Thus, the apparent shift of the $Dnmt1^{+/-}$ progeny average contribution toward yellow likely reflected the loss of methylation in somatic cells. To confirm that the coat color of

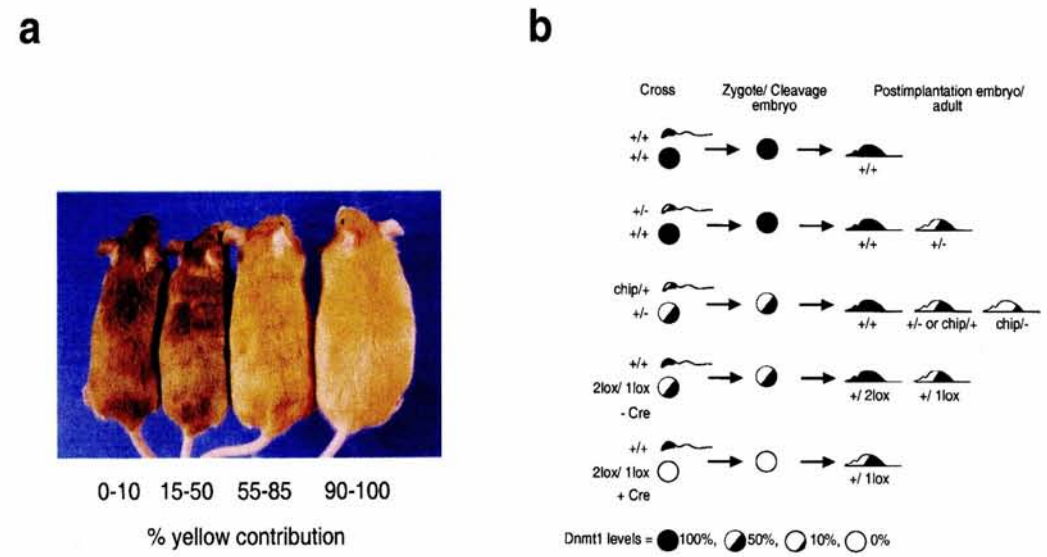

FIG. 1. Coat color of A^{iapy} mice. (a) A^{iapy} siblings show a wide degree of expressivity of the *agouti* gene, resulting in coat colors ranging from pseudoagouti (left) to completely yellow (right). Constitutive expression of the *agouti* gene also correlates with an obesity phenotype, which can be seen in the solid yellow mouse (28). (b) Relative levels of Dnmt1 in parental gametes and progeny from the crosses performed. The strength of the *Dnmt1* mutant alleles relative to wild type (set at 100%) is as follows: $Dnmt1^{+/2lox}$ (100%), $Dnmt1^{chip/+}$ (60%), $Dnmt1^{+/-}$ (50%), $Dnmt1^{+/1lox}$ (50%), $Dnmt1^{2lox/1lox}$ (50%), and $Dnmt1^{chip/-}$ (10%). Wild-type levels of Dnmt1 are indicated by a solid black color. For example, $Dnmt1^{+/-}$ mice, which contain only 50% of wild-type Dnmt1 levels, are depicted as 50% black. The genotypes of the gametes represent the genotype of the parental animals, as gametes are haploid and can carry either allele. The levels of Dnmt1 depicted in the sperm represent expected premeiosis levels, since mature sperm does not contain detectable Dnmt1. Those levels indicate the degree of Dnmt1 levels that the male gamete eventually contributes to the implantation embryo and adult when Dnmt1 expression is turned on. $Dnmt1^{+/-}$ and $Dnmt1^{chip/+}$ mice are depicted by a 50% black mouse for simplicity but contain, respectively, 50 and 60% of wild-type levels of Dnmt1.

our animals correlated with the methylation status of the *agouti* IAP LTR, as would be expected (28), we determined the extent of methylation at that locus in organs from A^{iapy} agouti, mottled, and yellow animals. We found that the methylation of the A^{iapy} insertion correlated with coat color and IAP-driven *agouti* RNA expression (Fig. 3).

If the loss of methylation at the A^{iapy} locus in somatic cells were directly linked to the level of Dnmt1, further reduction of Dnmt1 levels would be predicted to result in a more significant increase in yellow progeny. To test this hypothesis, we crossed males containing a hypomorphic allele of Dnmt1 ($Dnmt1^{chip}$) and the A^{iapy} allele with C57BL/6 females heterozygous for the Dnmt1 null allele to generate compound heterozygous $Dnmt1^{chip/-}$ mice, which express 10% of wild-type levels of Dnmt1 and are globally hypomethylated (7). Progeny from this cross had a coat color distribution of 26% agouti and 19% yellow (average yellow contribution = 45 to 50%) (Fig. 4A), a result similar to what was observed with $Dnmt1^{+/-}$ fathers and wild-type mothers (Fig. 2B). However, when the genotype of the offspring was taken into account, the distribution of coat color showed that the hypomethylated $Dnmt1^{chip/-}$ animals were on average more yellow than their wild-type or *Dnmt1* heterozygous siblings (Fig. 4B to E), consistent with $Dnmt1^{chip/-}$ mice being more hypomethylated. Specifically, 10% of $Dnmt1^{chip/-}$ animals were in the 0 to 10% agouti group and 52% were 90 to 100% yellow (average yellow contribution = 70 to 75%; $P = 0.001$). This cross yielded three additional genotypes, $Dnmt1^{+/+}$, $Dnmt1^{chip/+}$, and $Dnmt1^{+/-}$. As shown in Fig. 4C, the wild-type progeny had a coat color distribution similar to that in the control experiment shown in Fig. 2A, suggesting that halving the maternal store of Dnmt1 in early embryos did not have an effect on the paternally transmitted A^{iapy}. The $Dnmt1^{chip/+}$ and $Dnmt1^{+/-}$ mice had a slight bias towards the yellow color in comparison to wild-type progeny (50 and 45% average yellow color, respectively; Fig. 4D and E). These results are reminiscent of the *Dnmt1* heterozygotes in Fig. 2D, which also displayed a slight yellow bias. Thus, our results suggest that reduction of the longer Dnmt1 isoform levels affects the methylation of the *agouti* locus in embryonic cells during postimplantation and leads to a loss of A^{iapy} methylation.

The oocyte contains a large store of a shorter Dnmt1 isoform which is present during the cleavage stage of embryogenesis. To test whether this isoform plays a role in maintaining IAP methylation during that stage, we sought to reduce the levels of maternal Dnmt1 in oocytes to ensure minimal contribution of that protein to the early embryo. To do this, we used females containing a conditional allele of *Dnmt1* ($Dnmt1^{2lox}$ [15]) combined with a null allele ($Dnmt1^{1lox}$) and a Cre recombinase transgene driven by the *Msx2* promoter (37, 23), which expresses Cre in the oocytes. The $Dnmt1^{2lox}$ allele functions normally in vivo and produces wild-type levels of Dnmt1 (15). The Cre-mediated recombination of this allele in oocytes deletes exons 4 and 5, which contain the translational start site of the oocyte-specific form of Dnmt1. The effect of this rearrangement of the *Dnmt1* locus in reducing Dnmt1 levels in

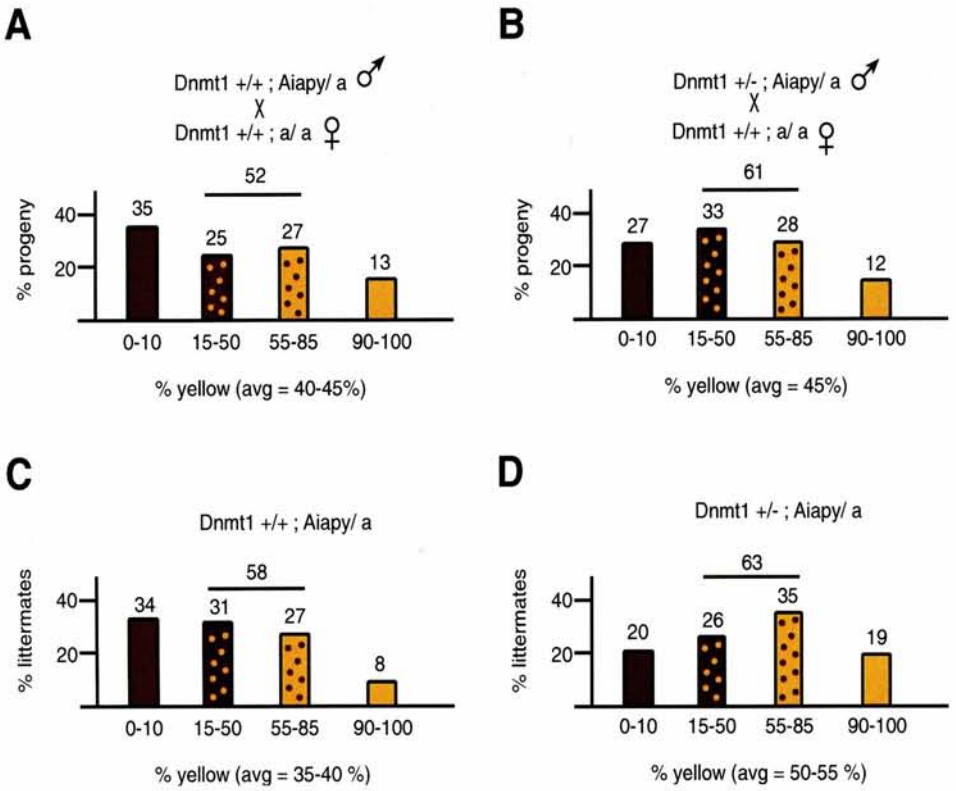

FIG. 2. Distribution of coat color of A^{iapy} progeny. All results are expressed in terms of the percentage of yellow coat color (*x* axis). The degree of yellow expression was scored in four different ranges of yellow contribution: 0 to 10%, 15 to 50%, 55 to 85%, and 90 to 100%. The *y* axis represents the percentage of the progeny which fell in a specific range and is indicated above each group. The total percentage of mottled contribution is indicated over the mottled ranges and represents the percentage of progeny falling in the 15 to 85% range. The average yellow contribution of each panel is indicated and is labeled "avg." The average determined was the arithmetic average, specifically, the sum of the estimated yellow contribution of each mouse divided by the number of mice. Coat color distribution are shown for progeny from fathers containing wild-type levels of Dnmt1 (A) or heterozygous levels of Dnmt1 (B) bred to C57BL/6 female mice. A wide range of coat color contribution is evident. The distribution in panel B was broken down according to whether the mice were $Dnmt1^{+/+}$ (C) or $Dnmt1^{+/-}$ (D). Sample sizes (*n*) were 96 (A), 133 (B), 60 (C), and 73 (D). The genetic background of all mice was C57BL/6.

oocyte was measured by immunoblotting of oocytes from hormone-primed $Dnmt1^{2lox/1lox}$ *Msx2Cre* females (Fig. 5A). Levels of the oocyte-specific Dnmt1 isoform were found to be much reduced in $Dnmt1^{2lox/1lox}$ oocytes containing *Msx2Cre* compared to $Dnmt1^{2lox/1lox}$ oocytes that did not contain *Msx2Cre*. Lower levels of smaller proteins were also detected in the *Msx2Cre* oocytes and may have represented products made from downstream translational initiation. Similar products were also observed in cells containing an N-terminal knockout of the *Dnmt1* gene in which the translational start site for the oocyte-specific form was deleted and resulted in an embryonic lethal phenotype in homozygous mutant animals ($Dnmt1^{n}$ allele) (22).

To test the effect of low maternal Dnmt1 levels on *agouti* expression in A^{iapy} progeny, we crossed $Dnmt1^{+/+}$; A^{iapy}/a males with black $Dnmt1^{2lox/1\ lox}$; *a*/*a* females carrying or not carrying the *Msx2Cre* transgene (Fig. 5C and D). All $Dnmt1^{2lox/1lox}$ females containing *Cre* transgenes transmitted a recombined *1lox* allele, showing that the Cre-mediated recombination in the oocyte was very efficient (data not shown). The litter size from the *Msx2Cre*-carrying mothers was normal (eight on average). These results contrasted with those of the knockout of the maternal store of Dnmt1 by deletion of the oocyte-specific promoter, which resulted in embryonic death in most fetuses during the last third of gestation (13), a difference which could be explained by the presence of low levels of truncated Dnmt1 in our experiment that might have provided residual activity and rescued the null phenotype. Interestingly, *Msx2Cre* transgenic females generated litters that were more yellow on average than the litters from females that didn't carry the *Msx2Cre* transgene. Specifically, progeny from *Msx2Cre* mothers were 33% agouti and 41% yellow (Fig. 5D; average yellow contribution = 55%) compared to 47% agouti and 4% yellow (Fig. 5C; average yellow contribution = 30%) when the mothers did not contain the *Msx2Cre* allele, a difference that was statistically significant ($P = 0.004$). To confirm that the results obtained in Fig. 5C were not biased by the *Dnmt1* genotype of the progeny, we plotted the pups in Fig. 5C according to whether they were $Dnmt1^{2lox/+}$ or $Dnmt1^{1lox/+}$ (Fig. 5E and F). The results showed that the color distribution between these

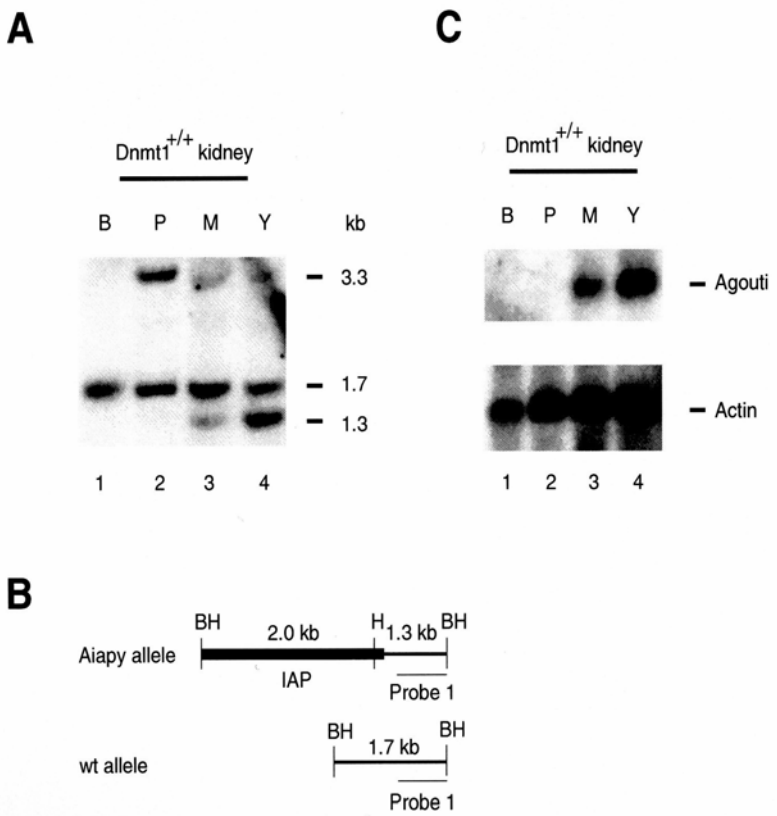

FIG. 3. Methylation status of the IAP allele correlates with agouti expression and coat color. (A) Southern blot analysis of kidney tissues from black (B), pseudoagouti (P), mottled (M), and yellow (Y) animals. The methylation-sensitive restriction enzyme *Hpa*II (H) in combination with *Bam*HI (BH) was used to detect the methylation status of the 5′ LTR of the IAP. The wild-type allele does not contain a *Hpa*II site yielding a fragment of 1.7 kb (see schematic diagram in panel B). Depending on the methylation status of the 5′ LTR, the A^{iapy} allele will yield a 1.3-kb fragment if unmethylated or a 3.3-kb fragment if methylated. Because the mice were heterozygous for the A^{iapy} allele, a wild-type 1.7-kb fragment was observed in all samples. All three fragments are indicated on the right side of the panel. A clear correlation between A^{iapy} hypomethylation and coat color can be observed. (B) Schematic diagram of the fragment sizes predicted from the A^{iapy} and wild-type alleles. (C) Northern blot analysis of total RNA from black (B), pseudoagouti (P), mottled (M), and yellow (Y) mice hybridized to an agouti probe. A clear correlation between methylation (panel A) and expression at the 5′ LTR of the IAP can be seen. The agouti mRNA is 692 bases. As a loading control, an actin probe was used on the same blot (lower panel).

two genotypes was similar ($P = 0.9$) and suggested that the effect seen in Fig. 5D was not simply the result of lower Dnmt1 levels in somatic cells ($Dnmt1^{1lox/+}$). Thus, in addition to maintaining imprinted gene methylation in early embryos (13), the maternal Dnmt1 may also play a role in maintaining IAP methylation.

DISCUSSION

In this work we have studied the role of Dnmt1 in the methylation and expression of IAP elements. In normal development, high levels of Dnmt1 are present during embryogenesis. We have investigated whether the level of Dnmt1 expressed during cleavage and after implantation is important for the maintenance of IAP methylation. Using coat color as the readout, we found that the level of Dnmt1 expression was important for the maintenance of IAP methylation during cleavage and early developmental stages. IAP methylation patterns established during early development are maintained throughout life and can profoundly affect gene expression and phenotype in the postnatal animal.

The most profound shift in coat color was seen when Dnmt1o expression was inhibited during oogenesis. This suggests that the maternal store of Dnmt1o not only maintains monoallelic methylation of imprinted genes but is also crucial for the maintenance of IAP methylation. It has been shown that Dnmt1o is present mostly in the cytoplasm during early embryogenesis but enters the nucleus at the eight-cell stage and immediately exits (2). Thus, Dnmt1o may play a role in keeping IAPs silent solely during this stage. Alternatively, it is possible that Dnmt1o is present in the nucleus throughout early development in levels that are below detection by fluorescence in situ hybridization but sufficient to maintain IAP silencing. Large amounts of Dnmt1o may be briefly ferried to the nucleus to resupply existing Dnmt1o levels. A previous study where Dnmt1o was deleted by genetic inactivation of the

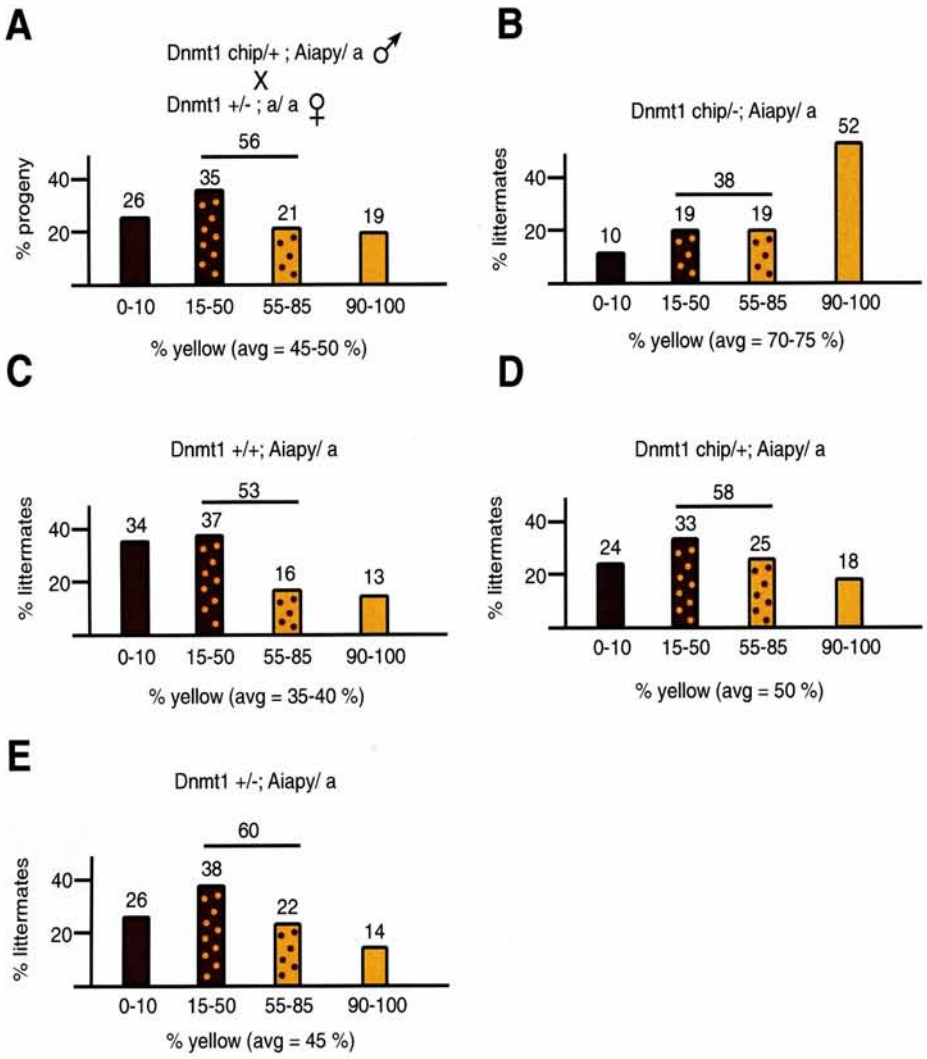

FIG. 4. Coat color distribution from parents containing low levels of Dnmt1. The progeny resulting from the cross in panel A was plotted according to the respective genotypes (B to E). Sample sizes (n) were 155 (A), 21 (B), 38 (C), 51 (D), and 45 (E). A clear switch to yellow coat color can be seen in $Dnmt1^{chip/-}$ animals (B). The genetic background of the mice was mostly C57BL/6 but contained small amounts of 129 and BALB/c, which came from the $Dnmt1^{chip}$ allele.

Dnmt1o promoter showed that demethylation did not occur at IAPs (13). However, in contrast to our experiments which analyzed a single IAP element, that previous study investigated the large number of IAP elements carried in the mouse genome. It is possible that Dnmt1o maintains methylation levels of only some but not most IAP elements. Additionally, the progeny described here is heterozygous for *Dnmt1* ($Dnmt1^{+/1lox}$), in contrast with previous experiments in which the mice contained wild-type levels of somatic Dnmt1 (13). The lower expression of the somatic form of Dnmt1 in $Dnmt1^{+/-}$ pups resulted in a slight increase in the fraction of mice with a yellow coat, whereas the reduction of the Dnmt1s level to 10%, as in $Dnmt1^{chip/-}$ mice, substantially increased the shift to yellow coat color. These observations suggest that the level of somatic Dnmt1 is also important for the maintenance of IAP methylation. Because Dnmt1s is not translated during cleavage (27), it likely functions after implantation, when Dnmt1o levels decrease. Although reduction of Dnmt1 levels in embryos resulted in yellower mice on average, some mice with Dnmt1o deleted or with 10% somatic Dnmt1 were completely agouti, indicating methylation at A^{iapy} in all cells of the animal. Thus, reduction of Dnmt1 levels in embryos does not always cause IAP demethylation and suggests that the loss of methylation at A^{iapy} is a stochastic event. The final color or degree of mottledness of the coat is likely a result of the existing IAP methylation state combined with the probabilistic event of losing additional CpG methylation during cleavage or after implantation as a result of lowered Dnmt1 levels.

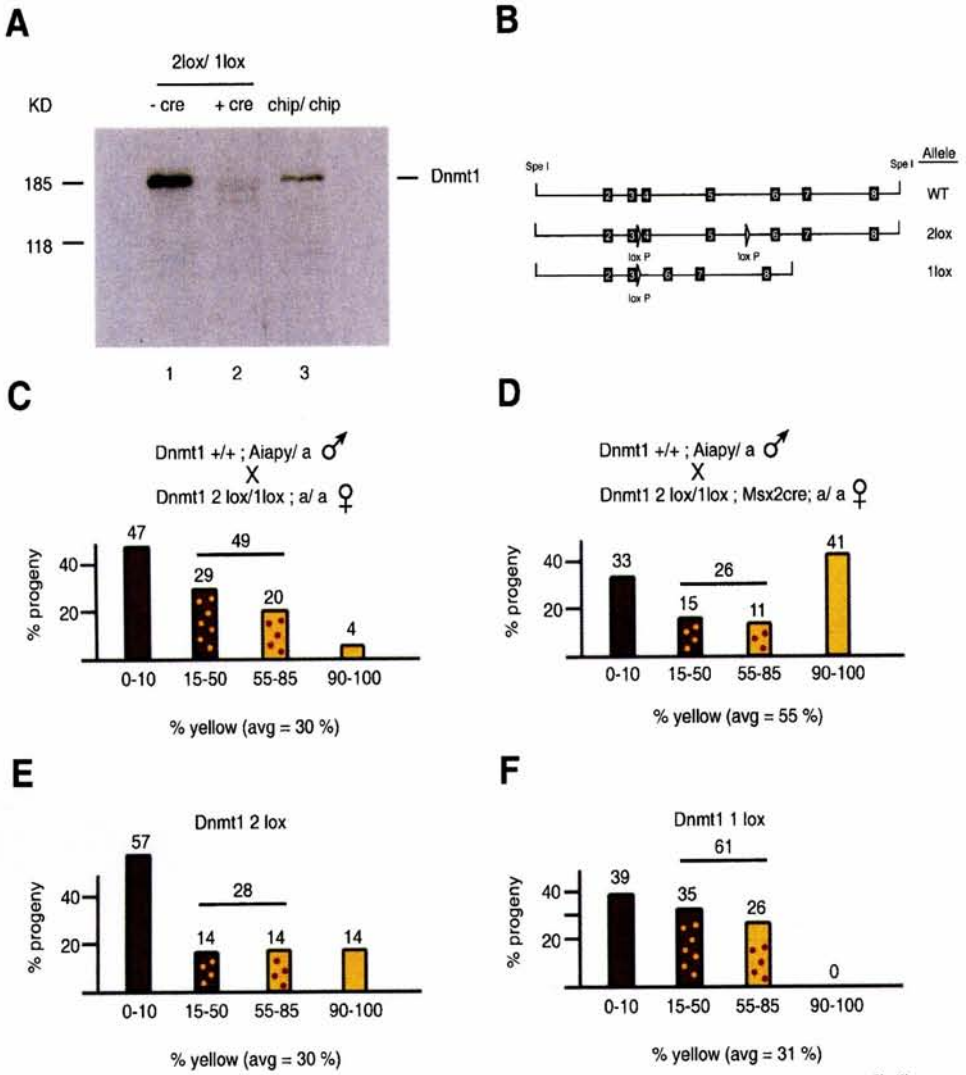

FIG. 5. Coat color distribution of progeny from mothers with reduced Dnmt1o. (A) Immunoblot of 10 oocytes from *Dnmt1*$^{2lox/1lox}$; *a/a* females without *Msx2Cre* (lane 1) or with *Msx2Cre* (lane 2) and from females homozygous for the hypomorphic *Dnmt1*^{chip} allele (lane 3). A C-terminal Dnmt1 antibody was used. The predicted size of the oocyte form is 170 kDa. (B) Schematic diagram of the *Dnmt1*^{2lox} (2lox) and *Dnmt1*^{1lox} (1lox) alleles compared to the wild-type allele (WT). The exons are indicated by the black boxes and corresponding exon numbers, and the loxP sites are indicated by arrowheads. The *1lox* allele contains a deletion of genomic sequences that includes exons 4 and 5. (C and D) Coat color distribution of progeny from *Dnmt1*$^{+/+}$; *A*^{iapy}*/a* fathers and *Dnmt1*$^{2lox/1lox}$; *a/a* mothers without *Msx2Cre* (C) or heterozygous for *Msx2Cre* (D). Sample sizes (*n*) were 45 (C) and 27 (D); $P = 0.004$. The background of the mice was mostly C57BL/6 but contained small contributions from FVB and 129, which came from the *2lox*, *1lox*, and *Msx2Cre* alleles. (E and F) The progeny in panel C were broken down according to whether they were *Dnmt1*^{2lox} (E) or *Dnmt1*^{1lox} (F).

In wild-type mice, IAPs are resistant to demethylation throughout embryonic development (19). Methylation of IAP in cleavage embryos is probably important in preventing IAP expression, which could lead to retrotransposition and insertional mutagenesis (41). Our results show that the shorter Dnmt1 isoform is important in keeping the IAPs silent during cleavage development. Changes of IAP methylation during embryogenesis are faithfully maintained in tissues of the adult, as evidenced by the direct correlation between coat color and the overall level of *A*^{iapy} methylation in the liver and kidney (Fig. 3 and data not shown). Proper establishment of these embryonic patterns of methylation are crucial in setting up chromatin states following remethylation of DNA during postimplantation development (11). Because structural patterns of chromatin established during this embryonic stage are maintained in the adult, changes of methylation prior to chromatin assembly may have long-lasting effects impacting all subsequent cell lineages.

Stochastic changes in methylation patterns in early embryogenesis induced by dietary or other factors can have far-reach-

ing effects in adulthood or in the next generation, such as the determination of coat color of A^{vy} mice or the kinked-tail phenotype of $Axin^{Fu}$ mice (29, 42, 44). Such modifications in genomic methylation patterns may lead to stable changes in gene expression that may have phenotypic consequences. For instance, the methylation and expression of the *reelin* gene, whose disruption leads to a phenotype similar to schizophrenia, has been shown to be influenced by an L-methionine-supplemented diet (38). It has also been suggested that cancer and a number of diseases such as multiple sclerosis, rheumatoid arthritis, and diabetes may have an epigenetic basis (32). The expression of disease genes could be affected by methylation directly, like the *reelin* gene, or indirectly by interference from nearby retroviral-like elements, such as the A^{iapy} allele in mice. Sequencing of the mouse and human genomes has revealed that a large proportion of their sequences, 37 and 42%, respectively, is derived from retrotransposable elements (18, 43) which are normally silenced by methylation. In humans, long interspersed transposable elements can alter the expression of neighboring genes (36) and short interspersed transposable elements are excluded from imprinted regions in the genome (9), suggesting that monoallelic expression of imprinted genes may be more vulnerable to methylation-mediated chromatin silencing occurring in short interspersed transposable elements. Our observations are consistent with the notion that changes of the epigenetic state of the genome can be induced early in development by genetic and environmental conditions. Such changes can have profound consequences for gene expression in the adult and for disease manifestation (16).

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Chapter 4

Genomic Hypomethylation Causes Tumors in Mice

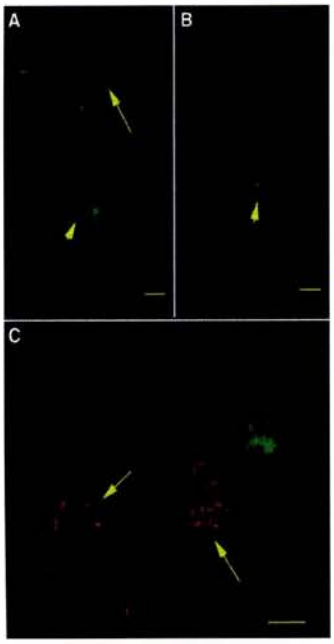

Fig. 3. Confocal immunofluorescence micrographs of human brain tissue. Sections from the entorhinal cortex of (**A**) an AD brain and (**B**) an age-matched control case were immunostained with oligomer-specific antibody (red) (arrows) and counterstained with thioflavin-S (green). Note the single thioflavin-S–positive plaque in the control case (arrowhead) and lack of oligomer-specific antibody fluorescence. (**C**) Oligomer-specific antibody positive deposits were observed in the AD case. When such deposits were found in association with a thioflavin-S–positive plaque, higher magnification and a Z-series indicated that the two deposits were spatially segregated. Scale bars, 20 μm.

have a conformation that is distinct from that of soluble monomers, low-MW oligomers, and fibrils. The fact that this epitope is common to amyloids of widely varying primary sequence further indicates that the epitope is formed from a specific conformation of the polypeptide backbone and is largely independent of the amino acid side chains in this region. A similar type of antibody specificity was recently reported by Wetzel and co-workers (*18*), but this antibody is specific for all types of amyloid fibrils and does not recognize soluble oligomers. Stefani and co-workers (*11*) have recently reported that soluble oligomers formed from non–disease-related proteins are inherently cytoxic, suggesting that they may have a common structure and function. Because the oligomer-specific antibody neutralizes the toxicity of oligomeric forms of all amyloids tested, they share a common structure, and they have a common mechanism of pathogenesis that is intimately associated with this common structure. This mechanistic commonality represents an important advance in our understanding of the mechanism of amyloid pathogenesis because it argues against a specific mechanism for one type of amyloid that is untenable for all of them. Because some amyloids, like Aβ, are in the extracellular space or the lumenal contents of the secretory and endocytic pathways, whereas other amyloids, like α-synuclein, reside in the cytosolic compartment, components that reside exclusively in either compartment are excluded as primary targets. In contrast, a common mechanism argues in favor of components that are accessible from both extracellular and cytosolic compartment, such as cell membranes as primary targets of amyloid pathogenesis.

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Supporting Online Material

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Materials and Methods
SOM Text
Figs. S1 to S4
References
Movie S1

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Induction of Tumors in Mice by Genomic Hypomethylation

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Genome-wide DNA hypomethylation occurs in many human cancers, but whether this epigenetic change is a cause or consequence of tumorigenesis has been unclear. To explore this phenomenon, we generated mice carrying a hypomorphic DNA methyltransferase 1 (*Dnmt1*) allele, which reduces Dnmt1 expression to 10% of wild-type levels and results in substantial genome-wide hypomethylation in all tissues. The mutant mice were runted at birth, and at 4 to 8 months of age they developed aggressive T cell lymphomas that displayed a high frequency of chromosome 15 trisomy. These results indicate that DNA hypomethylation plays a causal role in tumor formation, possibly by promoting chromosomal instability.

Human cancer cells often display abnormal patterns of DNA methylation. The role of aberrant hypermethylation in the silencing of tumor suppressor genes is now well documented (*1*). In contrast, the role of aberrant hypomethylation—which is observed in a wide variety of tumors (*2–5*), often together with regional hypermethylation—has remained unclear.

To investigate whether DNA hypomethylation has a causal role in tumor formation, we generated mice with highly reduced levels of Dnmt1, the enzyme that maintains DNA methylation patterns in somatic cells (*6*). Because mice homozygous for a Dnmt1 null allele ($Dnmt1^{c/c}$) die during gestation (*7, 8*), we combined a hypomorphic allele [$Dnmt1^{chip}$ (*9*)] with a null allele to generate $Dnmt1^{chip/c}$ (referred to here as $Dnmt1^{chip/-}$) compound heterozygotes with a substantially reduced level of genome-wide DNA methyl-

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ation. *Dnmt1*$^{chip/-}$ embryonic stem (ES) cells expressed 10% of wild-type levels (Fig. 1A). To test whether the reduced Dnmt1 expression affected DNA methylation in vivo, we generated mice carrying the different *Dnmt1* alleles and determined their global methylation levels with the use of a probe for endogenous retroviral A type particles (IAPs) (Fig. 1, B and D) (*10*) and centromeric repeats (Fig. 1C). Southern blot analysis of embryonic fibroblasts and adult tissues showed that the DNA from compound heterozygotes was hypomethylated relative to the DNA from *Dnmt1*$^{chip/chip}$ or *Dnmt1*$^{+/+}$ mice, although substantially less so than the DNA from *Dnmt1*$^{-/-}$ null ES cells. Mice carrying the different *Dnmt1* alleles were obtained at the expected Mendelian ratios, indicating that reduction of Dnmt1 expression to 10% was compatible with viability. However, compound heterozygotes (*Dnmt1*$^{chip/-}$) were runted and their weight at birth was only 70% that of *Dnmt1*$^{+/-}$ mice, in contrast to mice homozygous for the hypomorphic allele (*Dnmt1*$^{chip/chip}$), which were normal in size (Fig. 2A). *Dnmt1*$^{chip/-}$ mice, although remaining substantially underweight, were fertile and generated litters of nonrunted pups when bred with wild-type mice.

In addition to the runted phenotype, 80% of *Dnmt1*$^{chip/-}$ mice developed aggressive thymic tumors at 4 to 8 months of age. Cumulative survival of the *Dnmt1*$^{chip/-}$ mice is shown in Fig. 2B. Histological analysis classified the tumors as T cell lymphomas (*11*), and fluorescence-activated cell sorting (FACS) analysis revealed that most tumors were CD4$^-$/CD8$^+$ or CD4$^+$/CD8$^+$ (Fig. 2D). When tested for D-to-J rearrangements in the T cell receptor β locus, four of 10 tumors showed a predominant Dβ1-to-Jβ1 rearranged band (Fig. 2C, lanes 5, 9, 11, and 13) consistent with monoclonality. Tumors without Dβ1-to-Jβ1 recombination may have rearranged other D and J elements. Monoclonality suggests that hypomethylation induces cancer in a precursor cell, with subsequent events leading to malignant tumor formation. Consistent with frequent activation of the c-*myc* oncogene in mouse and human lymphoma (*12*), we found that c-*myc* was overexpressed in almost all hypomethylated tumors (15/18 *Dnmt1*$^{chip/-}$, Fig. 3C).

Genomic hypomethylation may contribute to lymphomagenesis by an epigenetic or a genetic mechanism. We considered three possible mechanisms.

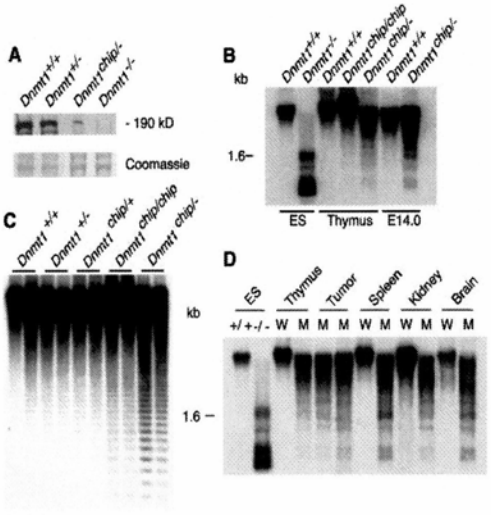

Fig. 1. Genomic hypomethylation in *Dnmt1* hypomorphic mice. (**A**) Immunoblot analysis of protein extracts from ES cells, using a C-terminal Dnmt1 chicken antibody (*34*). The level of Dnmt1 in *Dnmt1*$^{chip/-}$ ES cells was markedly reduced, demonstrating the hypomorphic nature of this allele. The *Dnmt1*$^{-/-}$ negative control extracts did not show any bands, as expected [c allele (*7*)]. A Promega antibody to chicken immunoglobulin Y (IgY), with horseradish peroxidase as secondary antibody, was used; detection was performed with the Amersham Pharmacia Biotech ECL kit. The Coomassie gel shows total protein levels in each sample. (**B**) Southern blot of methylation status of IAPs in *Dnmt1*$^{chip/chip}$ and *Dnmt1*$^{chip/-}$ mice. Genomic DNA was digested with the methylation-sensitive restriction enzyme Hpa II and probed with an IAP cDNA probe (*10*). Hypomethylation was detected in thymus from 6-week-old *Dnmt1*$^{chip/-}$ mice and in *Dnmt1*$^{chip/-}$ embryonic day (E) 14.0 fibroblasts but not in *Dnmt1*$^{chip/chip}$ homozygotes or wild-type controls, as evidenced by the presence of lower molecular weight DNA fragments. (**C**) Southern blot analysis of centromeric repeat methylation of E14.0 fibroblasts from mice containing various *Dnmt1* alleles. Hypomethylation is evident in the *Dnmt1*$^{chip/-}$ lanes. (**D**) Southern blot analysis of IAP methylation status in tissues from a 6-week-old *Dnmt1*$^{chip/-}$ mouse and in lymphomas. Significant hypomethylation is evident in all *Dnmt1*$^{chip/-}$ tissues. Controls for methylation (*Dnmt1*$^{+/+}$ ES cells) and hypomethylation (*Dnmt1*$^{-/-}$ ES cells) are also shown (ES, ES cells; W, *Dnmt1*$^{+/+}$; M, *Dnmt1*$^{chip/-}$).

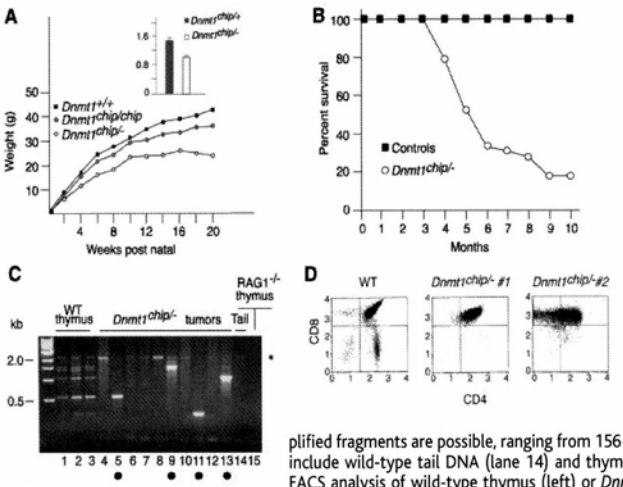

Fig. 2. *Dnmt1* hypomorphs are runted and develop T cell lymphomas. (**A**) Average weight of *Dnmt1*$^{chip/+}$ (n = 20) and *Dnmt1*$^{chip/-}$ (n = 20) male littermates at birth (inset). *Dnmt1*$^{chip/-}$ mice were 66% as large as *Dnmt1*$^{chip/+}$ mice. Females showed the same runt phenotype. The error bar indicates ±1 SD, $p < 0.0001$ (Student t test, StatView 5.0.1 software). Also shown are growth curves of *Dnmt1*$^{chip/chip}$, *Dnmt1*$^{chip/-}$, and wild-type male mice. Six to 10 mice of each genotype were used for each data point. (**B**) Cumulative survival of *Dnmt1*$^{chip/-}$ mice. Most *Dnmt1*$^{chip/-}$ mice became terminally ill between 4 and 8 months of age. Control mice were *Dnmt1*$^{chip/chip}$ (n = 18); experimental mice were *Dnmt1*$^{chip/-}$ (n = 33). Mice were autopsied when visibly ill. At autopsy, 23 of 33 *Dnmt1*$^{chip/-}$ mice had developed tumors (21 lymphomas and 2 fibrosarcomas). Autopsy of *Dnmt1*$^{chip/chip}$ mice at 6 months (n = 12) and 12 months (n = 6) showed no evidence of tumor formation. (**C**) Dβ1-to-Jβ1 rearrangement at the TCRβ locus was analyzed by the polymerase chain reaction as described (*35*), using primers 1 and 4 therein. The asterisk denotes the germline configuration [2171 base pairs (bp)]. When rearranged, five different amplified fragments are possible, ranging from 1561 to 381 bp (see wild-type thymus, lanes 1 to 3). Controls also include wild-type tail DNA (lane 14) and thymus DNA from a recombination-deficient RAG1$^{-/-}$ mouse. (**D**) FACS analysis of wild-type thymus (left) or *Dnmt1*$^{chip/-}$ tumors (middle and right) stained for CD4 and CD8 receptors, T cell–specific markers. Tumors analyzed (n = 16) contained either double-positive CD4^{high}/CD8^{high} cells (9/16, middle panel) or CD4^{low}/CD8^{high} cells (7/16, right panel).

(i) Hypomethylation may induce endogenous retroviral elements, leading in turn to insertional activation of proto-oncogenes (*13*). To test this idea, we hybridized RNA from randomly selected tumors with a Moloney murine leukemia virus (MMLV) cDNA probe and an IAP probe to detect endogenous retroviral and IAP expression, respectively. Of nine *Dnmt1*$^{chip/-}$ tumors, none showed C-type retroviral activation (Fig. 3A) (*14*) and only one of eight tumors showed a moderate increase in IAP expression (Fig. 3B, lane 7). In contrast, strong C-type retroviral expression was seen in a MMLV-induced lymphoma [Fig. 3A, slot a1 (*15*)] and IAP expression was highly activated in *Dnmt1*$^{-/-}$ fibroblasts [Fig. 3B, lanes 10 to 12 (*16*)]. Because c-*myc* is a frequent target for insertional activation by retroviral elements (*17*), we searched for inserted proviral elements in hypomethylated and MMLV-induced tumors. In 3 of 12 MMLV-induced tumors, an insertional rearrangement was seen in the vicinity of the c-*myc* locus, in agreement with previous observations (*17*). In contrast, no rearrangements were detected in hypomethylated tumors [0/18 (*11*)]. We conclude that the extent of hypomethylation in *Dnmt1*$^{chip/-}$ mice does not effectively activate endogenous retroviral elements and that virus insertions may not be a prevalent mechanism in hypomethylation-induced lymphoma.

(ii) Hypomethylation may activate proto-oncogenes through epigenetic effects (*18, 19*). Indeed, c-*myc* was overexpressed in most hypomethylated tumors (Fig. 3C). However, it is unlikely that activation of c-*myc* is a direct consequence of promoter demethylation because the gene is expressed at normal levels in thymuses from 2- and 4-week-old mice that show a level of hypomethylation identical to that of the tumors [Fig. 1D (*11*)]. In addition, c-*myc* was not activated in *Dnmt*$^{-/-}$ fibroblasts that are almost completely demethylated (*16*). Finally, if oncogene activation by hypomethylation stimulated T cell proliferation as a first step in transformation, one would expect the lymphomas to be polyclonal rather than monoclonal (Fig. 2C).

(iii) Hypomethylation may induce genomic instability. In fact, a significantly increased frequency of chromosomal rearrangements such as loss of heterozygosity (LOH) was observed in *Dnmt1* mutant ES cells, suggesting that normal levels of methylation are important for genomic stability (*20*). Defects in DNA methylation have been linked to genome instability in studies of colorectal tumor cell lines (*21*), mouse tumor models (*22, 23*), and patients with immunodeficiency–centromeric instability–facial anomalies (ICF) syndrome (*24, 25*).

To test whether DNA hypomethylation increases genomic instability in *Dnmt1*$^{chip/-}$ tumors, we performed array-based comparative genome hybridization [array CGH (*26*)] using thymic tumor genomic DNA prepared from *Dnmt1*$^{chip/-}$ and Mov-1 (*15*) and Mov-14 (*27*) MMLV transgenic mice (Fig. 3D). There was a statistically significant difference in chromosome gains between these tumor classes (Table 1). Ten of 12 hypomethylated tumors exhibited a gain of chromosome 15, whereas only 2 of 12 MMLV-induced tumors showed this change ($P = 0.004$). Relative to MMLV-induced tumors, hypomethylated tumors also displayed a gain of chromosome 14 (4/12 versus 0/12, $P = 0.05$) and a higher degree of duplicated and deleted chromosome regions (Table 1) (Fig. 3D).

Together with the centromeric hypomethylation we observed (Fig. 1C), these results suggest a causal link between DNA hypomethylation and chromosomal instability as one mechanism leading to tumorigenesis. The increased fluorescence ratios observed for chromosomes 14 and 15 are consistent with single-copy whole-chromosome gains throughout the tumor (Fig. 3D), which suggests that they are early events in the development of these monoclonal T cell lymphomas. Chromosome 15 is frequently duplicated in mouse T cell tumors (*28, 29*) and contains the oncogene c-*myc*, which when overexpressed causes T cell lymphomas (*17*). The fact that c-*myc* is overex-

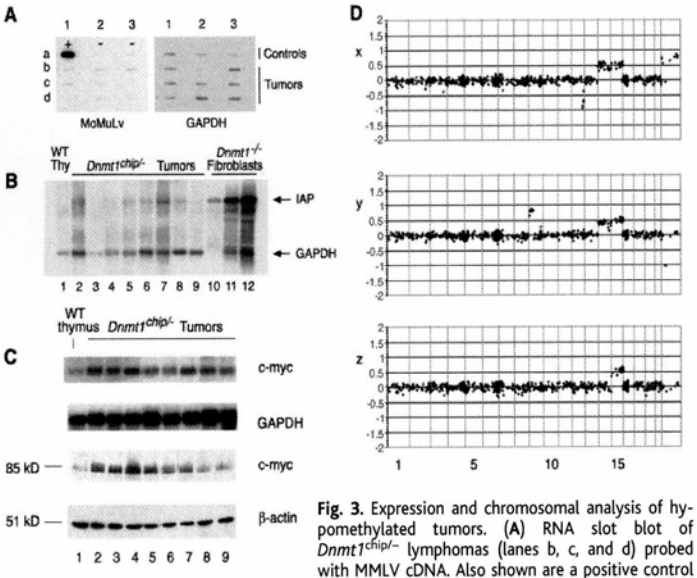

Fig. 3. Expression and chromosomal analysis of hypomethylated tumors. (**A**) RNA slot blot of *Dnmt1*$^{chip/-}$ lymphomas (lanes b, c, and d) probed with MMLV cDNA. Also shown are a positive control lymphoma from a Mov-1 mouse [slot a1 (*15*)] and negative control thymuses from wild-type 129/Sv (slot a2) and a wild-type littermate of a tumor-bearing mouse (slot a3). (**B**) Northern blot of IAPs in *Dnmt1*$^{chip/-}$ tumors. IAPs can be detected in most tumors, whereas wild-type thymus does not show IAP expression. Positive control (lanes 10 to 12, 1:3 serial dilutions) are *Dnmt1*$^{-/-}$ hypomethylated fibroblasts that have been shown to activate IAP expression (*16*). Comparison of IAP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels shows that most clones express much less IAPs than the positive control. (**C**) Expression levels of c-*myc* were assessed by Northern blot (top two panels) and by immunoblot (bottom two panels). Lanes 2 to 7 are tumors that showed chromosome 15 trisomy; lanes 8 and 9 are tumors that are diploid for chromosome 15. Probes used were c-*myc* exon 2 for the Northern analysis and a rabbit polyclonal IgG antibody to c-*myc* for immunoblots (Santa Cruz Biotechnology). (**D**) Array comparative genome hybridization (CGH) analyses of three *Dnmt1*$^{chip/-}$ tumors, showing clear single-copy, whole-chromosome gain of chromosome 15 (x, y, and z), whole-chromosome gains of 14 and loss on distal 12 (x), and gains of chromosome 14 and proximal 9 (y). The X gain (x) reflects a sex difference between tumor and control. Array CGH was performed as in (*26*). Fluorescence ratios (average of quadruplicate measurements) for each bacterial artificial chromosome are plotted as a function of genome location based on the February 2002 freeze of the assembled mouse genome sequence (http://genome.ucsc.edu). Vertical lines delimit chromosome boundaries.

Table 1. Gains or losses of chromosomes in *Dnmt1*$^{chip/-}$ and MMLV-induced tumors. The numbers indicate the number of times a particular event occurred in the *Dnmt1*$^{chip/-}$ or Moloney tumors. These events were not mutually exclusive; many tumors exhibited multiple chromosomal events.

Chromosomal changes	*Dnmt1*$^{chip/-}$ tumors ($n = 12$)	MMLV-induced tumors ($n = 12$)
Chr 15 gain	10	2
Chr 14 gain	4	0
Chr 10 gain	0	1
Partial Chr 9 gain	2	0
Partial Chr 4 gain	1	0
Partial Chr 16 loss	1	0
Partial Chr 12 loss	1	0

pressed (RNA and protein) in most hypomethylated tumors (Fig. 3C) is consistent with a mechanism in which a gain of chromosome 15 contributes, at least in part, to the elevated expression of c-*myc*. Moreover, c-*myc* expression was lower in the two tumors that did not show trisomy 15 than in the other tumors (Fig. 3C).

Our results show that genomic hypomethylation causes tumorigenesis in mice and is associated with the acquisition of additional genomic changes. Consistent with this, genomic hypomethylation was found to promote tumorigenesis in a different mouse tumor model and to increase the rate of LOH in cultured fibroblasts (*23*). However, it remains possible that DNA hypomethylation contributes to tumorigenesis through other mechanisms unrelated to chromosomal instability. The phenotype of hypomethylated mice is also consistent with that of *Suv39h* histone methyltransferase mutant mice; hence, DNA and histone methylation, pericentric chromatin structure, and the maintenance of chromosomal stability may be linked (*30*).

DNA methyltransferase inhibitors such as 5-aza-2′-deoxycytidine have been used successfully to treat cancer in humans (*19, 31*) and mice (*32, 33*). The efficacy of these drugs is presumably due to their ability to reverse the epigenetic silencing of tumor suppressor genes. In light of our results, however, this therapeutic strategy should perhaps be considered a double-edged sword: Genomic demethylation may protect against some cancers such as intestinal tumors in the Apc^{Min} mouse model (*32*) but may promote genomic instability and LOH (*20, 23*) and increase the risk of cancer in other tissues, as seen in hypomethylated mutant mice.

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Regulation of Elongating RNA Polymerase II by Forkhead Transcription Factors in Yeast

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The elongation phase of transcription by RNA polymerase II (RNAPII) is highly regulated and tightly linked to pre–messenger RNA (pre-mRNA) processing. Recent studies have implicated an early elongation checkpoint that facilitates the link to pre-mRNA processing. Here we show that the yeast forkhead transcription factors, Fkh1p and Fkh2p, associate with the coding regions of active genes and influence, in opposing ways, transcriptional elongation and termination. These events are coordinated with serine-5 and -2 phosphorylation of the heptad repeat of the carboxy-terminal domain (CTD) of RNAPII. Our results suggest that, in addition to their documented promoter function, Fkh1p and Fkh2p coordinate early transcription elongation and pre-mRNA processing. This may reflect a general feature of gene regulation in eukaryotes.

The winged-helix forkhead (Fkh) family of transcription factors is highly conserved in eukaryotes with roles in cell cycle control, cell death, proliferative responses, and differentiation (*1*). In yeast, *FKH1* and *FKH2* influence expression of a wide range of genes (*2*), including those that control the G_2–M phase transition of the cell cycle (*3*, *4*). Activation of genes of the *CLB2* cluster, expressed at G_2–M phase, requires cooperative binding of the Fkh2p and the Mcm1p transcription factors to the Swi Five Factor (SFF) site in the promoter. Fkh2p is the preferred component of the SFF factor, although Fkh1p substitutes in the absence of Fkh2p (*5*) and either factor is sufficient for activator recruitment (*4*). Previous studies have shown that the effects of ablation of each individual Fkh factor on the steady-state levels of *CLB2* mRNA are different; Fkh1p is required to repress expression, whereas Fkh2p activates expression to normal levels (*6–8*). This suggests additional opposing functions for Fkh factors in regulating *CLB2* expression. Structural and functional studies reveal a linker histone-like structure for the winged-helix Fkh domain and suggest interactions with chromatin other than sequence-specific DNA binding (*9*, *10*). In agreement with a more general effect on gene expression, our results demonstrate that Fkh factors differentially regulate the elongation phase of transcription at *CLB2* and at other loci.

We have analyzed the binding of Fkh factors to *CLB2* with the use of chromatin immunoprecipitation (ChIP) in strains expressing epitope-tagged Fkh factors (*11*). As expected, Fkh2p binds strongly and Fkh1p weakly to the upstream activating sequence (UAS) region on the promoter (*5*) (Fig. 1B). The majority of Fkh1p is associated with the first 600 base pairs (bp) of the transcribed region, whereas, in addition to UAS binding, a second peak of Fkh2p is present at the beginning of the coding region, extending toward the 3′ end of the gene. The unexpected presence of Fkh1p and Fkh2p in the *CLB2* coding region suggests a role in transcriptional elongation, in addition to their well-known function in *CLB2* activation involving UAS association.

We investigated the distribution of RNAPII over the *CLB2* gene (Fig. 1C; fig. S1). In wild-type (WT), the distribution of RNAPII (Rbp3-

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Chapter 5

Discussion

In this work, the effect of hypomethylation on mammalian development and cancer was investigated by lowering the levels of Dnmt1 in embryogenesis and in the adult. A new Dnmt1 isoform (Dnmt1o) was characterized in oocytes and cleavage embryos and carried DNA maintenance activity in ES cells. Functional analysis of this isoform in vivo suggested a role for Dnmt1o in maintaining IAP methylation in early embryogenesis, whereas the longer Dnmt1 isoform was important for maintaining IAP methylation in postimplantation embryos. Reduction of the activity of the *Dnmt1* gene in adult mice, which normally expresses the longer isoform, resulted in global genomic hypomethylation including at IAPs and development of tumors after a few months of age.

Thus, changes in the degree of methylation by reduction of Dnmt1 levels can lead to stable modifications of methylation patterns and disease. In the case of the *Dnmt1* $^{chip/-}$ mice, reduced Dnmt1 levels are present throughout the life of the animal and results in genomic hypomethylation. In addition, the methylation changes resulting from lowered Dnmt1 levels in early or late embryogenesis resulted in permanent changes in methylation patterns in the adult as evidenced by the coat color changes.

In addition to inherit such embryonic changes in methylation, mammals can also inherit changes in chromatin structure, as a result of altered DNA methylation. Indeed, postimplantation remethylation is followed by chromatin packaging which is

established based on the methylation state and is then stably inherited in the adult (Hashimshony, Zhang et al. 2003). Changes of methylation during the chromatin packaging stage or prior to that stage may impair chromatin assembly and proper execution of subsequent developmental programs.

Since adult cells originate from a limited number of cells in the embryo, changes in methylation patterns early on may be more crucial than during the adult stage. Thus, care must be taken that the levels of Dnmt1 be well regulated throughout the entire life of the animal as changes as early as embryogenesis may result in permanent changes that may cause disease later in life. Of importance is the effect of environmental factors on the integrity of embryonic genomic methylation.

5.1. Environmental factors influencing DNA methylation

It has been shown that methylation and expression of the A^{iapy} locus in embryos is sensitive to L-methionine intake by pregnant mothers (Wolff et al. 1998; Cooney et al. 2002). In humans, much of the evidence linking the diet to DNA methylation changes has been obtained from the adult stage. Dietary supplements such as folate or vitamins that affect the activity of enzymes that supply methyl groups for various cellular methylation processes can influence the rate of disease manifestation (Van den Veyver 2002) and the rate of colon cancer incidence (Giovannucci et al. 1993). Lower levels of folate intake has been associated with genomic instability (Blount et al. 1997; Jacob 1999) and genomic hypomethylation (Friso et al. 2002). Furthermore, methyl donor-

deficient diet has been shown to induce liver cancer associated with hypomethylation and overexpression of *c-ras, c-myc* or *c-fos* (Poirier et al. 1990; Dizik et al. 1991; Wainfan and Poirier 1992). An interesting study of the effect of diet on methylation changes in human pregnancy showed that during a winter famine in Holland in the Second World War, normal daughters born during that period were much likely to have smaller babies (John and Surani 1999). The latter has been postulated to have an epigenetic basis and might be consistent with a transgenerational inheritance of epigenetic state of the same sort observed at the A^{iapy} locus. A number of other diseases which are inherited but do not follow Mendelian inheritance have also been postulated to have an epigenetic component and include multiple sclerosis, diabetes, rheumatoid arthritis and cancer (Petronis 2001).

In contrast to embryogenesis, overall methylation patterns do not vary significantly in the adult. However, aging has been associated with both gains and losses of methylation. General hypomethylation is normally seen in adults (Mays-Hoopes et al. 1986; Wilson et al. 1987) and progressive losses of methylation are also observed in fibroblasts in vitro (Wilson and Jones 1983). CpG islands hypermethylation is also observed with aging such as the estrogen receptor, IGF2 and MYOD (Issa et al. 1994; Issa 2000). In fact, aging is one of the most important risk factor of cancer and CpG island hypermethylation may play a crucial role in the development of tumors in some individuals (Jones and Laird 1999; Toyota et al. 1999). Thus, while genetic mutations have been clearly shown to cause and explain diseases, it has never been shown whether epigenetic changes can actually initiate disease or whether these

changes simply represent a consequence of cellular malfunctions. Because methylation patterns, especially during embryogenesis, are liable to be altered by the diet, an understanding of the impact of such changes in development and disease would be relevant. Although highly speculative, it is possible that DNA methylation patterns may be altered by additional environmental stimuli. For example, temperature influences vernalization in plants growing at high altitudes and has been suggested to have an epigenetic component (Sheldon et al. 1999; Sheldon et al. 2000). Indeed, the FLOWERING LOCUS C (FLC) which appears to play an important role in the early flowering induced by low temperatures is thought to be regulated by DNA methylation (Sheldon et al. 1999; Sheldon et al. 2000).

5.2. DNA methylation, genomic instability and cancer

It has not been established whether DNA methylation plays a causal role in any of these diseases or whether it reflects the cellular diseased state. The best studied case in which DNA methylation abnormalities are consistently found is cancer.

The link between methylation changes and cancer has been established decades ago. However, it was never demonstrated whether hyper- or hypomethylation play a causal role in tumor formation. The results of this work clearly show that hypomethylation can induce tumorigenesis in mice. Hypomethylation induces chromosomal instability, resulting in the gain of chromosome 15 and additional gains of chromosome 14 in some tumors. Trisomy of chromosome 15 was correlated with *c*-

myc overexpression suggesting a role for this chromosome duplication in the oncogenic overexpression. Gene expression array analysis of tumor RNAs showed that the oncogene Notch-1 was also overexpressed in all tumors. The gene was found to contain IAP insertions which drive the expression of smaller species of oncogenic Notch-1. Thus, hypomethylation induced tumorigenesis in this model by 2 mechanisms: chromosomal instability and insertional mutagenesis. The potential for hypomethylation to induce tumor formation by a genomic instability mechanism was tested in a different study where the effect of genomic hypomethylation on a previously described tumor-prone model was tested (see Annex part A). In this model, animals heterozygous for null mutations of the linked tumor suppressor genes for p53 and NF1 (cis conformation) develop sarcomas after about 4 months of age. The mechanism of tumorigenesis in these mice has been shown to be LOH of the wild-type allele of *p53* and *NF1*. Because both genes are closely linked on chromosome 11, LOH at that locus results in the loss of both tumor suppressors genes. Induction of hypomethylation in this tumor model by breeding in the $Dnmt1^{chip}$ and *Dnmt1* null alleles resulted in a quicker formation of sarcomas suggesting that hypomethylation increases the rate of tumorigenesis in this model. Using markers specific for either allele, the mechanism of tumor formation was found to be whole chromosome LOH. To measure the mutation rate of tumor formation, embryonic fibroblasts were obtained from $Dnmt1^{chip/-}$; *p53*+/- / *Nf1* +/- (cis) and an assay was developed to measure the rate of foci formation on agar plates. The results showed that hypomethylation caused a 2 fold induction of the mutation rate. The results of these experiments emphasize that hypomethylation

induces chromosome instability in mice and are consistent with the results obtained in the *Dnmt1* $^{chip/-}$ mice.

These results are consistent with the chromosomal instability observed in ICF syndrome where DNMT3B is mutated and centromeres becomes hypomethylated (Xu et al. 1999; Ehrlich et al. 2001). *Dnmt3b* knockout in mice causes demethylation of the centromeric minor satellite repeats, suggesting that methylation of centromere repeats may play an important role in the maintenance of genomic stability (Okano et al. 1999). Similarly, induction of demethylation in cultured cells by 5-aza-2'-deoxycitidine causes elongation of the centromeric or juxtacentromeric regions of the same chromosomes that are affected in ICF (Jeanpierre et al. 1993). In addition, a homozygous null mutation of the *Dnmt1* gene in ES cells causes demethylation and a 5 to 10 fold increase in mutation rate (Chen et al. 1998) which was attributed to a higher rate of mitotic recombination.

5.3. Clinical relevance

Previous results have shown that in contrast to our results, hypomethylation was protective against tumor formation in an intestinal tumor model (Laird et al. 1995). Thus, hypomethylation may protect against tumorigenesis in certain cell types but at the same time may promote cancer in other cell types such as T cells. Hypomethylating agents such as 5-aza-2'-deoxycitidine (ex. Decitabine) have been used in the clinic and shown good promises for the treatment of myelodysplastic syndrome (MDS) and acute

myeloid leukemia (AML). In addition, inhibitors of histone deacetylases (HDAC) have also shown good promises (Ex. SAHA) and several groups are actively pursuing new drugs. Studies also suggest that a combination treatment of both 5-aza-deoxycytidine and TSA (trichostatin A), a drug that inhibits HDAC activity, cause a more effective reactivation of silenced tumor suppressor genes (Cameron et al. 1999). Many other histone- and chromatin modification enzymes have been shown to participate in tumorigenesis and new therapeutic approaches are being investigated.

Although some of these drugs, such as 5-aza-dC and HDAC inhibitors have shown exciting promises as cancer therapeutics, caution should be exercised in using them as prophylactics. While these compounds may have positive effects in treatment, it should be remembered that they might also have deleterious effects in other tissues and induce cancer. The combination of both hypomethylating agents and compounds which aim at modifying chromatin associated proteins might represent a better solution than a single drug approach since the concentration of each drug may potentially be lowered to reduce toxicity and prevent the development of diseases associated with methylation abnormalities. These drugs could also be used in combination with "smart" drugs, aimed at inhibiting specific oncogenes. The reversibility of epigenetic changes makes epigenetic therapy a sensible strategy. Although DNA hypomethylation agents are more likely to affect global levels of methylation, drugs aimed at histone modifying enzymes may display more restrained tissue-specific effects and therefore may prove less toxic. The combination of both may thus help to reduce

toxicity, improve effectiveness and increase tissue-specificity of drugs aimed at treating many diseases.

5.4. References

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Annex

Annex - A

Chromosomal Instability and Tumors

Promoted by DNA Hypomethylation

Chromosomal Instability and Tumors Promoted by DNA Hypomethylation

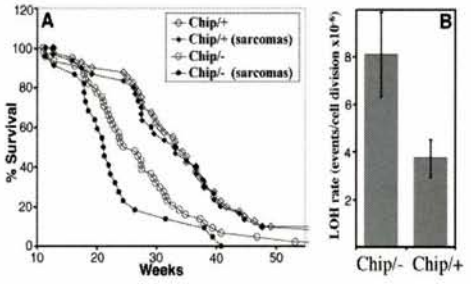
Amir Eden,[1] François Gaudet,[1,3] Alpana Waghmare,[1,2] Rudolf Jaenisch[1,2]*

Human tumors often display changes in DNA methylation, including both genome-wide hypomethylation and site-specific hypermethylation (*1*, *2*). In mice, DNA hypomethylation is sufficient to induce T cell lymphomas with consistent gain of chromosome 15 (*3*), indicating that genome-wide hypomethylation plays a causal role in cancer.

To explore further the link between DNA hypomethylation and chromosomal instability, we studied the effect of DNA hypomethylation on tumor-prone mice carrying mutations in both the Neurofibromatosis 1 (*Nf1*) and *p53* tumor suppressor genes. The *Nf1* and *p53* genes are closely linked on mouse chromosome 11, and double heterozygotes carrying the mutations on the same chromosome (NPcis) develop soft tissue sarcomas, which show simultaneous loss of heterozygosity (LOH) of *Nf1* and *p53* (*4*). When we induced genomic hypomethylation in the $Nf1^{+/-}$ $p53^{+/-}$ (NPcis) mice by introducing a hypomorphic allele of DNA methyltransferase 1 ($Dnmt1^{Chip/-}$) (*3*), the mice developed sarcomas at an earlier age compared with NPcis littermates with normal levels of DNA methylation ($Dnmt1^{Chip/+}$) (Fig. 1A). To determine whether hypomethylation promotes the initial LOH required for tumor development in the NPcis mice, we compared the rates of LOH in methylated and hypomethylated primary embryonic fibroblasts. We developed an assay to score for $Nf1^{-/-}$ $p53^{-/-}$ cells within a population of heterozygous cells (i.e., cells that have undergone LOH) (fig. S1). We then used this assay in a fluctuation analysis (*5*) to calculate the rate of LOH in hypomethylated versus methylated cells. This analysis revealed a significant increase in LOH rate in hypomethylated cells (2.2-fold; $P = 0.01$) (Fig. 1B), consistent with the hypothesis that hypomethylation promotes tumor development in NPcis mice by increasing the rate of LOH.

To investigate the chromosomal mechanism leading to LOH in the hypomethylated cells and whether specific chromosomal regions are involved, we analyzed LOH along chromosome 11 in single colonies representing independent LOH events (Fig. 1C). In methylated ($Dnmt1^{Chip/+}$) cells, LOH affecting the whole chromosome (including at position 1.5 cM from the centromere) occurred in 45% of analyzed events. The remaining 55% showed heterozygosity at 1.5 cM but were homozygous for markers at 20 cM or 39 cM and were, therefore, the result of mitotic recombination or of loss of the distal portion of the chromosome. Interestingly, the frequency of LOH events affecting the whole chromosome (including at 1.5 cM) was significantly higher in hypomethylated cells (77% compared with 45% in methylated cells) (Fig. 1C), suggesting that the increase in LOH rate in hypomethylated cells is the result of a specific effect of hypomethylation on the stability of the centromeric or pericentric regions. A link between hypomethylation and the stability of whole chromosome arms is also found in the human Immunodeficiency–Centromeric Instability–Facial Anomalies (ICF) syndrome, in hypomethylation-induced T cell lymphomas in mice (*3*) and in human hepatocellular and prostate carcinomas (*6*, *7*).

These results suggest that DNA hypomethylation promotes cancer through effects on chromosomal stability. Further characterization of the relations between DNA methylation, chromatin composition, and chromatin structure may allow a better understanding how DNA hypomethylation affects chromosome structure and integrity.



C

	1.5cM	20cM	39cM	46cM	66cM
Chip/+	D11Mit226	D11Mit270	*p53*	*Nf1*	D11Mit224
Embryo 1 (n=9)	44%	78%	100%	100%	100%
Embryo 2 (n=13)	38%	83%	100%	100%	100%
Embryo 3 (n=14)	53%	83%	86%	86%	86%
Total (n=36)	**45%**	**82%**	**95%**	**95%**	**95%**
Chip/-					
Embryo 1 (n=17)	71%	88%	100%	100%	100%
Embryo 2 (n=17)	82%	86%	100%	100%	100%
Embryo 3 (n=13)	77%	84%	99%	99%	99%
Total (n=47)	**77%**	**86%**	**99.7%**	**99.7%**	**99.7%**

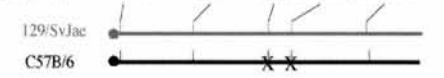


Fig. 1. (**A**) Survival curve of methylated [$Dnmt^{Chip/+}$ (Chip/+), $n = 42$] and hypomethylated [$Dnmt1^{Chip/-}$ (Chip/–), $n = 44$] $Nf1^{+/-}p53^{+/-}$cis (NPcis) mice. Survival curve for each genotype is plotted twice: One curve includes all mice (open symbols), and the second curve represents only mice that developed soft tissue sarcomas (solid symbols Chip/+), $n = 33$; Chip/–, $n = 22$). Mice of both genotypes typically developed one predominant, fast-growing sarcoma. Eighteen Chip/– mice developed T cell lymphomas [as described in (*3*)], including five mice that developed both a sarcoma and a lymphoma. (**B**) Increased rate of LOH events in hypomethylated mouse embryonic fibroblasts (MEFs), as determined by fluctuation analysis (*5*) (fig. S1). Graph shows the average rate in four independent experiments (±SD). The statistical significance was determined by Student's *t* test to be $P = 0.01$. (**C**) LOH analysis. Cells in this study were derived from C57/B6 × 129/svJae F_1 mice. The polymorphic markers indicated were used to examine heterozygosity along mouse chromosome 11 in cell colonies homozygous for *Nf1* and *p53* derived during fluctuation analysis. Only colonies from independent parallel cultures were considered independent events. We analyzed colonies obtained from three different fluctuation analyses (n = number of independent colonies). *Nf1* and *p53* mutant alleles (marked with "x") resided on the B6 chromosome, and LOH always resulted in the loss of the 129 allele. Results are shown as the percentage of colonies with only the B6 allele at the marked position.

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7. W. A. Schulz *et al.*, *Genes Chromosomes Cancer* **35**, 58 (2002).

8. We thank T. Jacks and K. Reilly for NPcis mice and V. Carey for advice on biostatistics. Supported by an EMBO fellowship ALTF 43-1999 (A.E.), by Boehringer Ingelheim (A.E.), and by an NIH grant CA87869 (R.J.).

Supporting Online Material

www.sciencemag.org/cgi/content/full/300/5618/455/DC1

Fig. S1

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Annex - B

Role of Notch-1 in Hypomethylation-Mediated Tumorigenesis

To look for factors that collaborate with c-myc in promoting tumorigenesis in *Dnmt1* $^{chip/-}$ tumors, we compared the expression profile of RNA from these tumors to that of either wild-type thymus RNA or CD4+/CD8+ wild-type cells using expression arrays. As expected, Dnmt1 levels were reduced (5 fold) in the hypomethylated tumors and c-myc was overexpressed (3 fold; Table 1). The results further showed that Notch-1, a protein which regulates T cell development and which has also been shown to act as an oncogene was one of the most overexpressed mRNA in all tumors (6-9 fold). In addition, two downstream targets of Notch-1, hairpin enhancer of split (HES-1) and Deltex were also overexpressed. These genes are normally activated by Notch-1 and their overexpression is consistent with Notch-1 overexpression.

Gene	Thymus WT	Thymus Mut	Dnmt1 null Fibroblasts	Thymomas Hypo CH3a	Thymomas Hypo CH3b	Thymomas Mov-1	CD4/ CD8 WT
c-myc	1	1	2	5	6	3	1
Notch-1	1	1	1	9	4	1	1
Deltex	1	1	1	16	12	1	1
Hes-1	1	1	1	10	3	2	1
Eta-1	1	1	1	11	4	1	1
IAP	1	2	100	2	2	2	1

Table 1. Relative gene expression in Dnmt1 chip/- tumors and pre-tumor thymuses. All gene expression levels are relative to the levels of wild type thymuses which have been arbitrarily set to 1. "Thymus mut" represents Dnmt1 chip/- pre-tumor thymuses, Dnmt1 null Fibroblasts" are mouse embryonic fibroblasts in which Dnmt1 was looped out by CRE resulting in the loss of all functional Dnmt1 alleles, "Thymomas Hypo CH3a" and "Thymomas Hypo CH3b" represent 2 independent experiments using 4 different Dnmt1 chip/- tumors in each case, "Thymomas Mov-1" are thymic lymphomas from viremic Mov-1 mice and "CD4/ CD8 WT" are CD4+/ CD8+ FACS-sorted cells from wild type mice. The numbers represent an average fold induction over 4-6 samples.

Elevated levels of Notch-1 were further confirmed by immunoblot analysis (Figure 1). In contrast, Notch-1 overexpression was not detected in hypomethylated pre-tumor thymuses, showing that hypomethylation by itself is not sufficient to trigger increased levels of Notch-1 (data not shown).

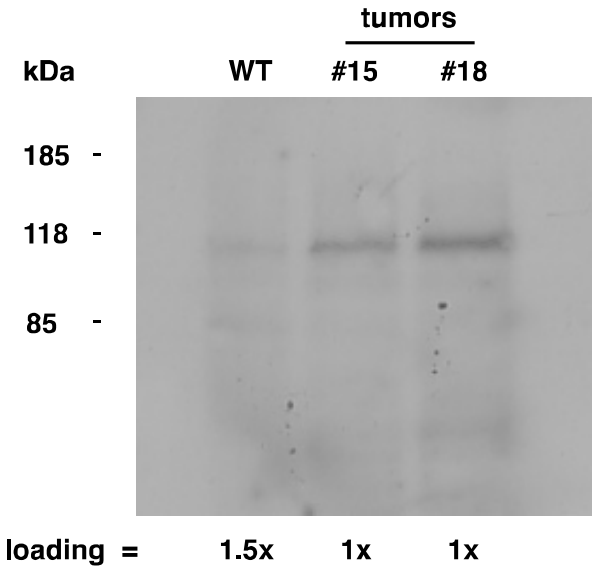

Figure 1. Overexpression of Notch-1 in Dnmt1 chip/- tumor cell lines. Western blot using a Notch-1 antibody against protein extracts from either wild type thymus (WT lane) or from Dnmt1 chip/- cell lines (#15 and #18 lanes). The Notch-1 signal is elevated in both cell lines even if the WT lane was overloaded by 50% (1.5x).

We next sought to characterize the nature of Notch-1 overexpression. Since the CGH analysis of the hypomethylated tumors suggested that the copy number of chromosome 2 (on which Notch-1 resides) was normal (see Chapter 4), we tested whether the Notch-1 locus might harbor smaller genomic rearrangements, undetectable by CGH. We isolated a mouse Notch-1 genomic BAC and used it as a probe against

interphase spreads of cell lines derived from hypomethylated tumors. DNA FISH analysis revealed the presence of 2 dots per cells on average, thus ruling out duplications or amplifications (Figure 2).

To test whether discrete genomic rearrangements occurred at the Notch-1 locus, we performed a restriction mapping analysis of the genomic locus. Southern analyses revealed the presence of abnormal restriction fragments in 6/19 tumors, consistent with genomic rearrangements (data not shown).

Since the band expected from the wild-type allele was found in all of these samples, only one allele would be expected to contain the rearrangement. To investigate whether these monoallelic rearrangements were responsible for monoallelic overexpression of Notch-1 expression, we performed a RNA FISH analysis of interphase spreads from hypomethylated tumor-derived cell lines using a Notch-1 BAC as a probe. Results showed that most cells contained a weaker and a brighter fluorescent dot compared with wild-type cells which contain 2 weak fluorescent dots. These results suggest that one of the two Notch-1 alleles in the tumor cells is expressed at higher levels than the other, a result that is consistent with the Southern data showing rearrangement at only one allele. The possibility that this rearranged allele is responsible for the increased expressed observed by FISH is supported by the Northern analyses which show that the smaller transcripts are found in larger amounts (A. Eden, personal communication).

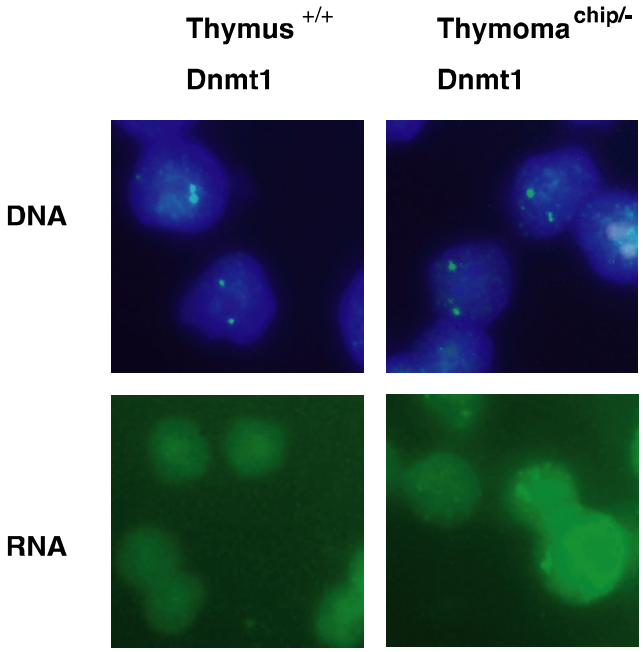

Figure 2. DNA and RNA FISH of Notch-1 on wild type and Dnmt1 chip/- tumor cells. A BAC containing the mouse Notch-1 locus was used as a probe for DNA FISH (upper panels) or RNA FISH (lower panels) against wild type thymus cells (left) or a Dnmt1 chip/- tumor cell line (right). On average, two dots of equal intensities were present in each cell nucleus in the DNA FISH. In contrast, Notch-1 mRNA signals were below detection in wild type cells but could be detected as 2 spots in the tumor cells: one weak spot and one brighter spot. The width of each field (each panel) is 25 micrometer.

To determine the nature of the transcripts resulting from these rearrangements, northern analyses were performed. The results showed that most tumors contained the wild-type transcript and an additional species of RNA, indicating rearrangement at one

allele (A. Eden, personal communication). In addition, these smaller RNA species were found in larger amounts than the endogenous transcript, suggesting that the rearranged allele is the one causing overexpression. To characterize the rearrangement, restriction mapping was used to fine-map these new changes within a 1 kb DNA fragment. A long-range PCR assay was developed to amplify this DNA segment and sequencing of 8 PCR-amplified DNA fragments from this rearranged region showed the presence of IAP insertions (A. Eden, personal communication).

Discussion

These results emphasize that endogenous non-replicative retroviruses may play a role in tumorigenesis in mice. Although IAP insertions have been characterized at various locus before in the mouse genome, this is the first demonstration that activation of an IAP by hypomethylation may lead to insertions that may play a role in tumorigenesis.

Induction of viremia in *c-myc* transgenic mice results in activation of Notch-1 in many cases and development of thymic tumors (1). Notch-1 translocations into the TCRb locus have also been observed in tumors in humans and the break point has been mapped to the same region upstream of the transmembrane domain. The region is very rich in non-contiguous repeats that might be less stable. It is also possible that the site is not less stable but rather is selected for because insertions in that specific locus may give a growth advantage. Although IAP expression is elevated in hypomethylated tumors, the degree of overexpression is not as overwhelming as in Dnmt1 null fibroblasts. This would thus suggest that the insertion site might be preferred and insertions can be detected even if IAP overexpression is not very high.

Insertional mutagenesis caused by retroviruses is not a common mechanism of tumorigenesis in humans. Nevertheless, the human genome contains 45% of retrotransposon-derived sequences and hypomethylation could lead to activation of

non-replicative retroelements that could participate in the tumor process or in other diseases. This interesting possibility remains to be further studied.

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Annex - C

Curriculum Vitae

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Education

Ludwig Maximilians Universität
Munich, Germany, Dissertation in the group of Prof. Dr. Heinrich Leonhardt at the Department of Biology and Prof. Dr. Rudolf Jaenisch at MIT.
Ph.D in Biology, 1996-

Université de Sherbrooke
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MS in Biochemistry, June 1995
BS in Biochemistry, June 1991

Experience

2004-present **Novartis Institutes for Biomedical Research, Epigenetics**
Cambridge, MA, *Senior Scientist*

1996-2003 **Whitehead Institute for Biomedical Research, MIT,** lab of Prof. Dr. Rudolf Jaenisch and LMU, lab of Prof. Dr. Heinrich Leonhardt
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1993-1995 **Harvard Medical School, Children's Hospital (Cardiology),** lab of Dr. Geoffrey S. Ginsburg
Boston, MA, *Graduate Researcher*

1991-1992 **Sherbrooke University, Québec, Canada.**
Instructor for graduates in Biochemistry (1992), Molecular Biology (1992) and Physical Chemistry (1991)

Honors

2001	Max-Delbrück-Center Symposium Award
1996-2001	Max-Delbrück-Center Scholarship
1995	1st place / Raymond Kalil Cardiovascular Research Award and Competition
1992-1992	Bristol-Myers-Squibb Scholarship

Publications

Eden, A., **Gaudet, F.**, Gribnau, J., Leonhardt, H. and Jaenisch, R., Activation of *Notch-1* by an IAP Element in Hypomethylation-Induced Tumors, in preparation.

Gaudet, F., Rideout, W.M.3rd., Meissner, A., Dausman, J., Leonhardt, H, Jaenisch, R., Maintenance of IAP Methylation by Dnmt1 in pre and postimplantation embryogenesis, *Mol. Cell Biol.*, **24**, 1640 (2004).

Gaudet, F., Hodgson J.G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J.W., Leonhardt, H., Jaenisch, R., Induction of Tumors in Mice by Genomic Hypomethylation, *Science*, **300**, 489 (2003).

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Gaudet, F., Ginsburg, G.S., Transcriptional Regulation of the Cholesteryl Ester Transfer Protein Gene by Orphan Nuclear Receptor Apolipoprotein AI Regulatory Protein-1, *J. Biol. Chem.*, **270**, 29916 (1995).

Annex - D

Contribution to Publications

Paper 1

A Short DNA Methyltransferase Isoform Restores Methylation In Vivo, *J. Biol. Chem*., 273, 32725 (1998), Gaudet, F., Talbot, D., Leonhardt, H., Jaenisch, R.

I layed out all project aims. I sequenced the promoter region of Dnmt1 (Fig. 1a-b) and identified new potential translation sites (Fig. 1c), tested the functionality of these ATG sites (Fig. 3a) and the ability of the Dnmt1 mutants to functionally complement Dnmt1 null ES cells (Fig3. b), evaluated the development potential of those rescued ES cells by generating teratomas in vivo (Fig. 4a-b). Finally I wrote the paper and made all the figures. D. Talbot did the immunoblots on Fig. 2. The sequencing of the N-terminus of Dnmt1 was performed by a core facility at MIT.

Paper 2

Dnmt1 Overexpression Causes Genomic Hypermethylation Loss of Imprinting, and Embryonic Lethality, *Mol. Cell. Biol.*, 22, 2124 (2002), Biniszkiewicz, D., Gribnau, J., Ramsahoye, B., Gaudet, F., Eggan, K., Humpherys, D., Mastrangelo, M.A., Jun., Z., Walter, J., Jaenisch.

I performed the immunoblots for Fig. 1b and Fig. 6b, both of which were critical to prove that the BACs were indeed overexpressing Dnmt1 in ES cells – a result on which the whole paper was based.

Paper 3

Induction of Tumors in Mice by Genomic Hypomethylation, *Science*, 300, 489 (2003), Gaudet, F., Hodgson J.G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J.W., Leonhardt, H., Jaenisch, R.

I designed and performed the vast majority of the work on this paper. I quantified the strength of the Dnmt1^{chip} allele (Fig.1a), evaluated the effect of reduced Dnmt1 levels on methylation of repetitive elements in mice (Fig. 1b-d), measured weight of all mutant animals over the course of 5 months (Fig. 2a), sacrificed and autopsied all sick animals for presence of tumors (Fig. 2b), estimated tumor nature and clonality (Fig. 2c-d), measured relative expression of various repetitive elements in hypomethylated tumors (Fig. a,b, c bottom 2 panels). Finally, I wrote the paper and made all the figures. G. Hodgson performed the CGH analysis from samples that I prepared (Fig. 3d), A. Eden performed the PCR on figure 2c and the northern blot on Fig. 3c and L. Jackson-Grusby contributed scientifically.

Paper 4

Chromosomal Instability and Tumors Promoted by DNA Hypomethylation, *Science*, 300, 455 (2003), Eden, A., Gaudet, F., Waghmare, A., Jaenisch, R.

I developed and characterized the Dnmt1 tumor model used in this paper and contributed scientifically to the project in general.

Paper 5

Maintenance of IAP Methylation by Dnmt1 in pre- and postimplantation embryogenesis, Mol. Cell. Biol., 24, 1640 (2004), Gaudet, F., Rideout, W.M.3rd., Meissner, A., Dausman, J., Leonhardt, H, Jaenisch, R.

I personally laid out all project aims and was the main force behind this project. I designed and performed all crosses and scored all coat colors (Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig. 5a-b). I made the immunoblot and southern. I did all statistical analyses. Finally, I wrote the whole paper and made all the figures. B. Rideout set up the Msx2Cre cross (Fig. 5c-f), A. Meissner made panel 3c and helped out with changes to the paper following initial submission and J. Dausman assisted with the management of the mouse colony and scoring of coat colors.

Paper 6

Activation of *Notch-1* by an IAP Element in Hypomethylation- Induced Tumors, *in preparation*, Eden, A.*, Gaudet, F.*, Gribnau, J., Leonhardt, H. and Jaenisch, R.

I designed and performed the vast majority of experiments. I performed the Affymetrix analysis on hypomethylated tumors which identified the Notch-1 pathway as being overexpressed in most samples, I identified DNA rearrangements within the Notch-1 gene, performed DNA and RNA FISH hybridization to better understand the nature and functionality of this rearrangement, I mapped the rearrangement to within 1 kb of the mutation point and demonstrated that Notch-1 is upregulated by western. A. Eden showed that Notch-1 transcripts are truncated and that the nature of the mutation is an IAP insertion.

Annex - E

Acknowledgments

I would like to thank my parents and my sister for their unconditional love and support throughout the years and my beloved Karen for being there and for making my life so much more beautiful.

Many thanks also go to Rudolf who has always been very supportive of my work and who provided me with an extraordinary environment to conduct my research and to Heinrich for welcoming me in his lab, for supporting me for all these years and for really wanting me to succeed. Many thanks also to Cristina for all the critical scientific comments (and non-scientific ones!). I've always enjoyed working with you all.

Finally, I would like to thank Laurie Jackson-Grusby for all those scientific discussions and advice (it's always been fun to talk to you), to Jessie and Ruthie for always being so nice and helpful and to all Jaenisch Lab and Leonhardt Lab members past and present for all your help, it was much appreciated.