The role of the *de novo* DNA methyltransferase
Dnmt3a in the nervous system

by

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Dedicated to my mother and father,

for understanding the unimportance of most things.

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ABSTRACT

DNA methylation is an important mechanism of gene regulation. Evidence is mounting that epigenetic mechanisms including that of DNA methylation operate in the nervous system. Genetic disruption of the *de novo* DNA methyltransferase Dnmt3a in mice were used to elucidate the role of the enzyme in the nervous system. Inactivating Dnmt3a in the nervous system resulted in a drastically shortened lifespan and in behavioral abnormalities that suggested a motor defect. Dnmt3a mutants also exhibited motor neuron loss and fragmented neuromuscular junction synapses. These results support a requirement for DNA methyltransferase activity in the neuromuscular system.

Thesis supervisor: Rudolf Jaenisch

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Chapter 1.

Introduction:

Epigenetics, methylation, and the nervous system

EPIGENETICS

To discern the full meaning of DNA, cellular machinery relies on much more information than can be provided by the DNA sequence alone. Just as text must be formatted with spacing and punctuation marks in order to be properly understood, so must mammalian DNA be formatted. The equivalent of spacing and punctuation for DNA – epigenetic modifications – comprise of heritable changes to the DNA molecule and to the proteins with which it is intimately connected. These modifications are reversible and do not involve a change to the DNA coding content itself. Such formatting enables the cell to understand which genes must be turned on or silenced.

One class of epigenetic regulation involves direct chemical modifications to the DNA molecule and was discovered by Hotchkiss (1948) in calf thymus DNA. Hotchkiss's discovery turned out to be the major form of direct modification of DNA in mammals: the addition of a methyl group to the C5 position of the cytosine in a 5'CpG3' dinucleotide (Figure 1). 5-CpG methylation is typically, though not exclusively, associated with gene silencing.

Methylation at CpGs in promoter regions can interfere with binding by transcription factors (Clark et al., 1997; Comb and Goodman, 1990; Mancini et al., 1999). Nevertheless, the predominant mechanism by which DNA methylation modulates gene expression is through the formation of a condensed chromatin structure inaccessible

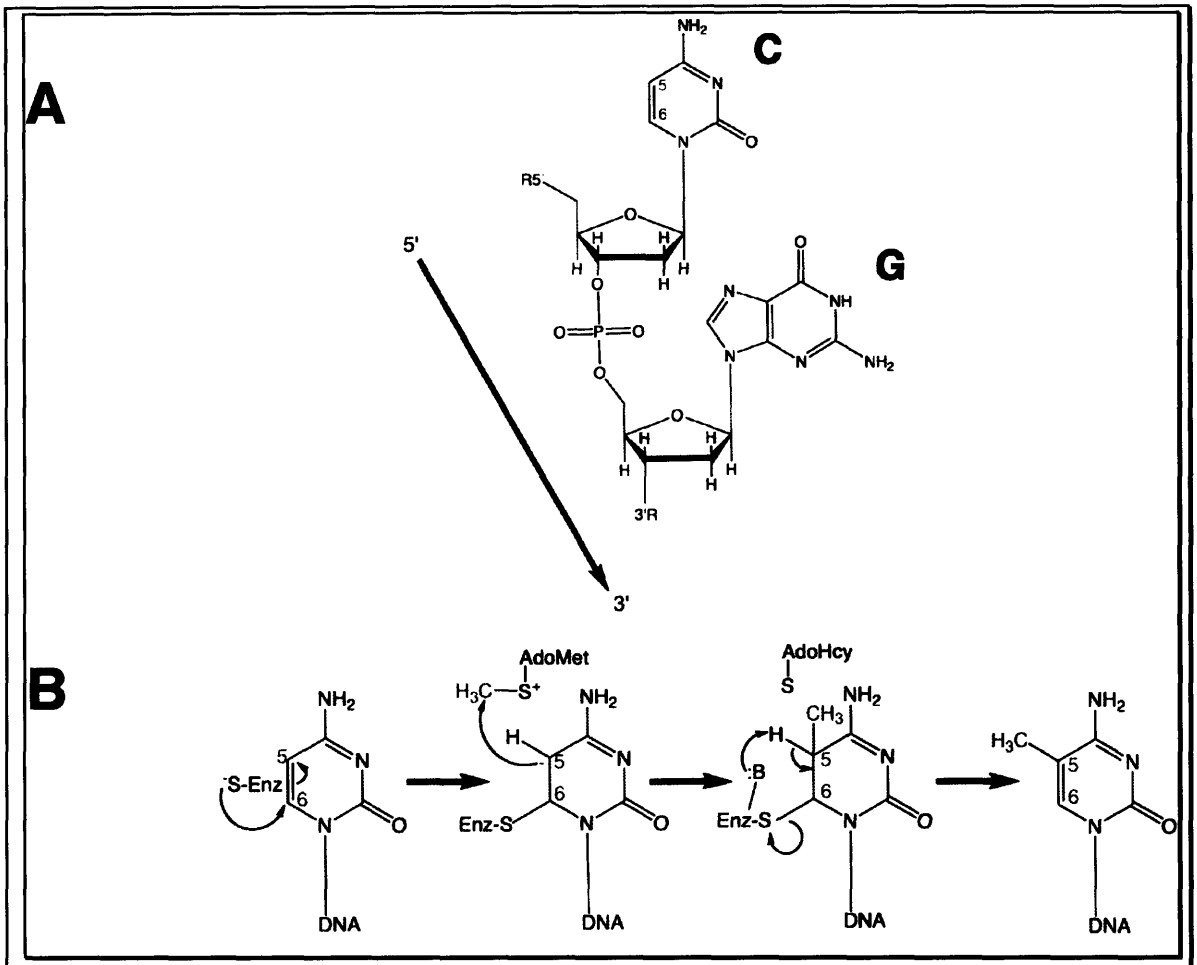

Figure 1. **(A)** Structure of a CpG dinucleotide. Methylation occurs at the position 5 carbon of the cytosine ring. **(B)** Simplified mechanism of the methylation reaction. For simplicity, only the cytosine ring is shown. The enzyme attacks the carbon at position 6 and forms a covalent bond with the cytosine ring. The intermediate attacks the methyl group of the methyl donor S-adenosy L-methionine (AdoMet). The result is methylation at the carbon at position 5 and a demethylated version of the donor molecule, S-adenosyl L-homocysteine (AdoHcy). Finally, the enzyme is released by beta-elimination. Though not shown, the mechanism also involves protonation of the nitrogen at position 3; abstraction of the proton from the cytosine-5 position is carried out by an unidentified enzyme base or water molecule.

Chemical structures were drawn using ChemDraw software (Cambridgesoft) and the mechanism was adapted from Kumar et al, 1994 and Goll et al, 2005.

to transcription complexes. Methylated CpGs can be bound by methyl-CpG-binding proteins, some of which act as transcriptional repressors (Ballestar and Wolffe, 2001; Wade, 2001). The methyl-CpG- binding protein MeCP2 not only possesses repressor activity independent of its ability to bind methylated DNA; it also recruits histone deacetylases, further contributing to transcriptional repression (Jones et al., 1998; Nan et al., 1998).

The methylation system interacts with another complex system of epigenetic modification that involves the posttranscriptional alteration of histones, the proteins around which DNA molecules are wrapped. Though there are many possible modifications of histones, a few have come under intense study as relevant to the study of epigenetics. Histone acetylation at lysine residues correlates with increased transcription of the associated DNA (Struhl, 1998). The H3 and H4 histones are mostly deacetylated in heterochromatic regions in fission yeast.

Histones H3 and H4 are also modified by mono-, di-, or tri-methylation at specific lysine and arginine residues. The lysine 27 site in histone H3 (H3K27) is methylated by E(Z)/EZH2 complexes, which contain polycomb group (PcG) proteins that act as transcriptional repressors. H3K27 methylation is associated with the inactive X-chromosome and possibly with *Hox* gene silencing (Cao and Zhang, 2004). Methylation at the lysine 9 residue in H3 (H3K9) is required for the interaction between Swi6, a trans-acting silencing factor, and heterochromatin (Nakayama et al., 2001).

In contrast, H3K4 di- and tri-methylation is associated with transcriptionally active genes. Factors known to interact with methylated H3K4, sich as CHD1, the NURF complex, and WDR5, are involved in transcriptional activation (Sims and Reinberg,

2006).

Both histone methylation and deacetylation act synergistically with DNA methylation, forming a self-feeding loop of epigenetic regulation. Methylated DNA is often associated with deacetylated histone tails, and there is a causal relationship between DNA methylation and histone methylation. Abrogating CpG methylation in plants and human cells affects methylation at H3K9, while methylation at H3K9 stimulates *de novo* DNA methylation in plants and fungi (Hermann et al., 2004).

The work in this thesis examines an enzyme capable of conferring CpG methylation, Dnmt3a, and its role in the nervous system. Several neurological diseases involve epigenetic changes to genes or mutations in components of the epigenetic machinery, suggesting that various processes in the nervous system are regulated epigenetically. In addition, methylation or methyl-binding proteins have been postulated to influence neuronal differentiation, neurogenesis and learning and memory. Examples of these are discussed in this chapter after an overview of cytosine methylation and the enzymes involved.

FUNCTIONS OF CYTOSINE METHYLATION IN MAMMALS

Genome defense

Many species use cytosine methylation as part of a defensive mechanism to protect the host genome from parasitic DNA elements. Prokaryotes methylate at both cytosine and adenine residues at sites in the endogenous genome to protect it from digestion by restriction enzymes. Thus, they can use restriction enzymes to degrade specifically foreign DNA that is unmethylated (Kumar et al., 1994).

In eukaryotes, methylation at cytosines is also used for defensive purposes, though it does not appear to function in such a restriction-modification system. In the haploid fungus *Ascobolus immersus*, MIP (methylation induced premeiotically) is targeted to repeat sequences (Wolffe and Matzke, 1999). MIP occurs at all cytosine residues (regardless of the sequence context) in the repeats (Goyon et al., 1996). The methylation can interfere with transcription elongation and prevent potentially deleterious recombination events between similar sequences (Matzke et al., 1999).

Plants similarly employ cytosine methylation to inhibit the expression of transgenic DNA, though the repression system plants use is more complicated. Transgene repression in plants takes place at both the transcriptional level (transcriptional gene silencing, TGS) and post-transcriptional level (post-transcriptional gene silencing, PTGS). Both these mechanisms involve DNA methylation at cytosines. In TGS, meiotically heritable methylation marks at promoters suppress the transgene. TGS in plants is not an exact duplicate of the system in *Ascobolus*. Not all high-copy transgenes are silenced in plants, indicating that the existence of repeats alone is not sufficient to trigger the silencing mechanism (Matzke et al., 1999). The role of methylation in PTGS is not fully understood, but in many cases of PTGS, methylated cytosines are found in transcribed regions of the transgene. Unlike the methylation in TGS, this methylation does not lead to meiotically heritable gene silencing (Matzke et al., 1999).

Repetitive elements are suppressed by methylation in mammals as well, though the methylation is specific for cytosine residues in CpG dinucleotides. Transposable elements comprise as much as 35% of the human genome (Wolffe and Matzke, 1999) Because they are capable of insertional mutagenesis, generating aberrant transcripts, and

improperly activating nearby genes (Chang-Yeh et al., 1991; Takahara et al., 1996; Yoder et al., 1997b), they threaten the integrity of the host genome. The host genome's primary mechanism of defense against this threat is CpG methylation of the elements. This modification attracts chromatin remodeling factors that induce a silenced state (Wolffe and Matzke, 1999). Most of the methylated CpGs in the mammalian genome are found at these repetitive elements, and demethylation of the genome in mice as well as plants leads to increased transposon activation (Goll and Bestor, 2005).

Retroviruses are also transcriptionally silenced in mammalian embryonic and hematopoietic stem cells (Asche et al., 1984; Challita and Kohn, 1994) by a mechanism that involves *de novo* methylation of the integrated provirus at cytosine residues in CpG dinucleotides (Hoeben et al., 1991).

Genomic imprinting

A function for cytosine methylation that is specific to mammals is the regulation of imprinted genes. Imprinted genes are a class of genes in mammals that are differentially expressed depending on the parental origin of the allele. Expression of these genes is controlled by cytosine methylation at differentially methylated regions (DMRs). The best-characterized imprinted genes are involved in fetal growth regulation, though not all have known functions.

To explain why imprinting occurs in mammals but not in egg-laying species, most hypotheses center around the unique mammalian relationship between mother and embryo and the need for controlling fetal growth as driving forces in the evolution of genomic imprinting (Jaenisch, 1997). The "parental conflict" hypothesis proposed by

Haig et al (Moore and Haig, 1991) posits that the genomic imprinting in mammals arose out of a conflict between the paternal and maternal genomes. If multiple males may father the offspring of one female, as occurs in mammals, then conflicting interests arise. It is in the interest of each father to direct as much of the available maternal resources as possible to his particular offspring. Conversely, distributing resources among all offspring equally is the best way for the mother to maximize the number of her surviving offspring.

Consistent with the predictions of the Haig hypothesis, the paternally expressed genes *Igf2* and *Ins2* enhance growth, while the maternally expressed genes *H19*, *Igf2r*, and $p57^{KIP2}$ restrict growth (Jaenisch, 1997). The interplay between some of these gene products illustrates the conflict around which the Haig hypothesis is built.

H19 encodes an untranslated RNA that represses transcription of the *Igf2* (insulin-like growth factor 2) gene, which is located about 100kb from the *H19* locus (Leighton et al., 1995). A "differentially methylated domain" (DMD) upstream of *H19* is methylated on the paternal allele during spermatogenesis, resulting in suppression of paternal expression of *H19* (Lewis and Murrell, 2004; Tremblay et al., 1995). Igf2 is a paternally expressed fetal growth factor that is also antagonized by the maternally expressed Igf2r. Igf2r opposes Igf2 function by acting as a scavenger receptor for Igf2 (Jaenisch, 1997). The *Igf2r* gene acquires methylation marks in intron 2 on the maternal allele during oogenesis (Stoger et al., 1993). These marks ensure maternal expression of Igf2r by inhibiting the transcription of Air, the antisense noncoding RNA that is expressed from a promoter located in intron 2 of the *Igf2r* gene (Sleutels et al., 2002).

X-chromosome inactivation

DNA methylation is also involved in the silencing of an entire chromosome in mammals. In each cell in female mammals, one of the two X chromosomes undergoes inactivation in order to match the gene dosage of the single X chromosome in cells from males. This process also relies on DNA methylation. *Xist*, which encodes an untranslated RNA involved in X-inactivation, is methylated on the X chromosome that remains active. *Xist* RNA coats the chromosome from which it is transcribed (the inactive X chromosome) and through a still unclear mechanism contributes to silencing the genes on that chromosome (Panning and Jaenisch, 1998). The maintenance of the silent state requires methylation (Lock et al., 1987).

Tissue-specific gene regulation

In addition to regulating imprinted genes and genes on the X chromosome, cytosine methylation is postulated to regulate tissue-specific genes during development. This role for cytosine methylation was hypothesized by Riggs (1975) and Holliday and Pugh (1975). According to their predictions, tissue-specific genes would be methylated and demethylated according to a developmental program. This hypothesis is attractive to many because it provides a mechanism for controlling gene expression in a tissue-specific manner.

Because convincing examples of tissue-specific genes that undergo demethylation during development were absent for many years, the hypothesis has also been met with skepticism. Nevertheless, the long-known heritability of methylation patterns (Wigler et al., 1981), correlation between methylation and transcriptional repression (Jones et al.,

1998; Nan et al., 1998), and correlation between demethylation and transcriptional activation ((Eden and Cedar, 1994) are all consistent with the hypothesis.

Many studies have correlated DNA methylation with the control of a cell type-specific gene. For example, Futscher et al (2002) studied methylation at the human maspin gene (SERPINB5) in cell culture. SERPINB5 is expressed in a subset of epithelial cells (those of the airway, breast, skin, prostate, and mouth) but not in other tissues and cell types (skin fibroblasts, lymphocytes, bone marrow, heart, and kidney). In cell types expressing SERPINB5, the SERPINB5 gene was found to be unmethylated at the CpG island in the promoter, in addition to having acetylated histones H3 and H4 in the corresponding region. Conversely, those not expressing SERPINB5 were heavily methylated at the CpG sites in the promoter and had hypoacetylated histones H3 and H4 in the differentially methylated region. Further, interfering with DNA methylation by using the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine relieved the transcriptional repression in the non-expressing cells. These results are consistent with the original hypothesis by Holliday, Riggs, and Pugh.

THE MAMMALIAN DNA METHYLTRANSFERASES

Enzymes known as the 5-CpG methyltransferases catalyze the transfer of a methyl group to the carbon at position 5 of the cytosine ring in a symmetric CpG dinucleotide (Goll and Bestor, 2005) (Figure 1A). The mammalian 5-CpG methyltransferases are part of a family of related proteins that includes members with no known function, or no demonstrable enzymatic activity. The protein family (depicted in Figure 2) and the non-enzymatic members of the family are also discussed briefly.

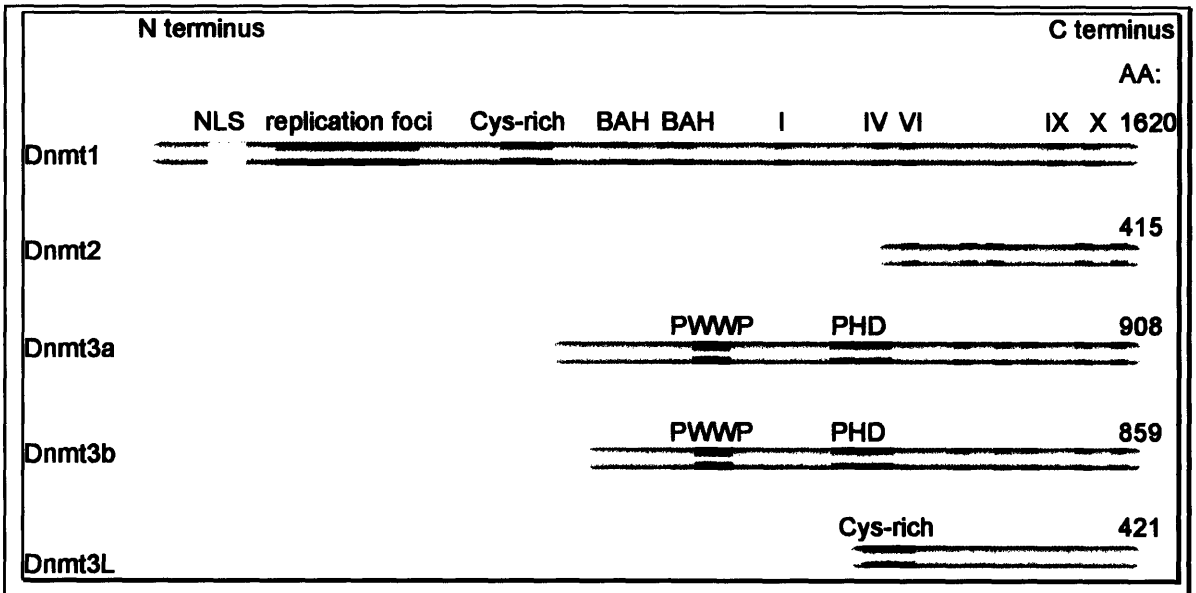

Figure 2. Schematic representation of the mammalian DNA methyltransferase family. Dnmt1, Dnmt3a, and Dnmt3b are known to have catalytic activity, wherease Dnmt2 (despite having catalytic domains) and Dnmt3L do not. Dnmt1 has an extensive N-terminal region with various domains including a nuclear localization sequence, a domain targeting the enzyme to replication foci, a cysteine-rich zinc-binding region, and bromo-adjacent-homology (BAH) domains.

Adapted from Goll et al, 2005 and Chen et al, 2003

Mammalian 5-CpG methyltransferases contain a catalytic region that bears striking homology to the bacterial cytosine methyltransferases. Ten characteristic sequence motifs comprise the catalytic domain (Lauster et al., 1989; Posfai et al., 1989). The catalytic mechanism of the cytosine methyltransferases was at first thought to be unusual: the cytosine substrate is flipped out of the double helix and inserted into the active site of the enzyme (Klimasauskas et al., 1994). As it turns out, chemical reactions in which bases are modified or excised from DNA duplexes commonly employ base eversion as a means of overcoming steric hindrances (Goll and Bestor, 2005).

During the so-called chemically improbable reaction of methylating a cytosine at position 5 (Chen et al., 1991), the methyltransferase forms a covalent bond with the target cytosine. A cysteine thiolate from the conserved prolylcysteinyl (PC) motif (located in motif IV) links to the carbon at position 6 of the cytosine ring. Subsequently, the newly activated carbon at position 5 attacks the carbon in a methyl group from the methyl donor, S-adenoysl-L-methionine (AdoMet). The hydrogen at position 5 is abstracted by an unknown base in the enzyme or by a water molecule. Finally, the enzyme is released from position 6 by beta-elimination (Goll and Bestor, 2005) (Figure 1B).

Dnmt1

The first member of this family to be identified and the only one so far isolated on the basis of its activity is Dnmt1 (Bestor et al., 1988). Dnmt1 is known as the "maintenance DNA methyltransferase." Its primary function is to follow the replication machinery and maintain patterns of methylation that would otherwise be progressively lost with each round of replication. The enzyme recognizes hemimethylated DNA (DNA methylated on one of the two strands) and methylates the nascent DNA strand, using the methylation pattern on the original parent strand as a template (Goll and Bestor, 2005).

Dnmt1 activity is essential for normal development. Mice homozygous for targeted null mutations in Dnmt1 have drastically reduced levels of global methylation and do not survive past midgestation (Li et al., 1992). Hypomethylation due to loss of Dnmt1 leads to de-repression of the intracisternal A particle (IAP) transposable elements (Walsh et al., 1998), biallelic expression of many imprinted genes (Li et al., 1993), and activation of Xist, the X-linked RNA molecule involved in X-chromosome inactivation

(Panning and Jaenisch, 1996).

Genetic studies of Dnmt1 have also pointed to a role for the enzyme in genome stability. Loss of Dnmt1 in embryonic stem cells elevates mutation rates for two distinct genes (the endogenous hypoxanthine phosphoribosyltransferase (*Hprt*) locus and a thymidine kinase (*TK*) transgene) (Chen et al., 1998). The mutations at both genes arise mostly from gene deletions. Mutations in the *TK* transgene result predominantly from mitotic recombination or chromosome loss followed by duplication of the remaining chromosome (Chen et al., 1998).

Other studies link Dnmt1 with tumorigenesis. Reducing the level of Dnmt1 in cells has a protective effect in a mouse model of intestinal neoplasia. Apc^{min} mice, which are predisposed to develop multiple intestinal polyps, develop fewer polyps when they are also heterozygous for a *Dnmt1* null mutation (Laird et al., 1995). This protective effect could be a result of the mutagenicity conferred by Dnmt1. Methylated cytosines can be deaminated to thymidines, resulting in C to T transitions.

Conversely, mutant mice expressing Dnmt1 at 10% of wild type levels develop aggressive T cell lymphomas in adulthood (Gaudet et al., 2003). The lymphomas are characterized by a high degree of trisomy in chromosome 15, indicating that hypomethylation could contribute to tumorigenesis by causing chromosome instability. Thus, with respect to tumorigenesis, there appears to be a delicate balance between the mutagenic effects of Dnmt1 and the protective effects the enzyme provides by keeping chromosomes stable.

The components comprising the enzyme have been studied as well. The N-terminal region of Dnmt1 contains several distinct domains. A nuclear localization

sequence allows entry of the protein into the nucleus, while one region targets Dnmt1 to replication foci during S phase (Goll and Bestor, 2005). A domain near the very end of the N-terminus is involved in degradation of the enzyme during G0 (Ding and Chaillet, 2002). The oocyte-specific form of Dnmt1 (Dnmt1o) lacks this domain and is resistant to degradation (Mertineit et al., 1998).

Not all of the domains in Dnmt1 have known functions. Dnmt1 contains a cysteine-rich region that binds to zinc ions and is also seen in other methyltransferases. The importance of this zinc-binding activity is unknown. Another region allows Dnmt1 to bind to DMAP-1 (DNA methyltransferase-associated protein 1) (Rountree et al., 2000). It is unclear what the function of this protein interaction is.

Situated in the C-terminal of Dnmt1 is a highly conserved catalytic domain. Though this domain contains all the characteristic methyltransferase motifs, it does not possess catalytic activity on its own. Its activity appears to require the presence of a large portion of the N-terminal region. It is hypothesized that the catalytic domain of Dnmt1 is made active by an intramolecular interaction with the N-terminal region. The interaction could induce a conformational change that transforms Dnmt1's catalytic region into an active state (Hermann et al., 2004).

The *de novo* DNA methyltransferases

Two other CpG methyltransferases, Dnmt3a and Dnmt3b, are classified as the major *de novo* DNA methyltransferases. Their ability to lay down new patterns of DNA methylation is supported by functional studies in knockout mice (Okano et al., 1999) and by their ability to methylate the mostly unmethylated genome of *Drosophila* (Lyko et al.,

1999).

Structurally, these enzymes are somewhat similar to Dnmt1 in that they are both comprised of an N-terminal region containing regulatory domains and a C-terminal region containing the classic catalytic motifs of a 5-CpG methyltransferase. A cysteine-rich zinc-binding region, similar to that found in Dnmt1, is found in both Dnmt3a and Dnmt3b. This ATRX-like cysteine-rich domain is also referred to as a PHD domain (plant homeo-domain) and is typically found in proteins that are involved in regulating eukaryotic transcription (Hermann et al., 2004). Dnmt3a and Dnmt3b also harbor a PWWP domain, which is thought to mediate targeting of the enzymes to pericentric satellite repeats (Chen et al., 2004).

Dnmt3b

Dnmt3b is the only methyltransferase directly linked to a human disease. Mutations in Dnmt3b occur in ICF syndrome (immunodeficiency, centromeric instability, and facial anomalies), a rare autosomal recessive disorder (Hansen et al., 1999). One major clinical manifestation of ICF syndrome is reduced serum immunoglobulin levels, though this reduction is variable both in extent and in the type of immunoglobulin classes affected. Patients usually succumb to infections before reaching adulthood (Ehrlich, 2003; Xu et al., 1999).

A major cytological finding in cultured lymphocytes from ICF patients is instability of chromosomes 1, 9, and 16. These chromosomes are decondensed in ICF lymphocytes, and susceptible to breakage and rejoining. Multiradiate structures composed of parts of the affected chromosomes can often be seen in lymphocytes from

ICF patients. The chromosome instability is a result of hypomethylation at CpG sites in the pericentric satellite regions on those chromosomes (Hansen et al., 1999).

Though not many ICF patients have been diagnosed to date worldwide, studies on the limited number of available patients show genetic diversity at the Dnmt3b locus. Not all ICF patients have mutations in Dnmt3b. For example, only 11 out of 14 ICF patients in a study by Wijmenga et al (2000) harbored mutations at the Dnmt3b locus.

The Dnmt3b mutations that have been characterized are heterogeneic. Most are missense and nonsense mutations, though no patients have ever been found to be homozygous for a non-sense mutation. The latter result suggests that complete absence of Dnmt3b function is incompatible with life. That Dnmt3b-nulls are inviable is also supported by the embryonic lethal phenotype of Dnmt3b-knockout mice (Okano et al., 1999).

While some patients harbor mutations in the catalytic domain, the mutations in other patients do not necessarily interfere directly with the catalytic activity of Dnmt3b. For example, the mutation in one Japanese patient mapped to a region in the N-terminal domain of the enzyme (Shirohzu et al., 2002), close to a PWWP domain thought to be important in targeting of the enzyme to pericentric satellite repeats (Chen et al., 2004).

What is the function of Dnmt3b? The features of ICF syndrome point to a role for Dnmt3b in maintaining chromosome stability, specifically in methylating satellite 2 repeat sequences. The equivalent minor satellite repeats in the mouse genome are greatly demethylated in mouse Dnmt3b knockout embryos and ES cells (Okano et al., 1999).

This specialized role for Dnmt3b fits with its processive mechanism, in which the unmethylated substrate is converted directly into the fully modified target without

allowing the accumulation of partially modified intermediates. That is, the enzyme does not dissociate from the DNA molecule between rounds of methylation reactions. Such processivity, which Dnmt3a does not possess, would allow Dnmt3b to methylate repeat sequences with multiple CpGs efficiently (Gowher and Jeltsch, 2002).

Though Dnmt3b is enzymatically active in isolation (Gowher and Jeltsch, 2002), it is known to interact with other proteins. Dnmt3b has been found in complexes with a minor form of Dnmt1 (Datta et al., 2003) and also interacts with SUMO-1 and Ubc9 through an N-terminal region. SUMO-1 covalently modifies its target proteins by sumoylation, and Ubc9 is a SUMO-1 conjugating enzyme (Kang et al., 2001). Dnmt3b also mingles with components of the condensin complex, which mediates genome-wide chromosome condensation (Geiman et al., 2004).

As with Dnmt1, the catalytic activity of Dnmt3b is localized to a C-terminal domain containing the classic cytosine methyltransferase motifs. Yet not all proteins generated from the Dnmt3b locus are catalytically active. Alternatively spliced forms of Dnmt3b have been documented in both human and mouse, and some of these isoforms do not include the key catalytic motifs (Figure 3). These isoforms arise from variations in splicing events at exons 10 and 11 and at exons 21 and 22. Exons 21 and 22 encode catalytic motifs, while exons 10 and 11 encode a region of unknown function between the PWWP and cysteine-rich domains (Weisenberger et al., 2004). Weisenberger et al identified additional transcripts of Dnmt3b in mice, generated by a splicing event that removes an untranslated region upstream of the ATG translation start site in exon 2.

It is still unclear what functions each of these Dnmt3b isoforms have, but their expression patterns have yielded some clues. Dnmt3b1 - the full-length form containing

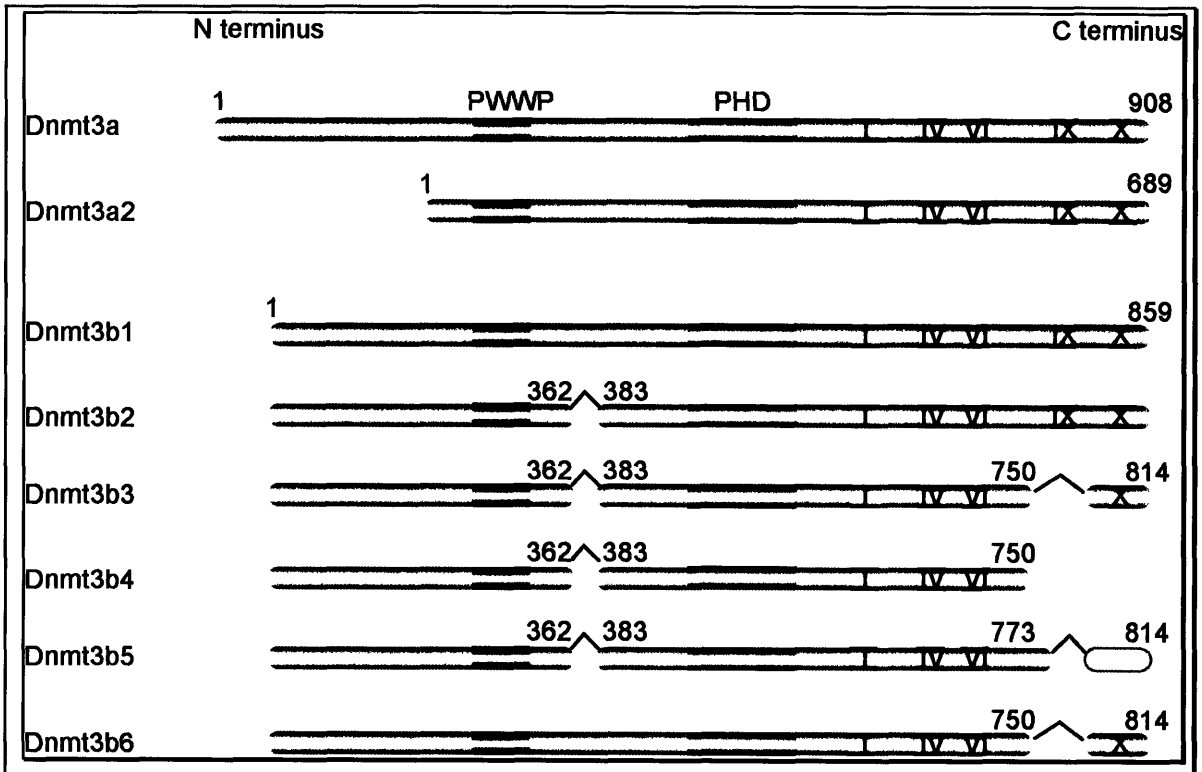

Figure 3. Schematic representation of the Dnmt3a and Dnmt3b isoforms. All isoforms share common PWWP and PHD domains. The methyltransferase catalytic domains are labelled with roman numerals. The C-terminal portion of Dnmt3b5 (denoted by a blank box) is out of frame.

Adapted from Chen et al, 2003

all exons – and Dnmt3b6 – an isoform missing both exons 21 and 22 – are the predominant forms expressed in mouse ES cells. While both these isoforms include exons 10 and 11, the isoforms expressed in somatic cells exclude exons 10 and 11. Human and mouse somatic cells express Dnmt3b2, Dnmt3b3, Dnmt3b4, and Dnmt3b5 transcripts, all of which lack exons 10 and 11 (Figure 3) (Weisenberger et al., 2004).

Though the isoforms lacking the catalytic motifs are presumed catalytically

inactive, they may still function as regulators of methylation. In human hepatocellular carcinomas, overexpression of DNMT3b4 (an isoform that is missing exon 21 as well as exons 10 and 11) is correlated with DNA hypomethylation at repeats in pericentromeric satellite regions (Saito et al., 2002). This hypomethylation was seen even in pre-cancerous stages and may contribute to tumorigenesis by promoting chromosome instability. Additionally, gene expression changes resulting from overexpression of DNMT3b4 have been documented by Kanai and Saito (2004). Messenger RNAs for the transcription factor STAT1 (signal transducer and activator of transcription 1), and for some of the proteins downstream in its pathway, were upregulated in human embryonic kidney cells transfected with DNMT3b4 cDNAs.

DNMT3b3, on the other hand, may be a positive regulator of methylation. In cultured T24 cells (a human cell line) treated with the methyltransferase inhibitor 5-aza-2'-deoxycytidine, re-methylation of the repetitive element D4Z4 occurs only when DNMT3b3 expression is detectable (Weisenberger et al., 2004).

Dnmt3a

The *de novo* DNA methyltransferase Dnmt3a resembles Dnmt3b in structure. Dnmt3a/Dnmt3b double knockout mice have a more severe phenotype than that of single Dnmt3a or Dnmt3b knockout mice, suggesting that the two enzymes partly compensate for each other. Nevertheless, the deleterious effects of lacking either enzyme indicate that they also have unique functions. Mice with homozygous null mutations in Dnmt3a typically die within several weeks of birth, due to as yet undetermined causes (Okano et al., 1999).

The Dnmt3a knockout phenotype suggests a role of Dnmt3a during development.

More recently, Dnmt3a has been implicated specifically in paternal and maternal imprinting. Kaneda et al (2004) generated a conditional knockout mouse model of *Dnmt3a* and crossed it to a transgenic line that expresses Cre recombinase in germ cells (TNAP-Cre). When such *Dnmt3a*-conditional knockout females were bred to wild type males, no live pups were obtained. Instead, all the embryos examined at E10.5 were either dead or dying. The differentially methylated regions (DMRs) of the maternally imprinted genes *Snrpn, Igf2r,* and *Peg1* were unmethylated at the maternal allele, indicating that Dnmt3a is necessary during oogenesis for maternal imprinting.

Dnmt3a is also required during spermatogenesis for paternal imprinting. Male conditional *Dnmt3a*/TNAP-Cre mice have smaller testes and reduced numbers of spermatogonia that diminish drastically as the mice age. Though these mice are infertile, Kaneda et al (2004) were able to study paternal imprinting by examining spermatogonia from P11 mutant mice. In these Dnmt3a-deficient spermatogonia, the normally methylated allele was unmethylated at two out of three paternally imprinted loci examined. The differentially methylated regions of the *H19* and *Dlk1-Gtl2* genes were unmethylated in Dnmt3a mutants, while that of *Rasgrf1* retained methylation.

In addition to having unique functions not shared by Dnmt3b, Dnmt3a also differs from Dnmt3b biochemically. Despite sharing 84% amino acid identity in their catalytic domains, Dnmt3a and Dnmt3b have distinct kinetic mechanisms. While the catalytic domain of Dnmt3b operates with a processive mechanism, both full length Dnmt3a and its isolated catalytic domain operate with a distributive kinetic mechanism (Gowher and Jeltsch, 2002). Dnmt3a treats target sites independently, dissociating from the DNA after each methylation reaction.

The mechanistic differences between Dnmt3a and Dnmt3b could be the basis of differences in preferred target sites. Dnmt3a cannot compensate for Dnmt3b in methylating satellite 2 repeat sequences, which contain many CpG dinucleotides and are undermethylated in ICF syndrome (Xu et al., 1999). It is possible that Dnmt3a is specialized for methylating single copy genes, consistent with its established role in methylating imprinted genes during gametogenesis.

Dnmt3a does not exclusively methylate CpGs. Though it is predominantly a CpG methyltransferase, Dnmt3a can also methylate CpA, and to a lesser extent CpT, dinucleotides (Ramsahoye et al., 2000). Such methylating ability for Dnmt3a was demonstrated in a transgenic *Drosophila* system. (The *Drosophila* genome is normally mostly unmethylated.) The function of non-CpG methylation is unknown, but Dnmt1 appears incapable of this methylation. While non-CpG methylation is rarely detected in somatic cells in the mouse, up to 20% of the methylated cytosines in ES cells were at non-CpG sites (Ramsahoye et al., 2000).

Like Dnmt3b, Dnmt3a interacts with a number of proteins involved in sumoylation, including Ubc9. The Dnmt3a protein itself is modified by SUMO-1, and this modification disrupts its ability to interact with histone deacetylases (Ling et al., 2004). In addition to complexing with histone deacetylase 1 (HDAC1), Dnmt3a associates with a histone H3 methyltransferase (HMTase) activity (Datta et al., 2003).

More than one form of Dnmt3a is known to exist in mice. The Dnmt3a isoform is a full-length version, and Dnmt3a2 is a truncated product missing the first 219 amino acid residues in the N-terminal domain (Figure 3). Chen et al (2002a) characterized Dnmt3a2 and found that is generated by transcription from a separate promoter located in

intron 7. Aside from missing exons 1 through 6, Dnmt3a2 transcripts differ from Dnmt3a transcripts by the inclusion of exon 7. Chen et al also identified human DNMT3A2, which differs from the mouse Dnmt3a2 by additional coding sequence in the 5' untranslated region from an extra exon downstream of exon 7.

Though it is truncated, Dnmt3a2 was found to be as catalytically active as the full-length form in *in vitro* assays with double-stranded synthetic substrates. Nevertheless, potential differences in function are suggested by differences in expression pattern, as assessed by Western Blot and RT-PCR. The full length isoform, Dnmt3a, is expressed at low levels in many tissues and cells including brain, liver, muscle, testes, ovary, heart, spleen, thymus, stomach, and ES cells. On the other hand, Dnmt3a2 expression is more tightly regulated and correlates with cell and tissue types associated with *de novo* methylation activity. Dnmt3a2 is the predominant form expressed in mouse ES cells and thymus, and is also detected in ovary, testes and liver (Chen et al., 2002a). In male germ cells, Dnmt3a2 expression correlates with stages of global DNA methylation, which occurs during postcoital days 16-18. It is the major methyltransferase expressed in gonocytes at postcoital days 14-18 (Sakai et al., 2004).

At the cellular level, the two isoforms exhibit differences in localization as well. In NIH3T3 cells transfected with GFP-fusion constructs, the Dnmt3a isoform appears to localize to nuclei. On the other hand, the Dnmt3a2-GFP signal excludes nucleoli and heterochromatin, though a low diffuse signal is detected in the cytoplasm. Results from ES cell subcellular fractionation experiments matched the data from the transfection experiments. The distinct localizations of each isoform suggest that they are responsible for methylating different components of the genome, with Dnmt3a targeting

heterochromatin and Dnmt3a2 targeting euchromatin (Chen et al., 2002a).

Additional variants of Dnmt3a were described by Weisenberger et al (2002). These novel transcripts included an alternate exon in the 5' region (exon 1ß), and a less abundant one including intron 4. Not much is known about the products of these alternative transcripts, though Weisenberger et al observed a difference in expression. Mouse ES cells express mostly transcripts containing exon 1ß (Dnmt3a-ß), while human and mouse somatic cells expressed transcripts containing the other version of exon 1 (Dnmt3a-α).

Maintenance versus *de novo* DNA methyltransferases

Though Dnmt1 is classified as the major maintenance DNA methyltransferase and Dnmt3a and Dnmt3b are designated the major *de novo* DNA methyltransferases, Dnmt1 has a higher specific activity for unmethylated DNA than Dnmt3a or Dnmt3b in *in vitro* experiments. Nonetheless, Dnmt1 exhibits up to a 30-fold preference for hemimethylated substrates over unmethylated substrates (Yoder et al., 1997a). Dnmt3a and Dnmt3b, on the other hand, have much lower specific activities for both kinds of substrate but do not exhibit a preference for either (Okano et al., 1998a). The division of these enzymes into separate roles is somewhat complicated by Dnmt1's direct physical interaction with Dnmt3a and Dnmt3b (Datta et al., 2003; Kim et al., 2002).

Dnmt2

Another member of the 5-CpG methyltransferase superfamily is the enigmatic Dnmt2. Dnmt2 is well conserved and more widely distributed across species than Dnmt1, Dnmt3a, and Dnmt3b. Though it lacks an N-terminal regulatory domain typical

of the other proteins, it contains all the catalytic motifs typical of 5-CpG methyltransferases, suggesting that it may function as a methyltransferase (Goll and Bestor, 2005). Though some labs report detectable enzymatic activity in biochemical assays (Hermann et al., 2003; Tang et al., 2003), Dnmt2 is not required for maintenance or *de novo* methylation of retroviral elements in mouse ES cells (Okano et al., 1998b).

On the other hand, a Dnmt2-like protein is responsible for the low-levels of cytosine methylation in *Drosophila.* Though its catalytic domains are similar to that of 5-CpG methyltransferases, the *Drosophila* homolog of Dnmt2 methylates CpA and CpT dinucleotides specifically. The function of this methylation is unknown. Disruption of *Drosophila* Dnmt2 by RNA interference had no effect on embryonic development (Kunert et al., 2003).

The puzzle about Dnmt2's function is complicated by the discovery that Dnmt2 can methylate RNA in *in vitro* assays. Purified human Dnmt2 protein was able to methylate RNA isolated from mice, *Drosophila,* and *Arabidopsis.* The RNA substrate of Dnmt2 was identified as $tRNA^{Asp}$, which was methylated at cytosine 38, located in the anticodon loop. Dnmt2 was only able to transfer a methyl group to RNA prepared from Dnmt2-/- mutant tissues, suggesting that wild type $tRNA^{Asp}$ may already be methylated *in vivo* (Goll et al., 2006). As with the Dnmt2-mediated methylation of DNA in *Drosophila,* the function of this methylation is unknown.

This dual RNA/DNA specificity of Dnmt2 evoked questions about the evolution of the eukaryotic DNA methyltransferases. Is Dnmt2 an intermediate enzyme along an evolutionary path from RNA methyltransferases to DNA methytransferases, as suggested by Goll et al (2006)? This proposed evolutionary path differs with the prevailing idea that

the eukaryotic DNA methyltransferases evolved from the prokaryotic DNA methyltransferases (Jeltsch et al., 2006).

Dnmt3L

Dnmt3L is closely related to Dnmt3a and Dnmt3b. Though it lacks catalytic activity of its own, it is required for the establishment of maternal imprints (Bourc'his et al., 2001; Hata et al., 2002) and is involved in methylation during spermatogenesis (Webster et al., 2005). Dnmt3L appears to act as a regulator: it interacts with isoforms of Dnmt3a and Dnmt3b and stimulates their activity (Chedin et al., 2002; Chen et al., 2005; Gowher et al., 2005).

EPIGENETICS AND THE NERVOUS SYSTEM

DNA methylation and other epigenetic mechanisms provide a means for establishing and maintaining cellular memory. In the case of X chromosome-inactivation, for example, cells in the embryo proper randomly choose an X chromosome to inactivate. Once an X has been chosen, that X chromosome stays inactivated and remains so in all progeny cells (Panning and Jaenisch, 1998). The progeny cells retain the memory of which X chromosome had been inactivated.

The heritability of epigenetic changes allows this form of cellular memory to persist through cell divisions. At the same time, the potential reversibility of epigenetic marks offers a mechanism for modulation. The attractive characteristics of this memory system lend the attractive idea that epigenetics may also be involved in organismal memory such as that of the nervous system. The mammalian nervous system is plastic, responding to experiences that often induce long lasting changes. These features may

lead one to speculate that heritable, yet reversible changes in gene expression are employed in the nervous system.

Over the years, the role of epigenetics in the nervous system in mammals has become more than pure speculation. It has been supported by evidence drawn from examples in normal biology and in disease.

Rett Syndrome and MeCP2

Rett Syndrome provided an early clue that epigenetic mechanisms operate in the nervous system. Rett syndrome is a neurodevelopmental disorder characterized by ataxia, mental retardation, stereotypical hand movements, seizures, and features of autism (such as social withdrawal and increased anxiety). The syndrome primarily affects and is a leading cause of mental retardation in girls. Patients are born seemingly healthy and become afflicted within the first year and a half of life, then undergo a rapid decline (Tucker, 2001).

Up to eighty-percent of Rett syndrome patients harbor mutations in *MECP2*, an X-linked gene (Amir et al., 1999; Hoffbuhr et al., 2001). *MECP2* encodes a transcriptional repressor that binds to methylated CpGs. An array of different missense, nonsense, and frameshift mutations in *MECP2* has been characterized in Rett syndrome patients. Most of them disrupt the function of the transcriptional repressor domain (Amir et al., 1999).

In addition to having its own transcriptional repressor activity, MeCP2 recruits chromatin-remodeling complexes to methylated CpGs. Other components of these complexes include Sin3A (a transcriptional repressor) and histone deacetylases (HDACs)

1 and 2 (Nan et al., 1998).

Though MeCP2 is expressed in most tissues throughout the body in both humans and mice, it is most highly expressed in neurons of the central nervous system (Akbarian et al., 2001; Shahbazian et al., 2002). Nearly all MeCP2-positive cells in the central nervous system are also positive for NeuN, a marker for mature neurons (Kishi and Macklis, 2004).

Results from genetic experiments in mice suggest that at least some of the symptoms of Rett syndrome originate from lack of MeCP2 function in postmitotic neurons. In mice, a deficiency of MeCP2 in postmitotic neurons is sufficient to mimic Rett syndrome. Chen et al (2001) and Guy et al (2001) excised a crucial exon of the *Mecp2* gene using the Cre-loxP conditional knockout system. Ablation of *Mecp2* by a CamKIIα promoter-driven Cre transgene caused Rett-like symptoms. This CamKIIα-Cre transgene is active during the first two weeks of postnatal life in postmitotic neurons of the forebrain, hippocampus, and brainstem. Such conditional *Mecp2* knockout mice had smaller brains (up to 18% decrease in brain weight) and smaller neuronal soma size (in the hippocampus and cerebral cortex), both of which are prominent clinical features in Rett patients. In addition to decreased nocturnal activity and increased weight, *Mecp2*/CamKIIα-Cre conditional knockout mice also exhibited an ataxic gait, reminiscent of the ataxia seen in Rett patients.

Though deletion of MeCP2 in a restricted set of neurons led to Rett-like symptoms, the symptoms were delayed and not as severe as those seen in the human patients. Male *Mecp2*/CamKIIα-Cre conditional knockout mice do not develop symptoms until approximately 3 months of age, an adult stage in mice (Chen et al.,

2001). On the other hand, the mutation in human males has such severe effects that few survive beyond two years of age.

Deletion of MeCP2 in mice using the Nes-Cre1 transgene led to similar but more severe symptoms with an earlier onset. The Nes-Cre1 transgene is active earlier and in a wider set of cells than the CamKIIα-Cre transgene. Activity by Nes-Cre1 is detected beginning at embryonic day 9.5, in glia as well as neurons, and in the cerebellum as well as the rest of the central nervous system. Still, most *Mecp2*/Nes-Cre1 knockout males survived until adulthood (Chen et al., 2001).

Further evidence that MeCP2 functions in postmitotic neurons came from a transgene rescue experiment by Luikenhuis et al (2004), who generated a mouse line in which *Mecp2* cDNA was integrated into the *tau* locus. The Mecp2 transgene was expressed under the control of the *tau* promoter, which is active specifically in postmitotic neurons. *Mecp2* knockout mutant mice that also bore one copy of this transgene exhibited none of the Rett-like symptoms typically found in *Mecp2* mutant mice, demonstrating that expression of MeCP2 in postmitotic neurons can rescue the phenotype of an *Mecp2* mutant mouse.

What is the role of MeCP2 in the nervous system? One proposal is that MeCP2 is required in later stages of brain maturation for events such as synaptogenesis and maintenance rather than in earlier processes such as neuronal migration and differentiation (Kaufmann et al., 2005). This hypothesis is supported by several lines of evidence: the neuroanatomic and clinical features of Rett Syndrome, MeCP2 expression patterns, and results from experiments in which the *Mecp2* gene has been manipulated.

The clinical features of Rett patients imply that the syndrome involves incomplete

postnatal brain development. Some of the manifestations of the disease point to a defect in maturation of the brainstem, which through some of its nuclei control several autonomic processes. Though Rett patients exhibit normal breathing rhythms during sleep, most exhibit abnormal breathing patterns at some point during the awake state. The breathing irregularities include apneusis (protracted inspiration), hyperventilation, non-epileptic vacant spells and breath holding (Julu et al., 2001; Kerr and Julu, 1999). Sudden respiratory arrhythmia is thought to be responsible for roughly a quarter of deaths in female Rett patients (Viemari et al., 2005). Similar breathing irregularities have also been documented in adult, symptomatic *Mecp2* mutant mice (Viemari et al., 2005).

The lack of integrative inhibitory control characteristic of these breathing abnormalities reveals a defect in the parasympathetic division of the autonomic nervous system. Other signs of parasympathetic dysfunction in Rett patients have been observed. Control of the sino-atrial node by the nucleus ambiguus, another brainstem nucleus, via the vagus nerve (cardiac vagal tone) is also depressed in Rett patients. This matches the low vagal tone present in neonates, which along with increased uptake of serotonin in some brainstem nuclei indicate an immature brainstem. Meanwhile, the sympathetic nervous system appears functional in Rett patients. For example, baseline blood pressure, which is under sympathetic control, is normal (Julu et al., 2001).

Disrupted brain maturation is also evident in other areas of the Rett brain. A major neuroanatomical finding in both human patients and in the *Mecp2* knockout mouse model is that of an overall decreased brain size. Head circumference of Rett syndrome patients is normal at birth, but their brains appear to stop growing at around twelve months. Thereafter the brain of a Rett syndrome patient is significantly smaller than of

others her age, though it remains at a steady size (Armstrong, 2001).

Volumetric studies of Rett syndrome patients using magnetic resonance imaging (MRI) demonstrate a preferential decrease in volume in certain regions of the brain. Though there is a global reduction in both grey and white matter, the largest reductions in grey matter are in the prefrontal, posterior, and anterior temporal regions. The caudate nucleus is also substantially smaller in volume (Subramaniam et al., 1997).

What could account for the smaller brain size? Total numbers of neurons appears to be preserved. Bauman et al (1995) have found that neurons in the hippocampus and the entorhinal cortex have smaller cell bodies, and there is an accompanying increase in the density of neurons per unit volume. The latter result contradicts Belichenko et al's results (1997) that there is no change in neuronal packing density, but different tissue preparation methods were used in the two studies.

Lacking from the volumetric and other studies on Rett brains is any consistent evidence of neurodegeneration. In the volumetric studies, no age-related decrease in cerebellum size was found. None of the major neuropathologic features associated with neurodegeneration - alterations in myelin, cell atrophy, and ventriculomegaly - have been consistently found in Rett patients (Subramaniam et al., 1997).

The other neuroanatomical features of Rett brains indicate no problems with neuronal migration either (Belichenko et al., 1997), but rather problems with synaptic function. In Rett brains, there is a selective decrease in dendritic branching in the same areas where reduced volume had been found. Though reduced dendritic arborization is found in many mental retardation disorders, there is a reduced branching in Rett brains even when compared to that in Trisomy 21 (Downs Syndrome), another mental

retardation disorder (Armstrong et al., 1998). Consistent with the less elaborate dendritic arborization, reductions in cortical thickness were also observed in Rett brains (Johnston et al., 2001).

When Belichenko et al (1995) examined the three-dimensional morphology of dendrites of pyramidal neurons in the frontal cortex, they found some regions that lacked dendritic spines, the structures onto which most synapses in the central nervous system anchor. Another indication that there is a synaptic defect in Rett patients is the slight but significant decrease in staining for synaptophysin - a marker for synapses - in areas of the frontal, temporal, and motor cortices (Belichenko et al., 1997).

Some of the morphological abnormalities found in Rett brains have also been found in MeCP2-deficient mice. Male adult *Mecp2* mutant mice exhibited decreased cortical thickness (not increasing after four weeks of age) in motor and somatosensory areas. In addition, they had increased neuronal packing density in some layers, notably layers II/III after four weeks of age. Paralleling the findings in human Rett brain samples, *Mecp2* mutant mice had smaller numbers of dendritic spines in layer II/III of somatosensory cortex (Fukuda et al., 2005). On the other hand, overexpression of MeCP2 in mouse cortical neurons enhanced dendritic complexity and increased axonal length (Jugloff et al., 2005).

In addition to the synapse-related defects in Rett syndrome and *Mecp2* mutant mice, another clue that MeCP2 is involved in synaptic function comes from the expression pattern of MeCP2. MeCP2 expression in several areas of the brain generally increases with developmental maturation. In the olfactory bulb, which mostly develops postnatally, MeCP2 protein is detected in neuronal nuclei as early as embryonic day 16.5

(E16.5). Thereafter, expression of MeCP2 increases with age until postnatal week 7, after which its expression remains elevated and constant through 20 weeks of age (Cohen et al., 2003).

A similar increase in MeCP2 expression is also observed in olfactory epithelium, though the overall amount of expression is much less than that observed in the olfactory bulb. Another difference is that in the epithelium, MeCP2 protein is not detected by either Western blot or immunostaining at the age of 7 weeks, though it is detected at earlier and later stages examined. As determined by immunocytochemistry on olfactory epithelium tissue, the bulk of MeCP2-positive neurons are also positive for olfactory marker protein (OMP), which is expressed in mature olfactory receptor neurons. (Cohen et al., 2003)

MeCP2 expression was also studied using an experimental paradigm in which the olfactory epithelium was damaged chemically. MeCP2 immunoreactivity disappears as olfactory receptor neurons in the epithelium are ablated, and then returns to pre-lesion levels as new cells repopulate the epithelium. In contrast, when an olfactory bulb was removed, MeCP2 expression in the corresponding epithelium disappears and does not return to the same levels seen before the bulbectomy.

The bulbectomy removes the target of the olfactory receptor neurons in the epithelium, causing their eventual degeneration. As cycles of neurogenesis in the epithelium generate new cells, MeCP2 expression is detected again. Yet these new cells lack targets in the bulb onto which they can synapse and they eventually degenerate (Cohen et al., 2003).

These experiments demonstrate that MeCP2 can be expressed without

synaptogenesis, though not at levels comparable to that seen in neurons that have made synaptic contacts. The steady increase in MeCP2 expression observed in normally developing olfactory bulb and epithelium coincides with the timing of synaptogenesis, which in those areas of the brain takes place during the first two postnatal weeks in mice (Cohen et al., 2003).

A similar coincidence between the timings of MeCP2 expression and synaptogenesis has also been observed in the rat hippocampus, cerebellum, and neocortex (Mullaney et al., 2004). In the dentate gyrus region of the hippocampus, the peak period of synaptogenesis occurs between postnatal days 4 and 11. MeCP2 expression increases during that time window. Similarly, in the cerebellum, MeCP2 is not expressed in granule cells until P21, the age after which the majority of the synapses involving those cells form. Changes in MeCP2 expression in the neocortex also appear to follow stages of maturation: MeCP2 immunostaining increases during first few postnatal days.

MeCP2 expression appears to correspond more closely to the formation of synapses rather than developmental age. The granule cells in the cerebellum differentiate in waves postnatally. The earliest differentiating neurons populate the deepest part of the granule cell layer, while those differentiating later populate the outer half. Yet MeCP2 expression is roughly the same in all those cells. Meanwhile, Purkinje cells are the first to make synaptic connections and also the first to express MeCP2. In the neocortex, though cells in layer V are born after those in layer VI, cells in layer V stain more darkly for MeCP2 than those in layer VI until P14 (Mullaney et al., 2004). This appears to match the pattern of postnatal synaptogenesis in rats: a higher density of synapses in layer V than layer VI during the first week of postnatal life (Blue and Parnavelas, 1983).

Is the coincidence between MeCP2 expression and periods of synaptogenesis meaningful? The evidence of impaired synaptic function in Rett patients suggests so. Abnormal electro-encephalograms (EEGs) and seizures are commonly observed in young Rett patients, and evidence for enhanced excitatory activity in the motor cortex was found in electromagnetic stimulation experiments. Neurotransmission mediated by glutamate, an excitatory amino acid neurotransmitter, is likely to be disrupted in Rett patients. Glutamate levels were found to be elevated in studies examining the cerebral spinal fluid from patients and in a magnetic resonance spectroscopy study (Johnston et al., 2001).

Similarly, synaptic function is disrupted in mouse models of Rett syndrome. Long term potention (LTP) in the CA1 region of the hippocampus is reduced in slices from symptomatic *Mecp2* null mice, while long term depression (LTD) is absent (Asaka et al., 2006). Similar impairments in synaptic plasticity were also found in another mouse model of Rett syndrome, this one involving a truncated allele of *Mecp2* that recapitulates some of the social behavioral and motor difficulties in Rett syndrome (Moretti et al., 2006).

Given MeCP2's function as a transcriptional repressor, investigators have been interested in finding its gene targets, especially ones whose functions may be relevant to Rett Syndrome. While gene expression profiling experiments in *Mecp2* mutant mice have not yielded convincing gene targets (Tudor et al., 2002), several genes have been found to have altered expression in postmortem tissue from Rett patients. One class of such altered genes is involved in synaptic function. Messenger RNAs for the N-methyl-D-aspartate (NMDA) type glutamate receptor NR1 subunit and metabotropic mGluR1 receptor were increased more than 1.5-fold in postmortem cerebral cortex from young

Rett patients. Synapse-related proteins microtubule-associated protein (MAP2), synapsin II, synaptogyrin 3, synaptotagmins 1 and 5, and syntaxin 1A are decreased at least 1.5-fold at the messenger RNA level (Johnston et al., 2001). Immunoreactivity of MAP2, changes of which are an essential component of the excitatory synaptic response, is reduced throughout the neocortex in postmortem Rett samples (Kaufmann et al., 2000; Kaufmann et al., 1995).

Though there is more to understand about how MeCP2 functions in the brain, its well-established importance in the nervous system provides a clear link between epigenetics and the nervous system.

Schizophrenia and reelin

Another disease that suggests a role for epigenetics in the nervous system is schizophrenia, a devastating psychiatric disorder afflicting an estimated 1% of the population. An additional 2-3% likely have a milder form, schizotypal personality disorder. In this disorder of thought, the cognitive and emotional sides of the mind are thought to be split, such that patients either express inappropriate or no emotions while talking about events typically invoking emotions. During the psychotic episodes characteristic of schizophrenia, patients are unable to check their perceptions against reality. Symptoms during the psychotic episodes typically include hallucinations, delusions (often paranoid), and incoherent and disordered thoughts. In one subtype of schizophrenia, patients may exhibit catatonia, a muted or abnormal posture (Kandel et al., 1991).

The etiology of the disease is complex, and involves many genetic components as

well as other factors. A genetic contribution is strongly suggested by studies of monozygotic and dizygotic twins, in the former of which there is between 30% and 50% concordance for the disease. Meanwhile, the concordance rate is only 15% in the dizygotic twins (as it is for other siblings) and only 1% in the general population (Kandel et al., 1991).

That epigenetic alterations are involved in schizophrenia etiology is suggested by the altered expression of reelin, an extracellular matrix protein secreted by neurons expressing receptor for Gamma-amino-butyric acid (GABAergic neurons). Reelin is important in many aspects of nervous system development and function, including neuronal migration, axon branching, synaptogenesis, and cell signaling (Abdolmaleky et al., 2005). In postmortem samples from schizophrenia patients, decreased levels (~50% of controls) of reelin mRNA were detected in all areas examined: areas from prefrontral cortex (Brodmann's area 10 and 46), Brodmann's area 22 in temporal cortex, hippocampus, caudate nucleus, and cerebellum (Impagnatiello et al., 1998).

Reelin expression was found to be influenced by epigenetic changes at the *Reln* gene promoter (Chen et al., 2002b). A candidate CpG island in the *Reln* promoter region extending from –1200 to +200bp of the transcription start site was identified. Administering drugs that interfere either with methylation (the non-methylatable cytosine analog, 5-aza-2'-deoxycytidine) or with histone deacetylation (trichostatin A and valproic acid) resulted in a 20-fold increase in the endogenous expression of reelin mRNA. In contrast, artificially methylating all of the CpG sites in a promoter-reporter construct caused the complete silencing of expression from the *Reln* promoter in a cell transfection assay.

This development on the regulation of reelin was followed by two independent studies documenting aberrant methylation of the *Reln* promoter in schizophrenia. Abdolmaleky et al (2005) examined postmortem tissue from patients. Using methylation-specific PCR and sequencing after treatment with sodium bisulfite (which converts nonmethylated cytosines to thymidine), they found that there was increased methylation at CpG sites within the *Reln* promoter compared to controls. This increased methylation was found in DNA from cortical regions (Brodmann's areas 9 and 10).

Grayson et al (2005) corroborated these findings with their own studies on postmortem brain tissue. Analyzing DNA samples by sodium bisulfite sequencing, they also found increased methylation at cytosines in various occipital and prefrontal cortex regions from schizophrenia patients. The two sites that exhibited the greatest increase in methylation were non-CpG sites: positions –139 (CpApG) and –134 (CpTpG) with respect to the transcription start site of *Reln.*

Veldic et al (2004) found that GABAergic interneurons in the telencephalon of schizophrenia brains overexpress Dnmt1 mRNA. Overexpression of this methyltransferase could lead to hypermethylation at gene promoters and subsequent downregulation of proteins like reelin.

Downregulation of reelin is hypothesized to contribute to schizophrenia pathophysiology. This idea is supported by some similarities (summarized by Costa et al (2001)) in the neuroanatomical and neurochemical findings in schizophrenia patients and in heterozygous reeler mice, which have haploinsufficient mutations in *Reln.*

The brains of both schizophrenia patients and heterozygous reeler mice have decreased cortical neuropil density, increased cortical neuronal packing density, and

decreased dendritic spine density on pyramidal cells of layer III. The decrease in dendritic spine density is linked to defects in sensorimotor gating, which is manifest as a decrease in prepulse inhibition (PPI) that worsens with age. This defect in prepulse inhibition has long been characterized in schizophrenia patients (Braff et al., 1992) and has also been documented in the heterozygous reeler mouse (Tueting et al., 1999).

The expression of some proteins is altered in schizophrenia patients and in heterozygous reeler mice. Not only is total reelin mRNA and protein reduced, but also the number of cells expressing reelin is decreased. Messenger RNA and protein levels of glutamic acid decarboxylase 67 (GAD_{67}), an enzyme expressed by GABAergic neurons, is reduced (Costa et al., 2001).

The epigenetic modulation of reelin and its possible involvement in the pathogenesis of schizophrenia suggest a possible avenue for pharmacological intervention using drugs that interfere with methylation or histone deacetylation.

Epigenetic mechanisms in cell differentiation in the nervous system

In the developing nervous system, astrocytes are generated much later than neurons are. Mouse neuroepithelial cells cultured from E14.5 embryos can differentiate into astrocytes, while those from E11.5 embryos cannot (Takizawa et al., 2001). Glial fibrillary acidic protein (Gfap) is implicated in this so-called neurogenic to gliogenic switch. Gfap is expressed in astroglia but not in neurons, and is regulated by the transcription factor STAT3. STAT3 binds to elements in the *Gfap* promoter and induces *Gfap* transcription (Teter et al., 1996).

STAT3 expression by itself is not adequate for expression of Gfap though. For

example, STAT3 activation alone cannot induce Gfap expression in mouse postmitotic neurons and E11.5 neuroepithelial cells. This restriction suggests that there is an additional mechanism that regulates Gfap expression (Takizawa et al., 2001).

It is now known that that mechanism is epigenetic. Teter et al (1996) described the methylation of CpG sites within the *Gfap* promoter. These sites undergo a change in methylation: they are methylated early in development (at E11.5), and then demethylated at E14.5 in cells differentiating to become astroglia, then again remethylated postnatally. One differentially methylated CpG site lies within a STAT-binding element. Takizawa et al (2001) found that methylation at that site prevents binding by STAT3 and persists in neurons but not in astroglia. As the central nervous system develops, the CpG site becomes de-methylated in cells differentiating along the astroglia lineage.

Fan et al (2005) found that Dnmt1, the maintenance DNA methyltransferase, is important in regulating this differentiation process. Cultured neuronal precursor cells lacking Dnmt1 become hypomethylated and differentiate prematurely into astroglia. Consistent with these observations, cervical spinal cords from Dnmt1 mutant early embryos (E15.5 and E18.5 stages) exhibit enhanced staining of glial markers such as Gfap and S100ß.

Though DNA hypomethylation at the CpG site in the *Gfap* promoter leads to increased GFAP expression, the effects of hypomethylation in the Dnmt1 mutant cells are wider spread. Activation of the JAK/STAT signalling pathway, which is involved in controlling the neurogenic to gliogenic switch, is enhanced in Dnmt1 mutant cells. Not only is STAT1 mRNA upregulated in the mutant cells; there is more activated (phosphorylated) STAT1/3 protein in Dnmt1-/- mutant cells in response to activation by

LIF. Protein levels of gp130, another member of the JAK/STAT signalling pathway, are also increased in Dnmt1-/- mutant cells (Fan et al., 2005).

Another example of epigenetic regulation in neural differentiation is provided by the RE1-silencing transcription factor (REST/NSRF). This factor binds to neuron-restrictive silencer elements (NRSE), usually found in the promoters of genes that are expressed specifically in neurons (Levenson et al., 2004). REST mediates silencing of neuronal-specific genes in non-neuronal cell types. The chromatin remodeling that is required for silencing by REST includes decreased histone acetylation and increased DNA methylation. For example, sodium channel type II gene (*NaCh type II/ Nav1.2*), a target of REST, requires methylation at CpG sites to be silenced (Lunyak et al., 2002).

Epigenetic mechanisms in adult neurogenesis

Though it was a long-held belief that neurogenesis does not take place in adult mammals, recent evidence has shown that it occurs in distinct areas of the adult mammalian brain. In most mammals, new neurons are born in the sub-granular zone (SGZ) of the dentate gyrus region of the hippocampus and in the sub-ventricular zone (SVZ) of the lateral ventricle throughout life. In all mammals examined except for humans, newly generated neurons from the SVZ migrate along the rostral migratory stream to the olfactory bulb, where they later reside as interneurons (Ming and Song, 2005). Whereas thousands of neurons are actively generated each day in the SVZ and subsequently migrate to the olfactory bulb (Lois and Alvarez-Buylla, 1994), neurogenesis in the adult mammal normally does not take place outside these regions and the SGZ. In response to injury such as from ischemia, some neurons are generated, but they typically

cannot complete the final stages of maturation and do not survive in the long term.

The functions of adult neurogenesis are still being investigated. One possibility is that it is employed as a self-repair mechanism, consistent with the induction of neurogenesis observed after injury to the brain (Ming and Song, 2005). Increased neurogenesis is also evident in some neurodegenerative diseases such as Alzheimer's and Huntington's disease (Hagg, 2005). Some evidence points to a role in learning and memory. For example, increased neurogenesis in the dentate gyrus in rodents is associated with enhanced spatial learning in some behavioral tasks and enhanced LTP in the dentate gyrus. Meanwhile, disrupting adult neurogenesis in the SGZ leads to opposite effects (Ming and Song, 2005).

Epigenetic regulation in adult neurogenesis involves methyl-CpG binding protein 1 (MBD1). MBD1 binds to methylated gene promoters and represses transcription through its transrepression domain. In mice, MBD1 is expressed in the brain, and highly so in the hippocampus. A possible role for MBD1 in neurogenesis is suggested by its higher expression in a subpopulation of immature cells in the subgranular cell layer of the dentate gyrus (one of the sites of adult neurogenesis) than in the more mature granule cells (Zhao et al., 2003).

In cell culture assays, adult neural stem cells isolated from the dentate gyrus region in MBD1-/- mice exhibit reduced neuronal differentiation, while astrocytogenesis is unaffected. Neurons in the hippocampi of MBD1-/- mice show decreased survival but normal proliferation in BrDU assays. Thus, MBD1 homozygous null mice have a deficiency in adult neurogenesis in the hippocampus region. These defects are accompanied by increased aneuploidy, suggestive of genome instability. Another sign of

genome instability is derepression of the endogenous viral IAP element in adult neural stem cells isolated from MBD1-/- mice (Zhao et al., 2003).

Epigenetics in learning, memory, and behavior

As mentioned above, several studies have suggested there is a link between adult neurogenesis and learning and memory (Ming and Song, 2005). Consistent with this proposed link and the role of MBD1 in adult neurogenesis, there is also evidence that epigenetic modulation functions in learning, memory, and behavior. The MBD1-/- mice described above also underperform in a Morris water maze test, a learning and memory task. The decreased performance of the MBD1-/- mice indicates a specific deficiency in spatial learning, as motor coordination and locomotion (also required for the task) were normal on the basis of other behavioral tests. Long term potentiation (LTP) in the dentate gyrus is also impaired in slices from MBD1-deficient hippocampi, though LTP in the CA1 region is unaffected (Zhao et al., 2003).

Mice bearing a truncated *Mecp2* allele also exhibit defects in spatial learning, as indicated by underperformance in the Morris water maze test. These mice also showed impaired contextual fear conditioning. This type of learning and memory task depends on the function of the hippocampus and the amygdala. Consistent with these problems in learning and memory, long term depression was impaired and enhanced synaptic transmission was observed in area CA1 area of the hippocampus of these *Mecp2* mutant mice. Defects in other areas of the brain were also found: there was reduced long-term potentiation in motor and sensory cortex (Moretti et al., 2006).

Methyl-binding proteins are not the only components of the epigenetic regulatory

network implicated in learning and memory. Levenson et al (2004) documented alterations in histone acetylation during the formation of long-term memories in the hippocampus. In rats, contextual fear conditioning led to increases of histone acetylation at histone H3 on the lysine 14 residue, a modification that is associated with transcriptional de-repression. Stimulating the ERK pathway, a signalling pathway associated with memory formation, increased histone H3 acetylation as well. Conversely, artificially elevating the level of histone H3 acetylation by using the histone deacetylase inhibitors trichostatin A or sodium butyrate led to enhancement of LTP induction in area CA1 of the hippocampus.

Other studies show direct connections between epigenetic regulation and behavior. Weaver et al (2004) demonstrated one of the first concrete examples of epigenetic marking in response to early postnatal experiences. They demonstrated that in rats, CpG sites in the promoter of the glucocorticoid receptor (*GR)* gene are differentially methylated depending on how the pups are nursed and groomed by their mothers. In pups whose mothers nursed them with an "arched back" style and groomed them extensively, the sites were hypomethylated. In pups raised by mothers with a different grooming and nursing style, the sites were methylated.

The experiments by Weaver et al (2004) also illustrate that epigenetic modifications affect behavior and can be reversible. Methylation differences at the *GR* locus persisted into adulthood, and rats with hypomethylated sites responded to stress better than rats in which the sites were methylated. Histone acetylation changes were also observed between the two groups of rats, and histone deacetylase inhibitors reversed the epigenetic distinctions (including that of DNA methylation) between the two groups of

rats.

DE NOVO DNA METHYLTRANSFERASES IN THE NERVOUS SYSTEM

Given the mounting evidence that epigenetics is important in gene regulation in the nervous system, what is the role of the DNA methyltransferases in the nervous system? Studies on the maintenance methyltransferase Dnmt1 have shown that it is essential to cell survival in mitotic neural precursors (Fan et al., 2001), is involved in modulation of genes necessary for astrogliogenesis (Fan et al., 2005), and exacerbates damage to brain tissue in one type of ischemia (Endres et al., 2000). The function of the *de novo* DNA methyltransferases, Dnmt3a and Dnmt3b, in the nervous system had not yet been clarified.

The *de novo* DNA methylating ability of these enzymes might be employed to modulate gene expression in neuronal differentiation, or in response to environmental cues or new experiences. Dnmt3a and Dnmt3b are both expressed in the nervous system, though during different time windows. Dnmt3b is expressed in the embryo during neurogenesis. In transgenic mouse embryos expressing lacZ from a transgene integrated into the Dnmt3b locus, lacZ was detected in cells across the ventricular zone of the central nervous system between embryonic days E10.5 and E13.5, but not after E15.5 (Feng et al., 2005).

The expression of Dnmt3a, as assessed by staining for a lacZ transgene integrated into the Dnmt3a locus, begins in the mouse embryo around E10.5, increases during the first few weeks after birth, and then wanes but remains detectable in the adult animals. Between E10.5 and E17.5, Dnmt3a expression is mostly detected in neuronal precursor

cells of the ventricular and subventricular zones. In the adult central nervous system, it is expressed predominantly in postmitotic neurons of the olfactory bulb, cortex, hippocampus, striatum, and cerebellum (Feng et al., 2005).

For this thesis I concentrated on studying the function of Dnmt3a in the nervous system. Its continued expression in the adult nervous system raises the interesting possibility that it has a role in brain maturation and postnatal function in the nervous system. This putative role could be especially interesting, given that some of the known neurological diseases involving epigenetics, such as Rett syndrome and schizophrenia, are disorders of brain maturation or likely involve postnatal events.

The isolation of the Dnmt3a gene (Okano et al., 1998a) and the existence of established techniques of gene disruption in mouse embryonic stem cells afforded a way to study Dnmt3a. Using the Cre-loxP recombination system from bacteriophage, and Cre transgenic lines that express Cre in the nervous system, it was possible to restrict a Dnmt3a mutation in order to circumvent the perinatal lethality of Dnmt3a knockout mice. The generation and characterization of such a Dnmt3a conditional knockout is described in the following chapter.

REFERENCES

Abdolmaleky, H. M., Cheng, K. H., Russo, A., Smith, C. L., Faraone, S. V., Wilcox, M., Shafa, R., Glatt, S. J., Nguyen, G., Ponte, J. F., *et al.* (2005). Hypermethylation of the reelin (RELN) promoter in the brain of schizophrenic patients: a preliminary report. Am J Med Genet B Neuropsychiatr Genet *134*, 60-66.

Akbarian, S., Chen, R. Z., Gribnau, J., Rasmussen, T. P., Fong, H., Jaenisch, R., and Jones, E. G. (2001). Expression pattern of the Rett syndrome gene MeCP2 in primate prefrontal cortex. Neurobiol Dis *8*, 784-791.

Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., and Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet *23*, 185-188.

Armstrong, D. D. (2001). Rett syndrome neuropathology review 2000. Brain Dev *23 Suppl 1*, S72-76.

Armstrong, D. D., Dunn, K., and Antalffy, B. (1998). Decreased dendritic branching in frontal, motor and limbic cortex in Rett syndrome compared with trisomy 21. J Neuropathol Exp Neurol *57*, 1013-1017.

Asaka, Y., Jugloff, D. G., Zhang, L., Eubanks, J. H., and Fitzsimonds, R. M. (2006). Hippocampal synaptic plasticity is impaired in the Mecp2-null mouse model of Rett syndrome. Neurobiol Dis *21*, 217-227.

Asche, W., Colletta, G., Warnecke, G., Nobis, P., Pennie, S., King, R. M., and Ostertag, W. (1984). Lack of retrovirus gene expression in somatic cell hybrids of friend cells and teratocarcinoma cells with a teratocarcinoma phenotype. Mol Cell Biol *4*, 923-930.

Ballestar, E., and Wolffe, A. P. (2001). Methyl-CpG-binding proteins. Targeting specific gene repression. Eur J Biochem *268*, 1-6.

Bauman, M. L., Kemper, T. L., and Arin, D. M. (1995). Pervasive neuroanatomic abnormalities of the brain in three cases of Rett's syndrome. Neurology *45*, 1581-1586.

Belichenko, P. V., and Dahlstrom, A. (1995). Studies on the 3-dimensional architecture of dendritic spines and varicosities in human cortex by confocal laser scanning microscopy and Lucifer yellow microinjections. J Neurosci Methods *57*, 55-61.

Belichenko, P. V., Hagberg, B., and Dahlstrom, A. (1997). Morphological study of neocortical areas in Rett syndrome. Acta Neuropathol (Berl) *93*, 50-61.

Bestor, T., Laudano, A., Mattaliano, R., and Ingram, V. (1988). Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. J

Mol Biol *203*, 971-983.

Blue, M. E., and Parnavelas, J. G. (1983). The formation and maturation of synapses in the visual cortex of the rat. II. Quantitative analysis. J Neurocytol *12*, 697-712.

Bourc'his, D., Xu, G. L., Lin, C. S., Bollman, B., and Bestor, T. H. (2001). Dnmt3L and the establishment of maternal genomic imprints. Science *294*, 2536-2539.

Braff, D. L., Grillon, C., and Geyer, M. A. (1992). Gating and habituation of the startle reflex in schizophrenic patients. Arch Gen Psychiatry *49*, 206-215.

Cao, R., and Zhang, Y. (2004). The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev *14*, 155-164.

Challita, P. M., and Kohn, D. B. (1994). Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. Proc Natl Acad Sci U S A *91*, 2567-2571.

Chang-Yeh, A., Mold, D. E., and Huang, R. C. (1991). Identification of a novel murine IAP-promoted placenta-expressed gene. Nucleic Acids Res *19*, 3667-3672.

Chedin, F., Lieber, M. R., and Hsieh, C. L. (2002). The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. Proc Natl Acad Sci U S A *99*, 16916-16921.

Chen, L., MacMillan, A. M., Chang, W., Ezaz-Nikpay, K., Lane, W. S., and Verdine, G. L. (1991). Direct identification of the active-site nucleophile in a DNA (cytosine-5)-methyltransferase. Biochemistry *30*, 11018-11025.

Chen, R. Z., Akbarian, S., Tudor, M., and Jaenisch, R. (2001). Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nat Genet *27*, 327-331.

Chen, R. Z., Pettersson, U., Beard, C., Jackson-Grusby, L., and Jaenisch, R. (1998). DNA hypomethylation leads to elevated mutation rates. Nature *395*, 89-93.

Chen, T., Tsujimoto, N., and Li, E. (2004). The PWWP domain of Dnmt3a and Dnmt3b is required for directing DNA methylation to the major satellite repeats at pericentric heterochromatin. Mol Cell Biol *24*, 9048-9058.

Chen, T., Ueda, Y., Xie, S., and Li, E. (2002a). A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. J Biol Chem *277*, 38746-38754.

Chen, Y., Sharma, R. P., Costa, R. H., Costa, E., and Grayson, D. R. (2002b). On the epigenetic regulation of the human reelin promoter. Nucleic Acids Res *30*, 2930-2939.

Chen, Z. X., Mann, J. R., Hsieh, C. L., Riggs, A. D., and Chedin, F. (2005). Physical and

functional interactions between the human DNMT3L protein and members of the de novo methyltransferase family. J Cell Biochem *95*, 902-917.

Clark, S. J., Harrison, J., and Molloy, P. L. (1997). Sp1 binding is inhibited by (m)Cp(m)CpG methylation. Gene *195*, 67-71.

Cohen, D. R., Matarazzo, V., Palmer, A. M., Tu, Y., Jeon, O. H., Pevsner, J., and Ronnett, G. V. (2003). Expression of MeCP2 in olfactory receptor neurons is developmentally regulated and occurs before synaptogenesis. Mol Cell Neurosci *22*, 417-429.

Comb, M., and Goodman, H. M. (1990). CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res *18*, 3975-3982.

Costa, E., Davis, J., Grayson, D. R., Guidotti, A., Pappas, G. D., and Pesold, C. (2001). Dendritic spine hypoplasticity and downregulation of reelin and GABAergic tone in schizophrenia vulnerability. Neurobiol Dis *8*, 723-742.

Datta, J., Ghoshal, K., Sharma, S. M., Tajima, S., and Jacob, S. T. (2003). Biochemical fractionation reveals association of DNA methyltransferase (Dnmt) 3b with Dnmt1 and that of Dnmt 3a with a histone H3 methyltransferase and Hdac1. J Cell Biochem *88*, 855-864.

Ding, F., and Chaillet, J. R. (2002). In vivo stabilization of the Dnmt1 (cytosine-5)-methyltransferase protein. Proc Natl Acad Sci U S A *99*, 14861-14866.

Eden, S., and Cedar, H. (1994). Role of DNA methylation in the regulation of transcription. Curr Opin Genet Dev *4*, 255-259.

Ehrlich, M. (2003). The ICF syndrome, a DNA methyltransferase 3B deficiency and immunodeficiency disease. Clin Immunol *109*, 17-28.

Endres, M., Meisel, A., Biniszkiewicz, D., Namura, S., Prass, K., Ruscher, K., Lipski, A., Jaenisch, R., Moskowitz, M. A., and Dirnagl, U. (2000). DNA methyltransferase contributes to delayed ischemic brain injury. J Neurosci *20*, 3175-3181.

Fan, G., Beard, C., Chen, R. Z., Csankovszki, G., Sun, Y., Siniaia, M., Biniszkiewicz, D., Bates, B., Lee, P. P., Kuhn, R., *et al.* (2001). DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. J Neurosci *21*, 788-797.

Fan, G., Martinowich, K., Chin, M. H., He, F., Fouse, S. D., Hutnick, L., Hattori, D., Ge, W., Shen, Y., Wu, H., *et al.* (2005). DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development *132*, 3345-3356.

Feng, J., Chang, H., Li, E., and Fan, G. (2005). Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. J Neurosci Res

79, 734-746.

Fukuda, T., Itoh, M., Ichikawa, T., Washiyama, K., and Goto, Y. (2005). Delayed maturation of neuronal architecture and synaptogenesis in cerebral cortex of Mecp2-deficient mice. J Neuropathol Exp Neurol *64*, 537-544.

Futscher, B. W., Oshiro, M. M., Wozniak, R. J., Holtan, N., Hanigan, C. L., Duan, H., and Domann, F. E. (2002). Role for DNA methylation in the control of cell type specific maspin expression. Nat Genet *31*, 175-179.

Gaudet, F., Hodgson, J. G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J. W., Leonhardt, H., and Jaenisch, R. (2003). Induction of tumors in mice by genomic hypomethylation. Science *300*, 489-492.

Geiman, T. M., Sankpal, U. T., Robertson, A. K., Chen, Y., Mazumdar, M., Heale, J. T., Schmiesing, J. A., Kim, W., Yokomori, K., Zhao, Y., and Robertson, K. D. (2004). Isolation and characterization of a novel DNA methyltransferase complex linking DNMT3B with components of the mitotic chromosome condensation machinery. Nucleic Acids Res *32*, 2716-2729.

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

Goll, M. G., Kirpekar, F., Maggert, K. A., Yoder, J. A., Hsieh, C. L., Zhang, X., Golic, K. G., Jacobsen, S. E., and Bestor, T. H. (2006). Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science *311*, 395-398.

Gowher, H., and Jeltsch, A. (2002). Molecular enzymology of the catalytic domains of the Dnmt3a and Dnmt3b DNA methyltransferases. J Biol Chem *277*, 20409-20414.

Gowher, H., Liebert, K., Hermann, A., Xu, G., and Jeltsch, A. (2005). Mechanism of stimulation of catalytic activity of Dnmt3A and Dnmt3B DNA-(cytosine-C5)-methyltransferases by Dnmt3L. J Biol Chem *280*, 13341-13348.

Goyon, C., Rossignol, J. L., and Faugeron, G. (1996). Native DNA repeats and methylation in Ascobolus. Nucleic Acids Res *24*, 3348-3356.

Grayson, D. R., Jia, X., Chen, Y., Sharma, R. P., Mitchell, C. P., Guidotti, A., and Costa, E. (2005). Reelin promoter hypermethylation in schizophrenia. Proc Natl Acad Sci U S A *102*, 9341-9346.

Guy, J., Hendrich, B., Holmes, M., Martin, J. E., and Bird, A. (2001). A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat Genet *27*, 322-326.

Hagg, T. (2005). Molecular regulation of adult CNS neurogenesis: an integrated view. Trends Neurosci *28*, 589-595.

Hansen, R. S., Wijmenga, C., Luo, P., Stanek, A. M., Canfield, T. K., Weemaes, C. M., and Gartler, S. M. (1999). The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. Proc Natl Acad Sci U S A *96*, 14412-14417.

Hata, K., Okano, M., Lei, H., and Li, E. (2002). Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development *129*, 1983-1993.

Hermann, A., Gowher, H., and Jeltsch, A. (2004). Biochemistry and biology of mammalian DNA methyltransferases. Cell Mol Life Sci *61*, 2571-2587.

Hermann, A., Schmitt, S., and Jeltsch, A. (2003). The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity. J Biol Chem *278*, 31717-31721.

Hoeben, R. C., Migchielsen, A. A., van der Jagt, R. C., van Ormondt, H., and van der Eb, A. J. (1991). Inactivation of the Moloney murine leukemia virus long terminal repeat in murine fibroblast cell lines is associated with methylation and dependent on its chromosomal position. J Virol *65*, 904-912.

Hoffbuhr, K., Devaney, J. M., LaFleur, B., Sirianni, N., Scacheri, C., Giron, J., Schuette, J., Innis, J., Marino, M., Philippart, M., *et al.* (2001). MeCP2 mutations in children with and without the phenotype of Rett syndrome. Neurology *56*, 1486-1495.

Holliday, R., and Pugh, J. E. (1975). DNA modification mechanisms and gene activity during development. Science *187*, 226-232.

Hotchkiss, R. D. (1948). The quantitative separation of purines, pyramidines and nucleosides by paper chromatography. J Biol Chem *175*, 315-332.

Impagnatiello, F., Guidotti, A. R., Pesold, C., Dwivedi, Y., Caruncho, H., Pisu, M. G., Uzunov, D. P., Smalheiser, N. R., Davis, J. M., Pandey, G. N., *et al.* (1998). A decrease of reelin expression as a putative vulnerability factor in schizophrenia. Proc Natl Acad Sci U S A *95*, 15718-15723.

Jaenisch, R. (1997). DNA methylation and imprinting: why bother? Trends Genet *13*, 323-329.

Jeltsch, A., Nellen, W., and Lyko, F. (2006). Two substrates are better than one: dual specificities for Dnmt2 methyltransferases. Trends Biochem Sci *31*, 306-308.

Johnston, M. V., Jeon, O. H., Pevsner, J., Blue, M. E., and Naidu, S. (2001). Neurobiology of Rett syndrome: a genetic disorder of synapse development. Brain Dev *23 Suppl 1*, S206-213.

Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet *19*, 187-191.

Jugloff, D. G., Jung, B. P., Purushotham, D., Logan, R., and Eubanks, J. H. (2005). Increased dendritic complexity and axonal length in cultured mouse cortical neurons overexpressing methyl-CpG-binding protein MeCP2. Neurobiol Dis *19*, 18-27.

Julu, P. O., Kerr, A. M., Apartopoulos, F., Al-Rawas, S., Engerstrom, I. W., Engerstrom, L., Jamal, G. A., and Hansen, S. (2001). Characterisation of breathing and associated central autonomic dysfunction in the Rett disorder. Arch Dis Child *85*, 29-37.

Kanai, Y., Saito, Y., Ushijima, S., and Hirohashi, S. (2004). Alterations in gene expression associated with the overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, during human hepatocarcinogenesis. J Cancer Res Clin Oncol *130*, 636-644.

Kandel, E. R., Schwartz, J. H., and Jessell, T. M. (1991). Principles of neural science, 3rd edn (New York: Elsevier).

Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., and Sasaki, H. (2004). Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature *429*, 900-903.

Kang, E. S., Park, C. W., and Chung, J. H. (2001). Dnmt3b, de novo DNA methyltransferase, interacts with SUMO-1 and Ubc9 through its N-terminal region and is subject to modification by SUMO-1. Biochem Biophys Res Commun *289*, 862-868.

Kaufmann, W. E., Johnston, M. V., and Blue, M. E. (2005). MeCP2 expression and function during brain development: implications for Rett syndrome's pathogenesis and clinical evolution. Brain Dev *27 Suppl 1*, S77-S87.

Kaufmann, W. E., MacDonald, S. M., and Altamura, C. R. (2000). Dendritic cytoskeletal protein expression in mental retardation: an immunohistochemical study of the neocortex in Rett syndrome. Cereb Cortex *10*, 992-1004.

Kaufmann, W. E., Naidu, S., and Budden, S. (1995). Abnormal expression of microtubule-associated protein 2 (MAP-2) in neocortex in Rett syndrome. Neuropediatrics *26*, 109-113.

Kerr, A. M., and Julu, P. O. (1999). Recent insights into hyperventilation from the study of Rett syndrome. Arch Dis Child *80*, 384-387.

Kim, G. D., Ni, J., Kelesoglu, N., Roberts, R. J., and Pradhan, S. (2002). Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. Embo J *21*, 4183-4195.

Kishi, N., and Macklis, J. D. (2004). MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions. Mol Cell Neurosci *27*, 306-321.

Klimasauskas, S., Kumar, S., Roberts, R. J., and Cheng, X. (1994). HhaI

methyltransferase flips its target base out of the DNA helix. Cell *76*, 357-369.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R. J., and Wilson, G. G. (1994). The DNA (cytosine-5) methyltransferases. Nucleic Acids Res *22*, 1-10.

Kunert, N., Marhold, J., Stanke, J., Stach, D., and Lyko, F. (2003). A Dnmt2-like protein mediates DNA methylation in Drosophila. Development *130*, 5083-5090.

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

Lauster, R., Trautner, T. A., and Noyer-Weidner, M. (1989). Cytosine-specific type II DNA methyltransferases. A conserved enzyme core with variable target-recognizing domains. J Mol Biol *206*, 305-312.

Leighton, P. A., Ingram, R. S., Eggenschwiler, J., Efstratiadis, A., and Tilghman, S. M. (1995). Disruption of imprinting caused by deletion of the H19 gene region in mice. Nature *375*, 34-39.

Levenson, J. M., O'Riordan, K. J., Brown, K. D., Trinh, M. A., Molfese, D. L., and Sweatt, J. D. (2004). Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem *279*, 40545-40559.

Lewis, A., and Murrell, A. (2004). Genomic imprinting: CTCF protects the boundaries. Curr Biol *14*, R284-286.

Li, E., Beard, C., and Jaenisch, R. (1993). Role for DNA methylation in genomic imprinting. Nature *366*, 362-365.

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

Ling, Y., Sankpal, U. T., Robertson, A. K., McNally, J. G., Karpova, T., and Robertson, K. D. (2004). Modification of de novo DNA methyltransferase 3a (Dnmt3a) by SUMO-1 modulates its interaction with histone deacetylases (HDACs) and its capacity to repress transcription. Nucleic Acids Res *32*, 598-610.

Lock, L. F., Takagi, N., and Martin, G. R. (1987). Methylation of the Hprt gene on the inactive X occurs after chromosome inactivation. Cell *48*, 39-46.

Lois, C., and Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. Science *264*, 1145-1148.

Luikenhuis, S., Giacometti, E., Beard, C. F., and Jaenisch, R. (2004). Expression of MeCP2 in postmitotic neurons rescues Rett syndrome in mice. Proc Natl Acad Sci U S A *101*, 6033-6038.

Lunyak, V. V., Burgess, R., Prefontaine, G. G., Nelson, C., Sze, S. H., Chenoweth, J., Schwartz, P., Pevzner, P. A., Glass, C., Mandel, G., and Rosenfeld, M. G. (2002). Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. Science *298*, 1747-1752.

Lyko, F., Ramsahoye, B. H., Kashevsky, H., Tudor, M., Mastrangelo, M. A., Orr-Weaver, T. L., and Jaenisch, R. (1999). Mammalian (cytosine-5) methyltransferases cause genomic DNA methylation and lethality in Drosophila. Nat Genet *23*, 363-366.

Mancini, D. N., Singh, S. M., Archer, T. K., and Rodenhiser, D. I. (1999). Site-specific DNA methylation in the neurofibromatosis (NF1) promoter interferes with binding of CREB and SP1 transcription factors. Oncogene *18*, 4108-4119.

Matzke, M. A., Mette, M. F., Aufsatz, W., Jakowitsch, J., and Matzke, A. J. (1999). Host defenses to parasitic sequences and the evolution of epigenetic control mechanisms. Genetica *107*, 271-287.

Mertineit, C., Yoder, J. A., Taketo, T., Laird, D. W., Trasler, J. M., and Bestor, T. H. (1998). Sex-specific exons control DNA methyltransferase in mammalian germ cells. Development *125*, 889-897.

Ming, G. L., and Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci *28*, 223-250.

Moore, T., and Haig, D. (1991). Genomic imprinting in mammalian development: a parental tug-of-war. Trends Genet *7*, 45-49.

Moretti, P., Levenson, J. M., Battaglia, F., Atkinson, R., Teague, R., Antalffy, B., Armstrong, D., Arancio, O., Sweatt, J. D., and Zoghbi, H. Y. (2006). Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. J Neurosci *26*, 319-327.

Mullaney, B. C., Johnston, M. V., and Blue, M. E. (2004). Developmental expression of methyl-CpG binding protein 2 is dynamically regulated in the rodent brain. Neuroscience *123*, 939-949.

Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., and Grewal, S. I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110-113.

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature *393*, 386-389.

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247-257.

Okano, M., Xie, S., and Li, E. (1998a). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet *19*, 219-220.

Okano, M., Xie, S., and Li, E. (1998b). Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res *26*, 2536-2540.

Panning, B., and Jaenisch, R. (1996). DNA hypomethylation can activate Xist expression and silence X-linked genes. Genes Dev *10*, 1991-2002.

Panning, B., and Jaenisch, R. (1998). RNA and the epigenetic regulation of X chromosome inactivation. Cell *93*, 305-308.

Posfai, J., Bhagwat, A. S., Posfai, G., and Roberts, R. J. (1989). Predictive motifs derived from cytosine methyltransferases. Nucleic Acids Res *17*, 2421-2435.

Ramsahoye, B. H., Biniszkiewicz, D., Lyko, F., Clark, V., Bird, A. P., and Jaenisch, R. (2000). Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. Proc Natl Acad Sci U S A *97*, 5237-5242.

Riggs, A. D. (1975). X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet *14*, 9-25.

Rountree, M. R., Bachman, K. E., and Baylin, S. B. (2000). DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat Genet *25*, 269-277.

Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H., and Hirohashi, S. (2002). Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc Natl Acad Sci U S A *99*, 10060-10065.

Sakai, Y., Suetake, I., Shinozaki, F., Yamashina, S., and Tajima, S. (2004). Co-expression of de novo DNA methyltransferases Dnmt3a2 and Dnmt3L in gonocytes of mouse embryos. Gene Expr Patterns *5*, 231-237.

Shahbazian, M. D., Antalffy, B., Armstrong, D. L., and Zoghbi, H. Y. (2002). Insight into Rett syndrome: MeCP2 levels display tissue- and cell-specific differences and correlate with neuronal maturation. Hum Mol Genet *11*, 115-124.

Shirohzu, H., Kubota, T., Kumazawa, A., Sado, T., Chijiwa, T., Inagaki, K., Suetake, I., Tajima, S., Wakui, K., Miki, Y., *et al.* (2002). Three novel DNMT3B mutations in Japanese patients with ICF syndrome. Am J Med Genet *112*, 31-37.

Sims, R. J., 3rd, and Reinberg, D. (2006). Histone H3 Lys 4 methylation: caught in a bind? Genes Dev *20*, 2779-2786.

Sleutels, F., Zwart, R., and Barlow, D. P. (2002). The non-coding Air RNA is required for silencing autosomal imprinted genes. Nature *415*, 810-813.

Stoger, R., Kubicka, P., Liu, C. G., Kafri, T., Razin, A., Cedar, H., and Barlow, D. P. (1993). Maternal-specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. Cell *73*, 61-71.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes Dev *12*, 599-606.

Subramaniam, B., Naidu, S., and Reiss, A. L. (1997). Neuroanatomy in Rett syndrome: cerebral cortex and posterior fossa. Neurology *48*, 399-407.

Takahara, T., Ohsumi, T., Kuromitsu, J., Shibata, K., Sasaki, N., Okazaki, Y., Shibata, H., Sato, S., Yoshiki, A., Kusakabe, M., *et al.* (1996). Dysfunction of the Orleans reeler gene arising from exon skipping due to transposition of a full-length copy of an active L1 sequence into the skipped exon. Hum Mol Genet *5*, 989-993.

Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M., and Taga, T. (2001). DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell *1*, 749-758.

Tang, L. Y., Reddy, M. N., Rasheva, V., Lee, T. L., Lin, M. J., Hung, M. S., and Shen, C. K. (2003). The eukaryotic DNMT2 genes encode a new class of cytosine-5 DNA methyltransferases. J Biol Chem *278*, 33613-33616.

Teter, B., Rozovsky, I., Krohn, K., Anderson, C., Osterburg, H., and Finch, C. (1996). Methylation of the glial fibrillary acidic protein gene shows novel biphasic changes during brain development. Glia *17*, 195-205.

Tremblay, K. D., Saam, J. R., Ingram, R. S., Tilghman, S. M., and Bartolomei, M. S. (1995). A paternal-specific methylation imprint marks the alleles of the mouse H19 gene. Nat Genet *9*, 407-413.

Tucker, K. L. (2001). Methylated cytosine and the brain: a new base for neuroscience. Neuron *30*, 649-652.

Tudor, M., Akbarian, S., Chen, R. Z., and Jaenisch, R. (2002). Transcriptional profiling of a mouse model for Rett syndrome reveals subtle transcriptional changes in the brain. Proc Natl Acad Sci U S A *99*, 15536-15541.

Tueting, P., Costa, E., Dwivedi, Y., Guidotti, A., Impagnatiello, F., Manev, R., and Pesold, C. (1999). The phenotypic characteristics of heterozygous reeler mouse. Neuroreport *10*, 1329-1334.

Veldic, M., Caruncho, H. J., Liu, W. S., Davis, J., Satta, R., Grayson, D. R., Guidotti, A., and Costa, E. (2004). DNA-methyltransferase 1 mRNA is selectively overexpressed in telencephalic GABAergic interneurons of schizophrenia brains. Proc Natl Acad Sci U S A *101*, 348-353.

Viemari, J. C., Roux, J. C., Tryba, A. K., Saywell, V., Burnet, H., Pena, F., Zanella, S.,

Bevengut, M., Barthelemy-Requin, M., Herzing, L. B., *et al.* (2005). Mecp2 deficiency disrupts norepinephrine and respiratory systems in mice. J Neurosci *25*, 11521-11530.

Wade, P. A. (2001). Methyl CpG-binding proteins and transcriptional repression. Bioessays *23*, 1131-1137.

Walsh, C. P., Chaillet, J. R., and Bestor, T. H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat Genet *20*, 116-117.

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., and Meaney, M. J. (2004). Epigenetic programming by maternal behavior. Nat Neurosci *7*, 847-854.

Webster, K. E., O'Bryan, M. K., Fletcher, S., Crewther, P. E., Aapola, U., Craig, J., Harrison, D. K., Aung, H., Phutikanit, N., Lyle, R., *et al.* (2005). Meiotic and epigenetic defects in Dnmt3L-knockout mouse spermatogenesis. Proc Natl Acad Sci U S A *102*, 4068-4073.

Weisenberger, D. J., Velicescu, M., Cheng, J. C., Gonzales, F. A., Liang, G., and Jones, P. A. (2004). Role of the DNA methyltransferase variant DNMT3b3 in DNA methylation. Mol Cancer Res *2*, 62-72.

Weisenberger, D. J., Velicescu, M., Preciado-Lopez, M. A., Gonzales, F. A., Tsai, Y. C., Liang, G., and Jones, P. A. (2002). Identification and characterization of alternatively spliced variants of DNA methyltransferase 3a in mammalian cells. Gene *298*, 91-99.

Wigler, M., Levy, D., and Perucho, M. (1981). The somatic replication of DNA methylation. Cell *24*, 33-40.

Wijmenga, C., Hansen, R. S., Gimelli, G., Bjorck, E. J., Davies, E. G., Valentine, D., Belohradsky, B. H., van Dongen, J. J., Smeets, D. F., van den Heuvel, L. P., *et al.* (2000). Genetic variation in ICF syndrome: evidence for genetic heterogeneity. Hum Mutat *16*, 509-517.

Wolffe, A. P., and Matzke, M. A. (1999). Epigenetics: regulation through repression. Science *286*, 481-486.

Xu, G. L., Bestor, T. H., Bourc'his, D., Hsieh, C. L., Tommerup, N., Bugge, M., Hulten, M., Qu, X., Russo, J. J., and Viegas-Pequignot, E. (1999). Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature *402*, 187-191.

Yoder, J. A., Soman, N. S., Verdine, G. L., and Bestor, T. H. (1997a). DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. J Mol Biol *270*, 385-395.

Yoder, J. A., Walsh, C. P., and Bestor, T. H. (1997b). Cytosine methylation and the ecology of intragenomic parasites. Trends Genet *13*, 335-340.

Zhao, X., Ueba, T., Christie, B. R., Barkho, B., McConnell, M. J., Nakashima, K., Lein, E. S., Eadie, B. D., Willhoite, A. R., Muotri, A. R., *et al.* (2003). Mice lacking methyl-CpG binding protein 1 have deficits in adult neurogenesis and hippocampal function. Proc Natl Acad Sci U S A *100*, 6777-6782.

Chapter 2.

Ablation of *de novo* DNA methyltransferase Dnmt3a in the nervous system leads to neuromuscular defects and shortened lifespan

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ABSTRACT

DNA methylation is an epigenetic mechanism involved in gene regulation and implicated in the functioning of the nervous system. The *de novo* DNA methyltransferase Dnmt3a is expressed in neurons, but its specific role has not been clarified. Dnmt3a is activated around embryonic day 10.5 in mouse neuronal precursor cells and remains active in postmitotic neurons in the adult. We assessed the role of neuronal Dnmt3a by conditional gene targeting. Mice lacking functional Dnmt3a in the nervous system were born healthy, but degenerated in adulthood and died prematurely. Mutant mice were hypoactive, walked abnormally, and underperformed on tests of neuromuscular function and motor coordination. Loss of Dnmt3a also led to fewer motor neurons in the hypoglossal nucleus and more fragmented endplates in neuromuscular junctions of the diaphragm muscle. Our results implicate a role for Dnmt3a in the neuromuscular control of motor movement.

INTRODUCTION

In mammals, the mechanism of methylation of DNA at cytosines in CpG dinucleotides is employed to regulate genes epigenetically. Genomic DNA methylation patterns are established during development by the action of the *de novo* methyltransferases Dnmt3a and Dnmt3b and are subsequently maintained by the methyltransferase Dnmt1 (Li et al., 1992; Okano et al., 1999). Complete ablation of the activity of either Dnmt1 or Dnmt3b results in embryonic lethality, (Li et al., 1992; Okano et al., 1998) whereas deletion of Dnmt3a causes perinatal lethality (Okano et al., 1999). An important role of DNA methylation in the nervous system is indicated by Rett

syndrome, a neurodevelopmental disorder caused by mutations in MeCP2, a transcriptional repressor that binds to methylated CpGs (Amir and Zoghbi, 2000; Chen et al., 2001; Guy et al., 2001). Also, changes in methylation of the *reelin* promoter have been correlated with schizophrenia (Grayson et al., 2005).

DNA methylation affects directly the expression of CNS specific genes. For example, sites within the promoter of the glial fibrillary acidic protein (*Gfap*) gene, a marker involved in astrocyte differentiation, is methylated at E11.5 but then demethylated at E14.5 in cells differentiating into astroglia (Teter et al., 1996). In neurons, which do not express Gfap, a CpG site in a STAT3 binding element in the *Gfap* promoter remains methylated. This methylation interferes with STAT3 binding and disallows the cells from proceeding along the astroglia lineage (Takizawa et al., 2001). Deletion of *Dnmt1*, which encodes the maintenance DNA methyltransferase, has no obvious effect on postnatal brain function (Fan et al., 2001), but causes neural precursor cells to differentiate prematurely into astroglia (Fan et al., 2005).

Dnmt3a is activated in neuronal precusor cells in E10.5 embryos. Expression of the enzyme increases postnatally, but its role in the nervous system is unknown. In adult mice, Dnmt3a expression persists, albeit at lower levels, in postmitotic neurons of many regions including hippocampus, cortex, striatum, olfactory bulb, and also in the brain stem and cerebellum (Feng et al., 2005), two brain regions that are involved in modulating motor movement.

Conventional Dnmt3a knockout mice die at around 3 or 4 weeks of age (Okano et al., 1999). In order to study the role of Dnmt3a at later stages, and to analyze its function specifically in the nervous system, we generated conditional Dnmt3a-knockout mice.

Ablation of Dnmt3a function in the brain using the Nes-Cre1 transgene resulted in motor defects and premature death, suggesting that the *de novo* DNA methyltransferase Dnmt3a is required in the neuromuscular system for proper movement.

RESULTS

Generation of conditional Dnmt3a knockout mice

In order to control the ablation of Dnmt3a, we designed a conditional knockout version of the *Dnmt3a* gene that could be used in conjunction with the Cre-loxP recombination system (Sauer and Henderson, 1988). In the targeting construct, exons encoding the catalytic domains were flanked by loxP sites (Figure 1A). A neomycin resistance gene under the control of the phosphoglycerate kinase promoter and flanked by frt sites (Possemato et al., 2002) was inserted in an intron to facilitate selection of integrated clones. The frt sites allowed removal of the *neo* cassette in ES cells by transient lipofection with a flp recombinase-expressing plasmid (Senecoff et al., 1985). ES cells with a conditional allele of *Dnmt3a* (*Dnmt3a*^{2lox}) (Figure 1B) were injected into blastocysts to generate chimeras, which were bred to establish *Dnmt3a* conditional mutants. Mice homozygous for conditional alleles of *Dnmt3a* (*Dnmt3a*$^{2lox/2lox}$) were healthy and fertile, indicating that the *Dnmt3a*^{2lox} allele is functional. We obtained mice bearing the recombined allele of *Dnmt3a*, *Dnmt3a*^{1lox} (see Figure 1A), in crosses with the nestin-Cre transgenic line Nes-Cre1, which is active in the male germline as well as in the nervous system (Chen et al., 2001; Dubois et al., 2006). Mice heterozygous for the *Dnmt3a*^{1lox} allele were intercrossed to generate homozygous mutant mice. Similar to

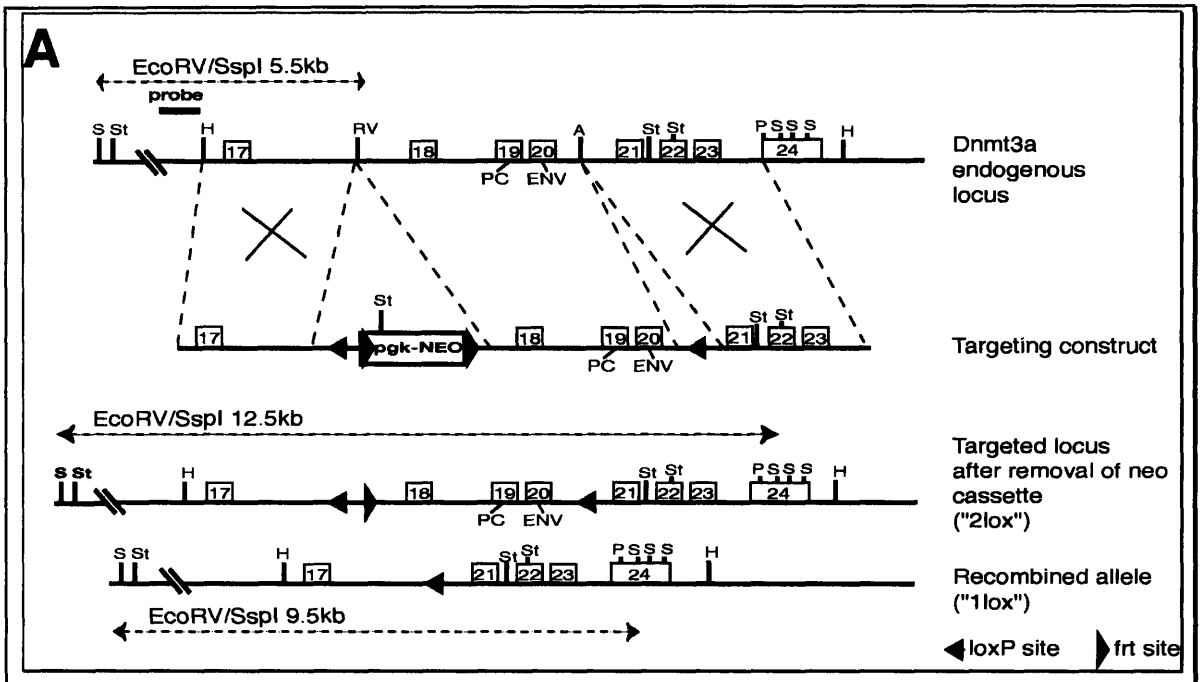

Figure 1. Conditional knockout of *Dnmt3a*. (**A**) Genomic locus, targeting construct, and modified alleles of *Dnmt3a*. Numbered boxes represent exons. The probe used for Southern blot analyses is indicated by a rectangular bar. Restriction sites: A, AseI; H, HindIII; P, PvuI; RV, EcoRV; S, SspI; St, StuI.

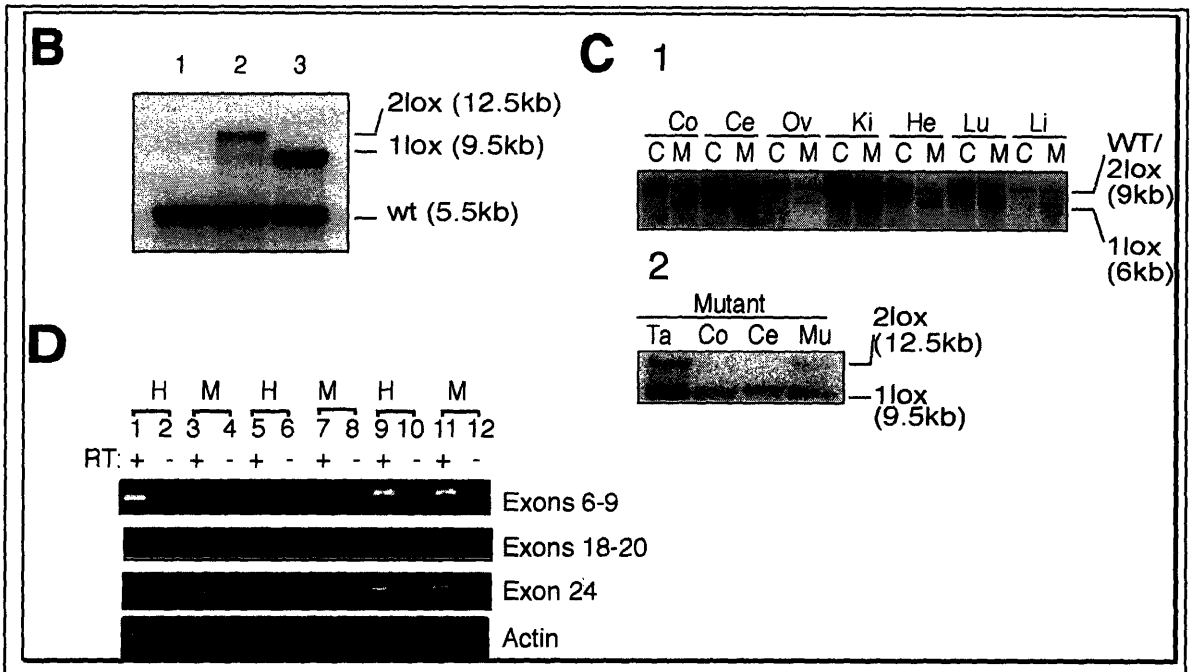

Figure 1 *continued.* Conditional knockout of *Dnmt3a*. **(B)** *Dnmt3a* Southern blot. Genomic DNA was digested by EcoRV and SspI. Lane 1: *Dnmt3a* +/+ (v6.5 ES cells used for targeting); lane 2: $Dnmt3a^{2lox/+}$ (targeted ES cell clone after removal of neo cassette by flp recombinase); lane 3: $Dnmt3a^{1lox/+}$ (targeted ES cell clone after transfection with Cre). **(C)** Southern blot analysis of recombination at *Dnmt3a* locus by Nes-Cre1 transgene. Blot 1: digestion by StuI. DNA from control ($Dnmt3a^{2lox/+}$, 'C') and mutant ($Dnmt3a^{2lox/1lox}$;Nes-Cre1, 'M') mice. Blot 2: digestion by EcoRV and SspI. DNA from mutant ($Dnmt3a^{2lox/1lox}$;Nes-Cre1) mice. Co, cerebral cortex; Ce, cerebellum; Ov, ovary; Ki, kidney; He, heart; Lu, lung; Li, liver; Ta, tail; Mu, muscle. **(D)** RT-PCR analysis of total cerebellum RNA from three pairs of heterozygous ('H') and mutant ('M') littermates. Primer pairs amplify *Dnmt3a* cDNA from regions before (exons 6-9), within (exons 18-20) and after (exon 24) the targeted deletion.

mice carrying a *Dnmt3a* deletion in the germ line (Okano et al., 1999) and consistent with the recombined $Dnmt3a^{1lox}$ allele representing a null mutation, $Dnmt3a^{1lox/1lox}$ mice were runted and most died within a few weeks of birth.

Ablation of Dnmt3a in the nervous system by the Nes-Cre1 transgene

In order to examine the role of Dnmt3a in the nervous system, we crossed *Dnmt3a* conditional knockout mice to the Nes-Cre1 transgenic line (Bates et al., 1999; Dubois et al., 2006; Trumpp et al., 1999), in which a modified Cre recombinase transgene is under the control of the rat nestin promoter and intron 2 enhancer. The Nes-Cre1 transgene has been shown to cause complete or near-complete deletion of various loci in the central nervous system by E15.5 (Dubois et al., 2006). Nes-Cre1 transgene-mediated recombination has been detected as early as E7.5 in some cells of the neuroectoderm and has also been observed outside of the central nervous system, possibly due to activity of the nestin promoter in an early multipotent stem or progenitor cell (Dubois et al., 2006).

To obtain *Dnmt3a*/Nes-Cre1 conditional knockout mice, we bred males bearing the Nes-Cre1 transgene and one copy of the $Dnmt3a^{2lox}$ allele to females homozygous for the $Dnmt3a^{2lox}$ allele. This breeding scheme was necessary because the Nes-Cre1 transgene is imprinted, and 95% or greater recombination in the brain is only observed when the Nes-Cre1 transgene is inherited paternally, whereas maternal transmission results in only 30% recombination (Fan et al., 2001). $Dnmt3a^{2lox/1lox}$;Nes-Cre1 (designated as "mutant") mice obtained from this breeding scheme inherit a recombined

(*Dnmt3a*^{1lox}) allele from the father because of Nes-Cre1 activity in the male germline (Chen et al., 2001; Dubois et al., 2006).

Because the extent of recombination in peripheral tissues varies depending on the locus being recombined (Dubois et al., 2006), it was important to determine the pattern of Nes-Cre-mediated recombination in conditional knockout mice by Southern blot analysis. Consistent with published data (Chen et al., 2001; Dubois et al., 2006; Trumpp et al., 1999), we observed near-complete recombination in the cortex, and complete recombination in the cerebellum (Figure 1C). Some recombination was observed in kidney and muscle, and little or none in other organs examined (ovary, lung, heart, and liver), as observed previously (Fan et al., 2001).

We investigated the expression of the mutant allele in the cerebellum, where Dnmt3a is highly expressed (Feng et al., 2005), by RT-PCR from mutant mice and their *Dnmt3a*$^{2lox/+}$;Nes-Cre1 (designated as "heterozygous") littermates. As shown in Figure 1D, we were unable to amplify a product from exons 18-20, which are expected to be deleted in mutants, from mutant samples. Transcripts containing exons 6-9 (upstream of the deletion) and exon 24 (downstream of the deletion) were detected, indicating the presence of truncated transcripts in which transcription proceeded past the excised region. Efforts to characterize protein products, if any, produced from the *Dnmt3a*^{1lox} allele failed due to the lack of specific antibodies.

To investigate whether global methylation was affected in mutant mice, we examined the methylation of IAP elements (Walsh, Chaillet et al. 1998). Genomic DNA from forebrain, cerebellum and heart samples from adult mice were digested with HpaII,

which recognizes 5'CCGG3' targets and is sensitive to methylation at the internal cytosine. Southern blot analysis revealed that IAP repeats remained methylated in mutant mice (Figure 2), suggesting that global methylation was unperturbed.

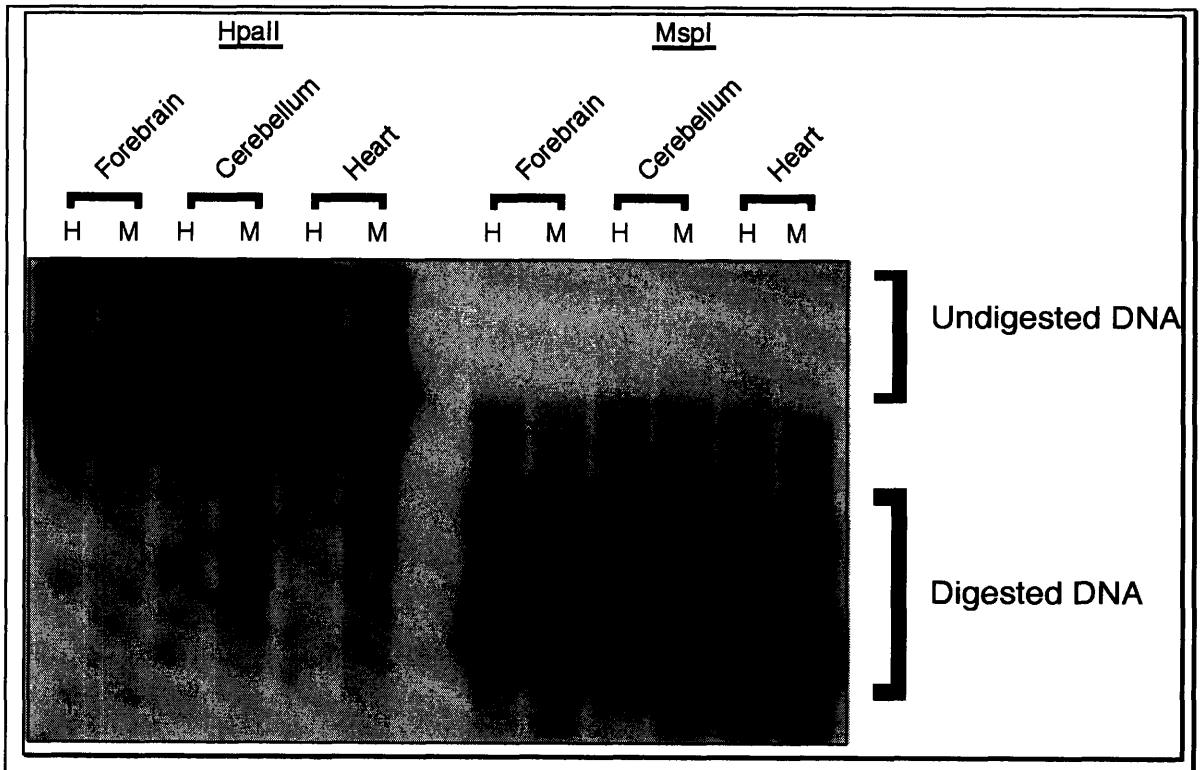

Figure 2. Global methylation analysis. Methylation at intracisternal A particle elements (Walsh et al, 1998). Southern blot of genomic DNA from heterozygous (H, *Dnmt3a*$^{2lox/+}$;Nes-Cre1) and mutant (M, *Dnmt3a*$^{2lox/1lox}$;Nes-Cre1) mice digested with HpaII, a methylation sensitive enzyme that cleaves at CCGGs. A set of samples was digested with the control enzyme MspI, a methylation-insensitive enzyme with the same recognition sequence as HpaII. Lower bands represent digested DNA, while upper bands represent undigested DNA. Blots were probed for intracisternal A particles.

Peripheral organs in *Dnmt3a*/Nes-Cre1 mutant mice appear normal

To assess the possible effect of recombination by the Nes-Cre1 transgene in peripheral tissues, we examined the tissues from symptomatic mutant mice. No gross structural abnormalities were observed in sections of kidney, liver, and skeletal muscle tissue stained with hematoxylin and eosin (Figure 3A). We did not observe signs of muscle degeneration, such as newly generated or missing patches of muscle fibers, in sections of muscle tissue.

We also determined blood serum levels of markers that are used as indicators of liver and kidney function. The serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), and total protein levels in mutant mice were not different than those of their $Dnmt3a^{2lox/+}$ littermates (designated as "controls") (Figure 3B). Blood urea nitrogen (BUN), altered levels of which can indicate various malfunctions including heart failure, low fluid intake, and kidney or liver damage, were also normal (Figure 3B). Creatinine levels, which can be altered in muscle degeneration, were normal in mutants, as was the BUN to creatinine ratio (B/C ratio) (Figure 3B).

Together, these data suggest that there were no major abnormalities in liver and kidney histology and function, and no overt signs of muscle degeneration in skeletal muscle.

Nes-Cre1-mediated deletion of *Dnmt3a* leads to decreased activity and shortened lifespan

Dnmt3a/Nes-Cre1 mutant mice were born healthy and in the expected frequency of 25% for the breeding scheme (95 mutants of 419 live pups; $p>0.5$, Chi-squared

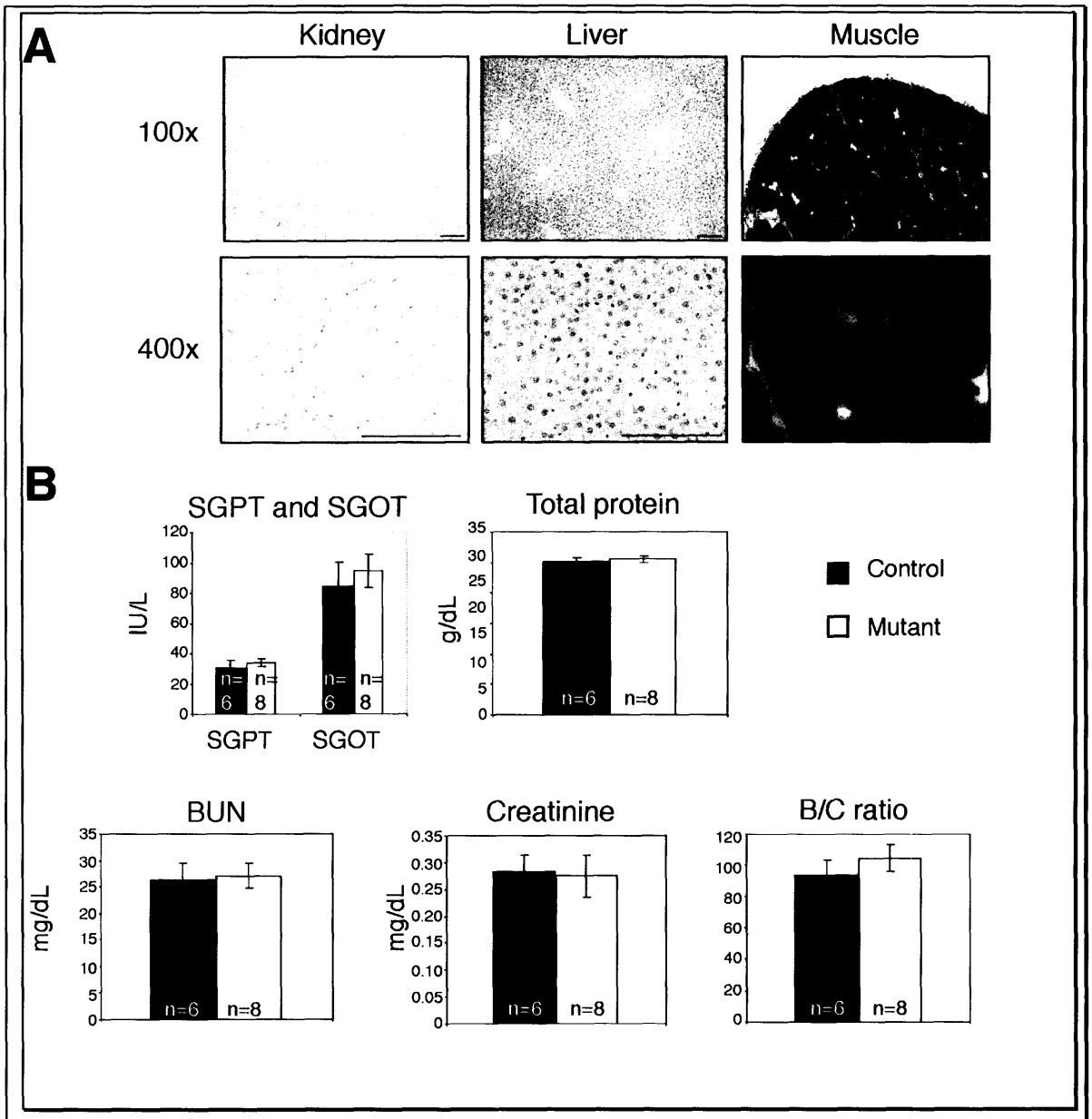

Figure 3. Peripheral organ histology and function in mutant mice. **(A)** Histological examination of tissues in mutant mice: 4um paraffin-embedded sections of kidney and liver and 30μm frozen section of quadriceps muscle stained with hematoxylin and eosin. Scale bars represent 100μm. **(B)** Blood serum levels of indicators of liver and kidney function and of muscle degeneration in mutant and wild type control mice. SGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamic oxaloacetic transaminase; BUN, blood urea nitrogen; B/C ratio, BUN to creatinine ratio. Error bars represent standard deviations of the mean.

analysis) suggesting that Nes-Cre1 mediated *Dnmt3a* deletion did not cause embryonic or perinatal lethality. Mutant males were underweight at 8 and 12 weeks (Figure 4A), whereas mutant females were of normal weights. At around two months of age, mutants became hyporesponsive, appeared to have altered hind limb posture, and exhibited an abnormal gait. Mutant mice had a median survival of approximately 18 weeks of age (Figure 4B).

To analyze the mutant defect in further detail, we performed a series of behavioral assays. Mice were placed in a laser cage apparatus, and baseline activity was quantitated by the number of laser beam interruptions per hour during the dark cycle. While there was no significant difference between the baseline activities of control and heterozygous mice, mutants exhibited a baseline activity that was only about 54% that of control levels (Figure 4C, $p<0.05$, single-factor ANOVA).

Because the mice were placed into a novel cage at the beginning of each experiment, activity during the first hour of the experiment could be used as a measure of exploratory behavior. Mice are usually more active during the first few hours in a novel environment, then become less active during an attenuation period until their activity rate reaches that of baseline. Both control and heterozygous mice exhibited greatly elevated activity during the first hour, while mutant mice were only about 40% as active as control mice (Figure 4D, $p<0.001$, single-factor ANOVA).

Voluntary running wheel activity was also measured. Though it appeared that the mutant animals ran on average less than their littermate counterparts (Figure 4E), this difference was not statistically significant, perhaps because there was a considerable degree of inter-animal variation in night time running wheel activity.

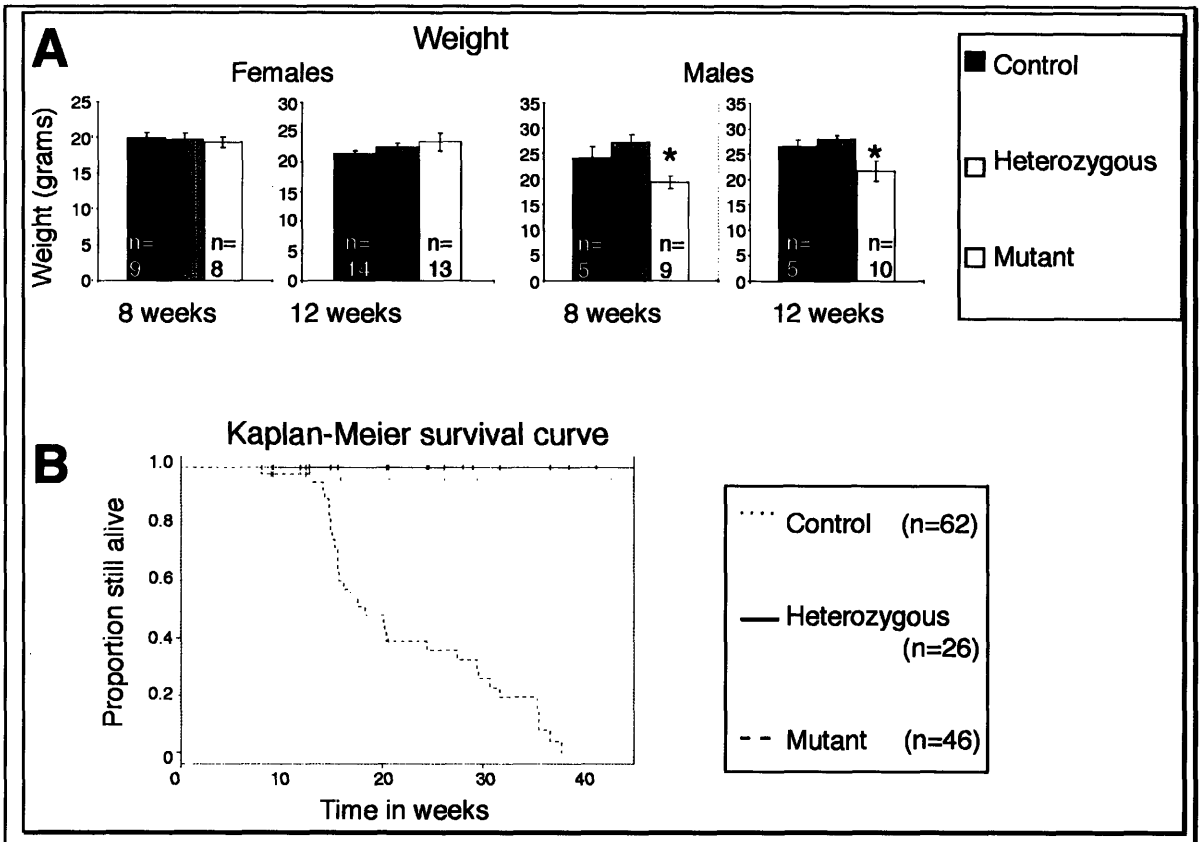

Figure 4. Weight, lifespan, and activity of *Dnmt3a*/Nes-Cre1 mice. (**A**) Weights of *Dnmt3a*/Nes-Cre1 mice at 8 and 12 weeks. (**B**) Kaplan-Meier survival curve for *Dnmt3a*/Nes-Cre1 mice. Upward ticks represent censored data. Error bars represent standard deviations of the mean. Asterisks indicate a significant difference.

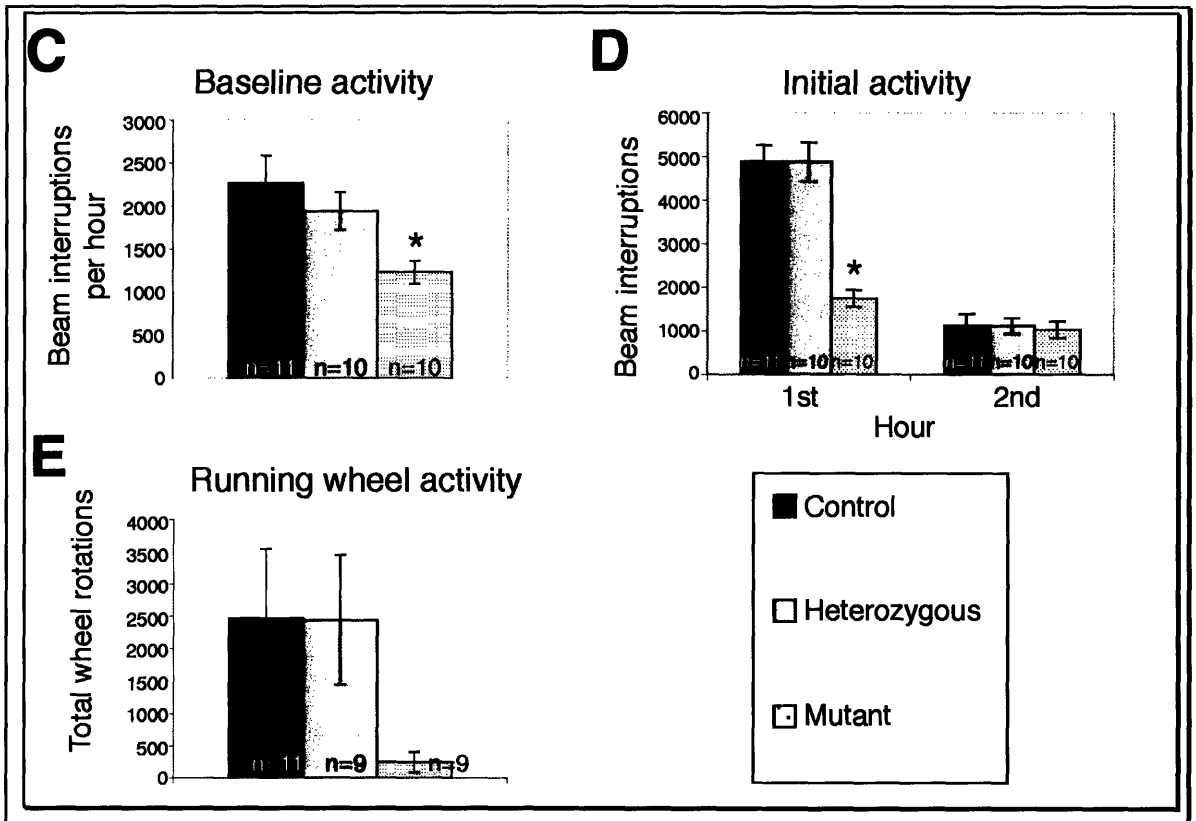

Figure 4 *continued.* Weight, lifespan, and activity of *Dnmt3a*/Nes-Cre1 mice. **(C-E)** Activity of 12-week old mice as measured either by a laser cage apparatus (C, D) or a running wheel setup (E). **(C)** Baseline (dark cycle) activity. **(D)** First and second hours of activity in a novel cage. **(E)** Voluntary running wheel activity. Error bars represent standard deviations of the mean. Asterisks indicate a significant difference.

Motor defects in behavioral tests of *Dnmt3a*/Nes-Cre1 mutant mice

Footprint analysis revealed a gait abnormality in *Dnmt3a*/Nes-Cre1 mutant mice (Figure 5A). At the age of twelve weeks, mutant mice had an approximately 40% smaller average step distance compared to control and heterozygous mice (Figure 5B, $p<0.001$, single-factor ANOVA) and had more variable gait widths than did the control and heterozygous mice (Figure 5C). Nevertheless, across all mouse genotypes, no significant differences were observed in the average gait width and linear movement (Figures 5C, 5E, $p>0.2$ for each of these parameters, single-factor ANOVA).

Grip strength, an indicator of neuromuscular function, was measured using the hanging wire test, in which mutant mice hang upside down from a cage top, holding onto the wires until they drop, or until the test is over. Mice typically used both fore limbs and hind limbs during the test. At both eight and twelve weeks of age, mutants hung on to the cage for about half as long as control and heterozygous mice did (Figure 6A, $p<0.01$ for both ages, single-factor ANOVA). The average latencies of heterozygous and control mice were not significantly different, and were close to or at the maximum cutoff (60 seconds) for the test.

We also tested the ability of mutant mice to tread on a rotating rod (rotarod), a task requiring motor coordination and balance. All mice were trained on the apparatus, then tested on two consecutive days on the rotarod. At eight weeks of age, mutant mice (Figure 6B) performed significantly worse on days 1 and 2 of testing, staying on the rods only about 40% as long as control and heterozygous mice did. This difference in performance widened by 12 weeks of age. There was no significant difference between

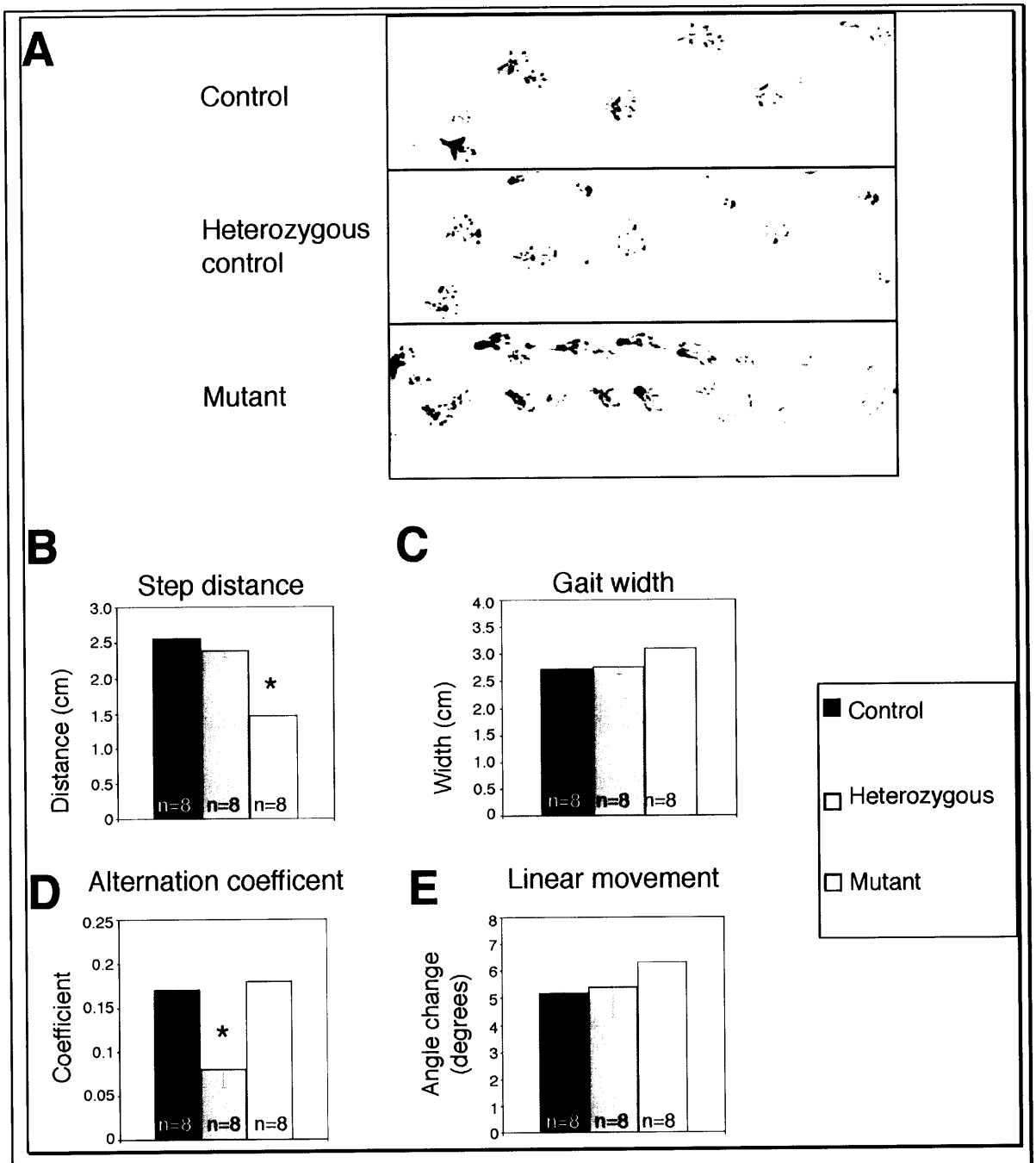

Figure 5. Footprint analysis of *Dnmt3a*/Nes-Cre1 mice. **(A)** Footprint patterns of 12 week-old mice. Front (red) and hind (green) paw prints are shown. Gait parameters – **(B)** average step length, **(C)** average gait width, **(D)** alternation coefficient, **(E)** and linear movement - measured from hind paw prints of 12 week old mice. Error bars represent standard deviations of the mean. The asterisk indicates a significant difference.

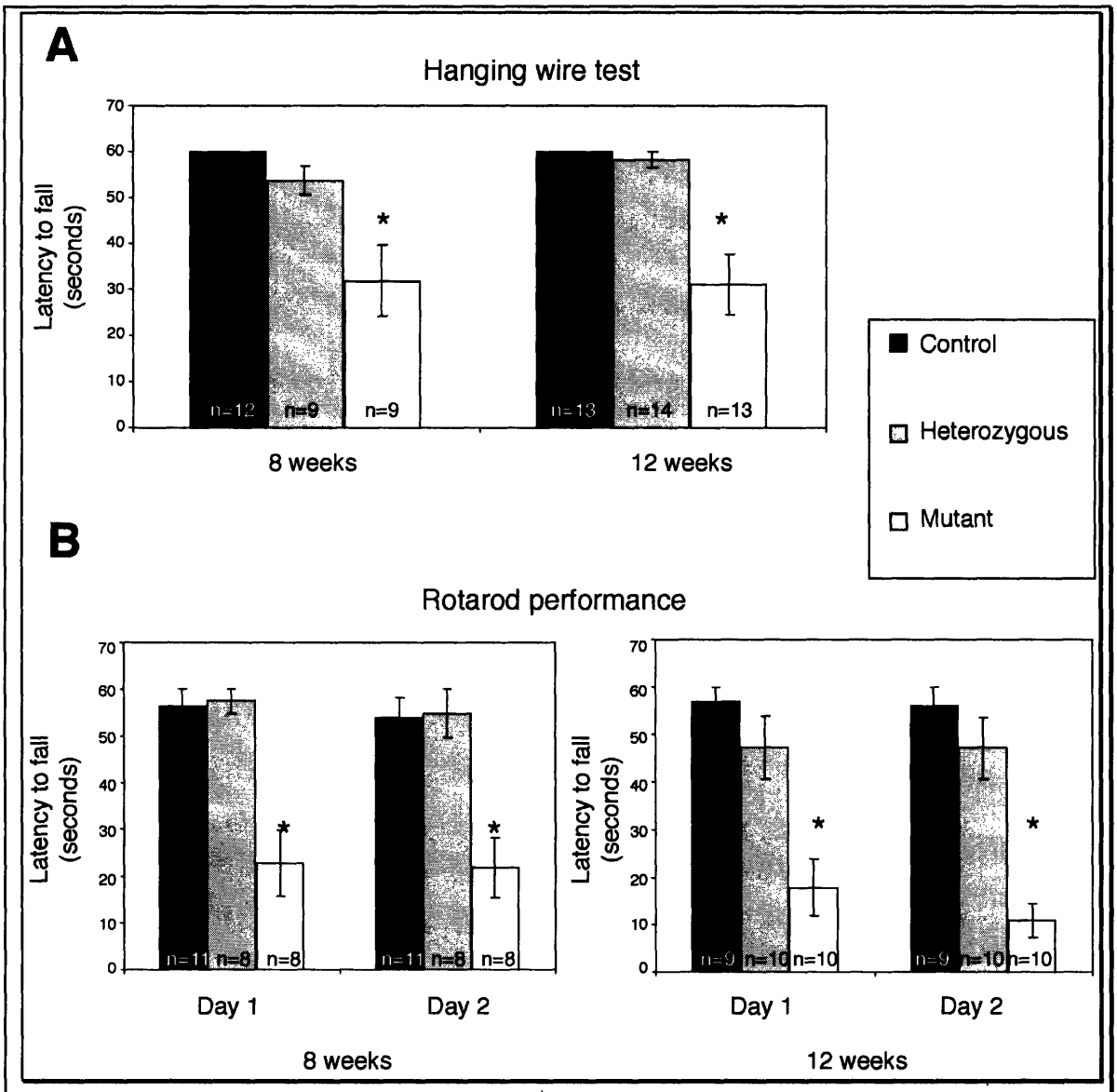

Figure 6. Muscle grip strength and motor abilities of *Dnmt3a*/Nes-Cre1 mice. Error bars represent standard deviations of the mean. Asterisks indicate a significant difference. **(A)** Latencies to fall in the hanging wire test at 8 and 12 weeks of age. **(B)** Latencies to fall in the rotarod test at 8 and 12 weeks of age.

the performances of control and heterozygous mice, both of which scored close to the maximum latency for the test at both ages (Figure 6B). Mutant mice at both ages showed no improvement from day 1 to day 2 of the trials.

Normal gross anatomy of the central nervous system

To investigate the possible causes of the behavioral defects, we examined the anatomy of the central nervous system. Coronal sections of forebrain and midbrain regions and sagittal sections of cerebellum were stained with Luxol Fast Blue and Cresyl Violet to visualize patterns of myelin and the gross distribution, size, and appearance of neuronal cell bodies respectively. Major structural abnormalities were not observed in any of the regions examined, including motor cortex, cerebellum, hippocampus, olfactory bulb, striatum and thalamic nuclei of mutant mice (Figure 7 and data not shown). Thus, gross anatomical alterations in the central nervous system cannot explain the behavior defects in mutant mice.

Decreased numbers of hypoglossal motor neurons

Because the gait abnormality and the underperformance on the hanging wire and rotarod tests suggested a motor defect, we examined motor neurons in 3-4 month old symptomatic mutant mice. Hypoglossal motor neurons located in the brainstem innervate the tongue and are easily identifiable in mice. Also, the small size of the entire hypoglossal nucleus facilitates quantification of total neuron numbers. We stained cross sections of brainstem with Cresyl Violet and identified neurons in the hypoglossal

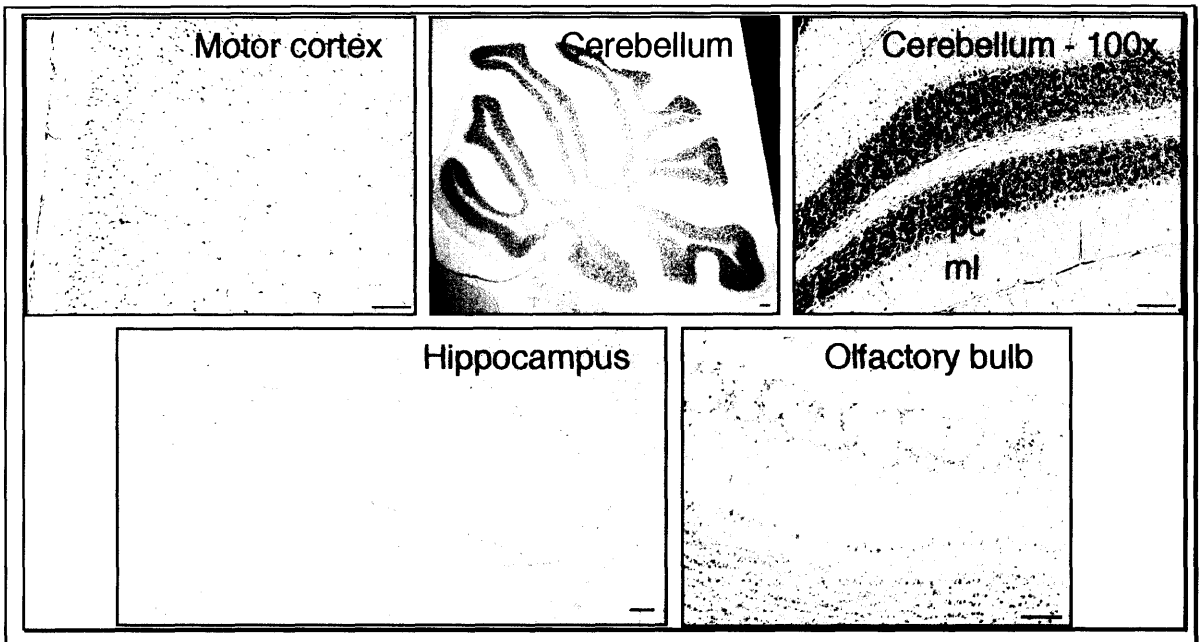

Figure 7. Neuroanatomy of mutant mice. 15μm paraffin-embedded sections of primary motor cortex, cerebellum, hippocampus, and olfactory bulb from 2 month-old symptomatic mice. Gr, granule cell layer; pc, Purkinje cell layer; ml, molecular layer. Sections were stained with Cresyl Violet and Luxol Fast Blue and cut through the coronal plane, except for the cerebellum sections, which were cut sagittally. Scale bars indicate 100μm.

nucleus in both mutant and control samples (Figure 8A). Though the estimates of total motor neuron counts varied greatly from mouse to mouse, mutant mice had on average fewer hypoglossal neurons than that of littermate controls (Figure 8B).

We also examined motor neurons in the spinal cord. In cross sections from areas innervating the limbs (cervical and lumbar regions) stained with Cresyl Violet, we identified normal-looking motor neurons in both mutant and control samples (Figure 8C).

Our results indicate that while motor neuron pools are not depleted in the spinal cord and hypoglossal nucleus, there are fewer hypoglossal motor neurons in symptomatic mutant mice.

Fragmentation of endplates at neuromuscular junctions in the diaphragm

We also analyzed neuromuscular junctions at synaptic sites in diaphragm muscles because of the high density of endplates in these muscles, and because of the relative ease of staining thin diaphragm muscles as whole mounts. Endplates were visualized using fluorescent-conjugated α-bungarotoxin (α-BTX), which binds to postsynaptic acetylcholine receptors (AChRs). We scored endplates for their integrity based on the continuity of the postsynaptic AChR regions, classifying them as either "fragmented" or "intact" (Figures 9). Though fragmented endplates were found in both littermate control and mutant samples, a higher percentage of the endplates in samples from mutant mice were fragmented (Table 1). On average, 50% of the endplates from mutant mice were fragmented, while only 18% of those from control mice were fragmented.

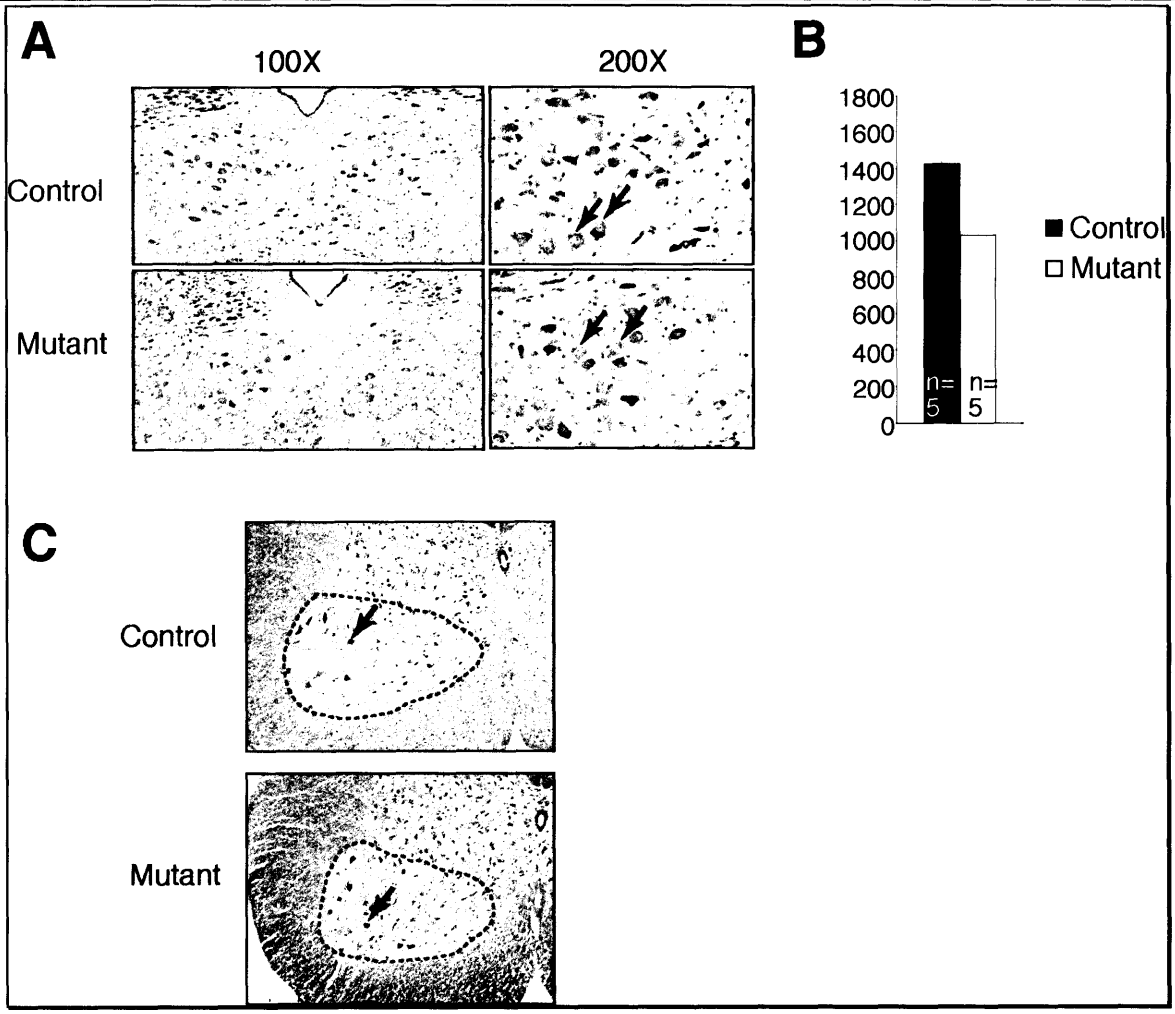

Figure 8. Motor neurons in control (*Dnmt3a*$^{2lox/+}$) and mutant (*Dnmt3a*$^{2lox/1lox}$;NesCre1) mice. **(A)** Coronal sections through the hypoglossal nucleus stained with Cresyl Violet. Arrows indicate some of the motor neurons meeting the criteria for counting. (See Materials and Methods.) **(B)** Estimates of total numbers of motor neurons in hypoglossal nuclei. **(C)** Cross sections of lumbar spinal cord tissue, stained with Cresyl Violet and Luxol Fast Blue. Ventral horns are encircled by dashed lines. Arrows indicate some motor neurons.

We also assessed how well the endplates were innervated by axon terminals. Axons and terminals were visualized by staining with antibodies against neurofilament and synaptophysin. Endplates were scored as either "fully innervated", "partially innervated", or "completely denervated" (Figure 9). In both control and mutant samples, many fragmented endplates were nevertheless well-innervated. There was no striking difference in the extent of innervation between control and mutant samples, with the exception of one mutant sample (Table 1), in which over half of the neuromuscular junctions that were scored were only partially innervated, and 7% were completely denervated.

In summary, in the absence of Dnmt3a, endplates at neuromuscular junctions in diaphragm muscle were less stable and more susceptible to disassembly, but innervation was not significantly perturbed.

DISCUSSION

Our data demonstrate a functional role for the *de novo* DNA methyltransferase Dnmt3a in the neuromuscular system. A role for Dnmt3a in the nervous system was predicted by its expression in the central nervous system, which begins in embryonic neuronal precursor cells and is sustained in postnatal CNS neurons (Feng et al., 2005). Though born normal, *Dnmt3a*/Nes-Cre1 mutant mice exhibit motor defects and die prematurely. Absence of Dnmt3a also leads to a decrease in the hypoglossal motor neuron pool and to fragmented neuromuscular junction endplates in diaphragm muscle.

Because the gene deletion resulted in a truncated RNA product, it seemed possible that a truncated protein product is generated and behaves as a dominant negative.

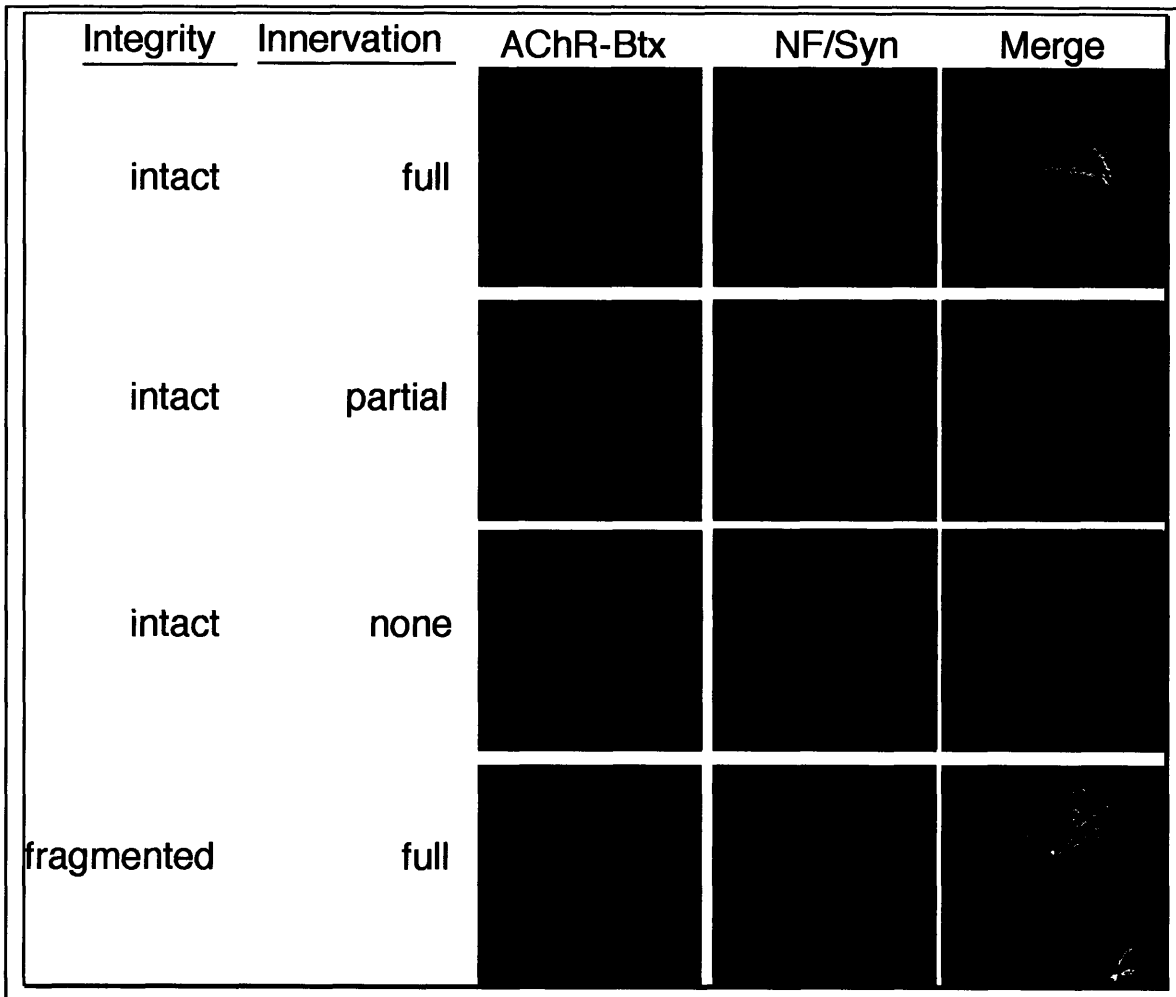

Figure 9. Examples of neuromuscular junction synapses imaged and scored for endplate integrity (either "intact" or "fragmented") and innervation ("full," "partial," or "none). Merged z-stack confocal images of synapses in diaphragms of mutant mice, stained with FITC-conjugated antibodies against neurofilament and synaptophysin (green), and Texas-Red-conjugated bungarotoxin for acetylcholine receptors (red). See Table 1 for quantification of synapses.

TABLE

	# of NMJs	Endplate integrity: intact	frag.	Innervation: full	partial	none
Controls						
1.	103	89 (86%)	14 (14%)	102 (99%)	1 (1%)	0 (0%)
2.	102	74 (73%)	28 (27%)	100 (98%)	2 (2%)	0 (0%)
3.	113	91 (81%)	22 (19%)	112 (99%)	1 (1%)	0 (0%)
4.	107	94 (88%)	13 (12%)	107 (100%)	0 (0%)	0 (0%)
Total	**425**					
Average		**82%**	**18%**	**99%**	**1%**	**0%**
Mutants						
1.	102	78 (76%)	24 (24%)	37 (36%)	58 (57%)	7 (7%)
2.	104	29 (28%)	75 (72%)	102 (98%)	2 (2%)	0 (0%)
3.	102	50 (49%)	52 (51%)	91 (89%)	9 (9%)	2 (2%)
4.	109	52 (58%)	57 (52%)	108 (99%)	1 (1%)	0 (0%)
Total	**417**					
Average		**50%**	**50%**	**80%**	**17%**	**2%**

Table 1. Quantification of neuromuscular junctions (NMJs) from diaphragm muscle stained with α-bungarotoxin and antibodies against neurofilament and synaptophysin. Endplates were scored as intact (comprised of 3 or fewer discrete AChR-positive regions) or fragmented (comprised of 4 or more discrete AChR-positive regions). Endplates were also categorized as fully, partially, or not at all occupied, depending on the extent of innervation by axon terminals. Diaphragms were dissected from littermate pairs of control (*Dnmt3a* $^{2lox/+}$) and mutant (*Dnmt3a*$^{2lox/1lox}$;Nes-Cre1) mice.

Two observations argue against this possibility. First, heterozygous (*Dnmt3a*$^{2lox/+}$; Nes-Cre1) mice have a normal lifespan (Figure 4B) and do not exhibit abnormalities in any of the behavioral tests we used (Figures 4-6), in contrast to mutant (*Dnmt3a*$^{2lox/1lox}$; Nes-Cre1) mice. Second, the phenotype of *Dnmt3a*$^{1lox/1lox}$ mice is similar to that of the Dnmt3a-null mice reported by Okano and colleagues (1999).

Although our results suggest a neuromuscular defect in *Dnmt3a*/Nes-Cre1 mutant mice, it is unclear which cells and tissues are affected by loss of Dnmt3a. In the nervous system, Dnmt3a is expressed largely in neurons, though some glial cells also express the enzyme (Feng et al., 2005). Expression of Dnmt3a in the brainstem and spinal cord is uncharacterized, but the enzyme is expressed in skeletal muscle (Chen et al., 2002; Okano et al., 1999). In the cerebellum, Dnmt3a protein is expressed consistently in cerebellar granule cells and in a fraction of Purkinje cells in adult mice (Feng et al., 2005). The cerebellum is involved in fine-tuning motor coordination and balance, and mice with cerebellar defects often exhibit gait abnormalities and problems on the rotarod test. Given the gait abnormality and motor defects in *Dnmt3a*/Nes-Cre1 knockout mice, it is possible that cerebellar dysfunction contributes to the mutant phenotype. Nevertheless, no gross anatomical changes were evident in the cerebellum, and further analysis of the cerebellum is needed to determine its contribution to the motor defect.

Recombination by Nes-Cre1 has been observed in all regions of the brain, including the cerebellum and the motor cortex, as well as in peripheral nerves (Dubois et al., 2006). In the central nervous system, the Nes-Cre1 transgene is active in both glia and neurons, leading to deletion of *Dnmt3a* in at least 95% of all cells. Lower levels of

recombination are seen in skeletal muscle tissue. We do not know to what extent the recombination in muscle contributes to the neuromuscular defect.

We also observed some recombination in the kidney, where Dnmt3a transcripts have been detected (Chen et al., 2002; Okano et al., 1998). Though we cannot rule out the possibility of a peripheral deficit contributing to the phenotype, we did not observe gross histological changes in the kidney. Also, blood serum tests did not reveal any signs of kidney dysfunction (Figure 3).

The impaired survival and motor defects in *Dnmt3a*/Nes-Cre1 mutant mice are reminiscent of those seen in SOD1 (superoxide dismutase) transgenics, a model for amyotrophic lateral sclerosis (Gurney, 1994; Gurney et al., 1994; Lalonde et al., 2005; Weydt et al., 2003). SOD1 transgenic mice develop motor neuron disease and lose innervation of muscle fibers (Gurney et al., 1994), though these symptoms are more severe than those seen in *Dnmt3a*/Nes-Cre1 mutant mice. Nevertheless, *Dnmt3a*/Nes-Cre1 conditional knockout mice die sooner than SOD1 G93A transgenic mice do (Gurney et al., 1994), suggesting that disruptions to the motor pathway alone may not account for the decreased survival of *Dnmt3a*/Nes-Cre1 mutants.

In conclusion, the *Dnmt3a*/Nes-Cre1 conditional knockout mice show that loss of Dnmt3a leads to a neuromuscular defect. Mutant mice have deteriorating motor function coupled with fragmented endplates in neuromuscular junction synapses. Motor neuron loss was also observed in the hypoglossal nucleus. Future work will be directed towards defining the molecular targets of Dnmt3a and their role in the neuromuscular system.

MATERIALS and METHODS

Construction of the Dnmt3a targeting construct

An 11kb HindIII genomic DNA fragment containing the catalytic domain and surrounding regions of the Dnmt3a locus was isolated by hybridizing a Dnmt3a cDNA probe to a 129/SvEvTac female mouse bacterial artificial chromosome library (Roswell Park Cancer Institute). The fragment was then subcloned into the pUC18 vector (Invitrogen), generating plasmid pUC183a.

To generate the Dnmt3a conditional knockout construct, a 4kb PvuI/AseI genomic fragment containing Dnmt3a exons 21-23 was inserted into a plasmid with a pBluescript II SK (Stratagene) backbone containing a loxP site. A 5.5kb HindIII/AseI genomic fragment containing the catalytic domain and an upstream region (exons 17-20) was then inserted on the other side of the loxP site. Separately, a pBluescript II SK-based plasmid was generated such that it contained a loxP site next to a neomycin resistance cassette under the control of the phosphoglycerate kinase 1 gene promoter (pgk-neo cassette, (Soriano et al., 1991) with the pgk-neo cassette being flanked by frt sites (Possemato et al., 2002).

The loxP/frt/pgk-neo/frt cassette was introduced into the Eco RV site in the 5.5kb Dnmt3a genomic arm of the Dnmt3a conditional construct, resulting in a final construct (pBS3alfN) with lox sites flanking the catalytic domain (exons 18-20) and genomic arms on either side.

Generation of Dnmt3a conditional knockout mice

pBS3alFN was linearized by NotI and introduced into F1 mouse embryonic stem cells (v6.5 line, (Rideout et al., 2000) by electroporation. Neomycin-resistant ES cell colonies were picked and screened by Southern blot analysis (Figure 1B). Approximately 5% of the ES cell colonies that were picked had properly integrated the conditional knockout allele of Dnmt3a.

To remove the neomycin resistance cassette, flp recombinase was introduced by transient lipofection using Cytofectene (Biorad) into a targeted ES cell clone. A subclone in which the cassette had been removed (as verified by PCR and death in G418) was expanded, and the ES cells were injected into blastocysts to make chimeras.

Mice

Mice were maintained in an AALAC-certified animal facility with a 12-hour light/12-hour dark cycle (7am and 7pm light on/off times). All procedures for use of animals were approved by the MIT Committee on Animal Care. The Nes-Cre1 transgenic mouse line was obtained from the laboratory of Andreas Trumpp and has been previously described (Dubois et al., 2006; Trumpp et al., 1999). The mutant mice used for study were of a mixed C57Bl/6 and 129 background due to the backgrounds of the ES cell lines and of Cre-transgenic mice used.

Southern blot analysis

Southern blot analysis was performed as previously described (Sambrook et al., 1989). To examine the *Dnmt3a* locus, 10-20μg genomic DNA was doubly digested with

EcoRV/SspI and blots were probed with probe '3ag10/11' (see Figure 1A), which recognizes an intronic region 5' to exon 17. *Dnmt3a* wildtype, *Dnmt3a*^{2lox}, and *Dnmt3a*^{1lox} alleles were distinguishable as 5.5kb, 12.5kb, and 9.5kb bands respectively.

Alternatively, genomic DNA was digested with StuI and probed with probe '3ag10/11.' With the StuI digest, the *Dnmt3a* wildtype and *Dnmt3a*^{2lox} alleles were both represented as 9kb bands, while the *Dnmt3a*^{1lox} allele was represented as a 6kb band.

To examine global methylation at IAP elements, 5μg genomic DNA was digested with HpaII, a methyl-sensitive restriction enzyme. As controls for digestion, another set of samples was digested with MspI, a methyl-insensitive restriction enzyme which recognizes the same target sequence as HpaII. The blots were probed with an 700bp probe that recognizes the LTR of an IAP element (Walsh et al., 1998).

Genotyping

Genotyping for the *Dnmt3a* locus was accomplished either by Southern blot as described above or by PCR with the following primers:

3a2LOXfor: 5'-CCT CTG GGG ATT AAA CTC TTG GCC AGC CC-3'

3a1LOXfor: 5'-CCT GAG AGA CTG GCC GGC TTT TCC TCA GAC-3'

3aCommon: 5'-CCT GTG TGC AGC AGA CAC TTC TTT GGC GTC-3'

Primers 3a2LOXfor and 3aCommon were used together to amplify products representing the *Dnmt3a* wildtype (~300 bp) or *Dnmt3a*^{2lox} alleles (~450 bp). Thirty cycles of amplification were carried out with a Perkin Elmer thermocycler using the

following conditions: denaturation at 95°C, 30s; annealing at 60°C, 30s; and extension at 72°C, 30s.

Recombination of a *Dnmt3a*^{2lox} allele to a *Dnmt3a*^{1lox} allele brings genomic regions together such that it is possible to amplify a ~500bp product using primers 3a1LOXfor and 3aCommon. When no *Dnmt3a*^{1lox} allele is present, no band is amplified. Thirty rounds of amplification were carried out using the following conditions: denaturation at 95°C, 30s; annealing at 68°C, 30s ; and extension at 72°C, 20s.

To detect the presence of a Cre transgene, primers

CreF: 5'TGG GCG GCA TGG TGC AAG TT3'

CreR: 5'CGG TGC TAA CCA GCG TTT TC3'

were used in amplification reactions with the following conditions: 30 cycles of denaturation at 95°C, 30s; annealing at 60°C, 30s; and extension at 72°C, 30s. In the presence of a Cre transgene, a ~500bp product is amplified.

RT-PCR analysis

RNA was extracted from freshly dissected tissues using Tri reagent (Molecular Research Center). Reverse transcription reactions were carried out using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) according to the kit protocol. Approximately 100ng total RNA from cerebellum tissue was used as template, and oligo dT primer was used for first strand synthesis. For amplification reactions, three separate pairs of primers with the following sequences were used:

3a1293sense: 5'-ACT GCG AGG TGG CTT GGG CTG GGA GTC CAG-3'

3a1800αsense:5'-CAC TGA GAA CTT GCC ATC TCC GAA CCA CAT G-3'

3a2800sense: 5'-GTG GGC ATG GTG CGG CAC CAG GGA AAG ATC-3'

3acat2αsense: 5'-GGC CAC CAC ATT CTC AAA GAG CCA GAA GAA GGG GCG-3'

3ag8: 5'-CGAGAGGACGGAGAAAAGTTCAGCACCC-3'

3ag9: 5'-CGGGGGGTGGGAGAGGAGACTCTGCCTCC-3'

To amplify DNA from a region 5' to the catalytic domain of Dnmt3a, primers 3a1293sense (which recognizes exon 6) and 3a1800αsense (which recognizes exon 9) were used. To amplify DNA from the catalytic domain, primers 3a2800sense (which recognizes exon 18) and 3acat2αsense (which recognizes exon 20) were used. To amplify DNA from a region 3' to the catalytic region, primers 3ag8 and 3ag9 (both of which recognize exon 24) were used.

Survival analysis

Kaplan-Meier survival analysis was accomplished using the survival package of the R project software for statistical biocomputing (http://www.r-project.org/). "Dead" mice included mice that were found dead as well as mice judged by experienced animal care technicians to be moribund. Moribund mice were expected to have died within a few days, and the dates used in survival analysis were the dates they were sacrificed. Censored data (indicated by upward tick marks in Figure 4B) represent mice whose outcomes were unknown at the time of the survival analysis. These included mice that were still alive at the time of analysis, as well as mice that were sacrificed for

experiments. (If data for a mouse is censored, it is removed from both the numerator and the denominator when calculating the survival ratio for that day.)

Blood serum chemistry tests

Blood was collected periorbitally from 10-15 week old symptomatic mutanat mice and their age-matched littermate controls. Serum was isolated and samples were analyzed by the Division of Comparative Medicine Pathology Laboratory at the Massachusetts Institute of Technology.

Behavioral analyses

For all tests, 12-week old female and male mice were used for behavioral tests, and their data were grouped and analyzed together. For the hanging wire and rotarod tests, 8-week old female and male mice were also tested. Control (*Dnmt3a*$^{2lox/+}$), heterozygous (*Dnmt3a*$^{2lox/+}$;Nes-Cre1), and mutant (*Dnmt3a*$^{2lox/1lox}$;Nes-Cre1) were compared and littermates were used.

Baseline activity and exploratory behavior

Mice were housed in standard Nalgene mouse caging, situated within a laser-cage array of the Opto-Varimex Mini (Columbus Instruments) activity counter. The activity counter was connected to a computer for automatic data collection. The software (Mini-Counter, Columbus Instruments) recorded activity and time information in five-minute intervals throughout the recording period. For each testing period, mice were introduced into a new cage at 3pm, at which time recording began. Recording continued until 7am

the next day. Baseline activity was calculated as the average number of beam interruptions per hour during the dark cycle (7pm-7am).

Running wheel activity

The data collection parameters and experimental paradigm for collecting running wheel activity data were as described above for baseline and exploratory activity, except for the cage setup. Mice were placed in a running-wheel cage setup (Nalgene) connected to a magnetic switch and counting system (Mini Mitter), connected to the automatic data collection system. Running wheel activity was defined as the total number of wheel rotations during the dark cycle.

Footprinting analysis

Mice were painted on their paws with nontoxic, removable paint, with green paint on the hind paws and red paint on the front paws. They were allowed to walk into a dark tunnel made of black acrylic with inside dimensions 7.5cm wide x 4.5cm height x 40cm long. Their footprints were captured on Whatman filter paper lining the bottom of the tunnel. Hind paw prints were used to calculate average step length, gait width, alternation coefficient, and linear movement values as described by Clark et al (1997) and as explained below.

Average step length is the average forward distance through the tunnel taken with each step. It is calculated by taking the distance travelled through the tunnel divided by the number of steps (counting each right and left step as separate steps) taken to cover that distance.

The *gait width* describes how wide the mouse's stance is as it walks. For each step, the gait width was measured as the perpendicular distance between one paw print and the next paw print. The average was taken for all measurable steps.

The *alternation coefficient* corresponds to how evenly the mouse alternates left and right feet as it walks. It is a value ranging from 0 to 0.5, with 0 corresponding to a perfectly alternating step and 0.5 corresponding to a "shuffle gait." In a shuffle gait, the mouse moves one foot forward and then moves the other foot only as far as the other foot had moved. The alternation coefficient is computed as the average of the absolute values of $(0.5\text{-}d_{lr}/d_{ll})$ for every left-right pair of steps, where d_{lr} is the left-right step distance and d_{ll} is the left-left step distance.

Hanging wire test

For the hanging wire test, a 15cm x 22cm section of a wire cage lid was sealed off using masking tape. The wires of the cage were about 1.5mm in diameter and spaced approximately 0.75cm apart. Eight or 12-week old mice were placed on top of the cage lid within the region confined by the tape. The lid was shaken gently three times, inverted, and held approximately 70cm above a mouse cage. The latency until fall was measured, with a maximum cutoff of sixty seconds. If latency during a first trial was less than ten seconds, the mouse was given a second trial, and its score was the longer latency of the two trials.

Rotarod test

Eight or 12-week old naive mice were trained on the rotarod apparatus (Ugo Basile/Stoelting) during two five-minute sessions on separate days. A day of rest was given between training periods and before the first test. After training, mice were tested

on two consecutive days. The rod rotated at a constant speed of 7.6 rpm during all training and trial sessions. The latency to fall was recorded, with a maximum cutoff of 60 seconds. The mouse was given a second trial if it fell within 10 seconds of being placed on the apparatus. In such a case, its score was the longer of the two latencies.

Statistical analyses

For comparing data from three groups (control, heterozygous, and mutant), data were analyzed using a single-factor analysis of variance (ANOVA) except in cases where the data were not normally distributed or in cases where the distribution could not be determined because of the small sample size ($n<8$). In those cases, the non-parametric Mann-Whitney U test was used in a pair-wise fashion. The cutoff for significance was defined as $p<0.05$. If a significant difference was found by ANOVA, groups were compared one pair at a time to determine which group was significantly (again defined as $p<0.05$) different than the others. For comparing data between two groups, a Student t-test was used on normally distributed data. The Mann-Whitney U test was used for data whose distribution was not normal or was indeterminate because of a small n ($n<8$).

Histology

Mice were perfused transcardially with PBS and then with 4% paraformaldehyde/PBS (Electron Microscopy Sciences) and their organs dissected and fixed in 10% formalin overnight. For all tissues except muscle, samples were processed in a Tissue-Tek VIP machine (Miles Scientific) and sections were cut using a microtome

(Leica) at 4μm thickness except for those cut for hypoglossal neuron analysis, which were cut into 15μm sections.

Deparaffinized brain sections were stained with Cresyl Violet and Luxol Fast Blue. Deparaffinized sections from the brain stem (used for hypoglossal neuron counting) were stained with Cresyl Violet alone. Deparaffinized liver and kidney sections were stained with hematoxylin and eosin.

For muscle tissue, the quadriceps muscle was dissected and then flash frozen in a liquid-nitrogen-cooled isopentane. 30μm sections were cut immediately using a cryostat (Leica CM 3050S), and then stained with hematoxylin and eosin.

Deparaffinization was carried out by incubation in xylene (3 times, 5 minutes each), 100% ethanol (2 times, 5 minutes each), 95% ethanol (2 times, 5 minutes each) and finally dH_20 (dipping for 10-15 seconds). Staining was accomplished using the following protocols. After staining, slides were cleared in xylene (3 times, 5 minutes each) and then mounted with a xylene-based mounting medium (Cytoseal-XYL, Richard-Allan Scientific).

Luxol fast blue staining

(If done, carried out before Cresyl Violet staining)

1. 95% ethanol, 5 minutes
2. Luxol fast blue solution, overnight at 37°C
3. 95% ethanol, 5 minutes
4. Distilled water rinse
5. 0.05% lithium carbonate, 10 seconds
6. 70% ethanol, 60 seconds

7. Distilled water rinse
8. (The differentiation steps 5-7 above are repeated until the nuclei are colorless and the background is very pale.)

Cresyl Violet Staining

(Where there is a time range specified, the same incubation time was used for sets of sections. For example, tissue sections from a mutant and its littermate control(s) were always stained together for the same period of time.)

1. 70% 1 hour to overnight
2. 95% ethanol, 3 minutes
3. 100% ethanol, 3 minutes
4. 95% ethanol, 3 minutes
5. 70% ethanol, 3 minutes
6. 50% ethanol, 3-5 dips
7. Cresyl Violet solution, 50-70 seconds
8. Distilled water, 3-5 dips
9. 70% ethanol, 5 minutes
10. 95% ethanol, 3 minutes
11. 95% ethanol/HAC, 5 minutes
12. 100% ethanol, 2 times, 5 minutes each

Hematoxylin and eosin staining

1. Freshly filtered Harris hematoxylin, 3 minutes
2. Tap water wash, 3 times, 1 minute each

3. Acid alcohol, 5 quick dips
4. Tap water wash, 3 times, 1 minute each
5. Ammonia water, brief dip (until color turns bright blue)
6. Tap water wash, 3 times, 1 minute each
7. 80% ethanol, 1-2 minutes
8. Eosin Y Solution, 1-2 minutes
9. 95% ethanol, 2 times, 2 minutes each
10. 100% ethanol, 2 times, 2 minutes each

Staining and other solutions

Luxol fast blue solution:

1 g Luxol fast blue MBSN (Sigma-Aldrich)

1 L 95% ethanol

5 mL 10% acetic acid

Cresyl violet solution:

4.15 g Cresyl violet

500 mL dH_2O

50 mL 1M sodium acetate

285 mL 1M acetic acid

(pH to 3.5 and vacuum filter before use.)

Hematoxylin solution and Eosin Y Alcohol working solution

Prepared commercially by Poly Scientific

Acid alcohol

1mL of concentrated HCl

99mL 70% Ethanol

Ammonia water

4mL 28% ammonium hydroxide

1000mL distilled water

Hypoglossal motor neuron counting

Symptomatic 3-4 month old mice were perfused and their brainstrems dissected and fixed in 4% paraformaldehyde. After processing and embedding in paraffin, brainstems were serially sectioned in the coronal plane through the entire hypoglossal nucleus. 15um sections were mounted onto glass slides and stained by Cresyl Violet, and every fifth section was imaged on an Olympus BH-2 microscope with an Olympus Plan 20x objective (numerical aperture=0.40) and using Pixelink software.

Counting was accomplished using the counting tool in Pixelink. Criteria for counting were: location within the hypoglossal nucleus (identified using the fourth ventricle as a landmark), positive staining with Cresyl Violet, clearly identifiable cell body, and clearly visible nucleoli. The raw count was multiplied by five for an estimate of the total number of hypoglossal neurons in the sample. Counting was conducted blind to the genotype of the mouse from which the sample was collected.

Staining of neuromuscular junctions

Symptomatic 3-4 month old mice were perfused transcardially with PBS and then with 4% paraformaldehyde/ PBS. Whole diaphragms were dissected and post-fixed in 4% paraformaldehyde for 30 minutes. The tissues were permeabilized in PBS/ 0.5% TritonX-100/ 4% BSA/ 0.1% sodium azide solution overnight at 4°C, then incubated in

primary antibody mix (1:1,000 IgG1 neurofilament 160 antibody and 1:1,000 rabbit synaptophysin antibody, both from Chemicon) for 5-7 days at 4°C. After washing 3 times for 20 minutes and then overnight in PBS, the diaphragms were transferred into secondary antibody mix (1:1,500 Alexa-Fluor 488-conjugated anti-IgG1 and 1:1,500 Alexa-Fluor 488-conjugated anti-rabbit IgG, both from Molecular Probes) for 5-7 days at 4°C. Both antibody solutions were made using PBS/ 0.5% TritonX-100/4 % BSA/ 0.1% sodium azide as the diluent. After another wash in PBS, tissues were incubated in a 5μg/mL solution of Alexa-Fluor 594-conjugated α-bungarotoxin (Molecular Probes) for 30 minutes at 4°C. After a final set of washes in PBS, diaphragms were whole-mounted onto glass slides using Vectashield Mounting Medium (Vector Laboratories).

Confocal imaging and analysis

Imaging and subsequent analysis of neuromuscular junctions were conducted blind with respect to sample genotype. Neuromuscular junctions were imaged on a laser scanning confocal microscope (Zeiss Axiovert 100M) using a Zeiss Plain Apochromat oil objective (100x, numerical aperture 1.40) in the W.M. Keck Imaging Facility at the Whitehead Institute for Biomedical Research. The 488nm wavelength line of an argon laser was used to excite the Alexa-Fluor 488 fluorochrome and a 543nm wavelength He-Ne laser was used to excite the Alexa-Fluor 594 fluorochrome. 512x512-pixel images were collected at 0.37μm intervals using LSM 510 software (Zeiss) to construct a Z-series, and the Z stacks were flattened using the software's projection algorithm.

At least 100 neuromuscular junctions from each diaphragm were analyzed. Junctions were scored for the extent of the endplate occupied (full, partial, or none) by

synaptophysin- and neurofilament- positive staining. Endplates were also evaluated for the integrity and continuity of the AChR-positive regions. "Fragmented" endplates were defined as endplates consisting of four or more discrete AChR-positive regions; all other endplates were scored as "intact."

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REFERENCES

Amir, R. E., and Zoghbi, H. Y. (2000). Rett syndrome: methyl-CpG-binding protein 2 mutations and phenotype-genotype correlations. Am J Med Genet *97*, 147-152.

Bates, B., Rios, M., Trumpp, A., Chen, C., Fan, G., Bishop, J. M., and Jaenisch, R. (1999). Neurotrophin-3 is required for proper cerebellar development. Nat Neurosci *2*, 115-117.

Chen, R. Z., Akbarian, S., Tudor, M., and Jaenisch, R. (2001). Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nat Genet *27*, 327-331.

Chen, T., Ueda, Y., Xie, S., and Li, E. (2002). A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. J Biol Chem *277*, 38746-38754.

Clark, H. B., Burright, E. N., Yunis, W. S., Larson, S., Wilcox, C., Hartman, B., Matilla, A., Zoghbi, H. Y., and Orr, H. T. (1997). Purkinje cell expression of a mutant allele of SCA1 in transgenic mice leads to disparate effects on motor behaviors, followed by a progressive cerebellar dysfunction and histological alterations. J Neurosci *17*, 7385-7395.

Dubois, N. C., Hofmann, D., Kaloulis, K., Bishop, J. M., and Trumpp, A. (2006). Nestin-Cre transgenic mouse line Nes-Cre1 mediates highly efficient Cre/loxP mediated recombination in the nervous system, kidney, and somite-derived tissues. Genesis *44*, 355-360.

Fan, G., Beard, C., Chen, R. Z., Csankovszki, G., Sun, Y., Siniaia, M., Biniszkiewicz, D., Bates, B., Lee, P. P., Kuhn, R., *et al.* (2001). DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. J Neurosci *21*, 788-797.

Fan, G., Martinowich, K., Chin, M. H., He, F., Fouse, S. D., Hutnick, L., Hattori, D., Ge, W., Shen, Y., Wu, H., *et al.* (2005). DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development *132*, 3345-3356.

Feng, J., Chang, H., Li, E., and Fan, G. (2005). Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. J Neurosci Res *79*, 734-746.

Grayson, D. R., Jia, X., Chen, Y., Sharma, R. P., Mitchell, C. P., Guidotti, A., and Costa, E. (2005). Reelin promoter hypermethylation in schizophrenia. Proc Natl Acad Sci U S A *102*, 9341-9346.

Gurney, M. E. (1994). Transgenic-mouse model of amyotrophic lateral sclerosis. N Engl J Med *331*, 1721-1722.

Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., Caliendo, J., Hentati, A., Kwon, Y. W., Deng, H. X., and et al. (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science *264*, 1772-1775.

Guy, J., Hendrich, B., Holmes, M., Martin, J. E., and Bird, A. (2001). A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat Genet *27*, 322-326.

Lalonde, R., Le Pecheur, M., Strazielle, C., and London, J. (2005). Exploratory activity and motor coordination in wild-type SOD1/SOD1 transgenic mice. Brain Res Bull *66*, 155-162.

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

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247-257.

Okano, M., Xie, S., and Li, E. (1998). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet *19*, 219-220.

Possemato, R., Eggan, K., Moeller, B. J., Jaenisch, R., and Jackson-Grusby, L. (2002). Flp recombinase regulated lacZ expression at the ROSA26 locus. Genesis *32*, 184-186.

Rideout, W. M., 3rd, Wakayama, T., Wutz, A., Eggan, K., Jackson-Grusby, L., Dausman, J., Yanagimachi, R., and Jaenisch, R. (2000). Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat Genet *24*, 109-110.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning : a laboratory manual, 2nd edn (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory).

Sauer, B., and Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A *85*, 5166-5170.

Senecoff, J. F., Bruckner, R. C., and Cox, M. M. (1985). The FLP recombinase of the yeast 2-micron plasmid: characterization of its recombination site. Proc Natl Acad Sci U S A *82*, 7270-7274.

Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell *64*, 693-702.

Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M., and Taga, T. (2001). DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell *1*, 749-758.

Teter, B., Rozovsky, I., Krohn, K., Anderson, C., Osterburg, H., and Finch, C. (1996). Methylation of the glial fibrillary acidic protein gene shows novel biphasic changes during brain development. Glia *17*, 195-205.

Trumpp, A., Depew, M. J., Rubenstein, J. L., Bishop, J. M., and Martin, G. R. (1999). Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. Genes Dev *13*, 3136-3148.

Walsh, C. P., Chaillet, J. R., and Bestor, T. H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat Genet *20*, 116-117.

Weydt, P., Hong, S. Y., Kliot, M., and Moller, T. (2003). Assessing disease onset and progression in the SOD1 mouse model of ALS. Neuroreport *14*, 1051-1054.

Chapter 3.

Perspectives

With the broader goal of understanding how epigenetic mechanisms operate in the nervous system, I focused on the *de novo* DNA methyltransferase Dnmt3a. Using mutant mice, I discovered a role for Dnmt3a in the neuromuscular system.

There are several possible avenues for further investigation of Dnmt3a's role in this system using the *Dnmt3a*/Nes-Cre1 conditional knockout mice. Though gross histological analyses in both the central nervous system and in the periphery did not reveal any changes, a closer examination of cellular morphology might. The motor defects of the *Dnmt3a*/Nes-Cre1 mutant mice and the expression of Dnmt3a in the cerebellum suggest that Dnmt3a could have a role in the cerebellum.

Aside from possible cerebellar defects, the decreased numbers of motor neurons in the hypoglossal nucleus I observed could be explored further. For example, one could examine cellular markers for apoptosis in the brainstem and the function of the tongue muscle innervated by the hypoglossal neurons. Since not all motor neuron pools even within the brainstem are affected equally in motor disorders, a complete analysis would require examination of other motor neuron populations in *Dnmt3a*/ Nes-Cre1 mutants. The morphology and function of the motor neurons in the spinal cord, especially the ones innervating the hindlimbs, may also yield some clues.

Another strategy for defining the requirement for Dnmt3a in the nervous system is to cross the Dnmt3a conditional knockout to more restricted Cre transgenic lines. A

recently established pan-neuronal Cre line (Banares et al., 2005) is active from day E11.5 and could be used to address the requirement of Dnmt3a in neurons. Specific ablation in cerebellar granule cells or in Purkinje cells is also possible because Cre lines restricted for each cell type have been established (Aller et al., 2003; Zhang et al., 2005).

A major gap in our understanding of Dnmt3a is which genes it regulates. The identification of these genes might clarify the role of Dnmt3a in neuromuscular function. Two common approaches to identify gene targets - assessing candidate genes and genome-wide transcriptional profiling of Dnmt3a mutant cells – could be used. Both have their limitations.

The candidate gene approach is limited by the paucity of candidates. Only a few genes have been identified as being regulated by methylation in the nervous system. One prominent example of such a gene is the the glial fibrillary acid protein (Gfap) gene. Studies on Dnmt3a's potential role in methylating the *Gfap* promoter would have to take into account the cycle of demethylation and remethylation at this promoter.

Methylation at the *Gfap* promoter is detected in embryos at E11.5 but is also detected at high levels in undifferentiated ES cells (Shimozaki et al., 2005; Teter et al., 1996). It is unclear when this methylation is established. The demethylation event at the *Gfap* promoter is better understood: it occurs at E14.5 in cells differentiating into astroglia (Takizawa et al., 2001; Teter et al., 1996). Finally, the *Gfap* promoter undergoes remethylation postnatally (Teter et al., 1996).

The ability to identify genes regulated by Dnmt3a by a microarray transcriptional profiling approach is also constrained. One constraint is the heterogeneity of cell types in the brain. Significant changes in gene expression may not be detected by such an

approach if those changes only occur in a low-abundance cell type and if RNA is extracted from whole brain tissue.

Purkinje cells, for example, comprise only a small fraction of the cells in the cerebellum but are tremendously important. They connect with the more populous cerebellar granule cells and are often affected in motor dysfunction disorders. There may well be genes misregulated only in Purkinje cells and whose misexpression contributes to the motor defects observed in Dnmt3a mutant mice.

Conceivably, one could circumvent the low-abundance issue by purifying for a particular cell population. In practice, this typically yields too little starting material. On the other hand, enriching for a particular cell type by cell culturing could introduce artifacts that would confound the analysis.

Genome-wide approaches such as reduced representation bisulfite sequencing (Meissner et al., 2005) could be used to assess methylation changes in Dnmt3a-deficient cells. Nevertheless, the same constraint of needing homogenous yet biologically-relevant starting material would operate here.

Yet another approach is suggested by the study of methyl-CpG-biding protein 2 (MeCP2). As MeCP2 is not only a transcriptional repressor but also a methyl-CpG binding protein, it was predicted to regulate gene expression. In the search for transcriptional repression targets of MeCP2, neither genome-wide approaches with microarrays nor candidate gene approaches yielded a consistent list of gene targets (Pescucci et al., 2005). The candidate gene approaches examined imprinted genes that were well known, missing the imprinted gene DLX5, which is misregulated in MeCP2-null cells (Horike et al., 2005).

DLX5 was discovered along with at least thirty other genes to have MeCP2-binding sequences based on chromatin immunoprecipitation experiments. The CpG island in the DLX5 gene is unmethylated and CpG sites in its MeCP2 binding sequence are not differentially methylated. DLX5 is more likely regulated by histone methylation rather than DNA methylation (Horike et al., 2005). Nevertheless, examining the other genes recovered in the screen for MeCP2 targets may yield targets of the Dnmt3a.

A similar chromatin precipitation assay to discover Dnmt3a-regulated genes could also work in principle. A current technical limitation is the lack of specific antibodies for Dnmt3a. Another consideration in designing such experiments is that Dnmt3a may need interactions with other proteins to bind its targets.

How Dnmt3a finds the genes it regulates is another puzzle to be solved. Dnmt3a might be guided to its targets through the proteins with which it complexes, such as histone deacetylase 1 (HDAC1) (Datta et al., 2003). An alternative possibility is that Dnmt3a may have restricted access to some genes, thus limiting the genes it can methylate. In this model of *de novo* DNA methylation, sites are methylated by default by indiscriminating methyltransferases unless protected (Bird, 2002). Accessibility to these genes might be blocked by factors or by sequestration in a protected locale. This idea, though extreme in nature, is supported by the observation that even normally unmethylated CpGs in the mammalian genome can be methylated in abnormal circumstances (Bird, 2002).

Alternatively, Dnmt3a may recognize its targets by clues left from the other components of the epigenetic machinery. Histone modification can be associated with either inactive or active chromatin and help inform the methylation machinery.

Methylation of histones at lysine 9 of histone H3 can attract DNA methylation to sites of active chromatin (Tamaru and Selker, 2001). Methylation at another site (lysine 4 of histone H3) and acetylation at histones H3 and H4 may inhibit DNA methylation at inactive chromatin (Bird, 2002). The interactions between DNA methylation and histone modification machinery is an important avenue of investigation, especially since histone modifications have been shown to be involved in learning and memory (Levenson et al., 2004; Weaver et al., 2004) and in neural differentiation (Lunyak et al., 2002).

While my studies focused on Dnmt3a, it would be interesting to determine the role of the other *de novo* DNA methyltransferase, Dnmt3b, in the nervous system as well. Dnmt3b is expressed in the developing nervous system across the ventricular zone between embryonic days E10.5 and E13.5 (Feng et al., 2005). Whatever role Dnmt3b has, if any, during this developmental time window remains to be elucidated.

As new tools are being developed to examine gene expression and methylation, many of the technical issues discussed here could be resolved. Such technological advances would create an exciting time in this field of research, as investigators continue to unravel the epigenetic mechanisms of gene regulation in the nervous system.

REFERENCES

Aller, M. I., Jones, A., Merlo, D., Paterlini, M., Meyer, A. H., Amtmann, U., Brickley, S., Jolin, H. E., McKenzie, A. N., Monyer, H., *et al.* (2003). Cerebellar granule cell Cre recombinase expression. Genesis *36*, 97-103.

Banares, S., Zeh, K., Krajewska, M., Kermer, P., Baribault, H., Reed, J. C., and Krajewski, S. (2005). Novel pan-neuronal Cre-transgenic line for conditional ablation of genes in the nervous system. Genesis *42*, 6-16.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. Genes Dev *16*, 6-21.

Datta, J., Ghoshal, K., Sharma, S. M., Tajima, S., and Jacob, S. T. (2003). Biochemical fractionation reveals association of DNA methyltransferase (Dnmt) 3b with Dnmt1 and that of Dnmt 3a with a histone H3 methyltransferase and Hdac1. J Cell Biochem *88*, 855-864.

Feng, J., Chang, H., Li, E., and Fan, G. (2005). Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. J Neurosci Res *79*, 734-746.

Horike, S., Cai, S., Miyano, M., Cheng, J. F., and Kohwi-Shigematsu, T. (2005). Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. Nat Genet *37*, 31-40.

Levenson, J. M., O'Riordan, K. J., Brown, K. D., Trinh, M. A., Molfese, D. L., and Sweatt, J. D. (2004). Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem *279*, 40545-40559.

Lunyak, V. V., Burgess, R., Prefontaine, G. G., Nelson, C., Sze, S. H., Chenoweth, J., Schwartz, P., Pevzner, P. A., Glass, C., Mandel, G., and Rosenfeld, M. G. (2002). Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. Science *298*, 1747-1752.

Meissner, A., Gnirke, A., Bell, G. W., Ramsahoye, B., Lander, E. S., and Jaenisch, R. (2005). Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. Nucleic Acids Res *33*, 5868-5877.

Pescucci, C., Meloni, I., and Renieri, A. (2005). Is Rett syndrome a loss-of-imprinting disorder? Nat Genet *37*, 10-11.

Shimozaki, K., Namihira, M., Nakashima, K., and Taga, T. (2005). Stage- and site-specific DNA demethylation during neural cell development from embryonic stem cells. J Neurochem *93*, 432-439.

Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M., and Taga, T. (2001). DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell *1*, 749-758.

Tamaru, H., and Selker, E. U. (2001). A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature *414*, 277-283.

Teter, B., Rozovsky, I., Krohn, K., Anderson, C., Osterburg, H., and Finch, C. (1996). Methylation of the glial fibrillary acidic protein gene shows novel biphasic changes during brain development. Glia *17*, 195-205.

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., and Meaney, M. J. (2004). Epigenetic programming by maternal behavior. Nat Neurosci *7*, 847-854.

Zhang, H., Fujitani, Y., Wright, C. V., and Gannon, M. (2005). Efficient recombination in pancreatic islets by a tamoxifen-inducible Cre-recombinase. Genesis *42*, 210-217.

Appendix A.

A critical role for Dnmt1 and DNA methylation in T cell development, function and survival

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Contribution: My contribution to this work was to analyze the status of IAP methylation by Southern blot (data presented in Figure 1F) and to verify the following data: recombination of Dnmt1 by Southern blot (presented in Figure 1C), FACS analyses of of B and T cells from mutant mice (presented in Figure 1A), and cell division analyses of mutant T cells using CFSE dye (corroborating data presented in Figure 7C).

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ABSTRACT

The role of DNA methylation and of the maintenance DNA methyltransferase Dnmt1 in the epigenetic regulation of developmental stage- and cell lineage-specific gene expression *in vivo* is uncertain. This is addressed here through the generation of mice in which *Dnmt1* was inactivated by Cre/loxP-mediated deletion at sequential stages of T cell development. Deletion of *Dnmt1* in early $CD4^-CD8^-$ double-negative thymocytes led to impaired survival of $TCR\alpha\beta^+$ cells and the generation of atypical $CD8^+TCR\gamma\delta^+$ cells. Deletion of *Dnmt1* in $CD4^+CD8^+$ double-positive thymocytes impaired activation-induced proliferation but differentially enhanced cytokine mRNA expression by naïve peripheral T cells. We conclude that Dnmt1 and DNA methylation are required for the proper expression of certain genes that define fate and determine function in T cells.

INTRODUCTION

During their differentiation from bone marrow progenitors, T lymphocytes make sequential cell fate choices: T cell rather than B cell; then TCRαβ or TCRγδ, CD4 or CD8, Th1 or Th2 lineage. These fate choices require the initiation of new programs of gene expression, and once initiated these programs must be faithfully propagated from parental cells to their progeny. With the exception of the T cell receptor (TCR), these changes in gene expression occur without a change in information encoded directly in the DNA sequence. This requires that patterns of gene transcription must be imposed in a heritable, epigenetic manner.

Transcription is initiated through the ordered assembly of relevant complexes of transcription factors on regulatory regions (enhancers and promoters) of the target gene,

so that the abundance, secondary modifications and location of transcription factors are one means by which transcription is controlled. A second level of transcriptional control is imposed epigenetically, through regulation of the accessibility of these transcription factors to the target gene within a highly ordered chromatin structure. Epigenetic mechanisms may act to set the threshold that transcription factors must exceed to activate gene expression (Bird and Wolffe, 1999; Reiner and Seder, 1999). In mammals, epigenetic regulation is mediated by changes in chromatin structure, which reflect in part reversible modification of histones, and by DNA methylation on cytosines in CpG dinucleotides (Bird, 1992; Bjorklund et al., 1999; Lewin, 1998; Mostoslavsky and Bergman, 1997; Panning and Jaenisch, 1998; Siegfried and H., 1997; Yoder et al., 1997). Methylation and demethylation within tissue-specific genes *in vivo* usually are associated with gene inactivation and activation, respectively. DNA methylation may repress gene expression directly by impeding the binding of *trans*-acting factors and indirectly through the recruitment of histone deacetylases by DNA methyltransferases and by methylated CpG binding proteins (Bird and Wolffe, 1999; Fuks et al., 2001; Kass et al., 1997; Nan et al., 1998; Robertson et al., 2000; Rountree et al., 2000; Siegfried and H., 1997); deacetylation of histones acts to compact chromatin and render it less accessible to *trans*-acting factors.

In mammals there are three known DNA methyltransferases. DNA methyltransferase 1 (Dnmt1) copies the pattern of CpG methylation from the parental DNA strand to the daughter strand during S phase (Bird, 1992; Panning and Jaenisch, 1998), and thereby maintains DNA methylation in mammalian cells (Beard et al., 1995; Lei et al., 1996; Li et al., 1992). Dnmt3a and 3b are *de novo* methyltransferases (Okano et

al., 1998; Okano et al., 1998). Each of these genes is essential in mammals, since mice in which either is disrupted in the germline die *in utero*. The maintenance of DNA methylation by Dnmt1 is also essential for proper X-chromosome inactivation, parental imprinting and silencing of parasitic retroelements in mammals (Bird and Wolffe, 1999; Csankovszki et al., 2001; Jaenisch, 1997; Li et al., 1992; Walsh et al., 1998). DNA methylation correlates inversely with the expression of many tissue-specific genes, suggesting that methylation may help to confer heritable patterns of gene expression in these and other somatic cells (Cedar and Bergman, 1999; Mostoslavsky and Bergman, 1997; Reiner and Seder, 1999). However, the extent to which methylation physiologically regulates or merely marks expression of these and other tissue-specific genes in lymphocytes and in other somatic cells is unclear and cannot be addressed in Dnmt1-deficient ($Dnmt^{N/N}$) mice, which die at gastrulation (Li et al., 1992).

In T and B lymphocytes, demethylation at the respective gene loci has been correlated with transcription and rearrangement of immunoglobulin and T cell receptor genes, with the cell lineage-specific expression of CD4, CD8, FcγR, and CD21, and with the transcription of cytokine genes including interferon-γ (IFN-γ), IL-3, IL-4 and IL-5 (Agarwal and Rao, 1998; Asnagli and Murphy, 2001; Bird et al., 1998; Carbone et al., 1988; Cedar and Bergman, 1999; Durum et al., 1998; Fitzpatrick et al., 1999; Fitzpatrick et al., 1998; Hamerman et al., 1997; Iezzi et al., 1999; Reiner and Seder, 1999; Sleckman et al., 1996; Young et al., 1994). Thus, DNA methylation may be one mechanism by which the expression of genes that help to determine the fate and function of T cells is controlled. To address the role of Dnmt1 and DNA methylation in T cells directly, we

generated mice with a loxP-*Dnmt1* conditional targeting allele and crossed them to mice expressing Cre-recombinase under the control of the lck proximal promoter or the CD4 enhancer-promoter-silencer. This resulted in the disruption of *Dnmt1* at sequential stages of T cell development. From these studies we conclude that Dnmt1 and DNA methylation are essential for normal T cell homeostasis and play distinct developmental stage-specific roles in the regulation of T cell lineage-specific gene expression.

RESULTS

Generation of lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} Mice

To address the role of Dnmt1 in T-lineage cells, we generated mice with a loxP-*Dnmt1* targeting allele (Figure 1A). Dnmt$^{2lox/2lox}$ cells have wildtype levels of Dnmt1 protein, but following Cre-mediated deletion Dnmt1$^{1lox/1lox}$ cells lack detectable Dnmt1 protein (Jackson-Grusby et al., 2001). Dnmt$^{2lox/wt}$ ES cell clones were used to generate Dnmt^{2lox} mice. F1 progeny were intercrossed to generate Dnmt$^{2lox/2lox}$ mice or were crossed to Dnmt$^{N/wt}$ mice (Li et al., 1992) to generate Dnmt$^{2lox/N}$ mice. Unlike the Dnmt^{1lox} allele, the Dnmt^{N} allele yields small amounts (1-2% of wildtype) of residual Dnmt1 protein (Jaenisch, 1997; Li et al., 1992). In Dnmt$^{2lox/N}$ mice, only one Dnmt^{2lox} allele must be rearranged to ablate *Dnmt1*, so the fraction of cells in a population in which *Dnmt1* has been ablated can be readily determined by comparing the ratio of 2lox to 1lox alleles. Since results with Dnmt$^{2lox/N}$ and Dnmt$^{2lox/2lox}$ mice were similar, we hereafter refer to them collectively as Dnmt^{2lox} mice.

We also generated transgenic mice in which Cre-recombinase was expressed under the control of the *lck* proximal promoter (Garvin et al., 1990) or CD4 enhancer-

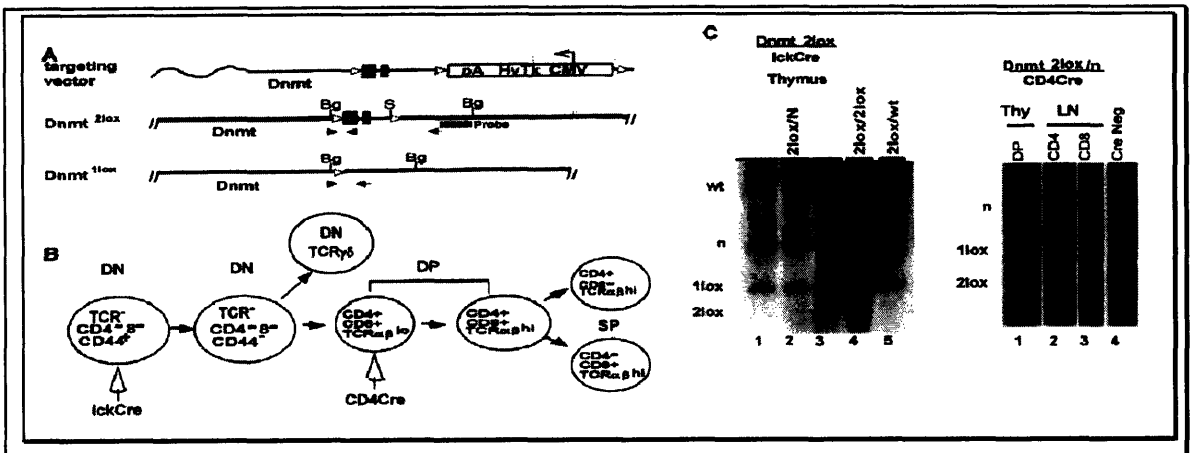

Figure 1. Targeted deletion of *Dnmt1* in thymocytes. **(A)** Targeting vector, targeted ES cells ($Dnmt^{2lox}$) and Cre-mediated deletion creating the $Dnmt^{1lox}$ allele. LoxP sites are shown as open arrowheads and exons 4 and 5 as filled boxes. The probe and PCR primers (closed arrows) used to detect 2lox and 1lox alleles are shown. **(B)** Intrathymic T cell development and stages of T cell development at which lckCre or CD4Cre transgenes are first expressed. **(C)** Southern analysis of *Dnmt1* deletion in thymus and lymph node (LN) cells from lckCre$Dnmt^{2lox}$ and CD4Cre $Dnmt^{2lox}$ mice. Densitometry indicated that the fraction of non-targeted $Dnmt^{2lox}$ alleles in total thymocytes from the lckCre$Dnmt^{2lox}$ mice was: lanes 1, 2, and 5, 0%; lane 3, 4%; lane 4, 6%, and for CD4Cre$Dnmt^{2lox}$ mice was: lane 1 ($CD4^+CD8^+$DP thymocytes), 15%, lanes 2 and 3 (lymph node $CD4^+$ or $CD8^+$ T cells), 0%, lane 4 (thymocytes from Cre-negative controls), 100%.

promoter-silencer (Sawada et al., 1994). Cre mRNA expression was restricted to lymphoid tissues of these mice (not shown). $Dnmt^{2lox}$ and Cre-transgenic mice were then crossed and analyzed.

Efficient Developmental-stage Specific Deletion of *Dnmt1* and Reduced DNA Methylation in lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} Mice

The murine *lck* proximal promoter and CD4 enhancer-promoter-silencer (Garvin et al., 1990; Sawada et al., 1994) become active at sequential stages of T-cell development in the thymus, the major stages of which can be delineated by the expression of CD4, CD8 and the T cell receptor (TCR), which define the $TCR^-CD4^-CD8^-$ (double-negative, DN), $TCR\alpha\beta^+$ $CD4^+CD8^+$ (double-positive, DP), $CD4^+$ or $CD8^+$ single-positive (SP) and $CD4^-CD8^-TCR\gamma\delta^+$ ($DNTCR\gamma\delta^+$) subsets (Kisielow and von Boehmer, 1995; Pénit et al., 1995) (Figure 1B). SP thymocytes emigrate to peripheral lymphoid organs as naïve T cells. Thymocytes from lckCreDnmt^{2lox} mice showed efficient deletion of loxP-flanked *Dnmt1* as determined on Southern blots (Figure 1C) or by PCR (Figure 1D). Deletion was >50% complete in the most immature $CD44^+$ DN cells, and non-deleted alleles were not detected by PCR in $CD44^-$DN or DP plus SP thymocytes (Figure 1D, lanes 2-4). Deletion was incomplete in peripheral T cells from some (Figure 1D, lane 5) but not other (not shown) lckCreDnmt^{2lox} mice, which appeared to reflect preferential survival of T cells derived from rare thymocytes in which deletion was incomplete (see below). As predicted, deletion began later in CD4CreDnmt^{2lox} mice, was >90% complete at the DP stage and non-deleted alleles were not detected in $CD4^+$ and $CD8^+$ SP thymocytes or T cells (Figure 1C,E). Thus, targeting of *Dnmt1* was highly

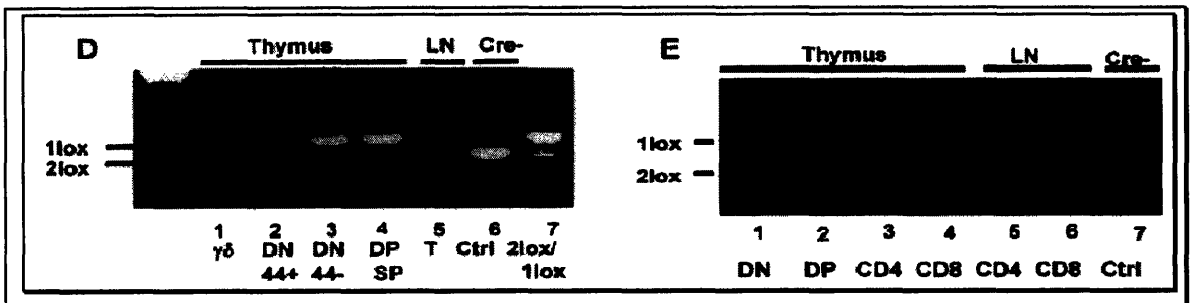

Figure 1 *continued.* Targeted deletion of *Dnmt1* in thymocytes. **(D)** PCR analysis of Cre-mediated deletion of *Dnmt1* in thymocyte subsets (lanes 1-4) and LN T cells (lane 5) from lckCreDnmt$^{2lox/N}$ mice; thymocytes from Cre-negative controls (lane 6); Dnmt$^{2lox/1lox}$ ES cells (lane 7); DNA markers are on the left. Densitometry indicated that the fraction of non-targeted Dnmt^{2lox} alleles was: lanes 1, 9%; lane 2, 39%, lanes 3 and 4, 0%, lane 5, 41%; lane 6, 100%. **(E)** PCR analysis of *Dnmt1* deletion in thymocyte subsets and LN T cells from CD4CreDnmt$^{2lox/N}$ mice and thymocytes from Cre-negative controls (lane 7). Densitometry indicated that the fraction of non-targeted Dnmt^{2lox} alleles was: lane 1, 90%; lane 2, 9%, lanes 3-6, 0%.

efficient both in lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} mice and occurred at sequential stages of T cell development.

Maturation of DN into DP thymocytes is accompanied by multiple rounds of cell replication, whereas proliferation of SP and TCR$\gamma\delta^{+}$ thymocytes and naïve T cells is limited (Pénit et al., 1995; Tough and Sprent, 1998). Since Dnmt1 maintains methylation during DNA replication, we reasoned that in lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} mice reduced DNA methylation should primarily occur in early thymocytes and after activation-induced proliferation of naïve T cells, respectively. Global demethylation was estimated by digesting DNA with the methylation-sensitive restriction enzyme HpaII and probing Southern blots with intracisternal A particle (Walsh et al., 1998) (Figure 1F) or centromeric repeat (Beard et al., 1995) (Figure 1G) probes. Thymus but not kidney DNA from lckCreDnmt^{2lox} mice was markedly demethylated (Figure 1F), whereas splenic DNA was less demethylated, reflecting the preponderance of non-T cells in spleen; demethylation in purified sub-populations of viable thymocytes and T cells (Figure 1G) paralleled the extent of *Dnmt1* deletion (Figure 1D). In CD4CreDnmt^{2lox} mice, DNA from total thymus and DP thymocytes was only slightly demethylated, and DNA from T cells but not B cells was partially demethylated (Figure 1F,G); this is consistent with replication-dependent demethylation , and limited replication of cells after deletion of *Dnmt1* at the DP stage.

Impaired Survival of TCR$\alpha\beta$-lineage Cells in lckCreDnmt^{2lox} Mice

Development of TCR$\alpha\beta$-lineage cells was profoundly affected in lckCreDnmt^{2lox} mice. Although, the numbers of DN thymocytes and DN thymocyte subsets were similar

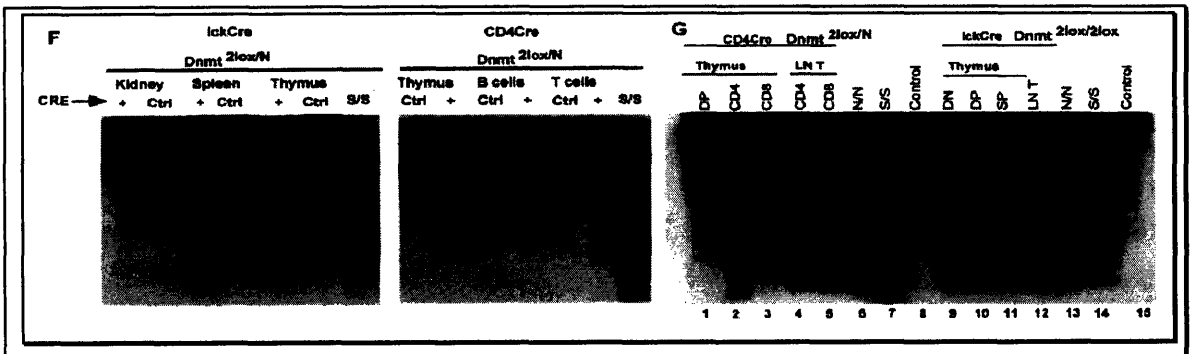

Figure 1 *continued.* Targeted deletion of *Dnmt1* in thymocytes. **(F)** Southern analysis using an intracisternal A particle probe (Walsh et al., 1998) and HpaII digested DNA from lckCreDnmt$^{2lox/N}$ (+), CD4CreDnmt$^{2lox/N}$ (+) and control (Ctrl) mice and from Dnmt-deficient (S/S) ES cells (Li et al., 1992). **(G)** Southern analysis using a centromeric repeat probe (Beard et al., 1995) and HpaII-digested DNA from thymus and LN cells of CD4CreDnmt$^{2lox/N}$, lckCreDnmt$^{2lox/2lox}$ and control (CD4CreDnmt$^{2lox/wt}$ and lckCreDnmt$^{2lox/wt}$) mice and from Dnmt1-deficient (N/N, S/S) ES cells (Lei et al., 1996; Li et al., 1992).

to littermate controls, the numbers of DP and SP thymocytes were reduced ~10-fold, and T cells in splenic, lymph node and gut-associated lymphoid tissues were greatly reduced (Figure 2A,B,C). To determine whether the reduction of thymocytes and T cells was due to a block in proliferation or to cell death, cells were pulse-labeled with BrdU *in vivo* or stained with Annexin V in *vitro*. The fraction of each thymocyte subset in cycle was at least as great in lckCreDnmt^{2lox} mice as in controls (Figure 3A), although the absolute numbers of DP thymocytes in cycle was reduced >3-fold. There was a marked increase in the fraction of dead (Annexin V^{+}) thymocytes in lckCreDnmt^{2lox} mice (Figure 3A). Thymocyte death did not appear to reflect an altered balance of pro- vs. anti-apoptotic Bcl-2 family gene expression, which was similar to controls (Figure 3B). Nonetheless, in lckCreDnmt^{2lox} mice a lckBcl-x_L transgene (Chao and Korsmeyer, 1997) substantially restored total, DP and SP thymocyte numbers ($45.8\pm9.3x10^6$, $28.9\pm5.3x10^6$ and $10.5\pm2.9x10^6$, respectively) (Figure 3C). These results suggest that impaired thymocyte development in lckCreDnmt^{2lox} mice is primarily due to reduced viability and not to a block in differentiation.

CD8αβ^{+}TCRγδ^{+} Thymocytes and T cells in lckCreDnmt^{2lox} Mice

In contrast to TCRαβ-lineage cells, TCRγδ^{+} cells were more abundant in the thymus, spleen and lymph nodes of lckCreDnmt^{2lox} mice (Figure 4A). Many TCRγδ^{+} cells in lckCreDnmt^{2lox} and lckCreDnmt^{2lox}Bcl-x_L mice atypically expressed CD8 (Figure 4B). In some of these mice, non-targeted Dnmt 2lox alleles were found in a fraction of peripheral T cells and thymic TCRγδ^{+} cells (an example is shown in Fig. 1D), but in other mice non-targeted alleles were not detected by PCR in these cell populations;

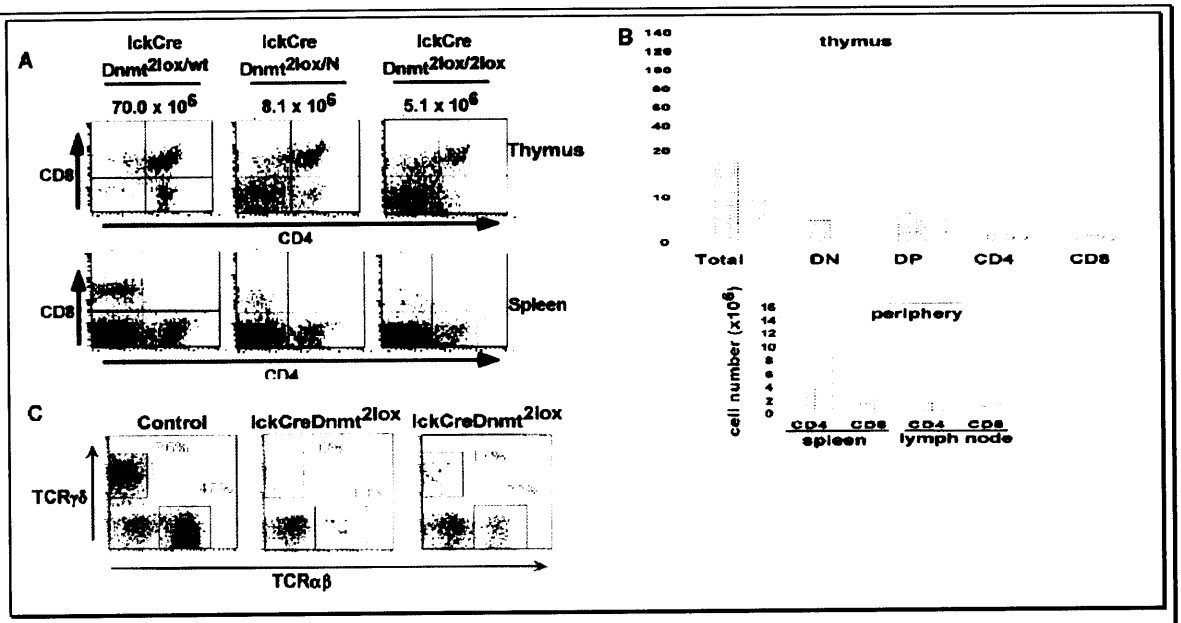

Figure 2. TCRαβ-lineage T cell development in lckCreDnmt^{2lox} mice. **(A)** Representative CD4 vs. CD8 dot plots of thymocytes and spleen cells; numbers of total thymocytes are indicated. **(B)** Numbers of thymocytes and spleen or lymph node T cells in lckCre Dnmt$^{2lox/wt}$ littermate control (open bars, n=10), lckCreDnmt$^{2lox/2lox}$ (filled bars, n=7), lckCreDnmt $^{2lox/N}$ (hatched bars, n=5) mice. **(C)** Gut-associated intraepithelial T cells (IEL). Results from one control and two lckCreDnmt^{2lox} mice are shown.

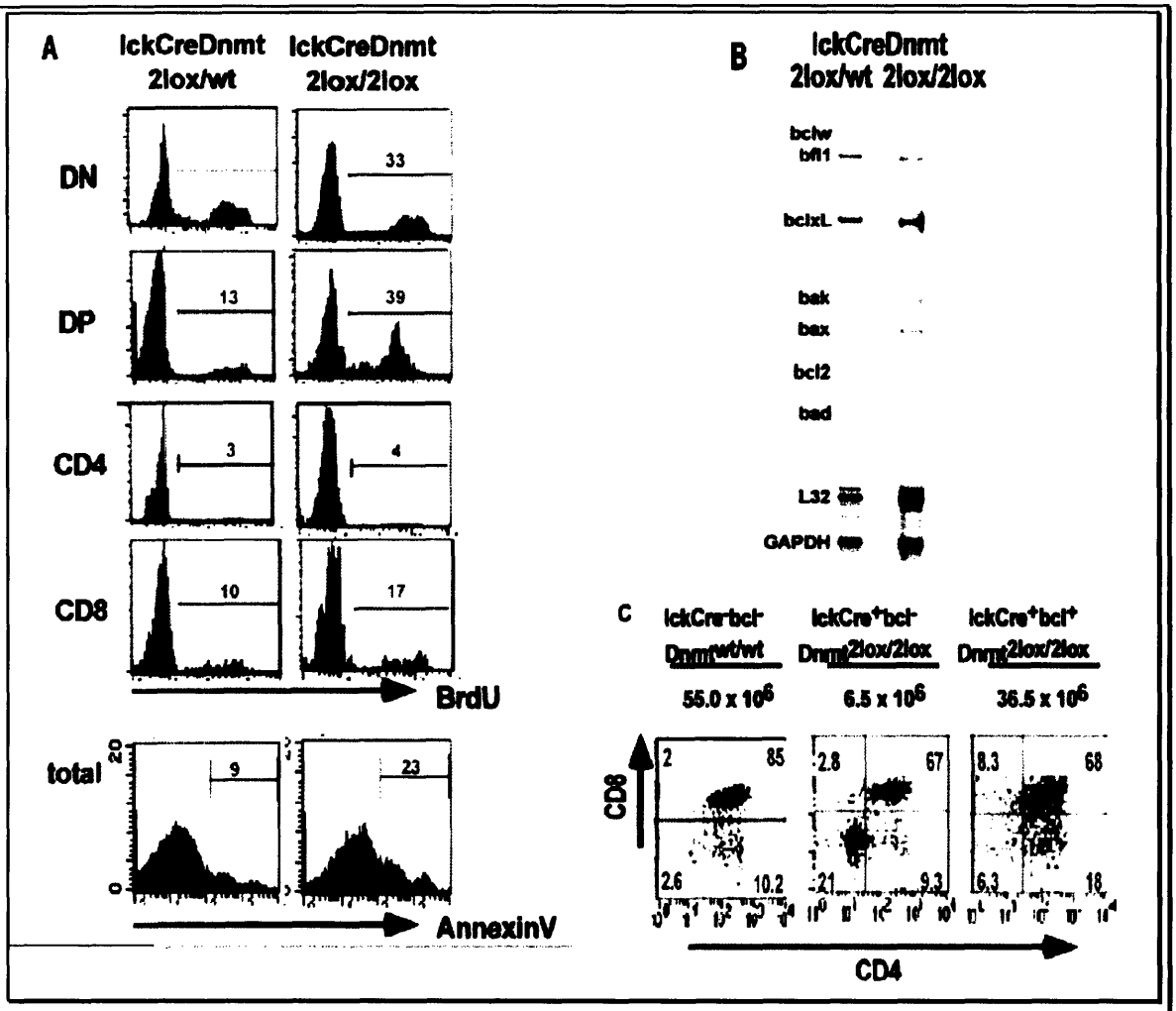

Figure 3. **(A)** *In vivo* thymocyte pulse-labeling with BrdU. Numbers are the proportion of BrdU-positive cells. Mice received two i.p. injections of BrdU (1mg each, 2 hour apart) and were sacrificed 1 hour after the second injection. Bottom panels show Annexin V staining of total thymocytes. **(B)** Expression of Bcl-2 family members by thymocytes from lckCreDnmt^{2lox} mice, as determined by multiprobe RNase protection analysis (Pharmingen). **(C)** An lckCreBcl-x_L transgene reduces cell death and restores thymocyte development in lckCreDnmt^{2lox} mice. Numbers of total thymocytes are indicated.

$CD8^+TCR\gamma\delta^+$ thymocytes and T cells were evident in mice in which non-targeted alleles were detected and in mice in which they were not. $CD8^+TCR\gamma\delta^+$ cells were not clonal, since they showed a diverse pattern of TCRβ and γ gene rearrangements, and, as expected, lacked TCRα gene rearrangements (Figure 4C). Most $CD8^+TCR\gamma\delta^+$ cells were $CD8\alpha^+CD8\beta^+$ (Figure 4D), which is characteristic of thymus-derived TCRαβ-lineage T cells, but not thymus- or gut-derived $TCR\gamma\delta^+$ T cells (Hamerman et al., 1997). Consistent with this, in contrast to the spleen and lymph nodes, $TCR\gamma\delta^+$ cells were markedly reduced in the gut-associated lymphoid tissues (Figure 2C). The expression of CD8 by $TCR\gamma\delta^+$ cells was selective, since expression of cell lineage-specific genes was not broadly derepressed in T-lineage cells from lckCreDnmt^{2lox} mice: CD8 was not aberrantly expressed on $TCR\alpha\beta^+CD4^+$ T cells (Figure 2A), nor did we observe the aberrant expression on T-lineage cells of proteins normally expressed on NK or B cells (NK1.1, FcγR, CD21, B220) but not on T cells (not shown).

To explore the mechanisms for the development of $CD8^+TCR\gamma\delta^+$ cells in lckCreDnmt^{2lox} mice, we first evaluated methylation of the respective gene loci. Methylation of CD8 α and β was examined in regions of the genes for which, by methylation-sensitive restriction enzyme analysis, demethylation has been correlated with expression (Carbone et al., 1988; Hamerman et al., 1997). To provide a more detailed and quantitative examination of CpG methylation in these regions than is possible with restriction enzyme analysis, we sequenced multiple clones of bisulfite-modified genomic DNA (Fitzpatrick et al., 1998). The CD8α locus was markedly demethylated in $CD8^+TCR\gamma\delta^+$ cells and partially demethylated in $DNTCR\gamma\delta^+$ cells from lckCreDnmt^{2lox} mice compared to $TCR\gamma\delta^+$ cells from lckCreDnmt$^{2lox/wt}$ littermate controls (Figure 4E),

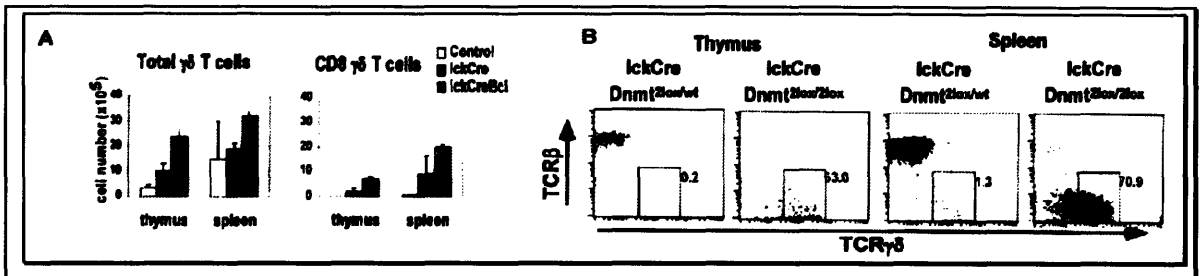

Figure 4. $CD8^{+}TCR\gamma\delta^{+}$ thymocytes and T cells in lckCre$Dnmt^{2lox}$ mice. **(A)** Increased numbers of $TCR\gamma\delta^{+}$ cells, a large fraction of which were $CD8^{+}TCR\gamma\delta^{+}$, in the thymus, spleens and lymph nodes of lckCre$Dnmt^{2lox/2lox}$ and lckCre$Dnmt^{2lox/2lox}$Bcl-x_L mice. **(B)** Representative TCRαβ vs. TCRγδ dot plots of $CD8^{+}$ SP thymocytes and T cells.

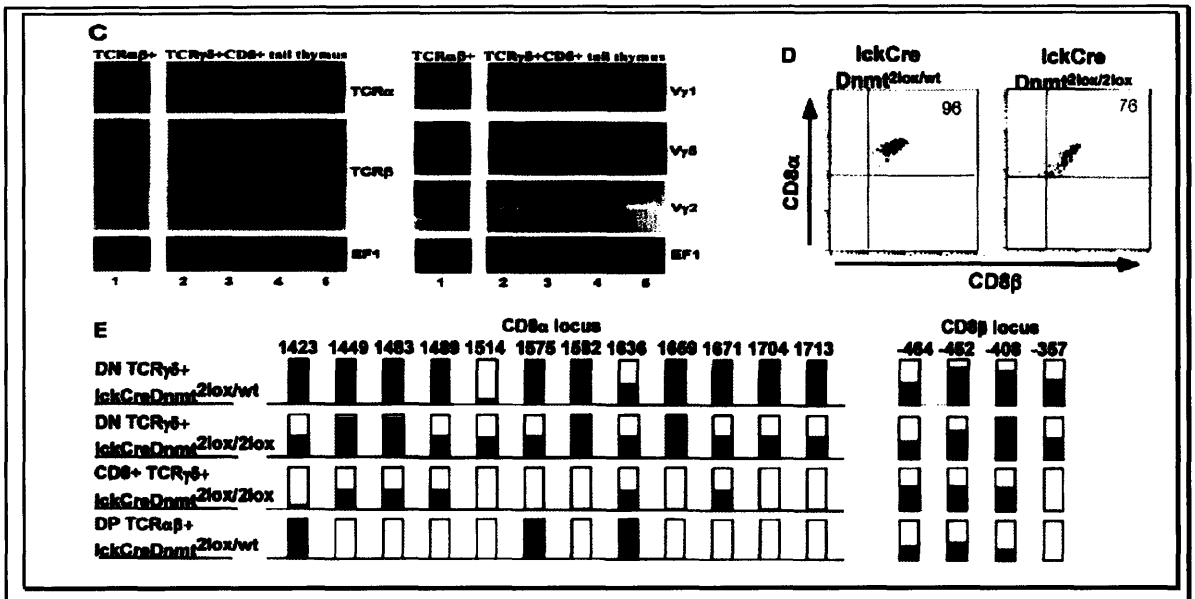

Figure 4 *continued.* $CD8^+TCR\gamma\delta^+$ thymocytes and T cells in lckCreDnmt^{2lox} mice. **(C)** PCR analysis of TCRα, β and γ gene rearrangements on DNA from $CD8^+TCR\gamma\delta^+$ T cells from lckCreDnmt^{2lox} mice (lanes 2 and 3 are from 2 representative mice) compared to $TCR\alpha\beta^+$ T cells, tail and thymocytes from controls (lckCreDnmt$^{2lox/wt}$). $V\gamma_2$ and EF1 (elongation factor-1α) are ethidium bromide stained bands and others are autoradiographs. Shown are rearrangements of VαF3JαD558A and Vβ5-Jβ2.1-2.5; similar results were obtained with Vβ8 and Vβ11. **(D)** CD8α vs. CD8β expression on $CD8^+TCR\alpha\beta^+$ T cells from controls and $CD8^+TCR\gamma\delta^+$ T cells from lckCreDnmt$^{2lox/2lox}$ mice. **(E)** Demethylation of CD8α and CD8β genes. The fraction of methylated (filled bar) and unmethylated (open bar) cytosines, determined by sequencing of bisulfite-modified DNA, is shown for the indicated CpG (Carbone et al., 1988; Hamerman et al., 1997). Results are from 11-12 genomic DNA clones from 2 or more cell preparations (DN and $CD8^+$ $TCR\gamma\delta^+$ cells) or 5-6 genomic DNA clones (DP thymocytes).

suggesting that progressive demethylation of this locus resulted in the expression of CD8α by TCRγδ$^+$ cells when methylation fell below a critical threshold. The CD8β locus was also less methylated in the CD8$^+$TCRγδ$^+$ cells from lckCreDnmt^{2lox} mice. Overall, the degree of demethylation of the CD8α and β genes in CD8$^+$TCRγδ$^+$ cells was similar to DP thymocytes from controls. These results are consistent with the notion that CD8αβ expression is normally repressed in TCRγδ$^+$ cells by methylation and was derepressed following the loss of Dnmt1 and the reduction of DNA methylation in *cis* at the CD8α and β loci.

Demethylation of sequences in the TCRγ enhancer (Eγ) and histone hyperacetylation of the enhancers (Eδ, Eα) in the TCRδ/α locus have been correlated with expression and V(D)J recombination at these loci in T cell progenitors (Durum et al., 1998; McMurry and Krangle, 2000; Schlissel et al., 2000). This raised the possibility that the loss of Dnmt1 enhanced demethylation at these loci in T cell progenitors of lckCreDnmt^{2lox} mice and thereby favored the generation of TCRγδ$^+$ cells. Since the CD25$^+$CD4$^-$CD8$^-$TCR$^-$ thymocyte population (CD25$^+$DN) includes precursors of both TCRγδ$^+$ and TCRαβ$^+$ cells, we assessed the methylation of CpG in these enhancers by sequencing of bisulfite-modified DNA and quantified V(D)J recombination intermediates in CD25$^+$DN thymocytes and, for comparison, in DP thymocytes. These enhancers were fully demethylated in CD25$^+$DN and DP thymocytes from lckCreDnmt^{2lox}, but were also largely demethylated (>85%) in cells from control mice. Consistent with these findings, the relative abundance of TCRJγ1, 2 and 3 and TCRDβ2 RAG-mediated V(D)J recombination intermediates (Schlissel et al., 2000) was similar in cells from

lckCreDnmt^{2lox} mice and controls (Figure 5), suggesting that rearrangements leading to the generation of TCR$\gamma\delta^+$ cells relative to TCR$\alpha\beta^+$ cells were not more common in these mice. There was also no evidence to suggest that the TCRγ locus remained accessible in DP thymocytes of lckCreDnmt^{2lox} mice, since unresolved recombination intermediates at the Jγ loci were not detected in these cells. Thus, the TCRγ locus closed normally as cells matured from the DN to DP stage, suggesting that rearrangement of TCRγ at the DP stage did not contribute to the generation of CD8$^+$ TCR$\gamma\delta^+$ cells in lckCreDnmt^{2lox} mice (Figure 5). Nor did we observe increased proliferation of TCR$\gamma\delta^+$ thymocytes, which was similar in controls (12 and 19% labeled) and in lckCreDnmt^{2lox} mice (8 and 12% labeled, n=2) by BrdU-labeling *in vivo*. Thus, the development of CD8$^+$TCR$\gamma\delta^+$ cells did not reflect or require biased generation or replication of TCR$\gamma\delta^+$ cells.

Normal T Cell Development but Altered T cell Function in CD4CreDnmt^{2lox} Mice

T cell development in the thymus and numbers of CD4$^+$, CD8$^+$ (Figure 6A) and TCR$\gamma\delta^+$ (not shown) T cells were similar in CD4CreDnmt^{2lox} mice and controls. This is consistent with the modest reduction in global methylation in thymocytes and resting peripheral T cells in CD4CreDnmt^{2lox} mice (Figure 1F,G). However, the fraction of CD44^{hi} memory T cells was reduced (Figure 6B). This suggested that replication-dependent maturation of naïve into memory T cells might be impaired. Consistent with this, proliferation of naïve T cells from CD4CreDnmt^{2lox} mice was reduced. The cloning frequency of naïve CD4$^+$ (not shown) and CD8$^+$ (Figure 6C) T cells from CD4CreDnmt^{2lox} mice was similar to controls, but the median number of cell divisions was 3 and none exceeded 5 (n >200), whereas control clones divided >7 times after 6

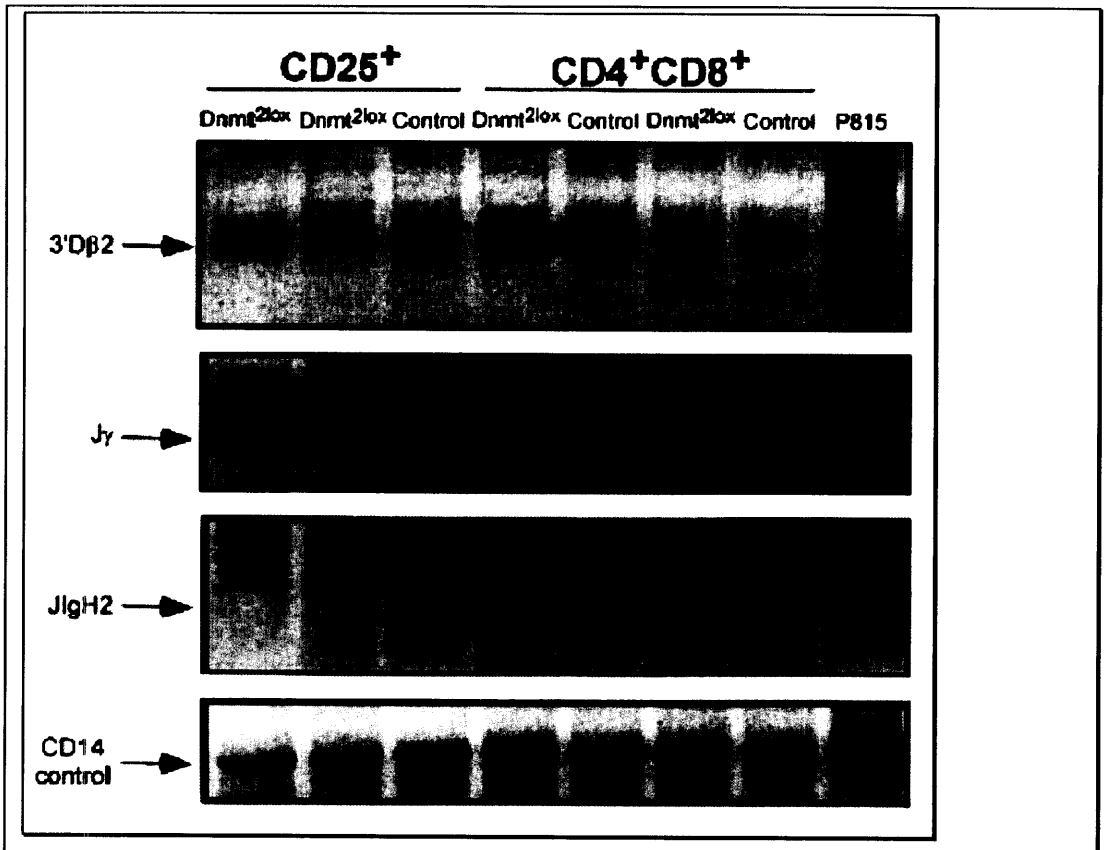

Figure 5. RAG-mediated dsDNA breaks in the TCRγ locus. Recombination intermediates at TCRJγ1,2 and 3 and for comparison at TCRDβ2 and immunoglobulin JH2 were determined by ligation-mediated PCR (Schlissel et al., 1993; Schlissel et al., 2000) on purified populations of $CD25^{+}CD4^{-}CD8^{-}TCR^{-}$ thymocytes ($CD25^{+}$) and DP thymocytes ($CD4^{+}CD8^{+}$) from $lckCreDnmt^{2lox}$ ($Dnmt^{2lox}$) or littermate control mice and from the P815 mast cell line (negative control). CD14 was amplified by PCR as a control for DNA abundance.

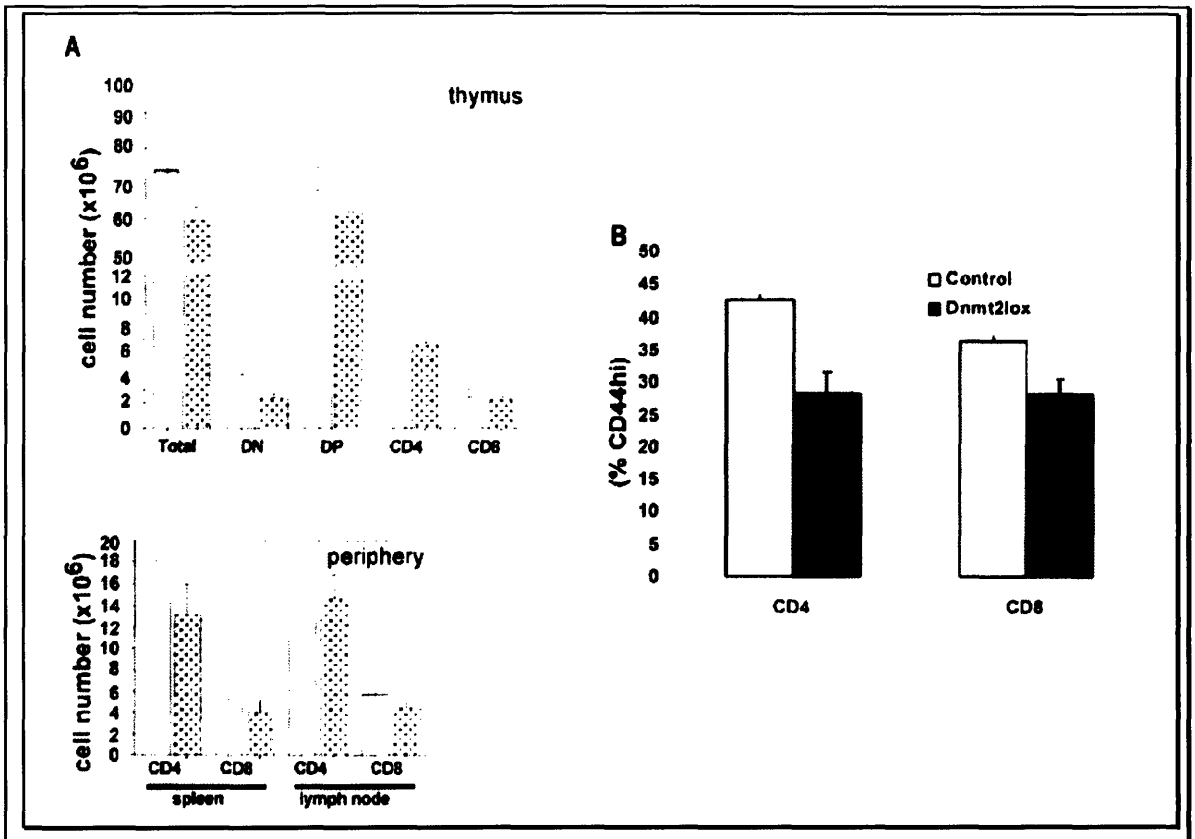

Figure 6. Normal development but impaired proliferation of T cells in CD4CreDnmt^{2lox} mice. **(A)** Numbers of thymocyte, splenic and lymph node T cells in control CD4CreDnmt$^{2lox/wt}$ (n=10, Cre-negative Dnmt^{2lox} mice gave similar results) and CD4CreDnmt^{2lox} (shaded bars, n=21) mice. **(B)** Reduced numbers of memory (CD44^{hi}) T cells in CD4CreDnmt^{2lox} mice.

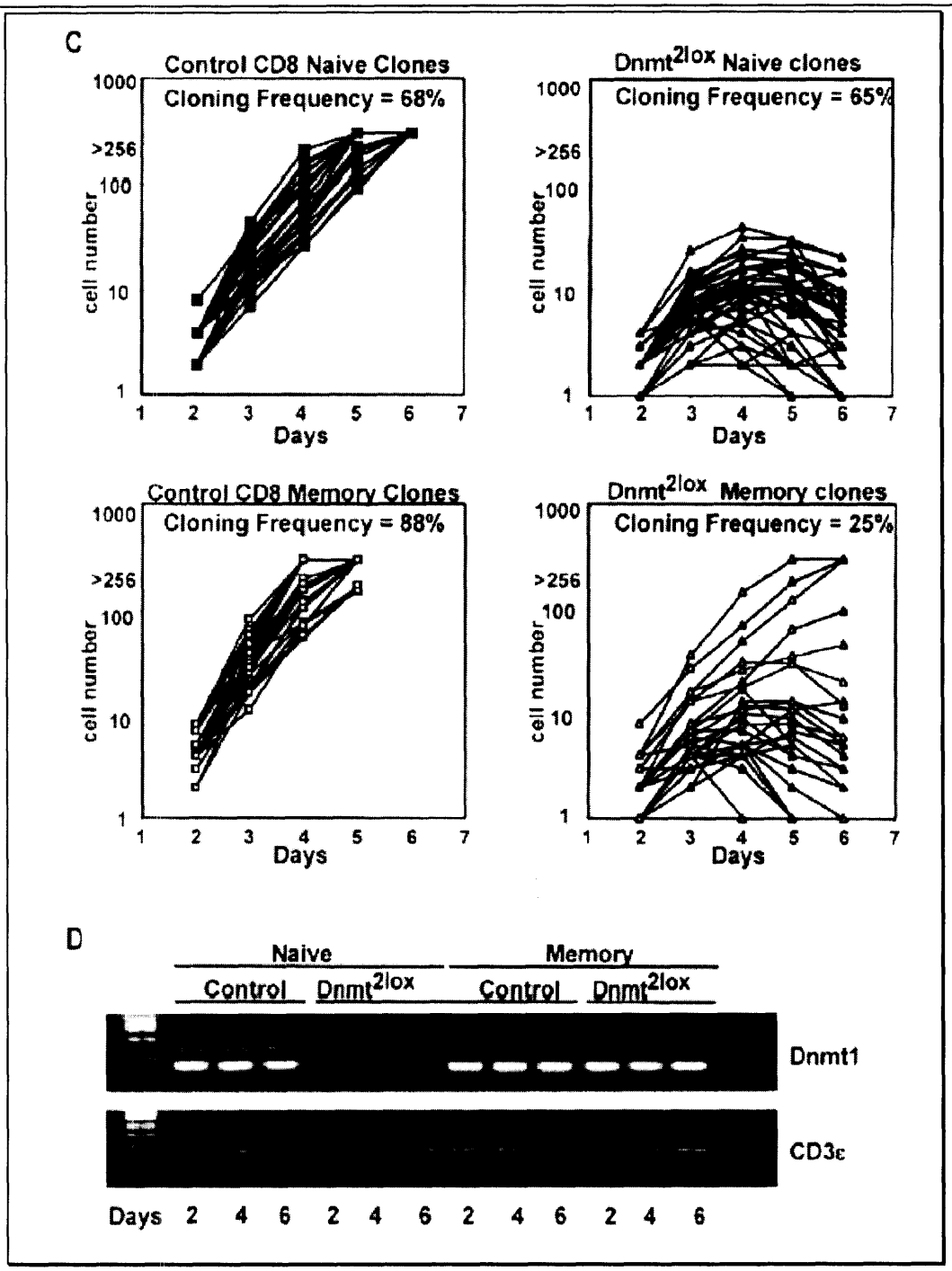

Figure 6 *continued.* Normal development but impaired proliferation of T cells in CD4CreDnmt^{2lox} mice. **(C)** Replication of single naïve or memory CD8^{+} T cells from CD4CreDnmt^{2lox} and control Cre-negative Dnmt^{2lox} mice stimulated under accessory cell-free conditions (Fitzpatrick et al., 1998). **(D)** RTPCR analysis of Dnmt1 and CD3ε mRNA from naïve and memory CD8^{+} T cells from cultures of the same cell preparations shown in **B**.

days. The cloning frequency and proliferation of memory $CD8^+$ T cells from $CD4CreDnmt^{2lox}$ mice was reduced, but ~3% replicated like control cells (Figure 6C). This 3% appeared to represent the progeny of rare cells that escaped Cre-mediated deletion, since Dnmt1 mRNA was detected by RTPCR in cultures of memory, but not naïve, T cells from $CD4CreDnmt^{2lox}$ mice (Figure 6D).

Cytokine expression by naïve T cells is facilitated by entry into S phase and cell replication, which have been proposed to act, at least in part, by facilitating demethylation and chromatin remodeling of cytokine loci (Agarwal and Rao, 1998; Bird et al., 1998; Fitzpatrick et al., 1998; Gett and Hodgkin, 1998; Hu-Li et al., 2001; Lee et al., 2001; Reiner and Seder, 1999; Richter et al., 1999; Ward et al., 1998). However, in marked contrast to their reduced replication, activation-induced cytokine mRNA expression by naïve $CD4^+$ and $CD8^+$ T cells from $CD4CreDnmt^{2lox}$ mice was differentially enhanced (Figure 7A). At 48 hours, IFN-γ, IL-2 and IL-3 mRNAs were consistently and substantially increased in naïve T cells from $CD4CreDnmt^{2lox}$ mice compared to naïve T cells from controls, IL-4 mRNA was modestly and variably increased (Figure 6B), whereas IL-5 mRNA abundance was low in controls and was not increased in Dnmt-1 deficient T cells (data not shown). By day 4, when replication of T cells from CD4CreDnmt2lox mice ceased (Figure 6C), viability and the expression of some cytokines declined (Figure 7A).

To determine if cytokine expression correlated with loss of methylation, we quantified cytokine mRNA (Figure 7C) and cytosine methylation (Figure 7D) over time within the IFN-γ and IL-3 genes, at sites that are fully methylated in non-expressing cell types and for which the degree of demethylation correlates with the magnitude of

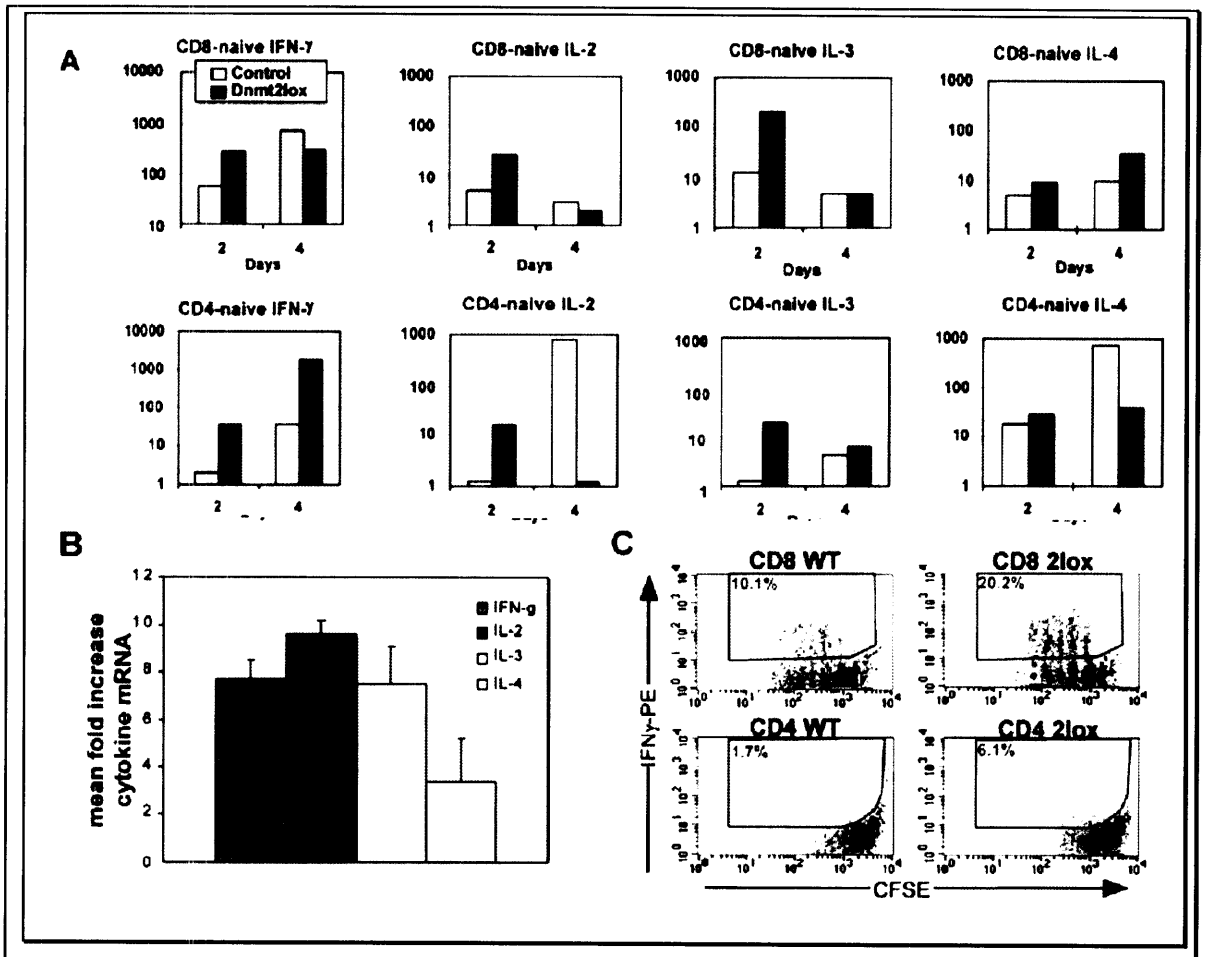

Figure 7. Enhanced cytokine mRNA expression by naïve T cells from CD4CreDnmt2lox mice. **(A)** Representative QC-PCR analysis of cytokine mRNA expression showing results for the naïve CD8+ T cells shown in Figure 6C and for naïve CD4+ T cells from the same mice. Results are expressed in arbitrary units relative to the expression of CD3e mRNA. **(B)** Mean ± SD ratio of cytokine mRNA in naïve T cells from CD4CreDnmt2lox mice compared to controls 48 hr after activation in vitro (n=3-5). **(C)** Representative intracellular IFN-g staining versus cell division determined by CFSE.

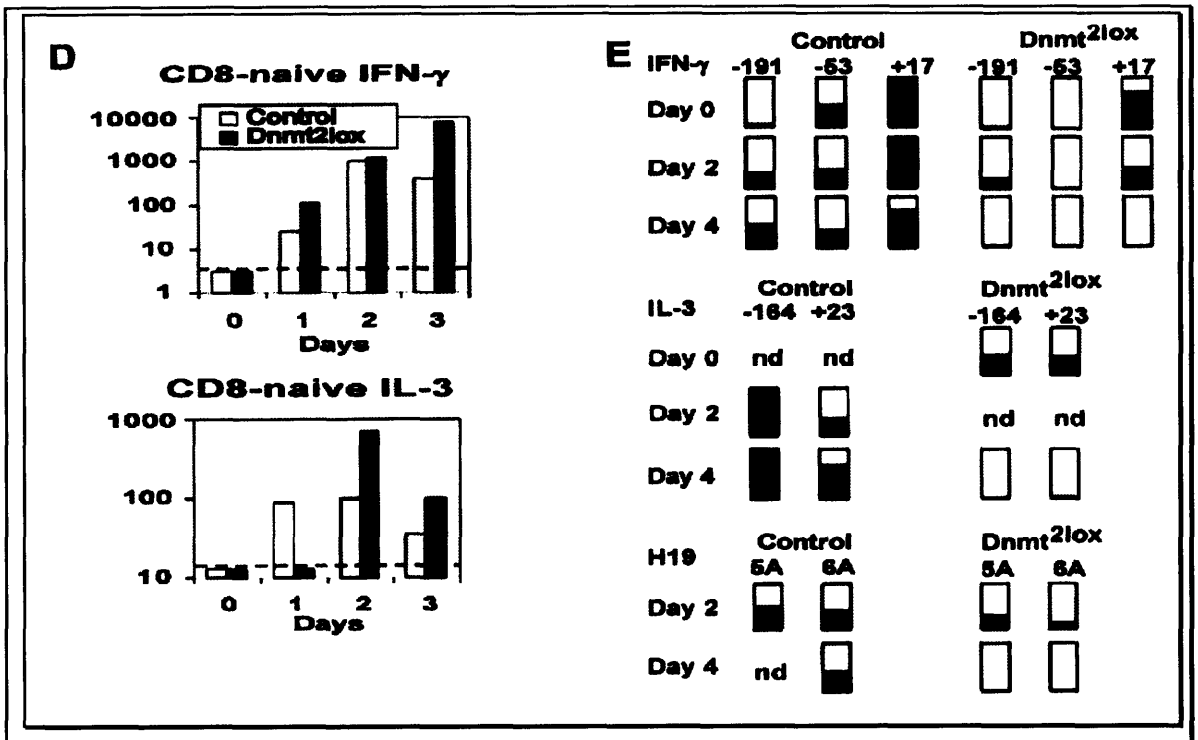

Figure 7. Enhanced cytokine mRNA expression by naïve T cells from CD4CreDnmt2lox mice. **(D)** IFN-g and IL-3 mRNA expression and **(E)** the fraction of methylated (filled bar) and unmethylated (open bar) cytosines at the indicated CpG in the IFN-g and IL-3 genes or in the imprinted region of the H19 gene, which was quantified by Ms-SnuPE using bisulfite-modified DNA as template; the dashed lines in **(D)** indicate the limit of detectability. nd – not done due to insufficient amounts of sample.

expression in T cells (Fitzpatrick et al., 1999; Fitzpatrick et al., 1998; Young et al., 1994). The imprinted H19 gene was assessed as an index of global demethylation (Warnecke et al., 1998). In naïve T cells from CD4CreDnmt^{2lox} mice but not from controls, methylation declined progressively with time after activation, and methylated CpG were undetectable at all loci at 4 days (Figure 7C), which coincided with the halt in cell replication (Figure 6C). The IFN-γ gene was more demethylated than the IL-3 gene in unstimulated Dnmt1-deficient T cells, and this correlated with the earlier increase in IFN-γ than IL-3 expression relative to controls (Figure 7C,D). This supports the notion that increased cytokine gene expression was due, at least in part, to demethylation in *cis*. To explore whether the increased cytokine mRNA expression by naïve T cells from CD4CreDnmt^{2lox} mice might be mediated in part by additional mechanisms acting in *trans*, we evaluated the expression of non-methylated reporter gene constructs after transient transfection. By contrast to the increased expression of the endogenous cytokine genes, transient transfection of reporter constructs into splenic T cells (Sweetser et al., 1998) from CD4CreDnmt^{2lox} mice revealed only a 2-fold increase (range, 1.4-2.8) in expression driven by the IL-2 and IFN-γ promoters and no increase in expression driven by the IL-4 promoter compared to controls. Together, these data suggest that demethylation in *cis* was the major mechanism for increased cytokine gene expression, but do not exclude *trans*-mediated influences on some cytokine genes.

DISCUSSION

Our observations in lckCreDnmt^{2lox} and CD4CreDnmt^{2lox} mice indicate an important but selective contribution of Dnmt1 and DNA methylation to cell lineage-

specific gene expression in developing and mature T cells. We also observe that DNA methylation is critical for normal T cell homeostasis.

An Essential Role for Dnmt1 and DNA Methylation in T Cell Homeostasis

Loss of Dnmt1 and of DNA methylation impaired proliferation and survival of T-lineage cells both at early and late stages of their development. In lckCreDnmt^{2lox} mice, deletion of *Dnmt1* preceded the period of brisk proliferation that follows the expression of TCRβ by CD44$^-$DN thymocytes, and marked demethylation and cell attrition were evident at subsequent stages of TCRαβ-lineage development. Similarly, replication of naïve T cells from CD4CreDnmt^{2lox} mice ceased 4 days after activation *in vitro* when methylation had fallen to undetectable levels. These findings suggest that impaired homeostasis of Dnmt1-deficient T cells and T cell progenitors was due to global demethylation, although we cannot exclude the possibility that loss of Dnmt1 had deleterious effects in addition to the failure to maintain DNA methylation.

These results in T-lineage cells closely parallel those we recently reported in mouse embryo fibroblasts (Jackson-Grusby et al., 2001) and in neuronal cells (Fan et al., 2001). Similarly, although the loss of Dnmt1 does not affect the survival and replication of undifferentiated ES cells, Dnmt1-deficient ES cells do not survive differentiation and Dnmt1-deficient embryos die at gastrulation (Beard et al., 1995; Lei et al., 1996; Li et al., 1992). Together these results suggest a general and essential role for Dnmt1 and DNA methylation in the homeostasis of differentiated mammalian cells. The basis for this is at present unclear. Replication and survival of Dnmt1-deficient fibroblasts can be enhanced

but not restored by introduction of a null p53 allele or by enforced expression of SV40 large T antigen, suggesting that disruption of cellular homeostasis is dependent in part on p53 (Jackson-Grusby et al., 2001). Similar findings have recently been reported in *Xenopus* embryos rendered deficient in xDnmt1 (Stancheva et al., 2001). Consistent with this, Bcl-X_L, which inhibits p53-induced, Bax-mediated cell death (Adams and Cory, 1998), partially restored thymocyte numbers in lckCreDnmt^{2lox} mice. However, Bcl-X_L also inhibits p53-independent pathways of cell death in thymocytes (Chao and Korsmeyer, 1997). Breeding of CD4CreDnmt^{2lox} mice onto a p53-null background did not restore the growth potential of naïve T cells following activation *in vitro* (data not shown), indicating that p53-independent mechanisms are sufficient to disrupt homeostasis of T-lineage cells lacking Dnmt1.

Selective Derepression of CD8α and CD8β on TCRγδ$^+$ Thymocytes and Peripheral T Cells in lckCreDnmt2lox Mice

In addition to the attrition of TCRab-lineage cells, the major phenotypic feature in lckCreDnmt^{2lox} mice was the atypical expression of CD8αβ$^+$ on many TCRγδ$^+$ cells, which correlated closely with demethylation of the CD8α and CD8β loci: these loci were largely methylated in DNTCRγδ$^+$ cells from controls, slightly demethylated in DNTCRγδ$^+$ thymocytes from lckCreDnmt^{2lox} mice and markedly demethylated in CD8αβTCRγδ$^+$ cells from lckCreDnmt^{2lox} mice. The most parsimonious interpretation of these findings is that demethylation in *cis* is both necessary and sufficient for CD8 expression by TCRγδ-lineage cells, although we cannot exclude a contribution by other indirect mechanisms. This provides experimental support for the notion, proposed on the

basis of correlative observations, that selective demethylation of CD8α but not CD8β in gut-derived TCRγδ$^+$ cells dictates their expression of CD8αα homodimers rather than CD8αβ heterodimers (Carbone et al., 1988; Hamerman et al., 1997). Demethylation of the CD8α and CD8β loci in TCRαβ-lineage cells correlates with the onset of CD8αβ expression as DN thymocytes mature to the DP stage and with continued CD8αβ expression by mature CD8$^+$ T cells, suggesting that demethylation is also necessary for CD8 expression in the TCRαβ-lineage (Carbone et al., 1988; Hamerman et al., 1997; Pestano et al., 1999). However, demethylation is not sufficient for continued expression of CD8αβ once commitment to the CD4 lineage occurs, since Dnmt1-deficient CD4SP thymocytes and T cells extinguished the expression of CD8 normally. Thus, in CD4$^+$ cells mechanisms other than methylation (Ellmeier et al., 1999) are sufficient to silence CD8 expression.

We also observed a modest increase in the total numbers of TCRγδ$^+$ thymocytes and splenic and lymph node T cells in lckCreDnmt^{2lox} mice. Their relative preservation and greater viability likely reflect in part the limited replication and already short life-span of TCRγ$^+$ thymocytes and peripheral T cells compared to TCRαβ-lineage cells (Pénit et al., 1995; Tough and Sprent, 1998). However, this does not fully explain the absolute increase in numbers of TCRγδ$^+$ cells in lckCreDnmt^{2lox} mice. Similar increases in the absolute numbers of TCRγδ$^+$ cells have been seen in other strains of mutant mice in which there is a marked reduction in TCRαβ-lineage cells in the thymus, including TCRβ, TCR β enhancer, pre-Tα and lck knockout mice (Leduc et al., 2000; von Boehmer et al., 1999). A common, and as yet unknown, mechanism may give rise to a

compensatory increase in the numbers of TCRγδ$^+$ T cells in these mice and in lckCreDnmt^{2lox} mice.

We found no evidence to suggest that the increased numbers of TCRγδ$^+$ cells in lckCreDnmt^{2lox} mice resulted from increased replication, which was similar to control cells by BrdU labeling, or from preferential V(D)J recombination at the TCRγ vs. TCRβ locus. Immunoglobulin and TCR loci are methylated in cells of the lymphoid lineage in which they are not recombined, and hypomethylated in lymphocytes in which they are (Berg and Kang, 2001; Durum et al., 1998; Mostoslavsky and Bergman, 1997; Siegfried and H., 1997; Whitehurst et al., 2000). Methylation of artificial recombination substrates blocks V(D)J recombination (Cherry and Baltimore, 1999; Sleckman et al., 1996). The failure of TCRγ rearrangement in IL-7 receptor α chain-deficient mice is associated with the failure to demethylate Eγ (Durum et al., 1998), suggesting that demethylation at this locus may be a barrier to recombination. We did not observe increased RAG-mediated cleavage at the TCRγ locus in DN thymocytes from lckCreDnmt^{2lox} mice, nor did we observe continued cutting at this locus in DP thymocytes. Eγ was completely demethylated in both of these subsets in lckCreDnmt^{2lox} mice and >85% demethylated in controls. Thus, the absence of increased TCRγ V(D)J recombination in Dnmt1-deficient DN thymocytes does not exclude the possibility that demethylation is necessary for recombination, but does indicate that demethylation is not the dominant mechanism limiting accessibility. We also found that methylation was not required for RAG-mediated cutting at this locus to be extinguished in DP thymocytes. Our findings are consistent with recent observations at immunoglobulin loci, which suggest that demethylation may be necessary but is not sufficient to activate V(D)J recombination

(Cherry et al., 2000; Engler and Storb, 1999). By contrast, treatment of fetal thymic organ cultures with the histone deacetylase inhibitor trichostatin A was sufficient to activate TCRγ V(D)J recombination in IL-7 receptor-deficient mice (Durum et al., 1998) and to allow V(D)J recombination of the fetal-type Vγ3 locus in adult T cell progenitors (Agata et al., 2001). Although trichostatin A may have had effects other than the induction of histone hyperacetylation at the TCRγ locus (Krangel, 2001), these data suggest that histone hypoacetylation may limit TCRγ V(D)J recombination to a greater extent than does DNA methylation.

Differential Enhancement of Cytokine Gene Expression in CD4CreDnmt^{2lox} Mice

We and others have shown previously that the capacity of T cells to produce IFN-γ (IFN-γ) and IL-3 is correlated with hypomethylation and an open chromatin structure within the IFN-γ gene (Agarwal and Rao, 1998; Fitzpatrick et al., 1999; Fitzpatrick et al., 1998; Katamura et al., 1998; Melvin et al., 1995; Young et al., 1994). Similar correlations have been made for the IL-2, IL-4 and IL-5 genes (Agarwal et al., 2000; Agarwal and Rao, 1998; Bird et al., 1998; Iezzi et al., 1999; Lee et al., 2000; Ouyang et al., 2000; Takemoto et al., 1998; Ward et al., 1998). Although naïve T cells appear capable of expressing each of these cytokines in the first hours after initial activation *in vitro* (Grogan et al., 2001), their capacity to produce effector cytokines increases as they differentiate into memory/effector T cells. This increase is paralleled by progressive demethylation and remodeling of chromatin structure at the respective cytokine loci, rendering them more accessible for engagement by transcription factors induced in response to T cell activation (Agarwal and Rao, 1998; Ben-Sasson et al., 2001; Bird et

al., 1998; Hu-Li et al., 2001; Iezzi et al., 1999; Reiner and Seder, 1999). Thus, robust expression of IL-2 is evident immediately upon activation of naïve T cells, and thereafter the expression of IFN-γ rises more rapidly than does the expression of IL-4 and IL-5 - full expression of these Th2 cytokines appears to be more dependent on DNA and cellular replication (Ben-Sasson et al., 2001; Bird et al., 1998; Gett and Hodgkin, 1998; Iezzi et al., 1999; Laouar and Crispe, 2000; Reiner and Seder, 1999; Richter et al., 1999). Thus, cytokine expression by naïve T cells may in principle be constrained by methylation, closed chromatin structure and the availability of key transcription factors. The relative importance of these mechanisms in the regulation of cytokine expression is a matter of current interest. CD4CreDnmt^{2lox} mice allowed us to explore the extent to which methylation limits cytokine gene expression by naïve T cells using a direct genetic approach.

Loss of Dnmt1 and declining DNA methylation in naïve T cells from CD4CreDnmt^{2lox} mice led to a marked and consistent increase in activation-induced expression of IFN-γ, IL-3 and also IL-2, suggesting that methylation plays an important role in limiting the expression of these cytokines by naïve T cells. Two types of evidence suggest that the augmented expression of IFN-γ was mediated predominantly by demethylation in *cis*: increased expression of IFN-γ correlated with early and progressive demethylation of the IFN-γ gene, and the expression of a non-methylated IFN-γ reporter construct in Dnmt1-deficient T cells was similar to that in control cells. The increased expression of IL-2 and IL-3 also appeared to be mediated, at least in part, in *cis*. Compared to these 3 cytokines, loss of Dnmt1 had a more modest effect on the

expression of IL-4 and did not enhance the expression of IL-5 in naïve T cells, suggesting that the absence of necessary *trans*-acting factors, a more repressive chromatin structure or both are relatively more important than methylation in limiting the expression of IL-4 and IL-5 by naïve T cells. Thus, although cell cycle progression acts in part as an opportunity for demethylation of cytokine gene loci (Agarwal and Rao, 1998; Bird et al., 1998; Iezzi et al., 1999; Reiner and Seder, 1999), our results indicate that loss of methylation had the greatest impact on the expression of cytokines that naïve T are most competent to express early after initial activation (IL-2, IFN-γ and also IL-3) but was not sufficient to enable robust early expression of IL-4 and IL-5. Repressive chromatin may contribute over and above the repressive effect of methylation at these Th2 loci. Supporting this proposal is the finding that IL-4 expression by naïve T cells was modestly augmented *in vitro* either by the DNA methylation inhibitor 5-azacytidine or by the histone deacetylase inhibitor trichostatin A; together the two inhibitors had a more robust effect and were sufficient to allow IL-4 expression by naive STAT6-deficient T cells (Bird et al., 1998). The current results are also compatible with the suggestion by Paul and coworkers that the inefficient induction of Th2 compared to Th1 cytokine production by naïve T cells reflects not only limited gene accessibility but limitations in key transcription factors (Ben-Sasson et al., 2001; Hu-Li et al., 2001).

Conclusions

We conclude that Dnmt1 and DNA methylation are essential for proliferation and survival in T-lineage cells, and help to set thresholds for the differential expression of certain cell lineage- and developmental stage-restricted genes. Loss of Dnmt1

derepressed CD8α and CD8β expression by TCRγδ$^+$ cells in lckCreDnmt^{2lox} mice and differentially enhanced the expression of cytokine genes by T cells in CD4CreDnmt^{2lox} mice. These changes in gene expression resulted, at least in part, from demethylation in *cis*, although we cannot exclude a role for methylation-independent functions of Dnmt1 (Robertson et al., 2000; Rountree et al., 2000). To our knowledge, this is the first demonstration that Dnmt1 is required for the proper expression of genes that help to define lineage and dictate function of T cells or other specific cell populations in a mature mammal. By contrast, the expression of other lymphoid-specific genes examined and developmental stage- and locus-specific V(D)J recombination were faithfully maintained. This suggests that even when Dnmt1 is lost and methylation declines the fates a cell can adopt may be restricted, and that the changes observed reflect genes that are regulated physiologically and are particularly sensitive to the loss of Dnmt1 and DNA methylation at that stage of T cell development. Similarly, expression of only a small fraction of genes is affected when histone acetylation or Swi/Snf-mediated chromatin remodeling are perturbed in yeast (Biggar and Crabtree, 1999; Bird and Wolffe, 1999). The extent to which Dnmt1, DNA methylation and chromatin modification play independent or overlapping roles in the regulation of cell lineage-specific programs of gene expression is uncertain, but conditional Dnmt1-deficient mice provide a tool by which to explore this question.

EXPERIMENTAL PROCEDURES

Transgenic Mice

Generation of the $Dnmt^{2lox}$ targeting vector and of $Dnmt^{2lox/wt}$ ES cells and mice have been described (Jackson-Grusby et al., 2001). The lckCre construct was created by engineering a nuclear localization signal and optimum eukaryotic translation start site at the 5' end of Cre (tcg agc ATG gca ccc aag aag aag agg aag gtg), and inserting this downstream of the *lck* proximal promoter (Garvin et al., 1990); CD4Cre was constructed by replacing the *lck* proximal promoter with the mCD4 promoter/enhancer/silencer (Sawada et al., 1994). Southern blots of genomic DNA that had been digested with BglII and SpeI were probed to detect Cre-mediated deletion of exons 4 and 5 of *Dnmt1*. The probe was generated by PCR using a genomic *Dnmt1* clone as template and as primers (5′ – 3′) ACT GGA GTT ACC GAT GCT GG and TCT ATG AAC TGT GTG CCT G. PCR analysis to detect Cre-mediated deletion of exons 4 and 5 of *Dnmt1* used genomic DNA from the indicated cells as template, a common forward primer (GGG CCA GTT GTG TGA CTT GG) and two reverse primers (TGA ACC TCT TCG AGG GAC C and ATG CAT AGG AAC AGA TGT GTG C) to detect the 2lox vs. the 1lox alleles.

Cell Preparations and Flow Cytometry

Single cell suspensions of thymus, spleen, and lymph nodes were obtained as described (Fitzpatrick et al., 1998; Kay et al., 1995). To isolate IEL, the small intestine was removed and Peyer's patches were excised. After removing fecal matter, intestines were cut longitudinally and then cut into 1 cm pieces. These were suspended in 1mM EDTA in Hank's balanced salt solution (HBSS) at 37°C and vortexed repeatedly to

separate lymphocytes from the epithelial sheaths. Aliquots were removed over time as the sheaths settled. The first two aliquots were replaced with solution and subsequent aliquots were replaced with 5% FBS in HBSS. Aliquots were pooled, concentrated, passed over nylon wool columns, centrifuged on a 40%/75% Percoll (Sigma) gradient and the IEL were recovered from the interface. Cells were resuspended and then stained with various combinations of monoclonal antibodies (BD-Pharmingen or Caltag) as indicated. Cells were then analyzed using a FACScan or LSR flow cytometer or purified by flow cytometric cell sorting on a FACStar Plus or FACS Vantage SE (Becton Dickinson). In some experiments, enrichment for the desired population was first performed by negative-selection using magnetic beads (Dynal). Viability was assessed using FITC-Annexin-V. For analysis of thymocyte proliferation, mice received two i.p. injections of BrdU (Sigma) (1mg each, 2 hour apart) and were sacrificed 1 hour after the second injection. Cells were then fixed, permeabilized and stained with FITC-anti-BrdU (BD-Pharmingen).

T Cell Receptor V(D)J Recombination

TCRα, β and γ gene rearrangements were determined by PCR using primers described previously (Anderson et al., 1992; Asarnow et al., 1989; Kang et al., 1998). Recombination intermediates (signal sequences that have been cleaved but not yet religated) at TCRJγ1,2 and 3 and for comparison at TCRDβ2 and immunoglobulin JH2 were examined by ligation-mediated PCR as previously described (Schlissel et al., 1993; Schlissel et al., 2000).

Analysis of DNA Methylation

Global methylation was assessed by Southern blot using either an intracisternal A particle (Walsh et al., 1998) or centromeric repeat probe (Beard et al., 1995). Methylation in the CD8α, CD8β, Eγ, Eδ and Eα loci was quantified by sequencing of genomic DNA after bisulfite-modification and PCR-amplification (Fitzpatrick et al., 1998). Nested primers (5′ to 3′) used to amplify bisulfite-modified DNA from these regions were CD8α: (outer) forward tggaaaggggttggtgtatttataatttagt, reverse aaacaaaactctattctaaaaaattcccaaaa, (inner) forward cgtaggtattttattgtttttgtagtgaattt, reverse aacaaacacaaaactacaaactcctcctaaaa; CD8β: (outer) forward tttattattttagggttagttggattttgaat, reverse tacaacatcaaaaccttaacttttcaaaaaac, (inner) forward atatatacgtaaagtttttgttttttgttatt, reverse aaaaaaaataataaatctcaaactcaatcata; Eα: (outer) forward ggtataggatttttttttaagaattatatagatagt, reverse ccttaaactaaatatataaacttatctaatttaaa, (inner) forward atgggtaagttggttagatagtgaattaatagtt, reverse aaacaataaaaatcaaaaaaaaaaaaacttcttaaa ; Eδ: (outer) forward ttagtgattaatttatttagattggattatatttt, reverse atacctactactctccacaataaaactaaatcaaa, (inner) forward ttttgttttgtttgaataaatttttaatttttaat, reverse atcaaaaatatcctaaaaaaacaaactattcaaaa; Eγ: (outer) forward tagagagatagaaaggttttgttgtggtttaattt, reverse cctcaataaataacaaataacacaactatccttaa, (inner) forward gttgtatttttttaagttttttttggttatttgtt, reverse tcacttcttcattctcctatactactttattcaaa. Methylation in the IFN-γ and IL-3 loci and in the imprinted region of the H19 locus (Warnecke et al., 1998) was quantified by Ms-SNUPE (Gonzalgo and Jones, 1997). Genomic DNA was bisulfite-modified, PCR-amplified, and cytosine methylation quantified by Ms-SnuPE using FITC-labeled dCTP or dUTP for primer extension and anti-FITC ELISA for detection. Primers used to amplify bisulfite-modified DNA have

been described (Fitzpatrick et al., 1998). Primers used for primer extension were attatgttttatttttgga (H19 site 5A), gttgtggggtttata (H19 site 6A), cgatttttttgggttttttga (IFN-γ -171), tcgaggtttttcggatta (IFN-γ -53), tgtgtttttttttaggttagt (IFN-γ +17), agtagtttttttttttatgt (IL-3 -164), tttttagtatttaagattat (IL-3 +23). Standards were prepared from DNA that was methylated *in vitro* with Sss1 methylase (New England Biolabs) or was obtained from Dnmt1-deficient (S/S) ES cells (Lei et al., 1996).

Cell Proliferation and Cytokine mRNA Production.

Naïve ($CD44^{lo}$) $CD4^+$ and $CD8^+$ T cells were purified by flow cytometry (Fitzpatrick et al., 1998), cultured in low density bulk cultures ($5x10^3$ cells/ml) in DMEM (Life Technologies) supplemented with 15% FCS (Hyclone) and 600 units/ml recombinant IL-2, and activated with plate bound MAb to CD3, LFA-1, CD28 and CD4 or CD8 for the indicated times, as previously described (Fitzpatrick et al., 1998). Cytokine mRNA abundance was determined by quantitative, competitive RTPCR (QCPCR) using CD3ε mRNA as a reference (Fitzpatrick et al., 1998). Dnmt1, CD3ε, GAPDH and β-actin mRNA was evaluated on ethidium bromide stained gels after amplification for the indicated numbers of cycles using semi-nested primers in a two-round PCR (Dnmt1) or one set of primers in a single-round PCR (CD3ε, GAPDH and β-actin); results with GAPHD and β-actin were similar to those with CD3ε. The first round Dnmt1 primers were forward ctgcctatcttgtatccggc and reverse atccgttgtaggacctgtgg and in the second round the forward primer was gctgagacaaaaagaaatgc. The Dnmt1 PCR product identity was confirmed by sequencing.

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REFERENCES

Adams, J. M., and Cory, S. (1998). The Bcl-2 protein family: Arbiters of cell survival. Science *281*, 1322-1326.

Agarwal, S., Avni, O., and Rao, A. (2000). Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer in vivo. Immunity *12*, 643-652.

Agarwal, S., and Rao, A. (1998). Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity *9*, 765-775.

Agata, Y., Katakai, R., Ye, S.-K., Sugai, M., Gonda, H., Honjo, T., Ikuta, K., and Shimizu, A. (2001). Histone acetylation determines the developmentally regulated accessibility for T cell receptor g gene recombination. J. Exp. Med. *193*, 873-879.

Anderson, S. J., Abraham, K. M., Nakayama, T., Singer, A., and Perlmutter, R. M. (1992). Inhibition of T-cell receptor beta-chain gene rearrangement by overexpression of the non-receptor protein tyrosine kinase p56lck. EMBO J. *11*, 4877-86.

Asarnow, D. M., Goodman, T., LeFrancois, L., and Allison, J. P. (1989). Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. Nature *341*, 60-62.

Asnagli, H., and Murphy, K. (2001). Stability and commitment in T helper cell development. Curr. Opin. Immunol. *13*, 242-247.

Beard, C., Li, E., and Jaenisch, R. (1995). Loss of methylation activates Xist in somatic but not in embryonic cells. Genes Dev. *9*, 2325-34.

Ben-Sasson, S. Z., Gerstel, R., Hu-Li, J., and Paul, W. E. (2001). Cell division if not a "clock" measuring acquisition of competence to produce IFN-g or IL-4. J. Immunol. *166*, 112-120.

Berg, L. J., and Kang, J. (2001). Molecular determinants of TCR expression and selection. Curr Opin Immunol *13*, 232-41.

Biggar, S. R., and Crabtree, G. R. (1999). Continuous and widespread role for the Swi-Snf complex in transcription. EMBO J. *18*, 2254-2264.

Bird, A. (1992). The essentials of DNA methylation. Cell *70*, 5-8.

Bird, A. P., and Wolffe, A. P. (1999). Methylation-induced repression- belts, braces, and chromatin. Cell *99*, 451-454.

Bird, J. J., Brown, D. R., Mullen, A. C., Moskowitz, N. H., Mahowald, M. A., Sider, J. R., Gajewski, T. F., Wang, C.-R., and Reiner, S. L. (1998). Helper T cell differentiation is controlled by the cell cycle. Immunity *9*, 229-237.

Bjorklund, S., Almouzni, G., Davidson, I., Nightingale, K. P., and Weiss, K. (1999). Global rranscriptional regulators of eukaryotes. Cell *96*, 759-767.

Carbone, A. M., Marrack, P., and Kappler, J. W. (1988). Demethylated CD8 gene in CD4+ T cells suggests that CD4+ T cells develop from CD8+ precursors. Science *242*, 1174-1176.

Cedar, H., and Bergman, Y. (1999). Developmental regulation of immune system gene rearrangement. Curr. Opin. Immunol. *11*, 64-69.

Chao, D. T., and Korsmeyer, S. J. (1997). BCL-XL-regulated apoptosis in T cell development. Internat. Immunol. *9*, 1375-84.

Cherry, S. R., and Baltimore, D. (1999). Chromatin remodeling directly activates V(D)J recombination. Proc. Natl. Acad. Sci. USA *96*, 10788-10793.

Cherry, S. R., Beard, C., Jaenisch, R., and Baltimore, D. (2000). V(D)J recombination is not activated by demethylation of the kappa locus. Proc. Natl. Acad. Sci. USA *97*, 8467-8472.

Csankovszki, G., Nagy, A., and Jaenisch, R. (2001). Synergism of xist rna, dna methylation, and histone hypoacetylation in maintaining x chromosome inactivation. J Cell Biol *153*, 773-84.

Durum, S. K., Candeias, S., Nakajima, H., Leonard, W. J., Baird, A. M., Berg, L. J., and Muegge, K. (1998). Interleukin 7 receptor control of T cell receptor g gene rearrangement: role of receptor-associated chains and locus accessibility. J. Exp. Med. *188*, 2233-2241.

Ellmeier, W., Sawada, S., and Littman, D. R. (1999). The regulation of CD4 and CD8 coreceptor gene expression during T cell development. Annu Rev Immunol *17*, 523-54.

Engler, P., and Storb, U. (1999). Hypomethylation is necessary but not sufficient for V(D)J recombination within a transgenic substrate. Mol. Immunol. *36*, 1169-1173.

Fan, G., Beard, C., Chen, R. Z., Csankovszki, G., Sun, Y., Siniaia, M., Biniszkiewicz, D., Bates, B., Lee, P. P., Kuhn, R., Trumpp, A., Poon, C., Wilson, C. B., and

Jaenisch, R. (2001). DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. J Neurosci *21*, 788-97.

Fitzpatrick, D. R., Shirley, K. M., and Kelso, A. (1999). Cutting Edge: Stable Epigenetic Inheritance of regional interferon-g promoter demethylation in CD44high CD8+ T lymphocytes. J. Immunol. *162*, 5053-5057.

Fitzpatrick, D. R., Shirley, K. M., McDonald, L. E., Bielefeldt-Ohmann, H., Kay, G. F., and Kelso, A. (1998). Distinct methylation of the interferon-gamma (IFN-g) and

interleukin-3 (IL-3) genes in newly activated primary CD8+ T lymphocytes: Regional IFN-g promoter demethylation and mRNA expression are heritable in $CD44^{high}$ CD8+ T cells. J. Exp. Med. *188*, 103-117.

Fuks, F., Burgers, W. A., Godin, N., Kasai, M., and Kouzarides, T. (2001). Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. Embo J *20*, 2536-2544.

Garvin, A. M., Abraham, K. M., Forbush, K. A., Farr, A. G., Davision, B. L., and Perlmutter, R. M. (1990). Disruption of thymocyte development and lymphomagenesis induced by SV40 T-antigen. Internat. Immunol. *2*, 173-180.

Gett, A. V., and Hodgkin, P. D. (1998). Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. Proc Natl Acad Sci USA *95*, 9488-9493.

Gonzalgo, M. L., and Jones, P. L. (1997). Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucl. Acids Res. *25*, 2529-2531.

Grogan, J. L., Mohrs, M., Harmon, B., Lacy, D. A., Sedat, J. W., and Locksley, R. M. (2001). Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. Immunity *14*, 205-15.

Hamerman, J. A., Page, S. T., and Pullen, A. M. (1997). Distinct methylation states of the CD8 beta gene in peripheral T cells and intraepithelial lymphocytes. J. Immunol. *159*, 1240-6.

Hu-Li, J., Pannetier, C., Guo, L., Lohning, M., Gu, H., Watson, C., Assenmacher, M., Radbruch, A., and Paul, W. E. (2001). Regulation of expression of IL-4 alleles: analysis using a chimeric GFP/IL-4 gene. Immunity *14*, 1-11.

Iezzi, G., Scotet, E., Scheidegger, D., and Lanzavecchia, A. (1999). The interplay between the duration of TCR and cytokine signaling determines T cell polarization. Eur. J. Immunol. *1999*, 4092-101.

Jackson-Grusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E., and Jaenisch, R. (2001). Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nat Genet *27*, 31-9.

Jaenisch, R. (1997). DNA methylation and imprinting. Why bother? Trends Genet. *13*, 323-329.

Kang, J., Coles, M., Cado, D., and Raulet, D. H. (1998). The developmental fate of T cells is critically influenced by TCRgd expression. Immunity *8*, 427-438.

Kass, S. U., Landsberger, N., and Wolffe, A. P. (1997). DNA methylation directs a time-dependent represssion of transcription initiation. Curr Biol *7*, 157-165.

Katamura, K., Fukui, T., Kiyomasu, T., Iio, J. T., G., Ueno, H., Heike, T., Mayumi, M., and Furusho, K. (1998). IL-4 and prostaglandin E2 inhibit hypomethylation of the 5' regulatory region of IFN-gamma gene during differentiation of naive CD4+ T cells. Mol Immunol *35*, 39-45.

Kay, M. A., Holterman, A. X., Meuse, L., Gown, A., Ochs, H. D., Linsley, P. S., and Wilson, C. B. (1995). Long-term hepatic adenovirus-mediated gene expression in mice following CTLA4Ig administration. Nature Genetics *11*, 191-197.

Kisielow, P., and von Boehmer, H. (1995). Development and selection of T cells: facts and puzzles. Adv. Immunol. *58*, 87-209.

Krangel, M. S. (2001). V(D)J recombination becomes accessible. J. Exp. Med. *193*, F27-F30.

Laouar, Y., and Crispe, N. (2000). Functional flexibility in T cells: independent regulation of $CD4^+$ T cell proliferation and effector function in vivo. Immunity *13*, 291-301.

Leduc, I., Hempel, W. M., Mathieu, N., Verthuy, C., Bouvier, G., Watrin, F., and Ferrier, P. (2000). T cell development in TCR beta enhancer-deleted mice: implications for alpha beta T cell lineage commitment and differentiation. J Immunol *165*, 1364-73.

Lee, G. R., Fields, P. E., and Flavell, R. A. (2001). Regulation of IL-4 Gene Expression by Distal Regulatory Elements and GATA-3 at the Chromatin Level. Immunity *14*, 447-59.

Lee, H. J., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O'Garra, A., and Arai, N. (2000). GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. J Exp Med *192*, 105-15.

Lei, H., Oh, S. P., Okano, M., Juttermann, R., Goss, K. A., Jaenisch, R., and Li, E. (1996). De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development *122*, 3195-205.

Lewin, B. (1998). The mystique of epigenetics. Cell *93*, 301-303.

Li, E., Bestor, T. H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915-26.
McMurry, M. T., and Krangle, M. S. (2000). A role for histone acetylation in the developmental regulation of V(D)J recombination. Science *287*, 495-498.

Melvin, A. J., McGurn, M. E., Bort, S. J., Gibson, C., and Lewis, D. B. (1995). Hypomethylation of the interferon-gamma gene correlates with its expression by primary T-lineage cells. European Journal of Immunology *25*, 426-30.

Mostoslavsky, R., and Bergman, Y. (1997). DNA methylation: regulation of gene expression and role in the immune system. Biochimica Et Biophysica Acta *1333*, F29-50.

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacethlase complex. Nature *393*, 386-389.

Okano, M., Xie, S., and Li, E. (1998). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet *19*, 219-220.

Okano, M., Xie, S., and Li, E. (1998). Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res *26*, 2536-40.

Ouyang, W., Lohning, M., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A., and Murphy, K. M. (2000). Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity *12*, 27-37.

Panning, B., and Jaenisch, R. (1998). RNA and the epigenetic regulation of X chromosome inactivation. Cell *93*, 305-8.

Pénit, C., Lucas, B., and Vasseur, F. (1995). Cell expansion and growth arrest phases during the transition from precursor (CD4-8-) to immature (CD4+8+) thymocytes in normal and genetically modified mice. J. Immunol. *154*, 5103-13.

Pestano, G. A., Zhou, Y., Trimble, L. A., Daley, J., Weber, G. F., and Cantor, H. (1999). Inactivation of misselected CD8 T cells by CD8 gene methyaltion and cell death. Science *284*, 187-191.

Reiner, S. L., and Seder, R. A. (1999). Dealing from the evolutionary pawnshop: how lymphocytes make decisions. Immunity *11*, 1-10.

Richter, A., Loehning, M., and Radbruch, A. (1999). Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. J. Exp. Med. *190*, 1439-1450.

Robertson, K. D., Ait-Si-Ali, S., Yokochi, T., Wade, P. A., Jones, P. L., and Wolffe, A. P. (2000). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat Genet *25*, 338-42.

Rountree, M. R., Bachman, K. E., and Baylin, S. B. (2000). DNTM1 binds HDAC2 and a new co-repressor, DMAP-1, to form a comlex at replication foci. Nature Genet. *25*, 338-342.

Sawada, S., Scarborough, J. D., Killeen, N., and Littman, D. R. (1994). A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell *77*, 917-29.

Schlissel, M., Constantinescu, A., Morrow, T., Baxter, M., and Peng, A. (1993). Double-strand signal sequence breaks in V(D)J recombination are blunt, 5'-phosphorylated, RAG-dependent, and cell cycle regulated. Genes Dev *7*, 2520-32.

Schlissel, M. S., Durum, S. D., and Muegge, K. (2000). The interleukin 7 receptor is required for T cell receptor gamma locus accessibility to the V(D)J recombinase. J Exp Med *191*, 1045-50.

Siegfried, Z., and H., C. (1997). DNA methylation: a molecular lock. Curr Biol *7*, R305-307.

Sleckman, B. P., Gorman, J. R., and Alt, F. W. (1996). Accessibility control of antigen-receptor variable-region gene assembly: Role of cis-acting elements. Ann. Rev. Immunol. *14*, 459-481.

Stancheva, I., Hensey, C., and Meehan, R. R. (2001). Loss of the maintenance methyltransferase, xDnmt1, induces apoptosis in Xenopus embryos. Embo J *20*, 1963-73.

Sweetser, M. T., Hoey, T., Sun, Y. L., Weaver, W. M., Price, G. A., and Wilson, C. B. (1998). The roles of nuclear factor of activated T cells and ying-yang 1 in activation-induced expression of the interferon-gamma promoter in T cells. J Biol Chem *273*, 34775-83.

Takemoto, N., Koyano-Nakagawa, N., Yokota, T., Arai, N., Miyatake, S., and Arai, K. (1998). Th2-specific DNase I-hypersensitivity sites in the murine IL-13 and IL-4 intergenic region. Int. Immunol. *10*, 1981-1985.

Tough, D. F., and Sprent, J. (1998). Lifespan of gamma/delta T cells. J Exp Med *187*, 357-65.

von Boehmer, H., Aifantis, I., Feinberg, J., Lechner, O., Saint-Ruf, C., Walter, U., Buer, J., and Azogui, O. (1999). Pleitropic changes controlled by the pre-T-cell receptor. Curr Opin Immunol *11*, 135-142.

Walsh, C. P., Chaillet, J. R., and Bestor, T. H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nature Genet. *20*, 116-117.

Ward, S. B., Hernandez-Hoyos, G., Chen, F., Waterman, M., Reeves, R., and Rothenberg, E. V. (1998). Chromatin remodeling of the interleukin-2 gene: distinct

alterations in the proximal versus distal enhancer regions. Nucleic Acids Res *26*, 2923-34.

Warnecke, P. M., Mann, J. R., Frommer, M., and Clark, S. J. (1998). Bisulfite sequencing in preimplantation embryos: DNA methylation profile of the upstream region of the mouse imprinted H19 gene. Genomics *51*, 182-190.

Whitehurst, C. E., Schlissel, M. S., and Chen, J. (2000). Deletion of germline promoter PD beta 1 from the TCR beta locus causes hypermethylation that impairs D beta 1 recombination by multiple mechanisms. Immunity *13*, 703-14.

Yoder, J. A., Walsh, C. P., and Bestor, T. H. (1997). Cytosine methylation and the ecology of intragenomic parasites. Trends in Genetics *13*, 335-40.

Young, H. A., Ghosh, P., Ye, J., Lederer, J., Lichtman, A., Gerard, J. R., Penix, L., Wilson, C. B., Melvin, A. J., McGurn, M. E., Lewis, D. B., and Taub, D. D. (1994). Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. J. Immunol. *153*, 3603-3610.

Appendix B.

Suppression of Intestinal Neoplasia by Deletion of *Dnmt3b*

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Contribution: I generated the Dnmt3b conditional knockout strain (using a construct that Konstantinos Meletis made) and developed Dnmt3b Southern blot and PCR genotyping assays.

ABSTRACT

Aberrant gene silencing accompanied by DNA methylation is associated with neoplastic progression in many tumors that also show global loss of DNA methylation. Using conditional inactivation of de novo methyltransferase Dnmt3b in *ApcMin/+* mice, we demonstrate that the loss of Dnmt3b has no impact on microadenoma formation, which is considered the earliest stage of intestinal tumor formation. Nevertheless, we observed a significant decrease in the formation of macroscopic colonic adenomas. Interestingly, many large adenomas showed regions with Dnmt3b inactivation, indicating that Dnmt3b is required for initial outgrowth of macroscopic adenomas but is not required for their maintenance. These results support a role for Dnmt3b in the transition stage between microadenoma formation and macroscopic colonic tumor growth and further suggest that Dnmt3b, and by extension de novo methylation, is not required for maintaining tumor growth after this transition stage has occurred.

INTRODUCTION

Altered DNA methylation in the form of global hypomethylation and regional hypermethylation is one of the most consistent epigenetic changes in cancer (18). Global hypomethylation, which is frequently observed at early stages of tumorigenesis in human cancer (10, 11), promotes tumor development in several mouse models and causes chromosomal instability in cultured fibroblasts (9, 12). After the initial observation of DNA hypermethylation within the retinoblastoma tumor suppressor gene (14), dozens of genes have been shown to be hypermethylated and transcriptionally silenced in tumors (2, 20). Although the consequences of global hypomethylation and gene-specific

hypermethylation have been mechanistically connected to chromosome instability and transcriptional silencing, respectively, the causes of aberrant DNA methylation patterns are currently unknown.

DNA methylation is catalyzed by a family of three DNA methyltransferases: Dnmt1, Dnmt3a, and Dnmt3b. Although the three Dnmts partially cooperate to establish and maintain genomic methylation patterns (21), they also have distinctive functions. Dnmt1 has a preference for hemimethylated DNA (1, 15, 38), and indeed a hypomorphic allele of Dnmt1 has been shown to cause global DNA hypomethylation (12). Dnmt1 is therefore considered the major maintenance methyltransferase. Dnmt3a and -3b probably function as de novo DNA methyltransferases because these enzymes were shown to have equal preferences in vitro for unmethylated and hemimethylated DNA (25, 26). Furthermore, de novo methylation of a subset of the CpG sites on stable episomes is detected in human cells overexpressing the murine Dnmt3a or Dnmt3b1 protein (17). Consistent with these notions, inactivation of both *Dnmt3a* and *Dnmt3b* by gene targeting blocks de novo DNA methylation in embryonic stem (ES) cells and early embryos, as well as de novo methylation of imprinted genes in germ cells (25, 26). These findings support the view that Dnmt3a and Dnmt3b function primarily as de novo methyltransferases during normal development. Nevertheless, the role of the de novo Dnmts in cancer is unresolved.

Evidence from humans and mouse models has shown that Dnmt3b is important for maintaining methylation of pericentromeric repetitive elements (5, 25) and of single copy genes in cooperation with Dnmt1 in a human colon cancer cell line (29, 37). Mouse embryonic fibroblasts expressing large T antigen and Ras form soft agar colonies and

large tumors, but similarly treated fibroblasts from *Dnmt3b–/–* mice do not grow in soft agar and are much less tumorigenic in vivo (34). These findings suggest that deletion of *Dnmt3b* inhibits cell transformation. In addition, *Dnmt3b* deficiency promotes the chromosomal instability of mouse embryonic fibroblasts, which in turn promotes spontaneous immortalization or premature senescence (7). Furthermore, deletion of *Dnmt3b* was shown to induce apoptosis in a human cancer cell line (3). Taken together, these observations implicate a role for Dnmt3b in oncogenesis.

Despite these in vitro studies, there is no direct evidence in vivo to clarify the role of *Dnmt3b* in tumorigenesis. Here we show that *Dnmt3b* expression is elevated in colonic adenomas derived from the *ApcMin/+* mouse model. We therefore chose this in vivo tumor model to analyze the role of Dnmt3b in tumor initiation and progression by using Cre-Lox mediated conditional *Dnmt3b* gene deletion in the intestinal mucosa. We found that the removal of *Dnmt3b* does not affect tumor initiation in the form of microadenomas but does inhibit the formation of macroscopic tumors at an early stage of tumor development.

MATERIALS AND METHODS

Construction of the Dnmt3b conditional knockout targeting vector.

A 10-kb XhoI-EcoRV genomic DNA fragment containing the catalytic domain and surrounding regions from the *Dnmt3b* locus was isolated from a 129/SvEvTac female mouse bacterial artificial chromosome library (Roswell Park Cancer Institute) by hybridization to a Dnmt3b cDNA probe. The fragment was subsequently cloned into the pBluescript II SK vector (Stratagene), generating plasmid pBS3bXEV, which was used to generate the conditional knockout construct.

A loxP site was inserted into the intron just 3' of the exon 19, which encodes DNA methyltransferase motif VI. In addition, a drug selection marker in the form of a

neomycin phosphotransferase gene under the control of the phosphoglycerate kinase 1 gene promoter (pgk-neo cassette flanked by Frt sites) (28) was inserted into the intron between exons 15 and 16 of the Dnmt3b locus, which are 5' to the catalytic domain. The pgk-neo cassette was flanked by frt sites to facilitate removal by flp recombinase. A loxP site was also included just 5' to the pgk-neo cassette.

In the final targeting construct, pBS3blfN, the loxP sites flank exons 16 to 19, which encode the catalytic domains. Arms of genomic DNA from the Dnmt3b locus—a 1.9-kb HindII-EcoRV fragment on the 5' side and a 4.5-kb HindII fragment on the 3' side—surround the loxP-flanked region. The orientation of pieces in the targeting construct was confirmed by restriction digest analysis and sequencing.

Generation of ES cells and mice with conditional knockout alleles of Dnmt3b.

Plasmid pBS3blfN was linearized with SacII and electroporated into F_1 (129SvJae x C57BL/6; v6.5 line) mouse ES cells and selected for drug resistance against neomycin as described previously. Neomycin-resistant ES colonies were analyzed by Southern blotting. In ca. 45% of the picked colonies, the targeting construct had integrated properly into the Dnmt3b locus.

In order to remove the pgk-neo cassette, ES cells from a targeted clone were transiently transfected with enhanced flp (Flp-e) recombinase-encoding plasmids (a gift from Francis Stewart) (4) by lipofection with Cytofectene (Bio-Rad). Removal of the pgk-neo cassette was confirmed by PCR, Southern blotting, and by death in G418-containing medium (350 μg/ml). Sixteen of twenty-four subclones in this experiment no longer contained the neomycin resistance gene.

For injection into blastocysts, we used ES cells from one properly targeted subclone in which the neomycin resistance cassette was removed. The resulting chimeras were crossed with C57BL/6 mice to generate the founder colony.

Mice and polyp analysis.

Mice were maintained in the facilities of Whitehead Institute for Biomedical Research. The conditional Dnmt3b allele was backcrossed to the C57BL/6 strain prior to crossing with *ApcMin*/+ mice. All tumor bearing mice analyzed in this experiment were backcrossed at least six generations into C57BL/6 mice. For tumor analysis, the entire intestine was excised immediately after sacrifice and subjected to a systematic microscopic screen for tumor formation along the entire length of the intestine. The investigator counting the adenomas was blind with regard to mouse genotype.

Genotyping.

DNA was isolated from tail tips and amplified by PCR for the Dnmt3b locus as follows: Dnmt3bF (5'-GCTGAATTATACCCGCCCCAAGGAGGGCG-3'; located just 5' of the 3'loxP site) and Dnmt3bR (5'-CCCTTCAAAGGTGCATCGTGGCCAGTGTGC-3'; located in the 3' arm of the conditional construct). PCR products are 410 bp for the wild-type allele and 550 bp for the 2lox allele. For the Dnmt3b 1lox allele, Dnmt3bR was used in conjunction with Dnmt3b1F (5'-CCTGAGAGACTGGCCGGCTTTTCCTCAGAC-3'; located in the 5' arm of the conditional construct), generating a roughly 500-bp product from the Dnmt3b 1lox allele. C57BL/6 *ApcMin*/+ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Oligonucleotides RM21a (5'-TTCTGAGAAAGACAGAAGTTA-3'), RM20 (5'-TTCCACTTTGGCATAAGGC-3'), and RM19 (5'-GCCATCCCTTCACGTTAG-3' were used for genotyping. The Fabp-Cre mice (a gift from Jeffrey I. Gordon, Washington University, St. Louis, MO) were backcrossed to the C57BL/6 strain for more than 10 generations. Cre-positive mice were determined by PCR with the oligonucleotides CreF (5'-TGGGCGGCATGGTGCAAGTT-3') and CreR (5'-CGGTGCTAACCAGCGTTTTC-3').

RESULTS

Dnmt3b is activated in the intestinal tumors of *ApcMin*/+ mice.

Elevated expression of Dnmt3b has been observed in many human tumors (13, 23, 24, 30, 32). We therefore sought to identify a mouse tumor model that recapitulated Dnmt3b overexpression for in vivo analysis of the functional importance of de novo methyltransferase upregulation. Western blot analysis of intestinal macroscopic tumors from *ApcMin*/+ mice revealed that expression of Dnmt3b is substantially higher in tumors compared to normal epithelium (Fig. 1A). Consistent with this result, immunohistochemical staining of sections through polyps identified strong and specific staining with an anti-Dnmt3b antibody, in contrast to the adjacent normal epithelium, which showed weak staining (Fig. 1B). These results demonstrate that Dnmt3b expression is elevated in the intestinal tumors of *ApcMin*/+ mice.

Deletion of *Dnmt3b* in the intestine.

To investigate whether *Dnmt3b* overexpression plays a causal role in intestinal carcinogenesis, we generated a conditional *Dnmt3b* allele that would allow intestine-specific deletion of this gene, since *Dnmt3b*-null mutant mice do not survive past midgestation (25). Deletion of the exons encoding highly conserved PC and ENV DNA methyltransferase catalytic motifs (motifs IV and VI) of Dnmt3b has previously been shown to result in a null mutation in this gene (25). We therefore created an analogous conditional mutation in *Dnmt3b*, by placing loxP sites flanking exons 16 through 19 using homologous recombination in V6.5 ES cells (Fig. 2A and see also Materials and Methods). Successfully targeted ES cells bearing a conditional knockout ("2lox") allele were analyzed by Western blotting with a Dnmt3b antibody and shown to express normal levels of Dnmt3b protein, confirming that the *Dnmt3b2lox* allele is a silent mutation (Fig. 2B). Western analysis of *Dnmt3b1lox/1lox* ES cells, similarly confirmed that the deleted *Dnmt3b1lox* allele is null (Fig. 2B).

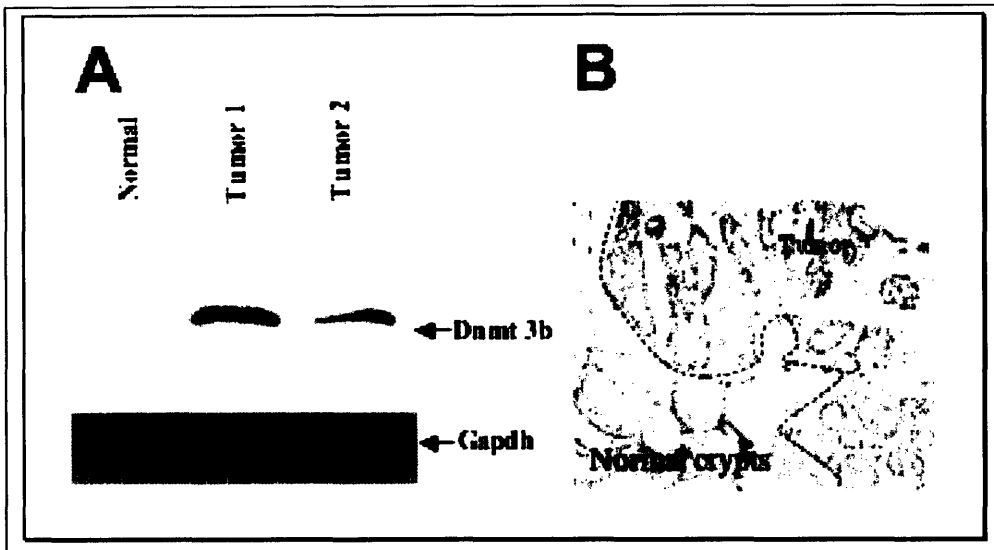

Figure 1. Expression level of Dnmt3b in intestinal tumors of *ApcMin/+* mice. **(A)** Western blot showing expression levels of Dnmt3b in intestinal macroadenomas and adjacent normal mucosa from *ApcMin/+* mice. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) serves as the loading control. **(B)** Immunohistochemical analysis with an anti-Dnmt3b antibody of a macroscopic colon tumor and adjacent normal crypts.

Dnmt3b2lox/+ ES cells were injected into blastocysts to generate chimeric mice, which were backcrossed to the C57BL/6 strain for subsequent crosses with *ApcMin*/+ mice. An intercross between *Dnmt3b2lox*/+ mice generated 13 2lox/2lox, 30 2lox/+, and 17 +/+ mice (total $n = 60$), a finding consistent with expected Mendelian ratios ($P < 0.05$, chi-square analysis) and, in contrast to the embryonic lethal phenotype observed for the *Dnmt3b*-null mutants (25), *Dnmt3b2lox/2lox* mice are healthy and fertile with no phenotype observed through 1 year of age.

To achieve intestine-specific deletion, a Cre transgene under the control of transcriptional regulatory elements from the fatty acid-binding protein gene (*Fabp-Cre*) (31) was used to generate *Dnmt3b2lox/2lox*, *Fabp-Cre* mice. Cre-mediated deletion of *Dnmt3b* was assessed by Southern blotting, which revealed ca. 50% recombination in the colon, but no recombination was detected in the small intestine from the same mice (Fig. 2C). To assess further the efficiency of Cre-mediated deletion within individual crypts, sections of the colon and small intestine of 8- and 21-week-old animals were stained with an anti-Dnmt3b antibody. Immunostaining showed ca. 50% of colon crypts stained positive for Dnmt3b in animals of both ages (Fig. 2D and 2E). Similar analysis of small intestinal crypts showed uniform expression of Dnmt3b in *Dnmt3b2lox/2lox Fabp-Cre* mice (data not shown), a finding consistent with the lack of recombination observed by Southern blotting in this tissue (Fig. 2C). Within the colon, immunostaining demonstrated that all cells of a given colon crypt were either Dnmt3b positive or negative, indicating that deletion of *Dnmt3b* likely occurred in crypt forming stem cells. Histological analysis did not reveal any significant phenotypic differences between Dnmt3b-positive and -negative crypts (Fig. 2D and E and data not shown).

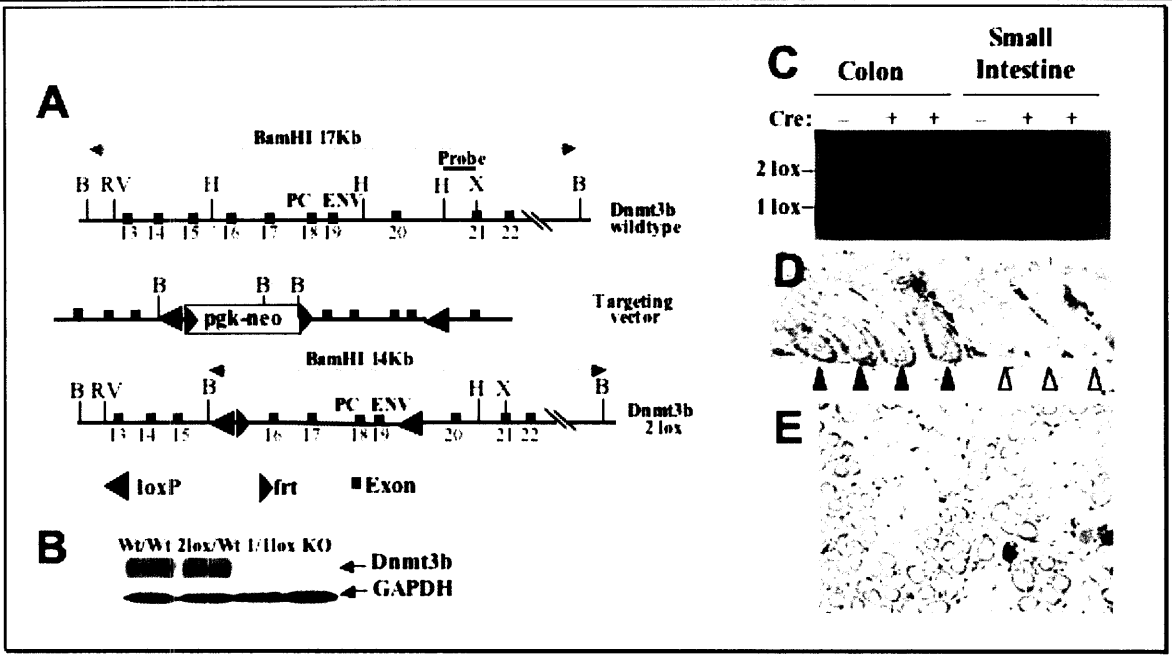

Figure 2. Targeting vector of conditional knockout and the frequency of Fabp-Cre mediated the recombination of conditional *Dnmt3b* allele in the intestine. (**A**) Restriction map of the Dnmt3b conditional knockout targeting vector. Restriction sites: B, BamHI; H, HindIII; RV, EcoRV; X, XhoI. (**B**) Western blot demonstrating the protein level of Dnmt3b in *Dnmt3b 2lox*/wild-type ES cells and *Dnmt3b 1lox/1lox* ES cells. The *Dnmt3b* knockout ES cell is a negative control (a gift from E. Li). (**C**) Southern blot demonstrating Fabp-Cre-mediated recombination of the conditional *Dnmt3b* allele in the colon and small intestinal mucosa harvested from *Dnmt3b2lox/2lox Fabp-Cre*-positive and -negative mice (14 kb for the *2lox* allele and 11 kb for the *1lox* allele). **(D and E)** Longitudinal section and cross section, respectively, of the colonic mucosa from a *Dnmt3b2lox/2lox Fabp-Cre*-positive and -negative mouse stained with an anti-Dnmt3b antibody, demonstrating the Fabp-Cre-mediated recombination of the conditional *Dnmt3b* allele.

Deletion of *Dnmt3b* decreases the number of colon tumors but not the formation of microadenomas.

To study the effects of *Dnmt3b* deletion on intestinal tumorigenesis in *ApcMin/+* mice, cohorts of 23 *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* and 18 *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre*$^-$ mice were sacrificed at 150 days of age to quantify intestinal tumors. Since Fabp-Cre has different recombination efficiencies in different regions of the intestinal tract (Fig. 2C), tumor numbers in the small intestine and the colon were scored separately. Conditional deletion of *Dnmt3b* reduced the total number of colonic tumors by ca. 40% ($P = 0.0012$; Fig. 3A) as shown by the average colonic tumor burden of 2.75 ± 0.6 polyps in *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* mice, whereas *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre–* mice had a mean macroscopic tumor load of 5.2 ± 0.7 colonic polyps. Based on the low recombination rate detected in the small intestine of Fabp-Cre mice, we anticipated little difference in tumor load in the small intestine of *Fabp-Cre*-positive and -negative cohorts. No significant difference was observed ($P = 0.89$, Fig. 3B), which shows the specificity of the result for Dnmt3b deletion in colon and supports the notion that Dnmt3b deficiency inhibits tumor formation in *ApcMin/+* mice.

Tumor formation in *ApcMin/+* mice occurs in two distinct stages, which are referred to as microadenoma and macroscopic adenoma. Microadenomas are considered to be the first stage of tumorigenesis and are detectable as histologically abnormal crypts that have mostly undergone loss of heterozygosity (LOH) at the *Apc* locus and therefore stain positive for ß-catenin (39). To discern whether Dnmt3b affects the initial stage of tumor formation, we compared the number of microadenomas in the colons of *ApcMin/+ Dnmt3b2lox/2lox* Fabp-Cre-positive and -negative mice. Microadenomas were scored on en face sections of the colonic mucosa by staining with anti-ß-catenin antibody. *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* mice had a mean microadenoma load of 7.2 ± 1.2/cm^2 in the colon and *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre–* mice had a mean

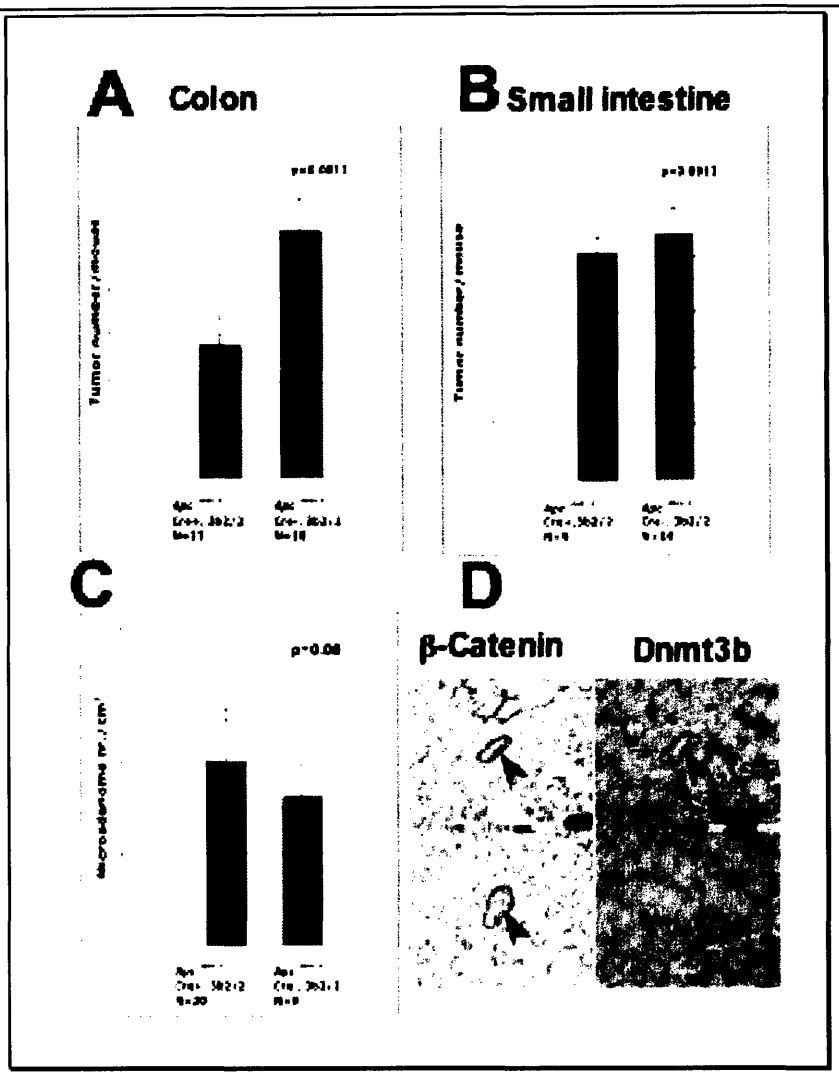

Figure 3. Effect of Dnmt3b deletion on the number of macroscopic tumors and microadenomas in the colon and small intestine of *Apcmin*/+mice. **(A and B)** Number of macroscopic tumors in the colons and small intestines of *Apcmin*/+ *Dnmt3b2lox/2lox* mice that either carried (*ApcMin*/+ *Dnmt3b2lox/2lox Fabp-Cre*+) or did not carry (*ApcMin*/+ *Dnmt3b2lox/2lox Fabp-Cre*–) the *Fabp-Cre* transgene. **(C)** The number of colonic microadenomas in *ApcMin*/+ *Dnmt3b2lox/2lox* mice that either carried or did not carry the *Fabp-Cre* transgene was determined by immunohistochemical analysis for ß-catenin. **(D)** Dnmt3b-positive and Dnmt3b-negative microadenomas in the colonic mucosa of *ApcMin*/+ *Dnmt3b2lox/2lox Fabp-Cre*+ mice. The left panel illustrates our method of microadenoma detection using an anti-ß-catenin antibody. The right panel illustrates immunohistochemical analysis of microadenomas with an anti-Dnmt3b antibody. The right upper picture shows a Dnmt3b-positive microadenoma, and the right lower picture shows a Dnmt3b-negative microadenoma.

microadenoma load of 6.5 ± 1.3/cm^2 (Fig. 3C). The marginal decrease in microadenomas observed was not statistically significant (P = 0.08). These results suggest that Dnmt3b deletion has no impact on the formation of microadenomas. To support this notion further, microadenomas of *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* mice were stained with anti-Dnmt3b antibody, revealing ca. 50% Dnmt3b-positive and 50% Dnmt3b-negative microadenomas (Fig. 3D). Since this ratio reflects the recombination frequency in the colonic mucosa (Fig. 2C), these results are consistent with the conclusion that Dnmt3b deficiency has no effect on microadenoma formation. Taken together, these data suggest that the inhibition of macroscopic tumor formation in *ApcMin/+* mice by Dnmt3b conditional mutants occurs later during either initiation or growth of macroscopic tumors.

Dnmt3b is not essential for tumor growth.

The consistency in the frequency of Cre-mediated recombination within the colon (50%, Fig. 2C to E), along with the nearly twofold reduction in colonic polyps in the Cre-expressing cohort (Fig. 3A), suggested that deletion of *Dnmt3b* might provide a strong block to macroscopic tumor formation. To assess whether loss of Dnmt3b was compatible with macroscopic tumor growth, immunohistochemical staining of tumor sections using a Dnmt3b antibody was performed to determine the number of Dnmt3b-positive and -negative tumors. This analysis revealed that polyps from *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* mice were predominantly composed of both Dnmt3b-positive and -negative sectors, with a few sections showing homogeneous wild-type or mutant staining patterns (Fig. 4A and Table 1). To quantitate the extent of recombination throughout the tumors, we developed a competitive PCR assay to measure the ratio of 1lox to 2lox *Dnmt3b* alleles in DNA isolated from individual whole colon tumors (Fig. 4B). DNA samples from 2lox/2lox and 1lox/1lox ES cells were mixed in defined ratios to standardize the PCR conditions. This analysis revealed that most macroscopic tumors contained 2lox as

well as 1lox *Dnmt3b* alleles, a few contained only the nonrecombined 2lox allele, and almost no tumors were observed with greater than 50% contribution of 1lox alleles.

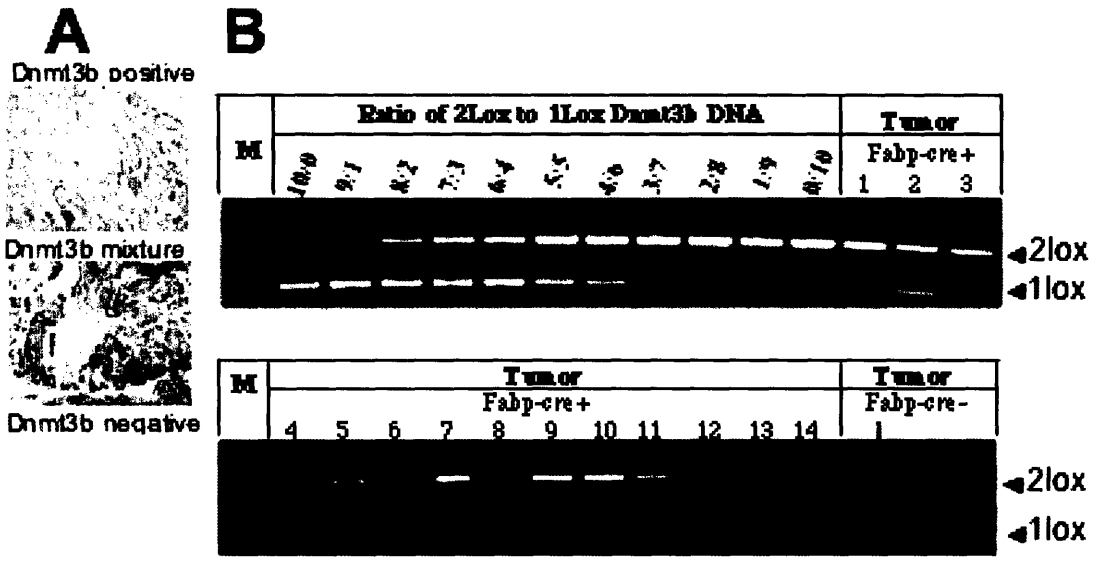

Figure 4. Most colon tumors from *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* mice are both Dnmt3b positive and negative. **(A)** Immunohistochemical analysis with an anti-Dnmt3b antibody illustrating expression of *Dnmt3b* in colon tumors of *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* mice. The left picture illustrates a purely Dnmt3b-positive section, the middle picture shows a mixed Dnmt3b-positive and -negative section, and the right picture shows a pure Dnmt3b-negative section. **(B)** PCR analysis shows that most colon tumors from *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* mice contain both *1lox* and *2lox* alleles of Dnmt3b. The top panel shows a reference PCR using different ratios of 2lox to 1lox Dnmt3b genomic DNA. The top right and lower panels show the PCR analysis of colon tumors from *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* mice.

TABLE 1. Loss of *Dnmt3b* observed in early-stage microadenomas is selected against in later-stage colonic tumors

		% Dnmt3b expression (*n*)[a]		
Tissue Analyzed	*n*	Positive	Mixed	Negative
Colon crypts	98	48 (49)		50 (51)
Microadenomas	23	11 (48)		12 (52)
Tumors	45[b]	14 (31)	26 (58)	5 (11)

[a]Dnmt3b expression was determined by immunohistochemistry on sections of Dnmt3b conditional mutant colonic tissue. Microadenomas were identified by beta-catenin staining of adjacent sections.

[b]These data include 14 tumors analyzed by PCR, using a genotyping assay for recombination at the *Dnmt3b* locus shown in Fig. 4B, and 31 tumors analyzed by immunostaining tumor sections.

The immunostaining and PCR analysis together demonstrated a partial contribution of Dnmt3b mutant cells in a majority of the macroscopic colon tumors in *ApcMin/+ Dnmt3b* conditional mutant animals (as summarized in Table 1). Since Cre-mediated recombination is irreversible and the majority of tumors in *ApcMin/+* mice are considered to be monoclonal (22, 27, 36), this suggests that Dnmt3b deletion occurred after tumor initiation during tumor growth. Two observations are consistent with this idea. First, we observed Cre expression in macroscopic tumors by reverse transcription-PCR (data not shown). Second, the areas lacking Dnmt3b expression were always seen as coherent patches (Fig. 4A, middle panel), indicating that the Dnmt3b-negative tumor sections were clonally derived from a tumor cell that had recombined *Dnmt3b 2lox* alleles during tumor growth.

The presence of clonal sectors of Dnmt3b mutant cells within the macroscopic tumor lesions suggested that Dnmt3b function is dispensable in an already-formed colon tumor. To assess whether Dnmt3b deletion affects the proliferation rate of tumors, histological sections of colon polyps were stained with a cellular replication marker (Ki67 antibody). Consistent with the notion that Dnmt3b is not required in adenomas, no significant difference in Ki67 staining was observed between Dnmt3b-positive (45.7% ± 1.4% positive cells per crypt) and -negative (42.8% ± 1.45% positive cells per crypt) tumor sectors (Fig. 5A, $P = 0.185$). In addition, TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) staining revealed 0.90% ± 0.2% and 0.82% ± 0.3% TUNEL-positive cells/crypt in Dnmt3b-positive and -negative areas, respectively ($P = 0.66$), indicating there was no effect of the *Dnmt3b* deletion on tumor cell apoptosis (Fig. 5B). Furthermore, we calculated the tumor volume from *ApcMin/+ Dnmt3b2lox/2lox* mice that either carried or did not carry the Fabp-Cre transgene. Figure 5C shows that the average volume of colon tumors from *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* mice (11.84 ± 1.5 mm^3) was not significantly different from the average tumor volume in *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre–* mice (11.04 ± 1.4

mm^3; $P = 0.68$). We therefore conclude that Dnmt3b deficiency impairs the initiation of macroscopic tumors but has no noticeable effect on later stages of tumor growth (Fig. 6).

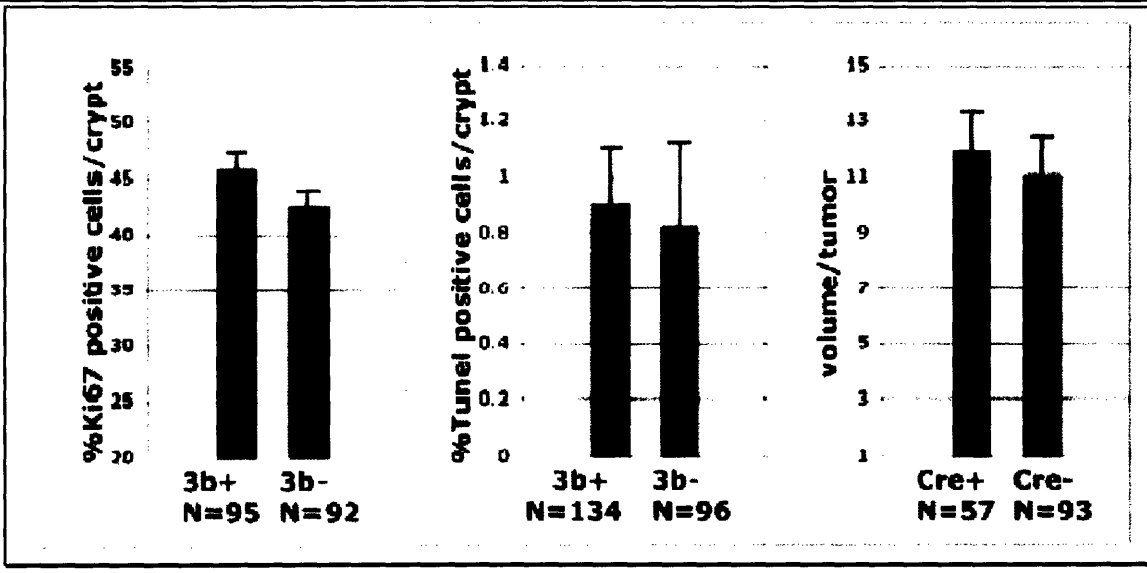

Figure 5. Effect of *Dnmt3b* deletion on tumor growth and apoptosis. **(A)** Percentage of Ki67-positive cells per crypt in either the Dnmt3b-positive or -negative region of the mixed tumor. **(B)** Percentage of TUNEL-positive cells per crypt in either the Dnmt3b-positive or the Dnmt3b-negative region of the mixed tumor. **(C)** Average volume (in cubic millimeters) of colon macroscopic tumors from *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* and *Fabp-Cre–* mice.

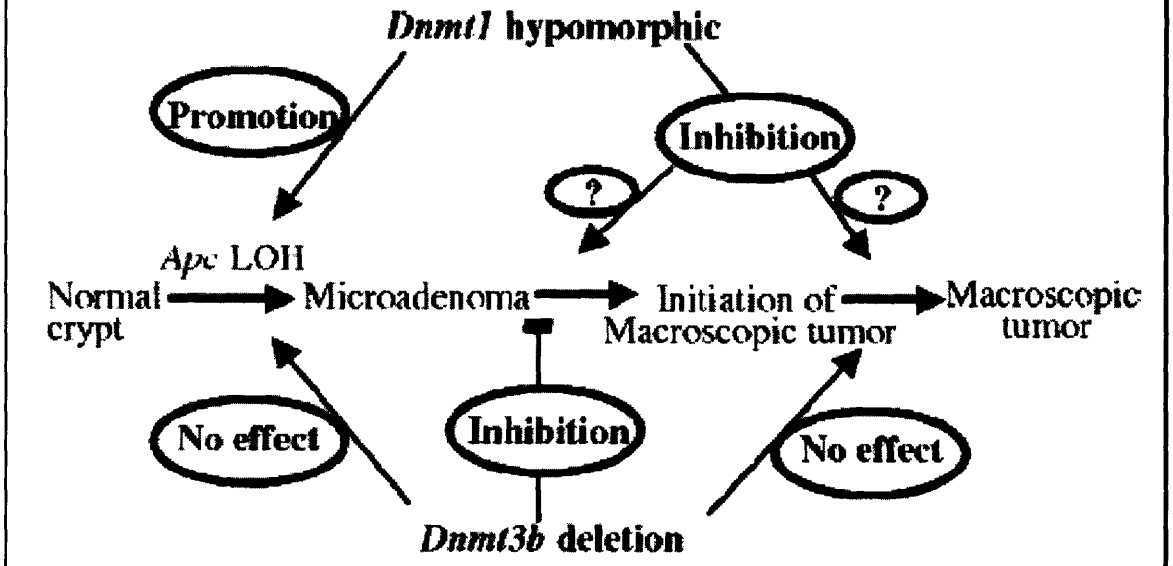

Figure 6. Model depicting the effects of Dnmt1 and Dnmt3b on various stages of colon tumorigenesis in *ApcMin* mice.

DISCUSSION

In this study we investigated the role of Dnmt3b in intestinal tumorigenesis of *ApcMin/+* mice. Colon tumorigenesis in *ApcMin/+* mice occurs in two distinct stages, with microadenomas representing the first stage, followed by the development of macroscopic adenomas. Western blot and immunohistochemical analyses indicated that Dnmt3b was overexpressed in adenomas of *ApcMin/+* mice compared to normal intestinal mucosa. To assess whether Dnmt3b was causally involved in tumorigenesis, we generated a conditional allele of *Dnmt3b* gene and used a Fabp-Cre transgene to delete this enzyme in the intestinal epithelium. The frequency of Cre-mediated *Dnmt3b* deletion was 50% in the colon crypts, with little or no deletion in the small intestine. When the number of microadenomas in the colons of *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* and *Fabp-Cre–* mice were compared, we found no differences, indicating that Dnmt3b deficiency had no effect on the formation of microadenomas. Consistent with this conclusion was the finding that 50% of all microadenomas in the colon of *ApcMin/+ Dnmt3b2lox/2lox Fabp-Cre+* were positive and 50% were negative for Dnmt3b, a ratio that corresponds to the Fabp-Cre-mediated recombination frequency in normal crypts. In contrast to microadenoma formation, *Dnmt3b* deficiency reduced the load of macroscopic adenomas in the colon by ca. 40%. No changes in tumor multiplicity were observed in the small intestine that could be attributed to Dnmt3b inactivation, because of the low to undetectable recombination frequency in this region. Together, these results support the notion that Dnmt3b deletion selectively inhibits the formation of macroscopic colonic tumors but does not affect the formation of colonic microadenomas.

Two findings indicate that Dnmt3b activity and, by extension, de novo methylation are required specifically at the transition from microadenoma to macroscopic tumor outgrowth. First, as summarized in Table 1, roughly 50% of microadenomas lacked Dnmt3b expression, which was equated to the recombination frequency in the colon. However, only 10% of macroscopic tumors developed without Dnmt3b, suggesting that

the outgrowth of the Dnmt3b deficient microadenomas was selected against. Second, the subsequent maintenance of a tumor outgrowth was not dependent upon Dnmt3b since both DNA and immunohistochemical analyses indicated that most tumors in *Fabp-Cre+* mice were mixed, being composed of cells carrying the unrecombined *2lox Dnmt3b* allele as well as the deleted *Dnmt3b 1lox* allele. Together, these two results strongly suggest that Dnmt3b deletion selectively inhibits the transition from microadenoma to macroscopic adenoma and that the majority of macroscopic adenomas in these mice were initiated in cells with a nonrecombined *Dnmt3b* conditional allele (2lox). Persistent or stochastic expression of Cre recombinase within an already formed tumor may have allowed recombination to occur at some later point after adenoma outgrowth, which resulted in clonal expansion of Dnmt3b-negative sectors in a given adenoma. This interpretation is supported by immunostaining, which showed that the region of the tumor lacking Dnmt3b expression always appeared as coherent patches of variable size. Although immunohistochemical analyses indicated that ca. 10% of the adenomas lacked Dnmt3b expression, these were analyzed on representative sections and may even be an overestimate of the number of tumors that formed entirely lacking Dnmt3b (Fig. 4A). In a less biased analysis, DNA isolated from whole tumor samples failed to confirm the presence of a tumor with greater than 50% contribution by Dnmt3b mutant cells (Fig. 4B), and in fact most of the mixed tumors had a contribution of less than 20% based on the competitive PCR analysis. Together, these results suggest that Dnmt3b deficiency resulted in a more than three- to fourfold reduction in macroscopic tumor incidence, owing to a selective requirement for de novo methylation at the transition from micro- to macroadenoma (Fig. 6).

It has been suggested, based on the analysis of chimeric *ApcMin/+* mice, that tumors in the small intestine arise by active interactions between crypts promoting loss of *Apc* and thus resulting in the formation of polyclonal adenomas (36). In that study, however, only 22% of all tumors were identified as polyclonal. A similar experiment

analyzing chimeric *ApcMin/+* mice, using lacZ as a reporter, yielded only 9% mixed tumors (22). Also, in our experimental system, an average of only three adenomas were observed in the colon, in contrast to an average of 17 tumors in the small intestine of the chimeric mice (36). Because the same frequency of recombination in crypts and microadenomas was observed in our study and the microadenomas were homogeneous in Dnmt3b expression (Table 1), our data indicate the initiating event of LOH at the APC locus must precede the local recruitment of a similarly mutated crypt to produce the mixed tumor class according to the collision model. The low incidence of lesions in our system virtually excludes the possibility that the random collision model accounts for a substantial fraction of the majority class of mixed tumors observed in the *ApcMin/+* Dnmt3b conditional mutants. Consistent with this conclusion are other studies that also demonstrated a monoclonal origin of colon tumors in mice (27). We think it more likely that persistent and stochastic expression of Cre recombinase within colonic tumors, which we observed by reverse transcription-PCR, explains the formation of the mixed class of tumors in these animals.

It has been shown previously that global DNA hypomethylation can protect as well as enhance tumor formation (8, 12, 19). For example, reduced Dnmt1 activity was demonstrated to inhibit adenoma formation in *ApcMin/+* mice but to promote formation of lymphomas in the thymus and of sarcomas in soft tissues (9, 12). Enhanced thymoma and sarcoma formation was due to increased genetic instability and elevated LOH frequencies of tumor suppressor genes. Consistent with this finding is our recent observation that reduced levels of Dnmt1 expression increased the number of microadenomas in *ApcMin/+* mice (39) by enhancing the rate of *Apc* LOH (Fig. 6). In contrast to reduced Dnmt1 activity, the somatic deletion of Dnmt3b would be predicted to have no effect on global methylation levels and thus would not be expected to affect APC LOH. In agreement with this prediction is the finding that microadenoma formation is unaffected by loss of Dnmt3b (Fig. 6).

Protection against macroscopic tumors by reduced Dnmt1 expression was suggested to be due to an epigenetic mechanism such as the impaired maintenance of methylation marks that are acquired during tumorigenesis. For example, de novo methylation of tumor suppressor genes of the *H19 DMR* has been shown to promote intestinal tumor load by loss of imprinting of *Igf2* (6, 33). Because the maintenance of methylation marks depends on Dnmt1 activity, a reduced Dnmt1 level may lead to loss of DMR methylation causing reduced *Igf2* expression and thus protect against intestinal tumors. Our results indicate that somatic deletion of Dnmt3b also protects against polyp formation, suggesting that this de novo methyltransferase is causally involved in tumor formation, possibly by promoting de novo methylation that mediates silencing of tumor suppressor genes (35) or loss of imprinting of genes such as *Igf2* (6, 33). This predicts that, once tumor growth is initiated, continuous expression of Dnmt3b would not be required for further tumor growth (Fig. 6). Indeed, we found that loss of Dnmt3b has no noticeable effect on the growth of established tumors as the proliferation and apoptosis rate of cells was similar in Dnmt3b-positive and -negative sectors of a given tumor. Also, we failed to detect any significant difference in the average tumor volume when comparing *Dnmt3b2lox/2lox ApcMin/+* mice with or without the *Fabp-Cre* transgene, which is consistent with tumor growth being unaffected by Dnmt3b. These studies are consistent with the results obtained by targeted disruption of the human *DNMT3B* gene in the human colon cancer cell line HCT116, which resulted in little to no change in the global DNA methylation or growth of these cells. Although the disruption of both *DNMT1* and *DNMT3B* caused DNA hypomethylation in the same colorectal cancer cell line, our results suggests that *Dnmt1* alone is sufficient to support the proliferation and maintenance of tumors in *ApcMin* mice (29, 37). Thus, although Dnmt3b is required during the initial outgrowth phase of macroscopic colonic adenomas in these mice, it has no effect on subsequent tumor growth and maintenance (Fig. 6).

Our previous observation that inhibition of Dnmt1 protected against intestinal tumorigenesis (19) sparked efforts to develop inhibitors of Dnmt1 that could be administered prophylactically to FAP patients carrying an *Apc* mutation. However, the later recognition that Dnmt1 inhibition can impair genomic stability and enhance tumor formation in other tissues such as the thymus or in soft tissues (9, 12) cautioned against long-term treatment with Dnmt1 inhibitors. Since Dnmt3b inhibition has no effect on microadenoma formation and, by implication, on genomic stability but strongly inhibits macroscopic tumor initiation, drug-mediated inhibition of this enzyme may be effective as a prophylactic agent to protect against tumors without the potential side effects of Dnmt1 inhibitors.

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REFERENCES

1. **Bacolla, A., S. Pradhan, J. E. Larson, R. J. Roberts, and R. D. Wells.** 2001. Recombinant human DNA (cytosine-5) methyltransferase. III. Allosteric control, reaction order, and influence of plasmid topology and triplet repeat length on methylation of the fragile X CGG.CCG sequence. J. Biol. Chem. **276:**18605-18613.

2. **Baylin, S. B., M. Esteller, M. R. Rountree, K. E. Bachman, K. Schuebel, and J. G. Herman.** 2001. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum. Mol. Genet. **10:**687-692.

3. **Beaulieu, N., S. Morin, I. C. Chute, M. F. Robert, H. Nguyen, and A. R. MacLeod.** 2002. An essential role for DNA methyltransferase DNMT3B in cancer cell survival. J. Biol. Chem. **277:**28176-28181.

4. **Buchholz, F., P. O. Angrand, and A. F. Stewart.** 1998. Improved properties of FLP recombinase evolved by cycling mutagenesis. Nat. Biotechnol. **16:**657-662.

5. **Chen, T., N. Tsujimoto, and E. Li.** 2004. The PWWP domain of Dnmt3a and Dnmt3b is required for directing DNA methylation to the major satellite repeats at pericentric heterochromatin. Mol. Cell. Biol. **24:**9048-9058.

6. **Cui, H., M. Cruz-Correa, F. M. Giardiello, D. F. Hutcheon, D. R. Kafonek, S. Brandenburg, Y. Wu, X. He, N. R. Powe, and A. P. Feinberg.** 2003. Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. Science **299:**1753-1755.

7. **Dodge, J. E., M. Okano, F. Dick, N. Tsujimoto, T. Chen, S. Wang, Y. Ueda, N. Dyson, and E. Li.** 2005. Inactivation of Dnmt3b in mouse embryonic fibroblasts results in DNA hypomethylation, chromosomal instability, and spontaneous immortalization. J. Biol. Chem. **280:**17986-17991.

8. **Eads, C. A., A. E. Nickel, and P. W. Laird.** 2002. Complete genetic suppression of polyp formation and reduction of CpG-island hypermethylation in Apc*Min*/+ Dnmt1-hypomorphic mice. Cancer Res. **62:**1296-1299.

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

10. **Feinberg, A. P., C. W. Gehrke, K. C. Kuo, and M. Ehrlich.** 1988. Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res. **48:**1159-1161.

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

12. **Gaudet, F., J. G. Hodgson, A. Eden, L. Jackson-Grusby, J. Dausman, J. W. Gray, H. Leonhardt, and R. Jaenisch.** 2003. Induction of tumors in mice by genomic hypomethylation. Science **300:**489-492.

13. **Girault, I., S. Tozlu, R. Lidereau, and I. Bieche.** 2003. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin. Cancer Res. **9:**4415-4422.

14. **Greger, V., E. Passarge, W. Hopping, E. Messmer, and B. Horsthemke.** 1989. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum. Genet. **83:**155-158.

15. **Gruenbaum, Y., H. Cedar, and A. Razin.** 1982. Substrate and sequence specificity of a eukaryotic DNA methylase. Nature **295:**620-622.

16. **Hochedlinger, K., Y. Yamada, C. Beard, and R. Jaenisch.** 2005. Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell **121:**465-477.

17. **Hsieh, C. L.** 1999. In vivo activity of murine de novo methyltransferases, Dnmt3a and Dnmt3b. Mol. Cell. Biol. **19:**8211-8218.

18. **Jones, P. A., and S. B. Baylin.** 2002. The fundamental role of epigenetic events in cancer. Nat. Rev. Genet. **3:**415-428.

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

hypomethylation. Cell **81:**197-205.

20. **Levine, J. J., K. M. Stimson-Crider, and P. M. Vertino.** 2003. Effects of methylation on expression of TMS1/ASC in human breast cancer cells. Oncogene **22:**3475-3488.

21. **Liang, G., M. F. Chan, Y. Tomigahara, Y. C. Tsai, F. A. Gonzales, E. Li, P. W. Laird, and P. A. Jones.** 2002. Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Mol. Cell. Biol. **22:**480-491.

22. **Merritt, A. J., K. A. Gould, and W. F. Dove.** 1997. Polyclonal structure of intestinal adenomas in ApcMin/+ mice with concomitant loss of Apc+ from all tumor lineages. Proc. Natl. Acad. Sci. USA **94:**13927-13931.

23. **Mizuno, S., T. Chijiwa, T. Okamura, K. Akashi, Y. Fukumaki, Y. Niho, and H. Sasaki.** 2001. Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood **97:**1172-1179.

24. **Nagai, M., A. Nakamura, R. Makino, and K. Mitamura.** 2003. Expression of DNA (5-cytosin)-methyltransferases (DNMTs) in hepatocellular carcinomas. Hepatol. Res. **26:**186-191.

25. **Okano, M., D. W. Bell, D. A. Haber, and E. Li.** 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell **99:**247-257.

26. **Okano, M., S. Xie, and E. Li.** 1998. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat. Genet. **19:**219-220.

27. **Ponder, B. A., and M. M. Wilkinson.** 1986. Direct examination of the clonality of carcinogen-induced colonic epithelial dysplasia in chimeric mice. J. Natl. Cancer Inst. **77:**967-976.

28. **Possemato, R., K. Eggan, B. J. Moeller, R. Jaenisch, and L. Jackson-Grusby.** 2002. Flp recombinase regulated lacZ expression at the ROSA26 locus. Genesis **32:**184-186.

29. **Rhee, I., K. E. Bachman, B. H. Park, K. W. Jair, R. W. Yen, K. E. Schuebel, H. Cui, A. P. Feinberg, C. Lengauer, K. W. Kinzler, S. B. Baylin, and B. Vogelstein.** 2002. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature **416:**552-556.

30. **Robertson, K. D., E. Uzvolgyi, G. Liang, C. Talmadge, J. Sumegi, F. A. Gonzales, and P. A. Jones.** 1999. The human DNA methyltransferases (DNMTs) 1, 3a, and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res. **27:**2291-2298.

31. **Saam, J. R., and J. I. Gordon.** 1999. Inducible gene knockouts in the small intestinal and colonic epithelium. J. Biol. Chem. **274:**38071-38082.

32. **Saito, Y., Y. Kanai, M. Sakamoto, H. Saito, H. Ishii, and S. Hirohashi.** 2002. Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc. Natl. Acad. Sci. USA **99:**10060-10065.

33. **Sakatani, T., A. Kaneda, C. A. Iacobuzio-Donahue, M. G. Carter, S. de Boom Witzel, H. Okano, M. S. Ko, R. Ohlsson, D. L. Longo, and A. P. Feinberg.** 2005. Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice. Science **307:**1976-1978.

34. **Soejima, K., W. Fang, and B. J. Rollins.** 2003. DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. Oncogene **22:**4723-4733.

35. **Takada, J., D. J. Baylink, and K. H. Lau.** 1995. Pretreatment with low doses of norethindrone potentiates the osteogenic effects of fluoride on human osteosarcoma cells. J. Bone Miner. Res. **10:**1512-1522.

36. **Thliveris, A. T., R. B. Halberg, L. Clipson, W. F. Dove, R. Sullivan, M. K. Washington, S. Stanhope, and M. A. Newton.** 2005. Polyclonality of familial murine adenomas: analyses of mouse chimeras with low tumor multiplicity suggest short-range

interactions. Proc. Natl. Acad. Sci. USA **102:**6960-6965.

37. **Ting, A. H., K. W. Jair, H. Suzuki, R. W. Yen, S. B. Baylin, and K. E. Schuebel.** 2004. CpG island hypermethylation is maintained in human colorectal cancer cells after RNAi-mediated depletion of DNMT1. Nat. Genet. **36:**582-584.

38. **Vilkaitis, G., I. Suetake, S. Klimasauskas, and S. Tajima.** 2005. Processive methylation of hemimethylated CpG sites by mouse Dnmt1 DNA methyltransferase. J. Biol. Chem. **280:**64-72.

39. **Yamada, Y., L. Jackson-Grusby, H. Linhart, A. Meissner, A. Eden, H. Lin, and R. Jaenisch.** 2005. Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc. Natl. Acad. Sci. USA **102:**13580-13585.

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