FUNCTIONAL CHARACTERIZATION OF THE DE NOVO DNA METHYLTRANSFERASE DNMT3B

By

SUHASNI GOPALAKRISHNAN

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To my family
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DNA methylation is an epigenetic mark that is required for transcriptional repression in mammalian development, imprinting, and in the maintenance of genome stability. Genome-wide methylation patterns are established and maintained by three DNA methyltransferases (DNMTs)-DNMT1, DNMT3A, and DNMT3B. DNMT3B is specifically involved in silencing the satellite repeats at the centromeric and pericentromeric regions. The role of DNMT3B at the centromeric region is also emphasized by mitotic defects arising due to chromosome instability observed in ICF syndrome, a disease caused by germline mutations in DNMT3B. Although the mechanism of DNA methylation is well known, the targeting of DNA methylation and DNMT3B to certain genomic loci remains poorly understood. DNMT3B is also regulated by alternative splicing. Several DNMT3B splice variants are overexpressed in tumor cells and negatively regulate normal DNMT3B mediated DNA methylation. Therefore it is important to understand the significance of DNMT3B splice variants in development and tumorigenesis.

In the present study, a yeast two-hybrid screening was performed and several novel DNMT3B protein interactions were identified. Of the several proteins identified, the interaction
of DNMT3B with the mammalian chromatin associated factor MCAF and the chromodomain helicase DNA binding protein CHD3 were confirmed, and need further characterization. The interaction between DNMT3B and the constitutive centromeric protein CENP-C was confirmed in mammalian cells. Results from siRNA knock downs, bisulfite genomic sequencing and ChIP, demonstrate that CENP-C recruits DNA methylation and DNMT3B to both centromeric and pericentromeric satellite repeats. CENP-C and DNMT3B influence the histone modifications in satellite repeat regions, including marks characteristic of centromeric chromatin and disruption of this interaction causes elevated transcription of centromeric repeats. Loss of CENP-C or DNMT3B leads to elevated chromosome misalignment and segregation defects during mitosis. Taken together, the interaction between CENP-C and DNMT3B suggests a novel mechanism by which DNA methylation is targeted to discrete regions of the genome and contributes to chromosomal stability.

In another study, a novel alternatively spliced form of DNMT3B lacking exon 5 was identified and characterized. This variant was termed DNMT3B3Δ5 because of its close resemblance with the ubiquitously expressed DNMT3B3 isoform. The novel splice variant lacking exon 5 is highly expressed in pluripotent cells and neural tissues, and is conserved in the mouse and is re-expressed on converting differentiated mEFs into pluripotent iPS cells. DNMT3B3Δ5 also displays altered expression in human tumor cell lines as well as an altered subcellular localization. Ectopic overexpression of DNMT3B3Δ5 resulted in repetitive element hypomethylation. Taken together, these results demonstrate that alternative splicing of exon 5 may play an important role in stem cell maintenance or differentiation and exon 5 could influence the functional properties of DNMT3B.
CHAPTER 1
INTRODUCTION
Regulation of Gene Expression by DNA Methylation

DNA methylation is the covalent chemical modification of DNA by the addition of a methyl group at the cytosine residues in the context of a CpG dinucleotide. Genome-wide DNA methylation is a stable epigenetic mark that is established by a family of enzymes known as DNA methyltransferases (DNMTs). DNA methyltransferases establish and maintain a repressive chromatin state and regulate gene silencing involved in processes such as X chromosome inactivation, imprinting, embryogenesis, gametogenesis, and silencing of repetitive DNA elements (Goll and Bestor, 2005). DNA methylation together with post-translational modifications on the N-terminal histone tails function as major epigenetic mechanisms that regulate gene expression. The post-translational modifications on histone tails include methylation, acetylation, phosphorylation, ubiquitination and sumoylation (Fischle et al., 2003). The modifications on histone tails are performed by a set of chromatin associated factors and recognized by an additional set of protein factors. In turn, these factors alter chromatin structure and consequently affect the transcriptional status of gene expression (Ruthenburg et al., 2007; Taverna et al., 2007). Histone modification patterns and cellular DNA methylation patterns are highly orchestrated during development. Aberrant DNA methylation leads to genetic diseases and cancer in humans. Alterations in genome-wide DNA methylation patterns leading to altered gene expression patterns and genomic instability are a hallmark of many transformed cells. Mechanisms by which DNA methylation patterns are regulated during development and disease states are not clearly understood. Despite compelling evidence of the cooperation of DNA methylation and the histone modification patterns, the mechanism and the roles of their coordination are yet to be delineated.
The DNA Methylation Machinery

The mammalian DNA methylation machinery consists of five known DNMT family members- DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Figure 1-1). Together, they are involved in the establishment of genome-wide DNA methylation patterns during embryonic development and coordinate in the maintenance of genomic DNA methylation in somatic cells. There are two general classes of DNA methyltransferases, de novo methyltransferases and maintenance methyltransferases. DNMT1 is the most highly expressed and catalytically active DNA methyltransferase. DNMT1 copies existing methylation patterns during cell division by specific methylation of hemimethylated CpG dinucleotides during DNA replication and hence is predominantly considered the maintenance methyltransferase (Bestor et al., 1992; Pradhan et al., 1999). DNMT1 is known to be associated with S phase replication foci via its interaction with PCNA (Leonhardt et al., 1992; Liu et al., 1998). Although in vitro studies with DNMT2 showed that it possesses very low enzymatic activity towards DNA (Hermann et al., 2003), recent studies demonstrated that it methylates tRNA^{Asp} in the wobble position of its anticodon loop (Goll et al., 2006). Interestingly, a Dnmt2 like protein was identified in Drosophila and has been shown to be necessary and sufficient for DNA methylation in Drosophila (Tang et al., 2003). Also, in Drosophila somatic cells, DNMT2 controls retrotransposon silencing in early embryos by initiating histone H4K20 trimethylation catalyzed by the SUV4-20 methyltransferase and is responsible for the maintenance of their methylated status (Phalke et al., 2009). Knockout of the Dnmt2 gene in mice produces no discernible phenotype (Okano et al., 1998b). DNMT3A and DNMT3B are responsible for establishing new DNA methylation patterns largely associated with retrotransposon sequences and satellite repeats in pericentromeric regions and differentially methylated regions (DMRs) of imprinted loci and are hence considered de novo methyltransferases (Goll and Bestor, 2005). DNMT3A and DNMT3B are closely related
proteins, which contain an amino terminal regulatory region consisting of a PWWP domain (Qiu et al., 2002), and a cysteine-rich ATRX domain also known as the PHD domain. The PWWP domain of DNMT3A and DNMT3B is required for targeting the enzymes to pericentromeric heterochromatin (Chen et al., 2004). These two regions contribute to the interaction of DNMT3A and DNMT3B with various chromatin remodeling factors such as HDAC1/2, hSNF2H, SETDB1, Lsh, and SUV(39)H (Bachman et al., 2001; Geiman et al., 2004b; Lehnertz et al., 2003; Li et al., 2006; Suzuki et al., 2006). Both DNMT3A and DNMT3B exhibit de novo type methylation activity in vitro (Aoki et al., 2001; Gowher and Jeltsch, 2001; Suetake et al., 2003; Yokochi and Robertson, 2002). While Dnmt3b preferentially methylates nucleosomal DNA, Dnmt3a was shown to preferentially methylate naked DNA (Takeshima et al., 2006). The mouse and the human DNMT3A and DNMT3B show 98% (DNMT3A vs Dnmt3a) and 94% (DNMT3B and Dnmt3b) similarity in their amino acid sequences (Xie et al., 1999).

Mice lacking both Dnmt3a and Dnmt3b exhibit global demethylation of their genomes and die at 8.5 dpc, although the extent of demethylation is less than in Dnmt1−/− mutant embryos (Okano et al., 1999). The double mutants are also unable to methylate newly integrated retroviral DNA (Okano et al., 1999). Mice lacking Dnmt3a developed to term, but died at about four weeks of age. In contrast, Dnmt3b null mice were not viable (embryonic lethality at E14.5-18.5) and showed multiple developmental defects, demonstrating that de novo methylation is an essential process for mammalian development (Li, 2002). Inactivation of Dnmt3b in mouse embryonic fibroblasts (MEF) resulted in DNA hypomethylation, chromosomal instability, and spontaneous immortalization (Dodge et al., 2005). It has been shown that Dnmt3a and Dnmt3b are essential for the stable inheritance, or ‘maintenance’, of DNA methylation patterns as well. Consistent with these findings, inactivation of both Dnmt3a and Dnmt3b in ES cells results in
progressive loss of methylation in various repeats and single-copy genes (Chen et al., 2003) revealing a role for Dnmt3a/3b in maintenance methylation. Evidence for an alternative DNA methylation maintenance mechanism comes from the finding that CpG island methylation is stably maintained even in the apparent absence of DNMT1 (Chen et al., 2003; Rhee et al., 2000). In other words, replication of methylation patterns by DNMT1 is only partially responsible for maintenance methylation and it is possible that features of certain DNA domains help maintain its methylated status through repeated de novo methylation (Beard et al., 1995). Therefore, although the DNA methyltransferases have been classified as maintenance and de novo, an increasing number of studies reveal that DNMT1, DNMT3A, and DNMT3B cooperate to establish and maintain genome-wide DNA methylation patterns (Fatemi et al., 2002; Gowher et al., 2005; Hata et al., 2002; Okano et al., 1999).

DNMT3L is a member of the DNA methyltransferase family that is highly expressed in germ cells and in testis (Aapola et al., 2004) and is homologous to DNMT3A and DNMT3B within the N-terminal regulatory region. Although catalytically inactive, DNMT3L regulates DNMT3A and DNMT3B by stimulating their catalytic activity in vivo (Suetake et al., 2004). Both DNMT3A and DNMT3B are known to interact with DNMT3L through their C-terminal catalytic regions. Mice deficient for Dnmt3L display genome-wide demethylation and developmental arrest at E8.5 and lack of Dnmt3L leads to a failure to establish maternal DNA methylation imprints in oocytes and male sterility due to defects in spermatogenesis (Bourc'his et al., 2001). DNMT3L is also known to bind to unmethylated lysine 4 of the histone H3 tail, therefore possibly inducing de novo DNA methylation by recruitment or activation of DNMT3A2 (Jia et al., 2007; Ooi et al., 2007). The growing list of diverse components that target de novo methylation include satellite repeat sequences, components of the RNAi pathway,
local histone methylation status, and catalytically inert DNA methyltransferase that have acquired regulatory roles (DNMT3L and DNMT3B splice variants) (Benetti et al., 2008; Geiman and Robertson, 2002; Sinkkonen et al., 2008; Suetake et al., 2004; Vire et al., 2006).

**The Coordination Between DNA Methylation and Histone Modifications**

Several studies have demonstrated that the DNMTs coordinate with chromatin-associated factors such as histone modifying enzymes (histone deacetylases (HDACs)), histone methyltransferases (SUV (39) H1/2 and EZH2) and ATP dependent chromatin remodeling enzymes (e.g. hSNF2H, LSH) to regulate chromatin structure across the genome (Goll and Bestor, 2005; Vire et al., 2006). DNMT1, DNMT3A, and DNMT3B interact with histone deacetylases (HDACs) which modify chromatin structure by deacetylating the core histones (Fuks et al., 2000; Fuks et al., 2001; Geiman et al., 2004b). The N-terminus of murine Dnmt1 (amino acids 686-812) contains a transcriptional repression domain that interacts with the histone deacetylase HDAC1 and is associated with deacetylase activity (Fuks et al., 2000). Further evidence that supports a link between DNMT1 and transcription comes from a study showing that DNMT1, HDAC1, Rb and E2F1 form a complex that can repress E2F1 regulated promoters (Robertson et al., 2000). DNMT1 also binds to histone H3K9 methyltransferase SUV39H1 and its interacting partner heterochromatin protein 1 beta (HP1β) (Fuks et al., 2003). DNMT1 associates with another H3K9 histone methyltransferase, G9a, at the DNA replication foci (Esteve et al., 2006). Like DNMT1, DNMT3B also interacts with HDAC1/2, SUV39H1 and the ATP dependent chromatin remodeling protein hSNF2H as detected by co-immunoprecipitation assays (Geiman et al., 2004b). In vitro interaction of DNMTs with the ATP-dependent chromatin remodeling enzyme hSNF2H alters their biological property by increasing their affinity for mononucleosomes (Robertson et al., 2004). Dnmt3b exhibited HDAC-dependent repression of a reporter gene through its N-terminal PHD domain (Bachman et al., 2001). Both
Dnmt3a and Dnmt3b interact with the transcription factor protein PU.1 through the PHD domain and Dnmt3b/PU.1 is a component of a larger complex that also includes the mammalian Sin3A (mSin3A), HDAC1 and methyl CpG binding protein MeCP2 (Suzuki et al., 2006). PU.1 (also known as Spi-1), is a member of the Ets transcription factor family, is predominantly expressed in myeloid (granulocytes, monocytes and macrophages) and B cells (Chen et al., 1995). Mammalian Sin 3 (mSin3A) is a protein closely related to the yeast SIN3 repressor protein and is the core component of a large multiprotein corepressor complex with associated histone deacetylase (HDAC) enzymatic activity (Hassig et al., 1997). The repression of PU.1 transactivation by Dnmt3a or Dnmt3b is dependent on their DNA methyltransferase activity (Suzuki et al., 2006).

An important link between histone H3K9 methylation and DNA methylation in mammals was identified when it was shown that Suv39h1 HMTase-directed H3K9 trimethylation was shown to be required for recruiting Dnmt3b-dependent DNA methylation to pericentromeric repeats. Mouse ES cells lacking Suv39h1/2 display an altered DNA methylation profile at pericentromeric satellite repeats, but not at other repeat sequences (Lehnertz et al., 2003). Studies have also demonstrated that de novo DNA methylation at the Oct4 and Nanog promoter regions was dependent on the initiation of the silencing by acquisition of histone H3K9 methylation, mediated by the SET domain containing histone methyltransferase protein G9a. The creation of heterochromatic structure is dependent on the recruitment of HP1 at H3K9 methylated chromatin, and is thought to be required for subsequent de novo methylation at the Oct4 and Nanog promoters (Feldman et al., 2006; Gu et al., 2006). In vitro differentiation of pluripotent cells lacking G9a or the orphan nuclear receptor family member GCNF (also a transcriptional repressor of Oct4 (Fuhrmann et al., 2001) leads to activation of Oct4 expression
due to promoter hypomethylation (Feldman et al., 2006; Gu et al., 2006). Also, the euchromatin specific histone H3 K9 methyltransferase SETDB1 associates with endogenous DNA methyltransferase activity and physically interacts with the de novo DNA methyltransferases DNMT3A and DNMT3B in vivo and in vitro. This interaction is mediated by the PHD domain of DNMT3A and SETDB1 does not interact with the maintenance methyltransferase DNMT1 (Li et al., 2006) emphasizing the specific link between histone methylation machinery and the de novo DNA methylation machinery. The intricate coordination between DNA methylation and histone modifications is also highlighted by finding that DNMT3L preferentially binds to the unmethylated lysine 4 of histone H3 (Jia et al., 2007; Ooi et al., 2007).

Lsh (lymphoid specific helicase), a member of the SNF2-helicase family of chromatin remodeling proteins, is involved in regulating DNA methylation patterns during embryonic development. Lsh is required for DNA methylation at select imprinted loci and silencing of IAP retrotransposons (De La Fuente et al., 2006). Deletion of Lsh perturbs DNA methylation patterns in mice causing reduced de novo methylation without any effect on maintenance methylation (Zhu et al., 2006). In vivo and in vitro pull down assays demonstrated that Lsh recruits Dnmt1, Dnmt3b, and HDACs to establish a transcriptionally repressive chromatin structure that is independent of the enzymatic activities of Dnmts (Myant and Stancheva, 2008). Recent studies indicate that Lsh physically associates with the Hox genes and regulates Dnmt3b binding, DNA methylation, and silencing of Hox genes during development. Lsh inactivation resulted in decreased DNMT3B and polycomb group (PcG) protein complex binding (Xi et al., 2007). Interestingly, DNMTs also physically interact with components of both PcG protein complexes PRC1 (composed of HPH, RING1, BMI1, HPC (1-4)) and PRC2 (composed of EED, EZH2, YY1, SU(Z)12) (Hernandez-Munoz et al., 2005; Li et al., 2007a; Vire et al., 2006). The
interaction of PRC2 components EZH2 and EED with the DNA methylation machinery (DNMT1, DNMT3A, and DNMT3B) was identified by co-immunoprecipitation assays (Vire et al., 2006). RNAi-mediated knockdown of DNMTs leads to loss of silencing at EZH2 target genes demonstrating that repression of some of EZH2 target genes requires both EZH2 and DNMTs (Vire et al., 2006). DNMT1, facilitates proper localization of PRC1 component BMI1 to polycomb complexes that are organized into discrete nuclear structures called PcG bodies (Hernandez-Munoz et al., 2005). Another PRC1 complex component, Cbx4 (hPC2), was identified as a SUMO (small ubiquitin like modifier) E3 ligase and interaction partner of Dnmt3a (Li et al., 2007a). As with DNMT3A, DNMT3B also interacts with SUMO-1 within its N-terminal domain and is sumoylated (Kang et al., 2001). Sumoylation of Dnmt3a disrupts its interaction with HDAC1/2 (Ling et al., 2004), while the regulatory implications of the sumoylation of DNMT3B are not clearly understood. A recent study also showed that Cbx4 also interacts with Dnmt3b and that DNMT3B functions as a corepressor of Cbx4 mediated transcriptional repression independent of its DNA methyltransferase activity (Kim et al., 2008b). Therefore, DNA methylation may help aid in the stable repression of genes by coordinating with other histone modifying enzymes. Disruption of the precise regulation of some of these interactions during development may lead to aberrant methylation and subsequent developmental defects or improper methylation patterns in somatic cells leading to tumorigenesis.

**DNA Methylation in Cancer**

Disruption of normal DNA methylation patterns is one of the most common features of transformed cells. Global genomic hypomethylation and locus-specific hypermethylation of CpG islands are hallmarks of epigenetic defects in cancer. Disruption of the DNA methylation machinery results in aberrant *de novo* methylation of CpG islands leading to transcriptional silencing of genes associated with tumor suppression, cell cycle regulation and apoptosis, as well
as genome-wide hypomethylation of repetitive elements (Baylin et al., 2001; Jones and Laird, 1999; Robertson, 2001). Hypomethylation of promoters associated with normally silenced genes, transposable elements and repetitive regions of DNA contribute to global hypomethylation and consequent genomic instability.

Global genomic hypomethylation is usually associated with chromosomal instability, aberrant activation of endogenous retroviral elements and oncogenes, and loss of imprinting (Wilson et al., 2007). A decrease in global methylation is largely attributed to loss of methylation at normally heavily methylated repeat elements including satellite repeats (Sat2) and retrotransposons (LINEs) as well as loss of methylation at the promoter regions of genes that are normally silenced. For example, the synuclein-γ gene (SNCG), which is usually expressed only in neurons, becomes demethylated in both breast and ovarian cancer (Gupta et al., 2003; Jia et al., 1999). Interestingly, the zinc finger protein and paralog of CTCF, Brother of the Regulator of Imprinted Sites (BORIS/CTCFL), a gene involved in epigenetic regulation, also becomes demethylated in epithelial ovarian cancers resulting in increased expression compared to normal ovarian tissue (Woloszynska-Read et al., 2007). Due to re-expression in cancerous state, BORIS/CTCFL, which is typically expressed only in testicular germ cells, is classified as a cancer-testes antigen/gene. Hypomethylation also contributes to loss of imprinting of IGF2 in colon cancer (Cui et al., 2002). Mice or humans with DNMT3B mutations are deficient in methylation of CpG islands on the inactive X chromosome and pericentromeric repetitive DNA sequences (Hansen et al., 2000; Kondo et al., 2000; Miniou et al., 1994; Okano et al., 1998a). In mice, DNA hypomethylation is sufficient to induce T cell lymphomas with consistent gain of an extra copy of chromosome 15 as well as activation of endogenous retroviral elements indicating
that genome-wide hypomethylation plays a causal role in cancer (Eden et al., 2003; Gaudet et al., 2003; Howard et al., 2008).

Hypermethylation of promoter CpG islands is frequent in tumors and is associated with aberrant gene silencing. There is clear evidence that DNMT1 and DNMT3B cooperate to silence genes in human cancer cells (Rhee et al., 2002). DNMT1 knockout in HCT116 colorectal carcinoma cell line resulted in loss of approximately 20% of DNA methylation while DNMT3B knockout resulted in loss of <3% of total methylation levels (Rhee et al., 2002; Rhee et al., 2000). Cells with DNMT1 and DNMT3B knockout (double knockout or DKO) displayed a much greater decrease in DNA methylation (>95%) demonstrating the overlapping and synergistic roles of DNMT1 and DNMT3B in maintaining cellular DNA methylation (Rhee et al., 2002). Double knockout cells also displayed chromosomal instability as evidenced by aneuploidy and a large increase in the number of novel chromosomal translocations (Karpf and Matsui, 2005). Interestingly, mouse embryonic fibroblast (MEFs) with a conditional knockout of Dnmt1 underwent apoptosis, differing in phenotype from the HCT116 DNMT1 knockout model. It was later determined that the original HCT116 DNMT1 knockout cells (Rhee et al., 2000) expressed a hypomorphic form of DNMT1 (lacking the PCNA binding domain) due to alternative splicing (Egger et al., 2006), and when a HCT116 cell line completely lacking the catalytically active DNMT1 was created, these cells underwent mitotic catastrophe (Chen et al., 2007). Depletion of DNMT3B, but not DNMT3A, induced selective apoptosis of cancer cells over normal cells, which was rescued by the ectopic expression of the catalytically active DNMT3B2 splice variant or the inactive DNMT3B3 demonstrating the essential role for DNMT3B in cancer cell survival (Beaulieu et al., 2002). Dnmt3b deficiency inhibits the formation of macroadenomas in the murine Apc Min/+ colon cancer model, indicating a
requirement for Dnmt3b in the transition from microadenoma to tumor (Lin et al., 2006). Together, these findings underscore the importance of regulation of DNA methylation and the DNMTs themselves in cancer and cell survival.

**DNA Methylation in Development**

In mammals, systematic regulation of DNA methylation occurs by genome-wide demethylation after fertilization, followed by waves of *de novo* methylation upon embryo implantation (Okano et al., 1999; Reik et al., 2001). Developmentally programmed DNA methylation regulates genomic imprinting and X chromosome inactivation (Panning and Jaenisch, 1996; Reik et al., 2001). Imprinting plays an important role in the pathogenesis of human disorders of growth and development (Paulsen and Ferguson-Smith, 2001). The expression of the *de novo* DNA methyltransferases is dynamically regulated during development with high levels in undifferentiated cells and reduced expression upon differentiation (Watanabe et al., 2002). DNMTs are required for cellular differentiation during early embryonic development to regulate the systematic transcriptional inactivation of developmental genes by promoter methylation. Dnmt3b is specifically expressed in the totipotent cells of early mouse embryos and also after the murine ES cells become committed progenitor cells (Watanabe et al., 2004). Analysis of gene expression patterns using cDNA microarrays have shown that DNMT3B is highly expressed in embryonic stem (ES) cells and pluripotent germ cells (Skotheim et al., 2005; Sperger et al., 2003).

DNMT3B is highly expressed in testis, suggesting a crucial function for DNMT3B in spermatogenesis (Okano et al., 1998a; Robertson et al., 1999; Xie et al., 1999). Studies in the central nervous system indicate that Dnmt3b may be important for the early phase of neurogenesis, while Dnmt3a is likely playing a role in regulating neurogenesis prenatally and the function and maturation of the central nervous system postnatally (Feng et al., 2005). The N-
terminal region of Dnmt3b regulates nerve growth factor (NGF)-induced differentiation of PC12 cells into neurons via its interaction with HDAC2 (Bai et al., 2005). Interestingly, suppression of truncated cadherin (T-Cad) promoter activity by Dnmt3b was independent of its catalytic activity, which was consistent with the insignificant change in T-Cad promoter methylation status in Dnmt3b-depleted cells. This process was mediated, at least in part, by the interaction of Dnmt3b with the T-cad promoter region via the ATRX and the PWWP domains of Dnmt3b, and it did not involve changes in the methylation status of the T-cad gene (Bai et al., 2006). Studies in mouse embryonic fibroblasts (NIH-3T3) showed that Dnmt3b, together with the erythroblastosis virus (Ets) family transcriptional factor PU.1, is involved in site-specific methylation and regulation of genes responsible for the differentiation of myeloid and B-cell lineages (Suzuki et al., 2006).

Key transcription factors, such as Oct4 and Nanog, form a transcriptional regulatory network that selectively activates genes essential for murine ES cell survival and proliferation while selectively repressing genes required for cell differentiation (Loh et al., 2006). The Oct4 enhancer/promoter region is hypomethylated in mouse ES cells and is hypermethylated in trophoblast stem cells, demonstrating epigenetic control of Oct4 expression during early embryogenesis by DNA methylation in a stage- and cell type-specific manner (Hattori et al., 2004). Interestingly, both Dnmt3a and Dnmt3b function synergistically within a protein complex and stimulate each other’s activity to methylate the Oct4 and Nanog promoters in differentiating mouse embryonic carcinoma (EC) and ES cells (Li et al., 2007b). A study of Oct4 transcription by injecting mammalian somatic cell nuclei into Xenopus oocytes, demonstrated that DNA demethylation is necessary for Oct4 transcription and subsequent epigenetic reprogramming, suggesting selective promoter demethylation precedes gene
reprogramming (Simonsson and Gurdon, 2004; Wernig et al., 2007) and that reprogramming deficiencies in cloned embryos arise from abnormal removal of repressive marks and impaired DNA methylation patterns (Boiani et al., 2002). Therefore, repression mediated by DNA methylation is likely to stabilize the silencing of pluripotency-associated genes (Schlesinger et al., 2007; Widschwendter et al., 2007).

The expression of DNMTs, as well as the establishment of DNA methylation patterns, is also regulated by the RNA mediated silencing machinery. The mammalian RNase III family nuclease, Dicer, initiates RNA interference (RNAi) by processing the small RNAs that determine the specificity of gene silencing pathways (Bushati and Cohen, 2007). Loss of Dicer compromises maturation of microRNAs (miRNAs) and leads to defects in gene silencing and differentiation (Bushati and Cohen, 2007). Dicer-deficient mice exhibit decreased expression of Dnmts and consequent global DNA methylation defects (Benetti et al., 2008). Transcriptome analysis of Dicer-null ES cells revealed downregulation of the miR-290 cluster leading to activation of the transcription factor retinoblastoma-like 2 (Rbl2) and a consequent repression of de novo DNA methyltransferases along with DNA methylation defects (Sinkkonen et al., 2008). Although Dicer-null ES cells initiate Oct4 silencing through the accumulation of repressive histone marks, subsequent Oct4 promoter de novo methylation is abolished, preventing stable repression of Oct4. Interestingly, the defective DNA methylation is rescued by ectopic expression of Dnmts or by transient introduction of the miR-290 cluster miRNAs, indicating that miRNAs control de novo DNA methylation in ES cells (Sinkkonen et al., 2008). Taken together, these studies suggest that DNA methylation is an essential regulatory mechanism that ensures proper establishment of gene expression patterns during epigenetic reprogramming in normal development.
The Immunodeficiency, Centromere Instability and Facial Anomalies (ICF) Syndrome

ICF syndrome is a rare autosomal recessive disorder caused by mutations in DNMT3B. Most of the point mutations cluster within the C-terminal catalytic domain of DNMT3B (Piirila et al., 2006) (Figure 1-2). Patients with ICF syndrome display variable levels of combined immunodeficiency, facial anomalies, developmental delay, and mental retardation (Ehrlich et al., 2006a). Centromeric instability, another hallmark of ICF B cells, is caused by loss of methylation within classical satellites (sat 2 and 3) at the pericentromeric regions of chromosomes 1, 9, and 16 (Ehrlich et al., 2006a). Satellite 2-rich 1qh and 16qh display high frequencies of abnormalities (Hansen et al., 1999; Maraschio et al., 1988; Xu et al., 1999) and exhibit multibranched chromosomes (Turleau et al., 1989). ICF patients are characterized by lymphoid-specific chromosome instability and by defective B cell negative selection and terminal differentiation (Blanco-Betancourt et al., 2004; Maraschio et al., 1989).

Lymphoblastoid cell lines of ICF patients show increased co-localization of the hypomethylated 1qh and 16qh sequences in interphase, abnormal looping of pericentromeric DNA sequences at metaphase, formation of bridges at anaphase, chromosome 1 and 16 fragmentation at the telophase to interphase transition, and, micronuclei with overrepresentation of chromosome 1 and 16 material in apoptotic cells (Gisselsson et al., 2005). Another source of anaphase bridging in the ICF cells was random telomeric associations between chromosomes (Gisselsson et al., 2005). In addition, genes on the inactive X chromosome, the highly restricted tissue specific/immunogenic cancer-testes genes, and non-satellite repeats D4Z4 and NBL2, are demethylated (Hansen et al., 2000; Kondo et al., 2000; Tao et al., 2002). Some of the DNMT3B catalytic domain mutations do not result in complete loss of DNMT activity in most cases examined; therefore, clinical variability of the disease may be related to the amounts of residual DNMT3B activity.
ICF patients also exhibit defective *de novo* methylation of cellular and viral DNA (Tao et al., 2002). Microarray expression analysis of B lymphoblastoid cell lines (LCLs) from ICF patients with diverse DNMT3B mutations using oligonucleotide arrays, showed a lymphoid lineage-restricted expression pattern suggesting *DNMT3B* mutations in ICF syndrome cause lymphogenesis-associated gene dysregulation by affecting the expression of genes that may regulate normal lymphocyte signaling, maturation, and migration (Ehrlich, 2003; Ehrlich et al., 2001). Microarray profiling studies have also identified several genes involved in developmental processes that display altered gene expression pattern accompanied by changes in the DNA methylation levels as well as the histone modification patterns in comparison to the normal parental cells (Jin et al., 2008). In a large proportion of ICF G2 nuclei, all heterochromatin protein 1 (HP1) isoforms show an aberrant signal concentrated into a prominent bright focus that co-localizes with the undercondensed 1qh or 16qh heterochromatin, and the SP100, SUMO-1, and other proteins from the promyelocytic leukemia (PML) (Luciani et al., 2005) suggesting although DNA hypomethylation does not prevent HP1 localization to the undercondensed chromatin, it causes inappropriate subcellular concentration and localization of these proteins that cause alterations in the structure of heterochromatin.

Mouse models of ICF syndrome also display many of the characteristics of ICF patients, including hypomethylation of repetitive sequences, distinct craniofacial anomalies, and defects in immune system, indicating that Dnmt3b plays an essential role at different stages of development (Ueda et al., 2006). Most of the mutant forms of DNMT3B in ICF cells showed decreased activity compared to the wild-type enzyme (Gowher and Jeltsch, 2002). Mutants with null alleles (catalytically inactive) were embryonic lethal, exhibiting multiple tissue defects. Those mutants with missense mutations, homologous to mutations in human ICF patients developed to
term and survived to adulthood, but showed facial abnormalities, T cell death, and hypomethylation of repetitive sequences. Studies using the mouse ICF model also indicate that some ICF mutations result in a loss of Dnmt3b function via disrupting protein-protein interactions with Dnmt3a or DNMT3L or by altering protein localization despite demonstrating DNA methyltransferase activity close to wild-type (Ueda et al., 2006; Xie et al., 2006). This also implies that most missense mutations in individuals with ICF may not be complete loss-of-function alleles. The striking resemblance of mutant mice with ICF like Dnmt3b mutations to individuals with ICF syndrome therefore suggests that these mice will serve as good models for understanding the etiology of ICF syndrome and will help in revealing the mechanisms of regulation of DNA methylation as well as identification of target genes that are regulated by DNA methylation during development.
Figure 1-1. The mammalian DNA methylation machinery. Shown are the schematic representations for each of the five known DNA methyltransferases (DNMT), DNMT1 (maintenance methyltransferase), DNMT2, DNMT3A and DNMT3B (de novo methyltransferases) and DNMT3L. Catalytic site PC (Proline-Cysteine) and motifs PWWP: proline-tryptophan-tryptophan-proline and ATRX – similar to Plant homeo-domain (PHD) are shown in patterned boxes. The PWWP and ATRX domain are found in many chromatin remodeling proteins and are thought to play an important role in their regulation. DNMT3L is a regulatory DNA methyltransferase with roles in imprinting.
Table 1-1. Protein-protein interactions of DNA methyltransferases

<table>
<thead>
<tr>
<th>DNMT</th>
<th>Protein</th>
<th>Function</th>
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<tr>
<td>DNMT1</td>
<td>PCNA</td>
<td>Targeting to DNA replication foci</td>
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<tr>
<td></td>
<td>HDAC1/2</td>
<td>Histone deacetylation</td>
</tr>
<tr>
<td></td>
<td>Rb, E2F1, HDAC1</td>
<td>Transcriptional repression of promoters of E2F1 regulated genes</td>
</tr>
<tr>
<td></td>
<td>SUV39H1, G9a, HP1</td>
<td>Histone H3K9 methylation</td>
</tr>
<tr>
<td></td>
<td>hSNF2H</td>
<td>Chromatin remodeling</td>
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<tr>
<td></td>
<td>DNMT3A, DNMT3B</td>
<td>Increase enzyme activity</td>
</tr>
<tr>
<td></td>
<td>EZH2</td>
<td>Histone H3K27 methylation</td>
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<tr>
<td></td>
<td>HDAC1/2</td>
<td>Histone deacetylation</td>
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<tr>
<td></td>
<td>SUV39H1,HP1</td>
<td>Histone H3K9 methylation, chromatin condensation</td>
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<tr>
<td></td>
<td>SUMO1, PIAS1</td>
<td>Sumoylation and transcriptional repression</td>
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<tr>
<td></td>
<td>DNMT1, DNMT3B</td>
<td>Increase enzyme activity</td>
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<td></td>
<td>PU.1</td>
<td>Hematopoietic specific transcription factor</td>
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<td></td>
<td>EZH2</td>
<td>Histone H3K27 methylation</td>
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<tr>
<td></td>
<td>DNMT3L</td>
<td>Stimulate methyltransferase activity, imprinting</td>
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<tr>
<td></td>
<td>SETDB1</td>
<td>histone H3K9 methylation, transcription repression</td>
</tr>
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</table>
Table 1-1. Protein-protein interactions of DNA methyltransferases (continued)

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<tr>
<th>DNMT</th>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
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<td>DNMT3B</td>
<td>HDAC1/2</td>
<td>Histone deacetylation</td>
</tr>
<tr>
<td></td>
<td>SUV39H1, HP1</td>
<td>Histone H3K9 methylation, chromatin condensation</td>
</tr>
<tr>
<td></td>
<td>hSNF2H</td>
<td>Chromatin remodeling</td>
</tr>
<tr>
<td></td>
<td>hCAP-C (SMC4), KIF4A, SIN3A, hCAP-E (SMC2), hSNF2H, HDAC1</td>
<td>Chromatin condensation</td>
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<tr>
<td></td>
<td>PU.1, mSIN3A, HDAC1, MECP2</td>
<td>Repress PU.1 transactivation</td>
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<tr>
<td></td>
<td>EZH2</td>
<td>Histone H3K27 methylation</td>
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<td>DNMT3L</td>
<td>Stimulate methyltransferase activity, imprinting</td>
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<tr>
<td></td>
<td>SUMO1</td>
<td>Sumoylation</td>
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<td>DNMT3L</td>
<td>HDAC1/2</td>
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</tr>
<tr>
<td></td>
<td>DNMT3A, DNMT3B</td>
<td>Enhance methyltransferase activity</td>
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</table>
Figure 1-2. DNMT3B mutations identified in ICF syndrome. Schematic representation of DNMT3B with mutations identified in patients with ICF syndrome. ICF syndrome is a rare autosomal recessive disorder caused by mutations in *DNMT3B*. Most of the point mutations cluster within the C-terminal catalytic domain of DNMT3B and have been mapped in this figure based on the immunodeficiency database (Piirila et al., 2006). The protein-protein interaction domains PWWP and PHD are represented in patterned boxes and the regions represented with roman numerals are conserved DNMT catalytic motifs.
CHAPTER 2
MATERIALS AND METHODS

Yeast Two-Hybrid Screening

The Matchmaker Two-Hybrid System 3 (Clontech) was used to screen a human adult testis cDNA library (Clontech) with full-length Dnmt3b1 as bait according to the manufacturer’s instructions. A commercially available human testis cDNA library with the cDNAs cloned into a yeast plasmid (fused to the GAL4 activation domain of pACT2) was purchased from Clontech. pACT2 (Catalog no. 638822) generates a HA tagged and GAL4 AD (amino acids 768–881) fusion of a protein of interest (or protein encoded by a cDNA in a fusion library) that is cloned into the multiple cloning site (MCS) in the correct orientation and reading frame. pACT2 is a shuttle vector that replicates autonomously in both E. coli and S. cerevisiae and carries the ampicillin resistance gene for E. coli. pACT2 also contains the LEU2 nutritional gene that allows yeast auxotrophs to grow on limiting synthetic media. To obtain sufficient quantities of library DNA to perform a library-scale yeast transformation, the library was amplified. Based on the initial titer of the library, the commercial library stock was amplified to give approximately 2-3 times the number of independent clones (or colonies). The amplified DNA was confirmed using restriction sites flanking the multiple cloning site of pACT2. The library was also tested with PCR primers flanking the cloning sites. The inserts in the restriction digests, as well as the PCR products, were of the size ranging from 0.4 kb-4 kb consistent with the expected size based on the initial size fractionation of the testis mRNA reported in the specification sheet for the library. The bait gene for this yeast two-hybrid screening project was murine Dnmt3b1, which was constructed to express as a fusion protein with the GAL4DBD in pGBKTT7 (Catalog no. 630489). The pGBKTT7 vector expresses proteins fused to amino acids 1–147 of the GAL4 DNA binding domain (DNA-BD). pGBKTT7 also contains the T7 promoter, a c-Myc epitope tag, and a
multiple cloning site (MCS) where the gene of interest is cloned. pGBKKT7 replicates autonomously in both *E. coli* and *S. cerevisiae* and contains the kanamycin resistance gene for selection in *E. coli* and the *TRP1* nutritional marker for selection in yeast. The yeast strain used in this screen was AH109. AH109 contains distinct *ADE2, HIS3, lacZ,* and *MEL1* reporter constructs that are only expressed in the presence of GAL4-based protein interactions. This strain virtually eliminates false positive protein interactions that arise during a typical GAL4-based two-hybrid screen because of tight regulation of gene expression. AH109 yeast colonies transformed with the plasmid containing the bait gene (full-length Dnmt3b1) were picked and screened for the expression of the GAL4-DBD fusion protein by western blotting with a mouse monoclonal anti-GAL4DBD and a mouse monoclonal Dnmt3b antibody. The successful transformants expressing the expected size protein (~120 kD) were used in the sequential transformation for library screening. The fusion proteins were also confirmed for an absence of auto-activation of the reporter genes on co-transformation with plasmid containing the GAL4 activation domain (AD). After transformation, the yeast strain was grown and maintained in the appropriate synthetic dropout (SD) media (SD/-Trp). The library screen was performed using yeast strain AH109 stably expressing full length Dnmt3b1 under high stringency conditions (Ade⁻, His⁻, Leu⁻, Trp⁻ media) using sequential transformation. A sequential transformation (large scale) was preferred because it uses significantly less plasmid DNA than simultaneous co-transformation and is 10-fold more efficient. Briefly, the AH109 yeast strain was propagated in YPD medium (20 g/L Difco peptone, 10 g/L Yeast extract) or on YPD agar plates (YPD containing 20 g/L agar). Selections were performed on SD/dropout plates (6.7 g/L yeast nitrogen base without amino acids, 20 g/L agar, and appropriate synthetic dropout supplement lacking specific amino acids). All media were supplemented with 2% glucose. A single colony (2–3
mm in diameter) was inoculated into 1 ml YPD and vortexed vigorously to disperse any clumps. The sample was transferred into a flask containing 50 ml of YPD or the appropriate SD medium and incubated at 30°C for 16–18 hours with shaking at 250 rpm to stationary phase (OD600>1.5). A 30 ml overnight culture was transferred to a flask containing 300 ml of YPD and incubated at 30°C with shaking at 250 rpm. When the OD600 of the culture reaches 0.4–0.6, the cells were harvested by centrifuging at 1,000 x g for 5 min at room temperature. The supernatants were discarded and the cell pellets were thoroughly resuspended in sterile TE and centrifuged at 1,000 x g for 5 minutes at room temperature. The supernatant was decanted and the cell pellet was resuspended in 1.5 ml of freshly prepared, sterile 1x TE/1x LiAc (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5, 0.1 mM LiAc). For each transformation, 100 ng plasmid DNA and 0.1 mg of herring testes carrier DNA were added to 0.1 ml of yeast competent cells in a sterile 1.5-ml tube and mixed well by vortexing. To each tube, 600 µl of sterile PEG/LiAc (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5, 0.1 M LiAc, and 40% PEG polyethylene glycol 3350) solution was added and vortexed at high speed for 10 seconds to mix. The mixture was incubated at 30°C for 30 minutes with shaking at 200 rpm. DMSO (70 µl per reaction) was added to the yeast cells and mixed by gentle inversion. The cells were heat shocked for 15 minutes in a 42°C water bath and chilled on ice for 1–2 minutes. The cells were then centrifuged for 5 seconds at 14,000 rpm at room temperature. The supernatant was discarded and the cells were resuspended in 0.5 ml of sterile 1x TE buffer and 100 µl of the cell suspension was plated on each SD agar plate to select for the desired transformants. The positive colonies (LacZ positive, blue-white selection) from the initial library transformants were restreaked on SD/-Ade/-His/-Leu/-Trp/X-α-gal 2-3 times to allow segregation in case they were transformed with multiple plasmids while maintaining the selective pressure on both the DBD and AD vectors. The restreaked and retested colonies were
collected and were assayed by liquid culture β-galactosidase activity (LacZ) after growth on the SD/-Leu/-Trp medium to verify the two-hybrid interactions. The transformants positive for interaction were identified by the appearance of blue colored colonies and liquid β-galactosidase assay. The positive colonies were sorted to eliminate duplicates by isolating the plasmid DNA from yeast (mixture of DBD/bait plasmid) and retransforming them into E.coli. The cDNA inserts were amplified by PCR to check for the product size and sequenced with primers flanking the multiple cloning sites (MCS) of the library plasmid using the DNA isolated form E.coli. The novel protein interacting partners were identified by sequencing the cDNA insert using the GAL4AD sequencing primer. The protein interaction was retested by co-transformation of the yeast recovered cDNA plasmid of the putative interacting protein or a full-length GAL4AD construct of the putative interacting protein (constructed in the laboratory) with the GAL4DBD-fused Dnmt3b1 in yeast followed by liquid β-galactosidase assay. Based on the transformation efficiency, the number of possible clones screened per transformation was estimated. The human testis cDNA library (Clontech) is indicated to have 2 x 10⁶ independent clones and the large scale screening was performed 2-3 times to ensure maximum coverage of the total possible number of independent clones screened.

**Beta-Galactosidase assay**

Approximately 10⁷ clones were screened and positive colonies (LacZ positive) from the primary screen were re-streaked on the same media for confirmation for LacZ positive phenotype and tested by performing a calorimetric β-galactosidase assay. Each new putative interaction was tested for β-galactosidase activity in comparison to the known interaction between p53 and T antigen that served as a positive control. Plasmid DNA from positive yeast colonies was directly transferred into E.coli by electroporation (Marcil and Higgins, 1992), and the transformed E.coli were sequenced using the GAL4AD sequencing primer in 96 well plates.
at the UF ICBR Genomics Sequencing core facility to identify the clones. Plasmids for the bait and positive library clone (identified by sequencing) were co-transformed into yeast strain Y190 (using Trp−, Leu+ media) and the interaction was verified with β-galactosidase assay. The Y190 host strain was used for β-galactosidase assay instead of the AH109 strain as the Y190 strain contains only two reporter constructs HIS3 and lacZ and is reported to have less stringent expression. The use of the less stringent Y190 strain enables good amplification of the reporter gene activity and helps the confirmation of the protein-protein interaction using the β-galactosidase assay in a quicker and easier way. A 5 ml overnight culture of desired transformants was grown in a minimal liquid SD selection medium (SD –Trp/Leu). The overnight culture was vortexed to disperse cell clumps and 1 ml of the overnight culture was transferred to 4 ml of YPD. The fresh culture was incubated at 30°C for 3–5 hr with shaking at 250 rpm until the cells are in mid-log phase (OD600 of 1 ml = 0.5–0.8). The exact OD600 was recorded when the cells were harvested. The substrate for the assay, o-Nitrophenyl-β-galactoside (ONPG) was dissolved at 4 mg/ml in Z buffer (16.1 g/L of Na2HPO4.7H2O, 5.50 g/L of NaH2PO4.H2O, 0.75 g/L of KCl, 0.246 g/L of MgSO4.7H2O and final pH 7.0) with shaking for 1–2 hours. For each transformant to be tested, 1.5 ml culture was placed into each of three 1.5-ml microcentrifuge tubes. The sample was centrifuged at 14,000 rpm (10,000 x g) for 30 seconds and the supernatant was discarded. The cells were washed once in 1.5 ml Z buffer and the supernatants discarded. Each pellet was resuspended in 300 µl of Z buffer. For each reaction, 100 µl cell suspension was transferred to a fresh microcentrifuge tube and the cells were lysed by placing the tubes in liquid nitrogen until the cells were frozen (0.5–1 min), and then thawed by placing the frozen tubes in a 37°C water bath for 0.5–1 minutes. The freeze/thaw cycle was repeated two more times to ensure that the cells have lysed. Each β-galactosidase
assay reaction sample contained 0.7 ml of Z buffer + β-mercaptoethanol (0.27 μl β-mercaptoethanol per 10 ml Z buffer) that was added to the lysed yeast. A blank tube with 100 μl of Z buffer (without lysed yeast) along with the other reaction components was set as a negative control for the calorimetric assay. At this point, a timer was started followed by the addition of 160 μl of ONPG in Z buffer to the reaction and blank tubes. The tubes were placed in 30°C incubator. After the yellow color developed (30 minutes to overnight), 0.4 ml of 1 M Na₂CO₃ was added to the reaction and blank tubes and the elapsed time in minutes was recorded. The reaction tubes were centrifuged for 10 minutes at 14,000 rpm to pellet cell debris and the supernatants were transferred to clean cuvettes. The absorbance was measured at A₄₂₀ for the samples relative to the blank. The OD₄₂₀ should be between 0.02–1.0 to be within the linear range of the assay. β-galactosidase activity was calculated using the formula: 1000 × OD₄₂₀/(t × V × OD₆₀₀), where t = incubation time, V = 0.1 ml × dilution factor, and OD₆₀₀ = A₆₀₀ of 1 ml of culture and is represented in Miller units where 1 unit of beta- galactosidase is defined as the amount which hydrolyzes 1 μmol of ONPG to o-nitrophenol and D-galactose per minute per cell (Griffith and Wolf, 2002).

**Cell Culture**

The cell lines used in these studies were –human cervical carcinoma cell line- HeLa, human colorectal carcinoma cell line- HCT116 and the isogenic HCT116 cell line with a knockout of DNMT3B (3BKO) (Rhee et al., 2002), human embryonic kidney – HEK293T cells and human embryonic kidney derived 293FT (fast transform) cells (Invitrogen). All cell types were maintained at 37°C in a 5% CO₂ incubator. HCT116 cell lines were cultured in McCoy’s 5A media supplemented with 2 mM L-glutamine (Invitrogen) and 10% heat inactivated FBS. Human embryonic kidney 293T cells were cultured in Dulbecco’s modified eagles’s medium
(DMEM) supplemented with 2mM L-Glutamine (Invitrogen) and 10% heat inactivated FBS. 293FT cells were cultured according to the manufacturer’s protocols in Dulbecco’s modified eagles’s medium (DMEM) supplemented with 0.1 mM MEM non essential amino acids (NEAA), 1 mM sodium pyruvate, 2 mM L-Glutamine (Invitrogen) and 10% heat inactivated FBS and 500 µg/ml of G418.

**Transient Transfection and Expression**

HCT116 cells or HeLa cells (2 x 10^5 cells per well) were plated on 6-well plates 12-16 hours before transfection with LT-1 reagent (Mirus). For each transfection, 2 µg of plasmid was used and 6 µl of LT-1 reagent was used per 1 µg of plasmid DNA. Transfection was performed according to the manufacturer’s instructions. Briefly, the 12 µl of transfection reagent was incubated with 250 µl of serum free medium for 20 minutes at room temperature in a sterile eppendorf tube. Plasmid DNA (2 µg) was added to this mixture and mixed thoroughly by pipeting and the sample was incubated for another 20 minutes at room temperature. The cells to be transfected were replenished with fresh complete medium and the DNA-transfection reagent mixture was added dropwise on the cells. The cells were harvested for DNA, RNA or whole cell extract 48 hours post-transfection.

**Calcium Phosphate Transfection**

HEK293T cells were plated (5 x 10^6 cells per 60 mm dish) in complete DMEM medium on the day prior to transfection to attain 70-75% confluence. The next morning, cells were replenished with fresh media. The following reagents were added in a sterile 12 ml polypropylene tube and mixed: 20 µg DNA, 61 µl of 2 M CaCl₂, and water added to make the final volume to 500 µl. The reagents were mixed by pipeting and 2XHBS (50 mM HEPES, 270 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.0) was added dropwise to the mixture while being mixed
with vigorous bubbling by pipetting. The DNA-CaPO₄ mixture was allowed to sit at room temperature for 5 minutes and then added dropwise to the surface of the media containing the cells. The cells were washed twice with PBS and replenished with normal medium the next day. The transfected cells were harvested 48 hours post-transfection.

**Short Interfering RNA (siRNA) Transfection**

The human DNMT3B siRNA, CENP-C siRNA, GAPDH siRNA, control siRNA and DharmaFECT 1 transfection reagent were purchased from Dharmacon, Inc. On day 1, HCT116 cells were plated in 24-well or 6-well plates at a density of 2 x 10⁵ cells/well in complete media (McCoy’s 5A). Transfection was performed on day 2, according to the manufacturer’s instructions using 3 μl DharmaFECT-1 transfection reagent and 100 nM siRNA (final concentration) per well. Transfected HCT116 cells were cultured for 36 hours before being replenished with complete medium. The cells were transfected once more on Day 4, (48 hours after the initial transfection) with 100 nM of desired siRNA. At 72 hours after the first transfection (day 5), the cells were harvested for RNA, DNA or chromatin.

**Lentiviral Generation and Transduction to Create Stable Cell Lines**

The complete coding sequence of DNMT3B1, DNMT3B3 and DNMT3B3Δ5 (a DNMT3B spliced variant that will be discussed in Chapter 5) were cloned into EcoRI- BamHI sites of the pLVX-Puro plasmid (Clontech). pLVX-Puro is an HIV-1 based, lentiviral expression vector. pLVX-Puro contains all of the viral processing elements necessary for the production of replication-incompetent lentivirus, and transgene expression when transfected into 293FT (Invitrogen) cells. The pLVX-Puro plasmid also contains a puromycin resistance genes allowing selection of stable transductants. The 293FT cells (Invitrogen) were transfected with the above mentioned lentiviral constructs in pLVX-Puro using the Lentiphos-HT packaging system (Clontech) according to the manufacturer’s instructions. Approximately 24 hours before
transfection, 4–5 x 10^6 293FT cells (Invitrogen) were plated in a 100 mm plate in 10 ml of growth medium and incubated at 37°C, 5% CO₂ overnight. The lentiviral construct plasmid DNA (3 µg) and the Lenti-X HT packaging mix (15 µl) were diluted to a final volume of 438 µl using the sterile H₂O supplied with the kit in a sterile 12 x 75 mm round bottom polystyrene tube. The Lenti-X HT packaging mix consists of pre-mixed plasmids that are necessary viral packaging components (Pol, Tat, Rev, Gag and VSV-G lentiviral genes) and is provided by the manufacturer (Clontech). To the diluted DNA, 62 µl of Lentiphos1 solution was added and the mixture was vortexed thoroughly. While vortexing the DNA/Lentiphos1 solution, 500 µl of Lentiphos2 was added dropwise into the tube. The mixture was incubated at room temperature for 5–10 min. The DNA/transfection mixture was vortexed and the entire contents of the tube (1 ml), were added dropwise, to the 293FT cells. The plates were moved back and forth to distribute the transfection solution evenly and then the plates were incubated at 37°C for a minimum of 8 hours to overnight in a CO₂ incubator. The next day, the media was changed and replenished with 10 ml fresh complete growth medium and the cells were incubated at 37°C for an additional 48 hours. The supernatants of the 293FT cells contain the lentivirus and were harvested at 48 hours post-transfection for subsequent lentiviral transduction. The supernatant was centrifuged briefly (500 x g for 10 min) and filtered through a 0.45 µm filter to remove cellular debris. HCT116 and 3BKO cells were split a day before the transduction (2 x 10^5 cells/well in a 6-well plate). Virus stock freshly prepared from 293FT cells was diluted with complete medium (1 ml virus + 2 ml complete medium) and 4 µg/ml polybrene was added to the mixture. Polybrene is a cationic polymer added to increase the efficiency of viral transduction. The viral supernatant was added to the cells and transduction induced for 24 hours. The culture was centrifuged at 1500 rpm for 90 minutes to improve infection efficiency. Twenty four hours
after transduction, the virus-containing transduction medium was removed and replaced with fresh growth medium. The cells were incubated for 24 hours and then subjected to selection with 2 μg/ml Puromycin (Sigma). The antibiotic selection concentration was halved once the control untransduced cells died. Subsequently, the surviving cells were diluted and plated to select for colonies. Surviving colonies were isolated by placing a sterile cloning cylinder around the cells and dislodged from the plate by the addition of trypsin to the cylinder. The cells were incubated until they detach from the dish and collected with a pipette and transferred to well. The cells were regrown in a new well under antibiotic selection. The clones were selected by screening for the gene expression by RT-PCR and western blotting.

**Preparation of Nuclear Extract**

Nuclear extracts were prepared as described in Geiman. et. al. HeLa cells were washed with PBS and the packed cell volume (PCV) was determined. 5 x PCV hypotonic buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.2 mM PMSF) was added to resuspend cells. The cells were incubated on ice for 10 minutes after which they were pelleted at 1500 rpm for 10 minutes at 4°C and the supernatant was decanted. The nuclear pellet was resuspended in 2 x PCV of hypotonic buffer A and transferred to a 30 ml dounce homogenizer. The cells were dounced with 15 plunges with a tight pestle and the sample was transferred into new tubes. Buffer B (50 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM EDTA, 75% sucrose, 0.5 mM DTT, 0.2 mM PMSF) was added to the sample to a final volume of 1/10 PCV and incubated on ice for 10 minutes and then centrifuged at 1500 rpm for 10 minutes 4°C. The supernatant was saved as the cytoplasmic fraction and the nuclei were resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 600 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.2 mM PMSF) at 1 ml per 10⁹ nuclei. The sample
was incubated in a rotor for 30 minutes at 4°C and then centrifuged at 15,000 rpm for 30 minutes. The supernatant was saved and dialyzed into a buffer containing no KCl (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.2 mM PMSF) over a period of 1-2 hours until the conductivity of the dialyzed sample was equal to that of a standard 100 mM KCl solution. The dialyzed sample was centrifuged at 10,000 rpm for 30 minutes to remove debris and the supernatant was saved at -70°C.

**Preparation of Micronuclear Extract**

A micronuclear extract was prepared instead of a conventional nuclear extract (discussed above) when the starting number of cells was low, usually from 1-2 150 mm dishes (Tsai and Carstens, 2006). HeLa cells (1x10⁷) were harvested by scraping them into 1.5 ml of cold PBS. The cell suspension was then transferred to a microfuge tube. Cells were pelleted by centrifuging at 4,000 rpm for 5 minutes and resuspended in 400µl of cold Buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mm MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) by flicking the tube. The cells were allowed to swell on ice for 10 minutes, and then vortexed for 10 seconds. Samples were centrifuged for 10 seconds, and the supernatant fraction was discarded. The pellet was resuspended in 5 x PCV of cold Buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 minutes for high-salt extraction with brief vortexing every 5 minutes. Cellular debris was removed by centrifugation at 13,000 rpm for 2 minutes at 4°C and the supernatant fraction was stored at -70°C.

**Preparation of Soluble Chromatin by Micrococcal Nuclease Digestion**

The soluble chromatin fraction was prepared as described in (Ando et al., 2002). HeLa cells (1x10⁷) from one or two 150 mm dishes were harvested by scraping them into 1.5 ml of cold PBS. The cells were allowed to swell on ice for 10 minutes in 5 x PCV of swelling buffer.
(10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mm MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF), and then vortexed for 10 seconds. Samples were centrifuged at 4,000 rpm for 5 minutes, and the supernatant fraction was discarded. The nuclear pellet (1x10$^7$ nuclei) was dissolved in 5 ml of ice-cold wash buffer (20 mM HEPES pH 8.0, 20 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.05 mM PMSF) containing 0.3 M NaCl to a concentration of approximately 2x10$^6$ nuclei equivalent/ml. The nuclear suspension was digested with 6U/ml MNase at 37°C for 30 minutes after the addition of CaCl$_2$ to a final concentration of 2 mM. The reaction was stopped by the addition of EGTA to a final concentration of 5 mM with quick chilling on ice. The digests were centrifuged at 10,000 x g for 10 min at 4°C. The solubilized chromatin was recovered in the supernatant and stored at -70°C and used in co-immunoprecipitation assays.

**Preparation of Whole Cell Extract and Immunoblotting**

Cells were washed twice in the dish with ice-cold PBS and lysed by adding ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/ml each of aprotinin, leupeptin, pepstatin) (0.25 ml per well in 6 well plate). The cells were scraped off the dish with a plastic cell scraper. The cell suspension was transferred into a centrifuge tube and placed in a rotor for 30 minutes at 4°C to lyse cells. The lysate was centrifuged at 13,000 rpm in a precooled centrifuge for 20 minutes. The supernatant was transferred to a fresh centrifuge tube and the pellet was discarded. The protein concentration of the cell lysate was determined using the Bradford’s assay. The sample was stored at –20°C. For immunoblotting analysis, whole cell extracts, nuclear extracts, micronuclear extracts, soluble chromatin or eluates from co-immunoprecipitations were prepared in sample loading buffer (2% SDS, 125 mM Tris-HCl pH 6.8, 20% glycerol and 0.01% bromophenol blue, 5% 2-mercaptoethanol) and electrophoresed by SDS-PAGE and electro-
transferred onto a PVDF membrane overnight in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). After transfer, the membranes were rinsed once with Tris buffered saline (TBST) containing 50 mM Tris-HCl pH 7.5, 0.05% Tween-20, 150 mM NaCl for 10 minutes at room temperature. The membrane was blocked with 5% non-fat dry milk in TBST for 1 hour at room temperature in a shaker. Immunoblotting was performed by incubating the membrane with primary antibody diluted in 5% milk (blocking solution) to a final concentration of 0.5-1.0 µg/ml for 1 hour at room temperature with rotation. The membrane was washed four times for 10 minutes with 0.5% milk in TBST on a shaker and then incubated with the appropriate peroxidase-conjugated secondary antibody (1:5000 dilution) for 45 minutes at room temperature. The membrane was washed four times for 10 minutes with 0.5% milk in TBST on a shaker. The bound secondary antibody was detected using an enhanced chemiluminescence kit (Pierce) by exposure of the blot to film (Mid Sci). The Pierce Supersignal West ECL kit is a highly sensitive nonradioactive, enhanced luminol-based chemiluminescence based assay for detection of horseradish peroxidase (HRP) on immunoblots. The Pierce Supersignal West Pico ECL kit was used for detecting signal in most immunoblots. The Pierce Supersignal West Dura ECL kit was used when higher sensitivity and longer signal duration are required for detecting the protein.

**Co-immunoprecipitation**

Approximately, 1x10^7 cells were harvested for each immunoprecipitation. Cells were washed twice in the 150 mm dish with ice-cold PBS and were processed for protein extraction as described above. For each co-immunoprecipitation, 600 µl of immunoprecipitation dilution buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, 0.5% Triton-X 100) was added to 200 µl of protein extract sample and precleared with 50 µl of the Protein A/G beads slurry (Santa Cruz) by incubating on rotator at 4°C for 2 hours. The
sample was centrifuged at 4000 rpm for 10 minutes at 4°C and the supernatant transferred to a fresh tube. To the eppendorf tube containing the cold precleared lysate, 10 µg primary antibody (Table 2-1) was added and incubated overnight at 4°C on a rocker. The next day, the immunocomplex was captured by adding 50 µl of washed Protein G agarose slurry by incubating the sample for 4 hours at 4°C on a rotator. The sample was centrifuged at 4000 rpm for 10 minutes at 4°C and the supernatant discarded. The beads were washed five times for 10 minutes each with 800 µl of wash buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, aprotinin (1 µg/ml), leupeptin (1 µg/ml), and pepstatin (1 µg/ml)). After the last wash, supernatant was removed and the beads were resuspended in 40 µl of 2x sample loading buffer, vortexed and denatured by boiling for 5 minutes at 95°C to dissociate the immunocomplexes from the beads. The sample was centrifuged at 13,000 rpm for 10 minutes, and the supernatant was loaded onto an SDS-PAGE gel to be analyzed by immunoblotting.

**RNA Isolation and RT-PCR**

Cells were lysed directly in a culture dish by adding 1 ml of TRIZOL reagent (Invitrogen) per 3.5 cm diameter dish or per one well of a 6-well plate. The cell lysate was passed several times through a 1 ml pipette tip. For each 1 ml of TRIZOL reagent, 200 µl of chloroform was added and the sample was incubated at room temperature for 2-3 minutes. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the upper aqueous phase was transferred carefully without disturbing the interphase into a fresh tube and the RNA was precipitated from the aqueous phase by mixing it with 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL reagent used for the initial homogenization. The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 10 ml of 75% ethanol.
in DEPC treated water. The samples were mixed thoroughly and centrifuged at 7,500 x g for 10 minutes at 4°C. All leftover ethanol was removed. RNA pellet was air-dried for 5-10 minutes and dissolved in DEPC-treated water and quantitated for further use. RNA to be used in analysis of satellite repeat regions was prepared using the QIAGEN RNAeasy kit with DNase treatment. DNase treated RNA was extracted by following the manufacturer’s protocols. cDNA synthesis was performed using the high capacity cDNA kit from ABI with approximately 2 µg of RNA, 2 µl of 10X reverse transcription buffer, 2 µl of 10X random primers (50 µM), 1 µl of RNase inhibitor, 0.8 µl of 25X dNTPs (100 mM stock), 1 µl of Multiscribe reverse transcriptase (50u/µl) and nuclease free water to a total reaction volume of 20 µl per reaction. PCR cycling conditions for reverse transcription were set according to the manufacturer’s instructions. Semi-quantitative RT-PCR was performed with 1 µl of cDNA, 2 µl 10X Taq gold reaction buffer (ABI), 2.5 mM MgCl₂ (ABI), 100 nM dNTPs, 0.25 µl Taq gold polymerase (ABI), 200 nM of each forward and reverse primer (Table 2-2) and nuclease free water to a final volume of 20 µl per reaction. The PCR reactions were incubated at 95°C for 10 minutes to activate the Taq polymerase and PCR amplification was performed for 35 cycles for 95°C for 30 seconds, X°C (Table 2-2) for 90 seconds. Quantitative real-time RT-PCR was performed using the Bio-Rad MiniOpticon Real-Time PCR System (Bio-Rad Laboratories). Serial dilutions of one-fourth concentration were performed to generate a standard curve starting with 1X, to give final concentrations of 1/4, 1/16, 1/64 and 1/256 of the cDNA sample from a control cell line. The standard curve was used determine the relative amount of product and set the threshold cycle (Cₗ) values for each primer for run to run consistency. Each 20 µl reaction mixture consisted of 1X SYBR Green PCR Master Mix (ABI) and 200 nM concentrations of forward and reverse primers. Duplicates for both the standards and the samples were simultaneously amplified using
the same reaction conditions. The threshold cycle (C_T) values were determined using the Opticon Monitor 3 software (Bio-Rad Laboratories). After PCR, melting curves were acquired by stepwise increase in the temperature from 55°C to 95°C to ensure that a single product was amplified in each reaction. Samples from at least three independent reactions were analyzed.

**DNA Isolation**

Approximately, 10^7 cells were trypsinized and harvested per cell line of interest/ siRNA knockdown cells. The cell pellet was rinsed twice with PBS and resuspended in 2 ml of TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). SDS (10% stock solution) and proteinase K (10 mg/ml stock solution) were added to a final concentration of 0.5% and 200 µg/ml, respectively. The mixture was incubated at 37°C overnight. The following day, the sample was extracted once with equal volumes of each of the following: buffered phenol (Invitrogen), phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) in that order. The DNA in the aqueous phase was then precipitated by adding 1/10 volume of 3M sodium acetate (pH 5.2) and 1.5 volume of absolute ethanol. The sample was centrifuged at 10,000 rpm for 10 minutes to pellet the DNA. The DNA pellet was washed once with 70% ethanol. The pellet was air dried and resuspended in TE and quantitated before use.

**Chromatin Immunoprecipitation**

Chromatin immunoprecipitation was performed as described in Methods in Molecular Biology (Lefevre and Bonifer, 2006). Approximately 8-10 x10^6 HCT116 cells were plated per 150 mm dish. Approximately 1x10^7 cells were required per immunoprecipitation. The cells were crosslinked by adding 37% formaldehyde to the plate (1% final concentration) and incubated for 10 min at room temperature in a shaker. The crosslinking was stopped by adding 2 M glycine to a final concentration of 0.125 M, and the mixture incubated for additional 5 minutes at room temperature to stop the crosslinking reaction. The cells were scraped off and
washed twice with ice cold PBS containing protease inhibitors (1 mM PMSF, aprotinin (1 µg/ml), leupeptin (1 µg/ml), and pepstatin (1 µg/ml)). The formaldehyde cross-linked cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl\(_2\) and 0.2% NP40, 1 mM PMSF, aprotinin (1 µg/ml), leupeptin (1 µg/ml), and pepstatin (1 µg/ml)) for 1 hour at 4°C. The supernatant was discarded and the nuclei were resuspended in glycerol buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 5 mM MgAc\(_2\) and 25% glycerol). To generate nucleosomal material, the nuclei were resuspended in an equal volume of 2X MNase buffer containing 50 mM KCl, 8mM MgCl\(_2\), 2 mM CaCl\(_2\), 100 mM Tris-HCl, pH 7.4 and treated with 100 u/ml for micrococcal nuclease 15 minutes at 37°C. The reaction was stopped by adding EDTA to a final concentration of 10mM. The digested nuclei were resuspended in immunoprecipitation buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2mM EDTA, 1% Triton-X 100 and 0.1% SDS, 1 mM PMSF, aprotinin (1 µg/ml), leupeptin (1 µg/ml) and pepstatin (1 µg/ml)). The sample was cleared of debris by centrifugation at 4000 rpm for 5 minutes and the supernatant was quantitated and used as the chromatin template for ChIP. For each ChIP, 25 µg of chromatin was used. If the volume of the chromatin preparation was less than 1 ml, the sample was diluted with ChIP dilution buffer (0.01% SDS, 1.0 % Triton X-100, 1.0 mM EDTA, 20 mM Tris-HCl, pH 8.0) to bring the volume to 1 ml per immunoprecipitation. Each immunoprecipitation sample was precleared by incubating with 50 µl Protein A-sepharose beads (Upstate) (50% slurry in TE+0.05% sodium azide, 50 µl per ml) by rotating at 4°C for 4 hrs. The sample was centrifuged at 4,000 rpm for 10 min and the supernatants were transferred to a new tube. For each ChIP, 5 µg of antibody (Table 2-1) was added to the lysate and incubated overnight on the rotator in 4°C. Protein A sepharose beads were resuspended to 50% slurry in BSA blocking buffer (3% BSA, 0.05% sodium azide, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.2 mg/ml salmon sperm DNA, (1
μg/ml) aprotinin, (1 μg/ml) leupeptin, (1 μg/ml) and pepstatin, (1 μg/ml)) to be blocked overnight. Each sample was immunocaptured by adding 60 μl of BSA blocked Protein A-Sepharose slurry and rotated for 4 hours at 4°C. The sample was centrifuged and 100 μl of non-specific antibody supernatant (mouse IgG; rabbit IgG) was saved in new tubes as the ‘Input’. The input for each sample was diluted with ChIP dilution buffer (500 μl final volume) for further processing. The pelleted beads were washed twice for 10 minutes at 4°C in a rotator, with 1 ml of each - low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP 40, 1% sodium Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) in that order. After the final wash, the chromatin bound to the beads was eluted with 2 washes (275 μl each) of freshly made elution buffer (1% SDS, 0.1 M NaHCO₃). The sample was vortexed after the addition of elution buffer and incubated in the bacterial culture shaker (37°C) for 15 minutes per elution. A total of 500 μl of eluate was collected per sample. The reverse crosslinking was performed by adding 20 μl 5M NaCl to each tube, including the input for each sample, and the sample was incubated for 4 - 5 hours at 65 °C. The contaminating RNA was removed by adding 1 μl of RNase A (from 10 mg/ml, DNase-free stock) to each sample and incubating at 37°C for 1 hour. Each sample was then treated with proteinase K (10 μl 0.5M EDTA, 20 μl 1M Tris-HCl, pH 7.0, and 1 μl 20 mg/ml proteinase K) and incubated for 1 hr at 37°C after which the immunoprecipitated samples were cleaned using the Qiagen miniprep columns. Each immunoprecipitated sample (500 μl) was diluted with 2 ml PB buffer provided in the Qiagen miniprep kit. Each input sample (100 μl) was diluted with 2 ml PB buffer provided in the Qiagen miniprep kit. The samples were mixed and run through one
MiniPrep column per sample. The column was washed once with PE buffer (containing ethanol). The DNA was eluted from the column with 100 μl TE (pH 8.0). Purified, immunoprecipitated DNA was analyzed by quantitative real-time PCR (qPCR) with primers amplifying specific regions of interest. The enrichment was represented as fold enrichment in comparison to the input signals ($2^{(Ct(Input)-Ct(sample))}$). Quantitative real-time PCR was performed using the Bio-Rad MiniOpticon Real-Time PCR System (Bio-Rad Laboratories). Each 20 μl reaction mixture consisted of 1x SYBR Green PCR Master Mix (ABI) and 200 nM concentrations of forward and reverse primers (Table 2-3). Each 20 μl PCR reaction contained 4 μl each of immunoprecipitated sample or 2 μl each of input samples (0.5% of input). The reactions were incubated at 95°C for 15 minutes to activate the polymerase, followed by amplification at 95°C for 15 seconds and X°C (Table 2-3) for 60 seconds for 35 cycles. After PCR, melting curves were acquired by stepwise increase in the temperature from 55° to 95°C to ensure that a single product was amplified in each reaction. Samples from at least three independent reactions were analyzed.

**Immunofluorescence**

HeLa cells or HCT116 cells were grown in 22-mm$^2$ glass coverslips in six well plates. Forty eight hours after transfection with plasmid or 72 hours after the transfection of siRNA, cells were washed twice times with PBS at room temperature. The cells were fixed on the coverslips by adding 2.0 % paraformaldehyde in PBS (pH 7.0) for 10 - 15 min. The fixative was removed and the cells were rinsed twice with PBS. The cells were permeabilized for 10 minutes with 0.5% Triton X-100 in PBS at room temperature. The cells were washed thrice with PBS and blocked for 30 minutes with 0.2% fish skin gelatin solution in PBS at room temperature. The gelatin solution was prepared by dissolving gelatin powder (Sigma) in sterile MilliQ water.
by gently swirling the mixture for 15 minutes in a 60°C water bath. The 0.2% gelatin solution was cooled at room temperature, and, while still warm (~37°C), filtered through a 0.45 µm syringe filter and stored at 4°C. The coverslip with the cells facing down was incubated on the primary antibody (1µg of total antibody in 50 µl of PBST (PBS with 0.1% Tween-20)) by placing on a parafilm for 1 hour at room temperature. The cells were washed four times with PBST. The cells were incubated with the appropriate fluorescent-labeled secondary antibody (0.002 µg of total secondary antibody in 50 µl of PBST) for 1 hour at room temperature. The cells were washed four times with PBST. The cells were then counterstained for DNA with 1 µg/ml Hoechst 33258 dye (10 mg/ml stock solution) in PBS for 1-5 minutes and washed 5 times with PBST. The excess buffer was removed by touching the edge of coverslip onto absorbent paper and mounted on the glass slide by placing the coverslips on fluromount (Southern Biotech) and allowed to solidify in the dark overnight. To enrich for mitotic cells, HeLa cells or HCT116 cells were transfected (as described above). Twenty four hours post-transfection, the cells were subjected to a cell cycle block by the addition of thymidine (Sigma) (250 mM stock solution prepared by dissolving thymidine in sterile PBS) to a final concentration of 2.5 mM. After 17 hours the cells were washed twice with sterile PBS and allowed to recover in complete medium. After 8–9 h of recovery time, cells were fixed with 2% paraformaldehyde and subjected to immunostaining as described above. The images were captured using a Nikon TE2000 inverted microscope and deconvolved using Nikon Elements advanced software.

**Bisulfite Genomic Sequencing**

Genomic DNA (5–10 µg) was denatured in 0.3 M NaOH at 37°C for 15 minutes. Denatured DNA was mixed directly with 333 µl of bisulfite solution (2.4 M sodium metabisulfite (Sigma)/ 0.5 mM hydroquinone (Sigma)) and treated in darkness for 4-hours. The bisulfite
treated DNA was captured by adding the QIAEX II agarose suspension and incubated at room temperature for 1 hour. The DNA bound beads are desalted and purified using the Qiaex II gel extraction kit (Qiagen) by rinsing twice each with buffer QX1 and PE (in that order) provided in the kit. After the washes, the QIAEX II suspension was resuspended in 50 µl TE and incubated at room temperature for 10 minutes. The beads were then treated by adding 0.3 M NaOH at 37°C for 15 minutes. The DNA was then precipitated by adding 9 M ammonium acetate and 3M sodium acetate (pH 5.2) and purified again using the Qiaex II gel extraction kit (Qiagen) as described above. After the beads were washed the DNA was eluted in 100 µl TE (pH 8.0) and stored at -20°C. The bisulfite treated DNA was used as a template to amplify regions of interest using Taq gold polymerase (ABI). The PCR product amplified from the bisulfite treated DNA was gel purified from the agarose gel using the Qiax II gel extraction kit (Qiagen) according to the manufacturer’s instructions and TOPO cloned using the TA Cloning Kit (Invitrogen). The cloned products in the TOPO clones were sequenced in a 96-well plate format using the M13 reverse primer at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) and the methylation levels were analyzed. The statistical significance was determined using the Chi-square test.
Table 2-1. Antibodies used in ChIP, IP and/or immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer/Source</th>
<th>Catalog Number</th>
<th>Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rabbit IgG</td>
<td>Pierce</td>
<td>0031883</td>
<td>Rabbit</td>
<td>10µg (ChIP)</td>
</tr>
<tr>
<td>Normal mouse IgG</td>
<td>Pierce</td>
<td>0031884</td>
<td>Mouse</td>
<td>10µg (ChIP)</td>
</tr>
<tr>
<td>DNMT3B</td>
<td>Novus Biologicals</td>
<td>NB300-516</td>
<td>Rabbit</td>
<td>10µg (ChIP)</td>
</tr>
<tr>
<td>Ac-H3K9/K18</td>
<td>Upstate/Millipore</td>
<td>07-593</td>
<td>Rabbit</td>
<td>10µg (ChIP)</td>
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<td>2XMe H3K4</td>
<td>Upstate/Millipore</td>
<td>07-030</td>
<td>Rabbit</td>
<td>10µg (ChIP)</td>
</tr>
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<td>3XMe H3K4</td>
<td>Abcam</td>
<td>ab8580</td>
<td>Rabbit</td>
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<td>ab24684</td>
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<td>ab6002</td>
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<td>ab1791</td>
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<td>CENP-C</td>
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<td>ab50974</td>
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<td>Rabbit</td>
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<td>ab13939</td>
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<td>FLAG M2</td>
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<td>F3165</td>
<td>Mouse</td>
<td>1:1000 WB</td>
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<tr>
<td>HA</td>
<td>Roche</td>
<td>1186742300150</td>
<td>Rat</td>
<td>1:1000 WB</td>
</tr>
<tr>
<td>HP1 alpha</td>
<td>Dr. Keith Robertson</td>
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<td>Primers</td>
<td>Sequence</td>
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<td>---------------------------------</td>
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</tr>
<tr>
<td><strong>Satellite alpha</strong> (Chr 1)</td>
<td>5'- TCATTCCCCACAAACTGCCTGTG -3' (F) 5'- TCCAACGAAGGCCACAAGA – 3’ (R)</td>
<td>59</td>
<td></td>
<td></td>
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<td><strong>Satellite 2</strong> (Chr 1)</td>
<td>5'- CTGCACTACCTGAAGAGGAC- 3’ (F) 5'- GATGGTTCAACACTCTTACA- 3’ (R)</td>
<td>56</td>
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<tr>
<td><strong>DNMT3B</strong></td>
<td>5'- GCTCTTACCTTACCATCAG -3’ (F)  5'- TGAACCTGTCTCCATCTCC 3’ (R)</td>
<td>58</td>
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<tr>
<td><strong>CENP-C</strong></td>
<td>5’ –CCAAAGAAGCAGATGATGCTCAG -3’ (F) 5’ – TACTTCCACTAATTCGAATCC – 3’ (R)</td>
<td>58</td>
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<tr>
<td><strong>DNMT3B WT</strong> (exon 4-6) specific</td>
<td>5’- AGAAAGCCAGGATGATCCGAAC-3’(F) 5’- AGGTGTGTCTCTGTGTCTCCTCTGT3’ (R)</td>
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<tr>
<td><strong>DNMT3B Δ5</strong> specific</td>
<td>5’- GAAAGCCGCCGATGATCCGAAC-3’(F) 5’- GACGCTCTAGGTGTCATCTCTCTCC 3’ (R)</td>
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<tr>
<td><strong>Dnmt3b WT</strong> (exon 5-7) specific</td>
<td>5’- CGCCACCATGTGCAAGAGGTGAC-3’ (F) 5’- GACGCTCTAGGTGTCATCTCTCTCC 3’ (R)</td>
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<tr>
<td><strong>Dnmt3bΔ6</strong> specific</td>
<td>5’- GAAAGCCCGCTCCTGAGAC-3’ (F) 5’- GACGCTCTAGGTGTCATCTCTCTCC 3’ (R)</td>
<td>65</td>
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<tr>
<td><strong>GAPDH</strong></td>
<td>5’– TTGTATCGTGGAAAAAAGACTC-3 (F) 5’– ACAGTCTTGTGGGCTGAGT-3’ (R)</td>
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<tr>
<td><strong>Gapdh</strong></td>
<td>5’– CACTTGAAGGTTGGAGCCAAAAG-3’ (F) 5’– GTGGATGCAAGGGATGATTTCTG3’(R)</td>
<td>60</td>
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<tr>
<td>Primers</td>
<td>Sequence</td>
<td>Annealing Temperature (°C)</td>
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| Satellite alpha (Chr 1) | 5’- TCATTCCCACAAACTGCGTTG -3’ (F)  
5’- TCCAACGAAGGCCACAAGA- 3’ (R)                  | 59                          |
| Satellite 2 (Chr 1) | 5’- CTGCACTACCTGAAGAGGAC- 3’ (F)  
5’- GATGGTCAACACTTACA- 3’ (R)                  | 56                          |
| WIFI          | 5’- AGCCCTTCGGCTCTTCTGTT -3’ (F)  
5’- CGGCAGAGACGTAAGACTGGCAAA -3’ (R)            | 65                          |
| GAPDH         | 5’- TCGTTCCCAAGTCTCCTGTTTCC -3’ (F)  
5’- TCCGCAGGCCGCTGGTC- 3’ (R)                  | 60                          |
<table>
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<th>Primers</th>
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<th>Annealing Temperature (°C)</th>
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| Satellite 2 (Chr 1)          | 5’-TTTCCGTTTTGGTGGTGATAC-3\’ (F)  
                                 | 5’-ACAGAATTGAATGGGATGC-3\’ (R)                                   | 60                         |
| DNMT3B\Δ5 specific           | 5’-CAGAGGGCCGAAGATCAAGCTC-3\’ (F)  
                                 | 5’-TCCACTGTCTGCCTCCACCTG-3\’ (R)                                   | 58                         |
| Dnmt3b\Δ6 specific           | 5’-CAAGCTCCCAGCCTCTAAGA-3\’ (F)  
                                 | 5’-CATACCCCTCTGATCTCATC-3\’ (R)                                   | 58                         |
Table 2-5. Primers used for BGS

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<td>Satellite alpha</td>
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<td>(Chr 4)</td>
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<td>Satellite 2</td>
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<td>56</td>
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<tr>
<td>(Chr 1)</td>
<td>5’- TAAATAATAACCTCCTTCTTT-3’ (R)</td>
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CHAPTER 3
IDENTIFICATION AND CHARACTERIZATION OF NOVEL DNMT3B INTERACTING PROTEINS

Introduction

Role of DNMT3B in DNA Methylation

Dnmt3b is required for mammalian development and for the establishment of maternal imprints in the oocyte (Okano et al., 1999). DNMT3B is also required for de novo methylation of specific genomic regions such as repetitive elements and proviral elements (Okano et al., 1999; Okano et al., 1998a). Knockout of Dnmt3b in ES cells resulted in loss of methylation at minor satellite repeats, which was not observed in Dnmt3a knockouts (Okano et al., 1999). Mice or humans with DNMT3B mutations are deficient in methylation of pericentromeric repetitive DNA sequences and at CpG islands on the inactive X chromosome (Hansen et al., 2000; Kondo et al., 2000; Miniou et al., 1994; Okano et al., 1998a). Conditional inactivation of Dnmt3b resulted in increased demethylation of the satellite repeats in murine embryonic fibroblasts (MEFs) (Dodge et al., 2005). Individuals with immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, which is an autosomal recessive disease caused by germ-line DNMT3B mutations, exhibit decreased satellite 2 repeat methylation making aberrant DNMT3B function as the causative agent for hypomethylation. In differentiating mouse embryonic carcinoma (EC) and ES cells, Dnmt3a and Dnmt3b function as a complex to stimulate each other’s activity independent of their catalytic activity and methylate the Oct4 and Nanog promoters, both genes known to regulate transcription and pluripotency during early development (Li et al., 2007b). DNA methylation and Dnmt3b binding, and consequent silencing of a subset of the Hox genes, (an evolutionarily conserved gene family known to regulate early development) is mediated by the association of Lsh (lymphoid specific helicase), a member of the SNF2-helicase family of chromatin remodeling protein (Xi et al., 2007).
**Known Protein Interactions of DNMT3B**

DNMT3B is known to interact with several chromatin associated factors via the PHD domain and the PWWP domain (Xie et al., 1999). These interactions have been identified by large scale protein purifications, co immunoprecipitation assays, and yeast two-hybrid screenings. DNMT3B was shown to coimmunoprecipitate with the histone deacetylases HDAC1/2 (Geiman et al., 2004b) and this interaction is mediated by the N-terminal PHD domain of DNMT3B (Bachman et al., 2001). DNMT3B also co-immunoprecipitates with the histone H3 K9 methyltransferase SUV39H1 and the heterochromatin protein HP1 (Geiman et al., 2004b), and the Suv39h1 HMTase directed H3K9 trimethylation was required for recruiting Dnmt3b-dependent DNA methylation to pericentromeric repeats (Lehnertz et al., 2003). Using mass spectrometry following biochemical purification, a novel complex of DNMT3B that includes components of the mitotic chromosome condensation machinery of hCAP-C (SMC4), KIF4A, SIN3A, hCAP-E (SMC2), hSNF2H, and HDAC1 was identified (Geiman et al., 2004a). DNMT3B also co-localizes with KIF4A, hCAP-C, and hSNF2H on condensed mitotic chromosomes and binds to sat2 and rDNA sequences (Geiman et al., 2004a). Both Dnmt3a and Dnmt3b interact with the transcription factor protein PU.1 through the PHD domain (Suzuki et al., 2006). PU.1 (Spi-1), a member of the Ets transcription factor family, is predominantly expressed in myeloid (granulocytes, monocytes and macrophages) and B cells (Chen et al., 1995). Dnmt3b along with the Ets family transcription factor, PU.1 forms a component of a larger complex that includes mSin3A, HDAC1 and the methyl CpG binding MeCP2 (Suzuki et al., 2006). Dnmt3b interacts with the chromatin remodeling enzyme Lsh both in vivo and in vitro independent of its enzymatic activity (Myant and Stancheva, 2008). DNMT3B co-immunoprecipitates with EZH2 and EED, that are components of the PcG protein complex PRC2 (Vire et al., 2006). DNMT3B also interacts with SUMO-1 within its N-terminal domain
and is sumoylated (Kang et al., 2001). A recent study using yeast two hybrid screening showed that the PRC1 component, Cbx4, also interacts with Dnmt3b (Kim et al., 2008b).

**Functional Roles for Known Protein Interactions of DNMT3B**

Dnmt3b exhibits histone deacetylase dependent repression of a reporter gene via its interaction with the histone deacetylase HDAC1 at its PHD domain (Bachman et al., 2001). The DNA methyltransferase activity of Dnmt3b is required for the repression of the Ets family transcription factor PU.1 mediated transactivation and site-specific methylation and regulation of genes responsible for the differentiation of myeloid and B-cell lineages (Suzuki et al., 2006). Recruitment of Dnmt3b, and consequent Dnmt3b dependent DNA methylation at the pericentromeric repeats, is dependent on the Suv39h1 HMTase directed H3K9 trimethylation at these regions (Lehnertz et al., 2003). ES cells lacking *Suv39h1* display an altered DNA methylation profile at pericentromeric satellite repeats but not at other repeat sequences (Lehnertz et al., 2003). The lymphoid specific helicase, Lsh a member of the SNF2-helicase family of chromatin remodeling proteins, is involved in regulating DNA methylation at select imprinted loci and silencing of IAP retrotransposons during development (De La Fuente et al., 2006). Deletion of *Lsh* perturbs DNA methylation patterns in mice causing reduced *de novo* methylation (Zhu et al., 2006). Lsh enables Dnmt3b binding at certain *Hox* gene loci during development and also facilitates the binding of Dnmt3b with the polycomb group proteins, which are known regulators of developmental genes (Xi et al., 2007). Lsh also establishes a repressive chromatin structure via its interaction with Dnmt3b although the creation of a transcriptionally non-permissive environment seems to be independent of the DNA methylation activity of Dnmt3b (Myant and Stancheva, 2008).

Although Dnmt3b and other DNMTs are known to physically associate with the components of the polycomb group of proteins PRC1 and PRC2 (Hernandez-Munoz et al., 2005;
Li et al., 2007a; Vire et al., 2006), the exact mechanism of gene expression regulation via this interaction is not clearly understood. The loss of silencing at EZH2 target genes after RNAi-mediated knockdown of DNMTs demonstrate that repression of EZH2 target genes requires both EZH2 and DNMTs and suggests that EZH2 mediates recruitment of DNMTs to regulatory regions of EZH2 target genes, although the mechanism of DNMT recruitment by EZH2 is not fully elucidated (Vire et al., 2006). A model similar to the recruitment of DNMTs to regions containing SUV39H1&2-mediated H3K9 trimethylation marks has been proposed wherein EZH2-mediated H3K27 methylation may be a requirement for the recruitment of DNMTs, suggesting a link between polycomb-mediated histone methylation and DNA methylation (Lehnertz et al., 2003; Taghavi and van Lohuizen, 2006). While sumoylation of Dnmt3a disrupted its interaction with HDAC1/2 (Ling et al., 2004), the implications of sumoylation of DNMT3B and the exact site of interaction with SUMO-1 itself (Kang et al., 2001) are unknown. The lysine residues that are the target of sumoylation in DNMT3B have not been identified. Similarly, while the PRC1 component, Cbx4 (hPC2), a was identified as a SUMO E3 ligase and interaction partner of Dnmt3a (Li et al., 2007a), Dnmt3b was shown to enhance Cbx4 mediated repression of fibroblast growth factor receptor 3 independent of its DNA methyltransferase activity (Kim et al., 2008b). It is conceivable that in some case DNMTs act as transcriptional repressors independent of their catalytic activity and can become activated later by an unknown regulatory signal that is brought about by their protein interactions (Taghavi and van Lohuizen, 2006) or by post-translational modifications such as sumoylation. The enzymatic activities of the interacting proteins of DNMT3B including histone methylation, ubiquitination, or the post-translational modifications such as sumoylation, may directly or indirectly alter DNMT activity
and/or recruitment to reinforce the stability of heterochromatin regions and thereby protect genome integrity.

**Role of MCAF in Chromatin Regulation**

MBD1-containing chromatin-associated factor (MCAF) (Figure 3-1), also known as ATFα-associated modulator (AM) and activating transcription factor 7-interacting protein (ATF7IP) was first identified and characterized as a protein with transcriptional repression activity that interacts with a member of the CREB/ATF family of transcription factors, murine ATFα (De Graeve et al., 2000). The mouse ATFα-associated Modulator (mAM) colocalizes and interacts with ATFα in mammalian cells, possesses ATPase activity, and demonstrates transcriptional repression activity in an ATPase-independent manner (De Graeve et al., 2000). mAM also interacts with several components of the basal transcription machinery (TFIIE and TFIH), including RNAPII itself (De Graeve et al., 2000). mAM displays a ubiquitous pattern of expression during mouse embryogenesis from 9.5-16.5 d.p.c. and is highly expressed in the adult mouse in specific areas of the brain like the hippocampus and the dentate gyrus, the thymus, lung, intestine, spleen and testis (De Graeve et al., 2000).

Interestingly, the SETDB1 (SuVar3-9, enhancer of Zeste, Trithorax domain bifurcated 1) /ESET (ets-related gene- (ERG) associated protein with SET domain) which is a histone H3 K9 methyltransferase specific to the euchromatic regions (Yang et al., 2002), tightly associates with the human homolog of mAM (Wang et al., 2003). mAM/hAM facilitates ESET/SETDB1-mediated histone methylation by facilitating the conversion of dimethyl to trimethyl state both *in vitro* and *in vivo* (Wang et al., 2003). The enzymatic activity of ESET/mAM complex is significantly higher than ESET itself, and this is brought about by an increase in the $V_{\text{max}}$ and decrease in the $K_m$ without any alterations in the substrate specificity of ESET by mAM (Wang et al., 2003).
hAM was also identified as the MBD1 (methyl domain binding)-containing chromatin-associated factor (MCAF), that interacts with the transcriptional repression domain of MBD1 (Fujita et al., 2003b). Further studies revealed that MCAF is an essential component of the complex that contains MBD1 and SETDB1 and is required for transcriptional repression and heterochromatin formation by MBD1 and SETDB1 (Ichimura et al., 2005). MCAF enhances the transcriptional repression by MBD1, together with SETDB1, and the expression of a MBD1 mutant that lacks its ability to interact with MCAF proteins, perturbs HP1- enriched heterochromatin formation at MBD1-containing chromosomal loci (Ichimura et al., 2005). MCAF also possesses a coactivator-like activity and interacts with enhancer-like transactivator Sp1 and facilitates Sp1-mediated transcription. The MBD1-MCAF complex blocks transcription by inhibiting Sp1 binding on methylated promoter regions (Ichimura et al., 2005). MCAF is characterized by the presence of evolutionarily conserved sequences Domain 1 (aa 562-817) and Domain 2 (aa 1154-1270), which are involved in protein-protein interactions (Ichimura et al., 2005). The transcriptional repression domain of MBD1 binds Domain 2 of MCAF. In addition, SETDB1 binds Domain 1 of MCAF, whereas Sp1 interacts with both Domains 1 and 2 of MCAF (Ichimura et al., 2005). The MBD1-MCAF-SETDB1 complex is recruited at CpG methylated tumor suppressor genes for transcriptional repression whereas the CpG island-associated promoter of telomerase subunit genes are transactivated by the MCAF-Sp1 complex coupled with the general transcriptional machinery (Fujita et al., 2003b; Ichimura et al., 2005; Liu et al., 2009). These findings suggest that MCAF can synergistically contribute to the cancer phenotype both as a co-activator and a co-repressor.

MCAF directly interacts with small ubiquitin like modifier (SUMO) via a sumo interaction motif (SIM) (Uchimura et al., 2006). Modification of MBD1 with either SUMO-2/3
or SUMO-1 facilitated the interaction between MBD1 and MCAF both *in vitro* and *in vivo* (Uchimura et al., 2006). Furthermore, depletion of SUMO pathway components perturbs the assembly of MCAF, H3-K9 trimethylation, and HP1 at MBD1-containing heterochromatin, indicating that posttranslational modifications such as sumoylation can act as epigenetic modulators for heterochromatin formation and gene silencing, at least in part by regulating the MCAF-MBD1 interaction (Uchimura et al., 2006).

**Role of Mi-2 alpha (CHD3) in Chromatin Regulation**

Mi-2 alpha belongs to the CHD (chromo-helicase DNA-binding) protein family (Woodage et al., 1997) (Figure 3-2). The human Mi-2 proteins were initially discovered as autoantigens in the connective tissue disease dermatomyositis (Ge et al., 1995; Seelig et al., 1995). The Mi-2 proteins fall within a subclass of the SWI/SNF family (Eisen et al., 1995) and are widely conserved across species. These large helicase-like ATPases contain conserved PHD fingers in addition to the chromodomains and a putative DNA-binding domain (Woodage et al., 1997). The ATPase activity of Mi-2 proteins from three different species (*D. melanogaster*, *X. laevis* and *H. sapiens*) is stimulated by nucleosomes, but not by free DNA or histones (Brehm et al., 2000; Guschin et al., 2000; Wade et al., 1998; Wang and Zhang, 2001). The Mi-2 ATPase is known to facilitate nucleosome mobility through a sliding mechanism and is essential to facilitate both translational movement of histone octamers relative to DNA and the efficient deacetylation of the core histones within a mononucleosome (Guschin et al., 2000). In *C. elegans*, the two Mi-2 homologs play a crucial role in a Ras signaling pathway critical to cell fate determination during hermaphrodite development (von Zelewsky et al., 2000). The Arabidopsis PICKLE gene, another Mi-2 homolog, acts as a component of an auxin signaling pathway required for lateral root formation (Fukaki et al., 2006) and also promotes histone H3 K27 tri-methylation (Zhang et al., 2008).
Mi-2 alpha and beta are integral components of the NuRD (Nucleosome remodeling and histone deacetylase) complex (Denslow and Wade, 2007). The Mi-2/NuRD complex is a large and abundant macromolecular complex with a broad cellular and tissue distribution that contains a chromatin remodeling ATPase and HDAC1/HDAC2 (Tong et al., 1998; Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998). It is unique in that this complex couples histone deacetylation and chromatin remodeling ATPase activities in the same complex and Mi-2/NuRD is the only known protein entity coupling two independent, chromatin-directed enzymatic functions. The consensus Mi-2/NuRD complex consists of the core subunits: HDAC1 and -2, RbAp46 and -48 (Becker and Horz, 2002), Mi-2 alpha and/or Mi-2 beta (Schultz et al., 2001; Xue et al., 1998), one or more MTA (metastasis-associated) proteins (MTA1, -2, or -3) (Denslow and Wade, 2007; Fujita et al., 2004; Fujita et al., 2003a), and methyl CpG-binding domain (MBD) family of proteins MBD2 or MBD3 (Le Guezennec et al., 2006; Wade et al., 1999; Zhang et al., 1999) and often p66 alpha and/or beta (Brackertz et al., 2006). The ATPase activity of Mi-2/NuRD resides in one or both of the two Mi-2 alpha or beta proteins (CHD3/CHD4) and the catalytic deacetylase subunits of Mi-2/NuRD are composed of HDAC1 and/or HDAC2 (Denslow and Wade, 2007).

Yeast two-hybrid screening identified Mi-2α as an interacting partner of Kruppel associated box (KRAB) domain-associated protein 1 (KAP1) (Schultz et al., 2001). KAP1 corepressor utilizes the Mi-2α-containing NuRD complex as a mediator of transcriptional repression (Schultz et al., 2001). Interestingly, the KAP-1 corepressor binds to the histone H3-K9-specific methyltransferase SETDB1 to coordinate histone methylation and the deposition of HP1 proteins to mediate silencing of genes regulated by the KRAB-KAP-1 repression system (Schultz et al., 2001). The KAP1 PHD domain is necessary for recruitment of the H3-K9-
specific HMTase SETDB1 and NuRD complex protein CHD3 to the promoter regions of 
KRAB-modulated genes (Schultz et al., 2001). The PHD domain of KAP-1 is required for both 
Ubc9 binding and sumoylation. KAP1 sumoylation, in turn, functions by directly recruiting the 
SETDB1 histone methyltransferase and the CHD3/Mi2 component of the NuRD complex via 
SUMO-interacting motifs (SIM) (Ivanov et al., 2007). SETDB1 and CHD3 bind to SUMO-modified KAP1 both \textit{in vitro} and \textit{in vivo}, interact with SUMO1 and SUMO2, and encode 
functional SIM motifs- SETDB1 (aa 122-IIEI-125) and CHD3 (aa 1995-VICI-1998) (Ivanov et 
al., 2007). Thus, sumoylation-mediated repression is required for KAP1-mediated gene 
silencing and occurs via direct recruitment of H3K9 HMTase and HDAC activities (Ivanov et al., 
2007).

Recently, the Mi-2/NuRD complex was found to associate with the pericentromeric 
heterochromatin consisting of satellite 2/3 DNA on human chromosomes 1, 9, and 16 in rapidly 
proliferating lymphoid cells (Helbling Chadwick et al., 2009). Mi-2/NuRD association occurred 
primarily during late S phase and was characterized by a unique pattern of histone modification 
consisting of HP1 proteins and histone H3 trimethylation at lysine 9 (H3K9me3), and its 
accumulation was independent of polycomb association (Helbling Chadwick et al., 2009). Mi-2 
alpha (CHD3) was also identified as an interaction partner for human c-Myb (cellular 
myeloblastosis) in a two-hybrid screen (Saether et al., 2007). The c-Myb protein belongs to a 
group of early hematopoietic transcription factors that are important for progenitor generation 
and proliferation. Mi-2 alpha enhanced c-Myb-dependent reporter activation and the Myb-Mi-2 
alpha co-activation is independent of the ATPase activity of Mi-2alpha (Saether et al., 2007). 
Mi-2 alpha harboring two activities; one repressive, helicase-dependent and one activating 
helicase-independent, suggests a dual function of the Mi-2 protein. The functional role for Mi-2
alpha in transcriptional activation is also consistent with data suggesting that Mi-2 alpha acts as a histone code ‘reader,’ because the Mi-2 alpha PHD finger shows preferential binding to trimethylated histone H3K36 (Shi et al., 2006). Methylated H3K36 is found enriched in active chromatin, where H3K36me3 generally accumulates toward the 3’-region of transcribed gene (Bannister and Kouzarides, 2005). Although the association of Mi-2 alpha with the NuRD complex suggests a repressive role for Mi-2 alpha, the novel activational role implicates an intrinsic transactivation function in the protein, and indicates an ability to recruit other co-activators depending on the chromatin context (Saether et al., 2007). Given the dual function of Mi-2, it is possible that protein associations as well as posttranslational modifications dictate mechanisms determining whether repression or activation should dominate.

In the current study, to gain a better understanding of the mechanisms that target DNA methylation and DNMT3B throughout the genome, a yeast two-hybrid screening was performed to identify novel interactions of DNMT3B. Several chromatin-associated proteins and DNA binding proteins were identified as new DNMT3B interacting proteins. Preliminary confirmation of their interactions was performed in yeast and by co-immunoprecipitation in mammalian cells. Preliminary results related to the studies of the interactions between DNMT3B and MCAF and CHD3 will be discussed in this chapter. Detailed characterization of another novel interaction of DNMTB with the constitutive centromeric protein CENP-C will be the focus of Chapter 4. The results from these studies demonstrate the complexity of the interplay between various epigenetic systems such as histone modifications, chromatin structure and remodeling and DNA methylation, and provide insights into the possible mechanism of DNMT3B targeting in the genome.
Results

Identification of DNMT3B interacting proteins by Yeast Two-Hybrid Screening

Although there is increasing evidence for the role of DNMT3B in regulating various cellular processes through its catalytic activity, as well as its interaction with other chromatin-associated proteins through its N-terminal regulatory domains, the mechanisms that dictate how DNMT3B is targeted throughout the genome are not well understood. It is also intriguing that subtle germline mutations in DNMT3B lead to chromosomal instability in ICF syndrome and mechanisms that contribute to this are also not fully understood. It is possible that specific proteins are involved in targeting of DNMT3B to certain genomic regions and contribute to the regulation of gene expression or maintenance of certain genomic loci. To gain a better understanding of mechanisms that are involved in targeting DNMT3B and DNA methylation in the genome, a large-scale yeast two-hybrid screening was performed using full-length DNMT3B as bait (Figure 3-3A) and a human testis cDNA library as the prey. It has been shown previously that DNMT3B is very highly expressed in human testis (Robertson et al., 1999) and it is conceivable that its interacting partners may also be highly expressed there. A human testis cDNA library (Clontech) was purchased and amplified. The amplified library was verified by restriction digestion (Figure 3-3A) and PCR (Figure 3-3B). The AH109 yeast strain was transformed with full-length murine Dnmt3b1 fused to the GAL4 DNA binding domain (GAL4-DBD), and the clones screened by western blotting for stable expression of the GAL4-DBD Dnmt3b1 fusion protein (Figure 3-3D). A positive clone was selected and tested for absence of self-activation of the selection markers in the absence of prey and eventually used to sequentially transform and screen the human testis cDNA library fused to the GAL4 activation domain (AD). Approximately 1x10^7 independent clones from the library were screened under high stringency conditions (-Ade/-His/-Leu/-Trp and blue/white screening) as described in Chapter 2. Positive
clones were restreaked to confirm growth and color phenotype (blue indicating LacZ positive) and then the plasmid containing the yeast library cDNA was isolated and sequenced.

A total of 104 positive clones were identified, and listed in Table 3-1. The positive clones included several proteins known to interact with DNMT3B such as DNMT3A, UBE2I (Ubc9) and the PIAS (Protein Inhibitors of Activated STAT) family member, PIAS2. Prominent among the novel interactions identified were the constitutive centromeric protein CENP-C, the ATF7 interacting protein (ATF7IP) also known as the MBD1 containing chromatin associated factor (MCAF), the Chromo-helicase DNA-binding protein (CHD3) also known as the Mi-2 alpha antigen protein, and the death domain interacting protein Daxx (Figure 3-4). Retransformation of AH109 or Y190 yeast strains with GAL4-DBD-Dnmt3b1 and the yeast recovered plasmids encoding the GAL4-AD fusion proteins yielded β-galactosidase activity comparable to positive controls further confirming this interaction in yeast. The interaction between p53 and T antigen as well as the self-interaction of Dnmt3b served as positive control with a strong yellow color that was observed in the calorimetric β-galactosidase activity assay. The interaction between p53 and lamin and Dnmt3b with the empty vector served as the negative control (no color). Further confirmation of interaction was also performed for some of the putative interacting proteins, where a full-length cDNA was generated and cloned into pGADT7 to generate a GAL4-AD fusion protein. This full-length construct of interacting protein was co-transformed with bait gene in parallel with the co-transformation of partial cDNA of interacting protein and the bait gene and the interaction assessed by β-galactosidase activity. The interactions of DNMT3B with MCAF and CHD3 are discussed below.
ATF7IP/MCAF interacts with DNMT3B

The activating transcription factor 7-interacting protein (ATF7IP) also known as the MBD1-containing chromatin-associated factor (MCAF) is a known modulator of transcriptional repression (De Graeve et al., 2000) and associates with other major players in epigenetic gene silencing, including the methyl domain binding protein MBD1 (Fujita et al., 2003b) and the histone H3-K9 methyltransferase SETDB1 (Wang et al., 2003). Preliminary results from the yeast-two hybrid screen in this study indicate that MCAF interacts with DNMT3B (Figure 3-4). To further investigate this interaction, the regions that mediate this interaction between DNMT3B and MCAF were mapped using yeast co-transformation assays (Figure 3-5). The Y190 yeast strain was co-transformed with GAL4-AD-MCAF (plasmid recovered from yeast with partial cDNA) and a series of Dnmt3b deletion constructs fused to the GAL4-DBD and the interaction was assessed by β-galactosidase assay (Figure 3-5A). Loss of the N-terminal region of Dnmt3b from amino acids 220-300 encoding the PWWP region and the exons 20 and 21 in the C-terminal region of Dnmt3b led to a moderate drop in the β-galactosidase activity. The interaction was also assessed using the same method when the Y190 yeast strain was co-transformed with GAL4-AD-MCAF (full-length) and a series of Dnmt3b deletion constructs fused to the GAL4-DBD (Figure 3-5B). Consistent with the previous experiment, loss of either the PWWP region of Dnmt3b or the exons 20 and 21 in the catalytic region led to a drop in the β-galactosidase activity. Presence of either one of the regions was not able to reconstitute full interaction with Dnmt3b as shown by the reduced β-galactosidase activity, suggesting that both the regions of DNMT3B may be important for between DNMT3B to interact with MCAF.

To confirm the interaction between MCAF and DNMT3B revealed by the yeast two-hybrid system, the ability of the two proteins to associate within their natural cellular context was
tested by co-immunoprecipitation following transient co-transfection of 293T cells with FLAG-tagged MCAF and GFP-tagged DNMT3B1. The whole cell extracts from cells co-expressing ectopic GFP-DNMT3B1 and FLAG-MCAF were subject to immunoprecipitation with anti-DNMT3B antibody and anti-FLAG M2 antibody (Figure 3-6). MCAF was detected only when immunoprecipitated with DNMT3B or in the self-immunoprecipitation (FLAG) assays, and was absent from the immunoprecipitate after IP with non-specific IgG. In the reciprocal co-immunoprecipitation DNMT3B was detected only the FLAG (MCAF) immunoprecipitates or the self (DNMT3B) immunoprecipitates and was absent in the non specific IgG immunoprecipitations (Figure 3-6). Taken together, the results indicate that DNMT3B and MCAF interact with each other \textit{in vivo}.

\textbf{CHD3/Mi-2α interacts with DNMT3B}

CHD3 (Mi-2α) is one of the very well studied CHD (\textit{chromo-}\textit{helicase} DNA-binding) family members, that belongs to the SWI/SNF family (Becker and Horz, 2002). CHD3 exhibits nucleosome dependent ATPase activity (Brehm et al., 2000; Guschin et al., 2000) and contains conserved PHD fingers in addition to the chromodomains and a putative DNA-binding domain (Woodage et al., 1997). Mi-2α is also an integral component of the macromolecular NuRD (\textit{N}ucleosome \textit{r}emodeling and histone \textit{de}acetylase) complex (Denslow and Wade, 2007). In a yeast two-hybrid screening performed in this laboratory, CHD3 was identified as a DNMT3B interacting protein by another lab member and confirmed in yeast with the preliminary β-galactosidase assay (data not shown). Given the interesting properties of CHD3, it was chosen for further confirmation alongside the experiments in the following studies. To further confirm the interaction between CHD3 and DNMT3B, the interaction was tested by co-immunoprecipitation following transient co-transfection of 293T cells with FLAG-tagged CHD3
and GFP-tagged DNMT3B1. The whole cell extracts from cells co-expressing ectopic GFP-DNMT3B1 and FLAG-CHD3 were subject to immunoprecipitation with anti-DNMT3B antibody and anti-FLAG M2 antibody (Figure 3-7). CHD3 was detected only when immunoprecipitated with DNMT3B or in the self-immunoprecipitation (FLAG) assays, and was absent from the immunoprecipitate after IP with non-specific IgG. In the reciprocal co-immunoprecipitation DNMT3B was detected only the FLAG (CHD3) immunoprecipitates or the self (DNMT3B) immunoprecipitates and was absent in the non specific IgG immunoprecipitations (Figure 3-7). Taken together, the results indicate that DNMT3B and CHD3 interact with each other in vivo.

Conclusions and Discussion

The results from this study, although preliminary, indicate that MCAF and CHD3 interact with DNMT3B. Further experiments are required to delineate the exact role of these interactions in the function and/or targeting of DNMT3B. While the role of MCAF in regulating gene silencing at methylated regions due to its association with MBD1 has been well studied, the identification of MCAF as a DNMT3B interacting protein provides yet another example of the intricate networking and coordination between the DNA methylation machinery and the histone methylation machinery because MCAF is associated with the histone H3 K9 methyltransferase SETDB1 and promotes histone trimethylation by SETDB1 (Wang et al., 2003). Although the exact mechanism of coordination between DNA and histone lysine methylation remains unclear, further studies of physical interaction between protein components of these two systems are expected to shed light on the nature of this system.

The association of DNMT3B with CHD3, which was recently identified to specifically associate with the pericentromeric regions of chromosomes in proliferating lymphoid cells similar to that of patients with ICF syndrome, sheds light on the epigenetic regulation of
silencing of satellite repeats (Helbling Chadwick et al., 2009). The finding that Mi-2/NuRD association was characterized by a unique pattern of histone modifications consisting of HP1 proteins and histone H3 trimethylated at lysine 9 (H3K9me3), and its accumulation was independent of polycomb association (Helbling Chadwick et al., 2009), suggests that although many epigenetic silencing mechanisms act together, some mechanisms and complexes can be more specific for certain genomic loci. It is possible that in this case DNMT3B recruits the Mi-2 alpha associated NuRD complex to reinforce gene silencing at these regions via recruitment of nucleosome remodeling activity of CHD3 and consequent deacetylation by the HDACs in the NuRD complex.

In addition to MBD1 (Fujita et al., 2003b), SETDB1 (Wang et al., 2003), and DNMT3B (our studies), MCAF also interacts with several transcriptional factors, including ATFα (De Graeve et al., 2000) and Sp1 (Fujita et al., 2003b; Ichimura et al., 2005) suggesting that it can act as an adaptor for a wide range of proteins that modulate gene regulation and chromatin structure. However, the mechanisms involved in regulating the assembly of macromolecular complexes containing MCAF, and how such chromatin complexes contribute to the regulation of gene silencing and heterochromatin formation remain largely, uncharacterized. In addition to modulating the enzymatic activity of ESET and enhancing its transcriptional repression activity, mAM is also likely to bridge interactions of ESET/SETDB1 with certain transcription factors and chromatin associated proteins. SETDB1 itself associates with endogenous DNA methyltransferase activity and physically interacts with the de novo DNA methyltransferases DNMT3A and DNMT3B in vivo and in vitro (Li et al., 2006). The interaction between SETDB1 and DNMT3A was characterized and shown to be mediated by DNMT3A’s PHD domain and DNMT3A enhanced SETDB1-mediated reporter gene repression (Li et al., 2006). Although,
SETDB1 also co-immunoprecipitated with DNMT3B is the same study, the precise role or outcome of this interaction was not characterized (Li et al., 2006). Whether MCAF itself can associate with DNA methyltransferase activity or whether it stimulates its DNA methyltransferase activity would be an interesting idea to test in future studies.

Interestingly, MCAF and CHD3, both identified as novel DNMT3B interacting proteins in this study, have also been shown to possess dual functions as a co-activator or a co-repressor depending on the genomic context (Fujita et al., 2003b; Ichimura et al., 2005; Saether et al., 2007). Both MCAF and CHD3 have demonstrated ATPase activity although the ATPase activity of MCAF was very weak (De Graeve et al., 2000; Woodage et al., 1997). Given these observations, both MCAF and CHD3 are likely to function as transcriptional modulators, depending on their interaction partners or the cell type they are expressed in, by modulating the enzymatic activity or substrate specificity of the proteins that they interact with. On the other hand, MCAF and CHD3 may also play important roles in targeting the chromatin associated enzymes to specific genes by mediating interactions between the enzymes like SETDB1 and DNMT3B and specific transcription factors or in altering the nucleosome positioning via their ATPase activity in response specific protein interactions. In the future, it would also be interesting to test if MCAF or CHD3 can modulate the DNA binding properties or DNA methyltransferases activity of DNMT3B itself.

It also appears that specific recognition of SUMO-conjugated transcriptional regulators by individual components of the repression machinery could be an important regulatory mechanism in chromatin regulation. It has been shown that MCAF and CHD3 contain a SUMO interaction motif (SIM) that facilitates its association with sumoylated MBD1 or SETDB1 (Ivanov et al., 2007; Saether et al., 2007; Uchimura et al., 2006). In the case of CHD3, inhibition
of the sumo pathway promoted c-myb-CHD3 transactivation (Saether et al., 2007), while sumoylation enhanced the co-repressor activity of the KAP1-CHD3-SETDB1 complex (Ivanov et al., 2007). These studies indicate that once modified by SUMO, these proteins can serve as a scaffold and recruit repression or activation machinery through the recognition of the conjugated SUMO moieties by the SIM motifs of their associated proteins. Sumoylation might, in turn, promote or inhibit protein–protein interactions and therefore have an effect on transcription indirectly, perhaps by preventing ubiquitin-mediated proteasomal degradation, or by altering the subcellular localization of interacting protein partners or the sumoylated protein themselves leading to differential recruitment of activities, that cause transcription repression or activation of target genes (Lyst and Stancheva, 2007). Interestingly, previous studies have shown that DNMT3B interacts with SUMO (Kang et al., 2001) and this was confirmed in the yeast two-hybrid screening performed in this study (Table 3-1) where several components of the SUMO pathway were identified. It is therefore possible that in this case, sumoylation of MCAF, CHD3 or DNMT3B itself may be a factor in regulating their interaction or play a causal role in their interaction. Future studies using in vitro and in vivo sumoylation assays or depletion of sumoylation mechanisms could provide insights into the involvement of this pathway in mediating the interaction between DNMT3B and MCAF or CHD3, and possibly its role in regulating the subcellular localization, DNA binding properties or DNA methyltransferase activity.

In summary, data from these experiments demonstrate the physical interaction and a possible functional connection between components of the gene regulatory network involved in the histone methylation, histone deacetylation, and nucleosome remodeling, and the DNA methyltransferase DNMT3B. These studies therefore contribute to a better understanding of the
complexity of the self-reinforcing heterochromatin machinery operating at silenced genomic loci. Ultimately, how the concerted action of these effector molecules leads to chromatin reorganization at specific genomic loci resulting in silent chromatin remains to be seen.
Figure 3-1. Schematic representation of MCAF (ATF7IP) and its domain structure. The evolutionary conserved domains –Domain 1 (aa 562-817) and Domain 2 (aa 1154-1270) are shown.
Figure 3-2. Schematic representation of CHD3 (Mi2-alpha) and its domain structure. The domain structure of Mi-2α according to the predictions made by SMART (Simple modular architecture research tool) (Schultz et al., 1998) is shown: HMG (high mobility group box); PHD-RING (PHD zinc finger or Ring finger); DEXD (DEAD-like helicase super family); HELIC (helicase superfamily).
Figure 3-3. Creation of a stable AH109 yeast strain expressing full-length GAL4-DBD-Dnmt3b1. A) Schematic representation of the bait gene Dnmt3b expressed as a fusion protein with the GAL4DBD, B) Restriction digestion of human testis library with BglII, C) PCR amplification of the cDNA library using primers flanking the MCS, D) Western blotting (WB) using a mouse monoclonal antibody for GAL4DBD was performed to screen for successful transformants expressing the GAL4DBD-Dnmt3b fusion protein. L-1 kb DNA ladder, UT-Untransformed, pGBK7 is the empty parental GAL4-DBD vector.
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<td>CTGLF4</td>
<td></td>
<td>Contains GTPase-like domain, activates GTPase PI3K</td>
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<td>RPS16</td>
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<td>Component of 40S subunit</td>
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<tr>
<td>KLRK1</td>
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<td>Transmembrane protein in natural killer cells</td>
<td>1</td>
</tr>
<tr>
<td>KIAA2026</td>
<td></td>
<td>IMP dehydrogenase/GMP reductase family protein. Unknown</td>
<td>1</td>
</tr>
<tr>
<td>AK075484</td>
<td></td>
<td>Similar to Sterile alpha motif domain containing protein 4. Unknown</td>
<td>1</td>
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<td>CKAP1-TCPBP</td>
<td></td>
<td>Tubulin folding factor</td>
<td>1</td>
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<tr>
<td>ZMYND19</td>
<td>MIZIP</td>
<td>Contains zinc finger and MYND domain</td>
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</tr>
<tr>
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<td>Testicular development, interacts with BRCA-1.Contains PHD and RING finger</td>
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<td></td>
<td>Actin binding, cytoskeleton</td>
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<td>SMAD5</td>
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<td>Transcriptional modulator activated by bmp receptor kinase.</td>
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</tr>
<tr>
<td>ARP2/3</td>
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<td>Actin related protein, action polymerization</td>
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<td>C1orf80</td>
<td></td>
<td>Calcium/lipid-binding region, CaLB domain containing protein.</td>
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<td>PIAS-G</td>
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<td>Kinesin light chain 3, microtubule associated, organelle transport</td>
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<td>CHD3</td>
<td>Mi-2 alpha</td>
<td>ATPase dependent chromatin remodeling factor, component of NuRD complex</td>
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Figure 3-4. Preliminary confirmation of putative positive clones identified in yeast two-hybrid screening by β-galactosidase activity assay. The AH109 yeast strain was transformed with either the yeast recovered partial cDNA (pACT2) of the putative interacting protein or the full-length cDNA (pGADT7) of the putative interacting protein cloned in the laboratory as a part of this study. The new interactions were tested in comparison to known control interactions. Positive control (p53+TAg and Dnmt3b1 with DNMT3B3) and negative control (p53+Lamin and Dnmt3b1+empty GAL4-AD vector) for interaction are also shown.
Figure 3-5. Mapping Dnmt3b regions involved in the interaction with MCAF. A) Y190 yeast strain was co-transformed with the murine Dnmt3b1 deletion constructs fused to the GAL4-DBD and a partial cDNA of MCAF fused to GAL4-AD (pACT2), B) Y190 yeast strain was co-transformed with the murine Dnmt3b1 deletion constructs fused to the GAL4-DBD and a full-length GAL4-AD-MCAF (pGADT7) and the interaction assessed by β-galactosidase activity. Positive control (p53+TAg and Dnmt3b1 with DNMT3B3) and negative control (p53+Lamin and Dnmt3b1+empty GAL4-AD vector) for interaction are also shown. Numbering refers to the amino acids of full-length murine Dnmt3b1. Boxed region in panels A and B indicates minimal interaction domains and the roman numerals represent conserved catalytic DNMT motifs.
Figure 3-6. Ectopically expressed DNMT3B and MCAF interact in mammalian cells. 293T cells were transfected with pCDNA3 FLAG MCAF and pEGFPC2-DNMT3B1. The whole cells extracts were subjected to immunoprecipitation with antibodies against FLAG and DNMT3B antibody and a control species matched non specific IgG. Input – whole extract prior to co-IP, IgG – negative control for co-IP. The immunoprecipitating antibody (IP Ab) is indicated along the top of the western panel and the antibody used in western blotting (WB) is indicated at the bottom.
Figure 3-7. Ectopically expressed DNMT3B and CHD3 interact in mammalian cells. 293T cells were transfected with pClneoB 3XFLAG CHD3 and pEGFPC2-DNMT3B1. The cells extracts were subjected to immunoprecipitation with antibodies against FLAG and DNMT3B antibody and a control species matched non specific IgG. Input – whole cell extract prior to co-IP, IgG – negative control co-IP. The immunoprecipitating antibody (IP Ab) is indicated along the top of the western panel and the antibody used in western blotting (WB) is indicated at the bottom.
Introduction

Characteristics of the Centromeric Chromatin

Reliable partitioning of the genome during cell division is an important cellular process in all higher unicellular and multi-cellular life forms. This function depends on the centromere, the primary constriction of eukaryote chromosomes. Centromeres are special structures of unique chromatin composition that forms a base upon which the kinetochore is assembled during entry into mitosis (Pidoux and Allshire, 2000). The kinetochore is a multi-protein assembly that mediates microtubule attachment between the centromere and mitotic spindle. Kinetochores sense errors in chromosome attachment to the mitotic spindle and activate the spindle assembly checkpoint to delay metaphase-to-anaphase transition until all chromosomes achieve bipolar spindle attachment. Defects in centromere function lead to genetic instability, with consequences in fertility, human disease, cancer and birth defects (Fodde et al., 2001; Martinez-Perez et al., 2001; Michel et al., 2001; Van Den Berg and Francke, 1993). Errors in chromosome segregation are also hallmarks of tumor progression (Hartwell and Kastan, 1994). A functional kinetochore and therefore a functional centromere is the fundamental chromosomal component responsible for the inheritance of genetic information by ensuring proper chromosome segregation during cell division and thus genetic stability.

Although the cytological function, position, and structure of the centromere have been conserved over several millions of years, the DNA component of centromere is highly diverse. Mammalian centromeres are composed of long arrays of tandemly repeated satellite DNA sequences (Sunkel and Coelho, 1995). Satellite alpha repeats are tandem repeats of 170 bp DNA sequences and represent the main DNA component of every human centromere (Lee et al., 1997)
Satellite 2 (Sat2) DNA sequences are found predominantly in pericentromeric heterochromatin (region adjacent to centromeres) in humans (Figure 4-1A). The mouse genome contains at least two types of repetitive elements at centromeres, the major satellite repeats and the minor satellite repeats. The major satellite repeats are located in the pericentromeric region, whereas the minor satellite repeats correspond to the centromeric region (Guenatri et al., 2004).

Centromeric chromatin is also defined by the unique incorporation of the histone H3 variant CENP-A (centromeric protein-A). Mutations in the centromere specific histone variant CENP-A in humans and its homologs in yeast, *C. elegans*, *Drosophila* and mouse, result in disrupted centromeric chromatin, disrupted kinetochore, defective chromosome segregation, and failure to recruit many kinetochore components to the centromere during interphase and mitosis indicating the requirement of CENPA for proper centromere and kinetochore function (Buchwitz et al., 1999; Henikoff et al., 2000; Saitoh et al., 1997; Takahashi et al., 2000). The protein components at the centromere fall under two classes. The first class comprise proteins that are constitutively associated with the centromere, such as CENP-A, CENP-B, and CENP-C, which are thought to have structural roles in kinetochore formation. The second class, known as passenger proteins, associate with the centromere transiently during the cell cycle and comprise proteins with diverse roles in cell division, such as spindle capture, metaphase-to-anaphase transition, and sister chromatin cohesion (Amor and Choo, 2002). CENP-A containing nucleosomes are formed predominantly on α-satellite DNA that contains CENP-B boxes (α1-type array) (Figure 4-1A) and together with CENP-B and CENP-C forms the CENP-A/B/C prekinetochore chromatin complex (Ando et al., 2002). Both CENP-A-null and CENP-C-null mice show severe mitotic defects, while CENP-B null mice appeared to be normal (Fukagawa and Brown, 1997; Howman et al., 2000; Hudson et al., 1998; Kalitsis et al., 1998; Perez-Castro
et al., 1998). Although CENP-B and CENP-C recognize the same sets of alpha – satellite they occupy separate alpha satellite domains (Politi et al., 2002). CENP-A chromatin also recruits a six-component CENP-A nucleosome-associated complex (CENP-A-NAC) that regulates assembly of other downstream centromere components and kinetochore formation during mitosis (Foltz et al., 2006; Okada et al., 2006). Together this provides the evidence that CENP-A specifies centromere position, and that its incorporation forms a substrate for kinetochore formation. Each kinetochore protein also associates with other kinetochore proteins as well as other proteins for full centromere function, suggesting that the organization of centromere and the kinetochore changes dynamically throughout cell cycle progression. Apart from CENP-A, centromeres contain a unique histone modification pattern consisting of both euchromatic and heterochromatic histone marks (Sullivan and Karpen, 2004) (Figure 4-1B) while the pericentromere, the region flanking the centromere, has epigenetic marks characteristic of compacted heterochromatin domains, including histone lysine trimethylation and DNA hypermethylation (Lehnertz et al., 2003; Maison and Almouzni, 2004). Maintenance of centromeric and pericentromeric heterochromatin has been proposed to be important for centromere function. In particular, loss of DNA methylation at pericentromeric regions caused by abrogation of the DNA methyltransferase enzymes Dnmt1 or Dnmt3a and Dnmt3b (Chen et al., 2004; Okano et al., 1999) results in defective centromere function (Chen et al., 2004; Dodge et al., 2005), although the mechanisms responsible for this are still largely unknown.

Role of the Constitutive Centromeric Protein CENP-C

CENP-C, is a constitutive kinetochore component in higher vertebrate cells and was originally identified as an antigen of anti-centromere antibodies from patients with various autoimmune diseases (Saitoh et al., 1992). CENP-C homologues have been identified in several species, including yeast, nematode, and fly (Brown et al., 1993; Heeger et al., 2005; Moore and
Roth, 2001; Oegema et al., 2001). CENP-C contains domains similar to those of the protein encoded by MIF2, a gene involved in chromosome segregation in budding yeast (Brown et al., 1993). The *C. elegans* homologue of CENP-C, HCP4, is involved in sister kinetochore resolution (Moore et al., 2005) and the *Drosophila* CENP-C homologue was identified to interact with Separase, which is essential for sister chromatid separation (Heeger et al., 2005).

Conditional knockout of CENP-C in chicken DT40 cells and disruption of the *CENP-C* gene in mice revealed that CENP-C is essential for proper mitotic segregation and cell survival (Fukagawa and Brown, 1997; Kalitsis et al., 1998). Analysis of DT40 cells with a conditional knockout of CENP-C revealed that the inactivation of *CENP-C* caused mitotic delay, chromosome missegregation, and apoptosis (Fukagawa and Brown, 1997; Fukagawa et al., 1999). Microinjection of anti-CENP-C antibodies into human HeLa cells causes mitotic arrest (Tomkiel et al., 1994) indicating the CENP-C is an essential component for proper cell division. Overexpression of CENP-C in chicken DT40 cells using an exogenous promoter causes the mislocalization of the protein in various regions of the chromosome arms but does not lead to formation of new centromeres or disrupt the native centromeres, indicating that overexpression of CENP-C alone is insufficient to cause centromerization (Fukagawa et al., 1999). *In vivo* expression of CENP-C truncation constructs demonstrate that the protein has an autonomous centromere-targeting domain within the region that displayed DNA-binding properties (aa 196-537) *in vitro* (Yang et al., 1996). The expression of CENP-C varies during the cell cycle, rising progressively from S phase through G2 and mitosis, with maximum levels at G1 (Knehr et al., 1996). Binding of the herpes simplex virus regulatory protein Vmw110 to the centromere causes specific degradation of CENP-C by the induction of the proteasome mediated degradation (Everett et al., 1999). Although cells deficient for CENP-C display poor signals for checkpoint
proteins, they progress to the next cell cycle after mitotic delay, suggesting that CENP-C–deficient cells have defective spindle checkpoint function (Kwon et al., 2007). Regions 426-537 and 638-943 of CENP-C are both capable of localizing at centromeres and binding alpha satellite suggesting CENP-C may target the centromere by establishing multiple contacts with both DNA and protein constituents of the kinetochore (Trazzi et al., 2002). Interestingly, amino acids 426-551 of CENP-C also bind to alpha satellite RNA in vitro (Wong et al., 2007). The binding of CENP-C to centromere satellite RNA directly facilitates the accumulation and assembly of CENP-C at the nucleolus and mitotic centromere (Wong et al., 2007). During interphase, CENP-C associates with the human death domain associated protein Daxx, a protein that is known to cause to cell death in response to Fas ligand binding, indicating the possible cause of induction of apoptosis in the absence of CENP-C (Pluta et al., 1998). Furthermore, a recent study shows that PTEN (Phosphatase and TENsin homolog) tumor suppressor associates with CENP-C to maintain centromere stability (Shen et al., 2007) although if and how PTEN interaction influences CENP-C function in kinetochore assembly or mitotic progression is yet to be determined.

**Role of the DNMT3B in the Maintenance of Genome Stability**

Several studies indicate an important role for DNA methylation and DNMT3B in particular, in maintenance of genome stability. Conditional inactivation of *Dnmt3b* but not *Dnmt3a* in murine embryonic fibroblasts (MEFs) results in demethylation of satellite repeats and an increase in anaphase bridging in mitotic cells (Dodge et al., 2005). These cells also displayed elevated frequency of aneuploid and tetraploid cells, indicating a role for DNMT3B in chromosome stability (Dodge et al., 2005). Interestingly, mouse ES cells deficient for *Dnmt3a* or *Dnmt3b* or both, exhibit elevated rates of centromeric recombination (sister chromatid exchange) as well as longer centromeric transcripts while the ES cells lacking *Dnmt1* displayed
no altered centromeric recombination in comparison to the wild-type ES cells, demonstrating the importance of de novo methyltransferase machinery at these genomic loci (Jaco et al., 2008). Moreover, defective DNA methylation due to loss of Dnmt3a and Dnmt3b did not affect global recombination frequencies, implying a specific role of these proteins in repressing illicit recombination at centromeric repeats of the genome. Both satellite alpha and Sat2 sequences are highly methylated in normal human postnatal tissues, and often hypomethylated in various cancers (Narayan et al., 1998; Qu et al., 1999a; Qu et al., 1999b).

Some of the strongest evidence supporting the role of DNMT3B in centromeric stability comes from the study of patients with ICF syndrome, a rare human genetic disease caused by germline mutations in the DNMT3B gene. While the common abnormalities associated with this disease include immunoglobulin deficiency that is usually seen in the presence of normal B and T-cell counts, immunodeficiency due to defective B cell function and maturation, facial anomalies, and a variable degree of mental retardation, the most characteristic feature of ICF patients is loss of DNA methylation from centromeric and pericentromeric repeat regions (alpha satellite and satellite 2/3 repeats, respectively) and loss of chromosomal condensation at these regions during mitosis (Ehrlich et al., 2006a; Ehrlich et al., 2008; Hansen et al., 1999; Hassan et al., 2001; Miniou et al., 1997; Xu et al., 1999). Some ICF patients also exhibit defective lymphogenesis (at a step after class switching) or lymphocyte activation although mechanisms responsible for this defect are unknown (Ehrlich et al., 2006a). The immunodeficiency aspect of ICF patients is largely responsible for patient’s frequent mortality in early childhood (Ehrlich et al., 2008). The pericentromeric heterochromatin on chromosomes 1, 9, and 16 contain megabase size blocks of satellite 2/3 DNA, representing one of the highest concentrations of methylated DNA in the human genome (Lubit et al., 1976) and are the most affected by DNA
hypomethylation in ICF patients. Hypomethylation of pericentromeric heterochromatin and consequent chromosome decondensation leads to characteristic chromosomal abnormalities in the vicinity of the centromere of chromosomes 1, 9, and 16, and, usually, the formation of multiradial chromosome involving one or more of the decondensed chromosomes, translocations, and telomeric associations (Ehrlich et al., 2006a; Ehrlich et al., 2008). The mouse model of ICF syndrome mirrors the human phenotype where the murine equivalents of the human centromeric and pericentromeric repeat regions (minor and major satellite, respectively) are hypomethylated (Ueda et al., 2006). The mutations in $DNMT3B$ in ICF syndrome most often occur on the catalytic region of $DNMTB$ and ICF patients often express hypomorphic forms of $DNMT3B$ (Ehrlich, 2003; Ehrlich et al., 2001; Hansen et al., 1999; Xu et al., 1999). Although $DNMT3B$ specifically associates with histone deacetylases, HP1, other DNMTs, chromatin remodeling proteins, condensins, and other nuclear proteins, the partial loss of catalytic activity due to mutations in the catalytic region of $DNMT3B$ has been largely attributed to be responsible for the disease (Ehrlich et al., 2008). It is possible that these protein-protein interactions of $DNMT3B$ have some downstream effect on maintaining the chromatin structure after DNA methylation at these regions, although the exact mechanism of how $DNMT3B$ is targeted to these regions of the genome, remain unclear. Taken together, these findings suggest that the centromeric and pericentromeric regions are specific targets of $DNMT3B$ and that $DNMT3B$, and possibly $DNMT3B$-induced DNA methylation, plays an important role in proper heterochromatin formation at its target regions, and therefore in proper centromere location, kinetochore formation, chromosome segregation, chromosomal stability, and maintenance of chromatin structure at these regions.
Results

DNMT3B and CENP-C Interact In Vivo in Yeast

Yeast two-hybrid screening using full-length DNMT3B as bait and a human testis cDNA library as the prey, generated a list of potential DNMT3B interacting proteins. The results from the yeast two-hybrid screening indicated several SUMO pathway related proteins such as Ubc9, PIAS4 and SUMO itself, apart from other chromatin associated proteins such as MCAF and CHD3 (discussed in Chapter 3). While some of the protein interactions of DNMT3B identified in this screen was novel, most of them were anticipated because of their functional properties (chromatin associated). The results from the yeast-two hybrid screening also yielded the constitutive centromeric protein CENP-C as an unanticipated candidate among several novel DNMT3B-associated proteins. Although, CENP-C is widely considered a structural protein associated with the centromere, given the important role of CENP-C in mitosis and the known association of DNMT3B in centromeric DNA methylation, this interaction appeared the most novel and therefore was pursued in greater detail. Several clones encoding portions of the constitutive centromeric protein CENP-C were identified in the yeast two-hybrid screen with the largest clone encoding the amino acids- 200-943 of CENP-C. The interaction was identified under high stringency conditions using the sequential transformation of AH109 host strain stably expressing GAL4-DBD Dnmt3b1 fusion protein with a human testis cDNA library. The novel interaction between CENP-C and DNMT3B was confirmed by co-transformation of GAL4-DBD-Dnmt3b1 with GAL4-AD-CENP-C (aa 200-943) plasmids in Y190 strain (Figure 4-2) followed by β-galactosidase activity assay. The β-galactosidase activity from this co-transformation assay was comparable to positive controls (interaction between p53 and T antigen) tested at the same time (Figure 4-2). Also, the GAL4-DBD fused human DNMT3B1 when co-transformed with the yeast recovered GAL4-AD fused partial CENP-C yielded β-
galactosidase activity comparable to the co-transformation of GAL4-AD-CENP-C with the mouse Dnmt3b1 (Figure 4-2). The same was true when GAL4-DBD fused human DNMT3B1 and the full-length GAL4-AD CENP-C were cotransformed and the interaction assessed β-galactosidase activity, indicating that both the human and mouse DNMT3B interact with CENP-C.

The region mediating the interaction of DNMT3B and CENP-C was mapped in yeast using co-transformation followed by β-galactosidase assay (Figure 4-3). The Y190 yeast strain was co-transformed with either a GAL4-AD-CENP-C recovered from yeast (Figure 4-3A) or a full-length GAL4-AD-CENP-C (Figure 4-3B) and a series of Dnmt3b deletion constructs fused to the GAL4-DBD and the interaction was assessed by β-galactosidase assay. Results indicate that the N-terminal region of Dnmt3b from amino acids 1-140 is required for interaction with CENP-C. A reciprocal mapping assay was also performed by co-transformation with full-length Dnmt3b1 and a series of CENP-C deletion constructs in Y190 yeast strain (Figure 4-4). The reciprocal mapping assay revealed that the C-terminus of CENP-C consisting of amino acids 638-943 contributed to the interaction of the two proteins, although this region did not reconstitute the full interaction activity with Dnmt3b1 as shown by the reduced level of β-galactosidase activity, in comparison to the full-length CENP-C, suggesting that other regions of CENP-C may also be involved.

**DNMT3B Interacts with CENP-C *In Vivo* in Mammalian Cells**

To further confirm and characterize this interaction that was identified in the yeast two-hybrid screening, co-immunoprecipitation assays were performed using several approaches. Initially, 293T cells were co-transfected with FLAG-tagged DNMT3B1 and HA-tagged CENP-C, and the cell extracts from transfected cells were subject to immunoprecipitation (Figure 4-5).
FLAG-DNMT3B1 immunoprecipitated HA-CENP-C and HA-CENP-C immunoprecipitated with FLAG-DNMT3B1 in the reciprocal co-IP assay, performed with antibodies against the respective tags (Figure 4-5A). In addition, antibody against the endogenous protein was also able to reciprocally co-immunoprecipitate the ectopically expressed DNMT3B or CENP-C (Figure 4-5B). When 293T cells were transfected with a single tagged construct of either FLAG-DNMT3B1 or HA-CENP-C, both tagged proteins were able to immunoprecipitate the endogenous interacting protein (Figure 4-6). To gain further evidence of this interaction in a physiological context, nuclear extract from untransfected HeLa cells were subjected to immunoprecipitation (Figure 4-7A). The interaction between CENP-C and DNMT3B was confirmed in vivo by immunoprecipitation using two different antibodies directed against CENP-C and one directed against DNMT3B. Both CENP-C and DNMT3B also co-immunoprecipitate with each other when the soluble chromatin derived from HeLa cells (prepared as described in Ando et al) was used in the immunoprecipitation assays (Figure 4-6B). Given that CENP-C is a protein involved in regulating mitotic progression, it is possible that the DNMT3B-CENP-C interaction could be cell cycle stage specific. To test this possibility, HeLa cells were enriched for mitotic cells using a double thymidine block. Cells were harvested for nuclear extracts either immediately after the second thymidine block (G1/S phase enrichment) or released for 8-9 hours after the second thymidine block and harvested (G2/M phase enrichment). For both extracts, the interaction between CENP-C and DNMT3B was tested by immunoprecipitation (Figure 4-8). Results indicated that the proteins interact in both the G1/S and G2/M conditions although it is possible that the level of association is variable with cell cycle stage. It would be of interest to test this idea more rigorously in the future studies.
Given that CENP-C is present in a nucleosomal complex with the centromere specific histone variant CENP-A, constitutive centromeric protein CENP-B, and the alpha satellite DNA (Ando et al., 2002), it is presumable that DNMT3B, via its interaction with CENP-C, is associated with the other centromere proteins as well. To test this possible interaction, cell extracts from 293T cells transfected with FLAG tagged CENP-A and HA-tagged DNMT3B1 were subject to immunoprecipitation. Indeed, FLAG-CENP-A and HA-DNMT3B1 readily immunoprecipitate each other (Figure 4-9A). As a positive control, the known interaction of CENP-A with CENP-C was confirmed by co-immunoprecipitation using nuclear extracts as well as transient transfection (Figure 4-6A, 4-9B). Also, DNMT3A and DNMT3B share several common protein interactions due to the significant homology in protein sequence and overlapping functions. To test if CENP-C could be one such candidate, 293T cells were transfected with HA-tagged DNMT3A and FLAG-tagged CENP-C and the transfected cell extracts were subject to immunoprecipitation (Figure 4-10A). Indeed, DNMT3A co-immunoprecipitated with CENP-C and vice versa. Unlike DNMT3A, the other active DNA methyltransferase DNMT1 was unable to co-immunoprecipitate with CENP-C (Figure 4-10B), suggesting that both that de novo DNA methylation and both the de novo methyltransferases have strong links to the centromere.

While the yeast two-hybrid assay clearly mapped amino acids 1-140 on Dnmt3b1 as the region interacting with CENP-C, none of the CENP-C deletion constructs in the reciprocal mapping reconstituted the activity of full-length CENP-C, indicating that more than one region of CENP-C might be involved or that there might have been low levels of protein expression in the yeast co-transformation experiments. To circumvent this issue, co-immunoprecipitation assays were performed in mammalian cells by co-transfecting a series of HA-tagged CENP-C
deletion constructs (Figure 4-11A), and FLAG-DNMT3B1, into 293T cells. All of the CENP-C deletion constructs tested expressed proteins at comparable levels in 293T cells (Figure 4-11B). Cell extracts were subject to immunoprecipitation with FLAG antibody and the interaction was detected by immunoblotting (Figure 4-11C). Immunoprecipitation analysis confirmed that the C-terminal region of CENP-C (aa 638-760) was involved in interacting with DNMT3B. Interestingly, the central region of CENP-C (aa 426-537) also interacted with DNMT3B. These two regions of CENP-C are required for CENP-C recruitment to centromeres (aa 426-537) and for its ability to bind alpha-satellite DNA (aa 638-943) (Trazzi et al., 2002) and alpha-satellite RNA (aa 638-943) (Wong et al., 2007). Given that both CENP-C and DNMT3B are known DNA binding proteins, it is possible that contaminating genomic DNA in the extracts might mediate the interaction between DNMT3B and CENP-C. To test this possibility, 50 μg of ethidium bromide was added to the nuclear extracts and incubated for 30 minutes at 4°C to precipitate any DNA present in the extracts. This is a method commonly employed to determine if DNA may be involved in mediating a protein-protein interaction. The DNA-free nuclear extract was then subject to immunoprecipitation using antibodies against endogenous DNMT3B or CENP-C (Figure 4-12). The results indicate that DNMT3B and CENP-C also interact in the absence of DNA. Taken together, these data reveal that DNMT3B interacts with two discrete functional domains of CENP-C and that the CENP-C and DNMT3B interaction is independent of DNA.

**Localization of CENP-C and DNMT3B During Cell Cycle**

Previous studies using immunofluorescence microscopy have demonstrated that DNMT3B displays a wide nuclear distribution and co-localizes with centromeric and pericentromeric regions in the mitotic chromosomes (Craig et al., 2003; Geiman et al., 2004a). CENP-C, on the
other hand, is present as distinct foci at the centromere of each chromosome (Saitoh et al., 1992; Tomkiel et al., 1994). In order to examine the extent of co-localization of DNMT3B and CENP-C, HeLa cells were transfected with green fluorescent protein (GFP)-tagged DNMT3B1 to visualize DNMT3B1 and they were stained for endogenous CENP-C (Figure 4-13). In interphase cells, DNMT3B1 displayed a wide nuclear distribution with a few foci in DAPI-dense heterochromatic regions, while CENP-C was present in distinct foci that co-localized with anti-centromere antibodies. Some of the DNMT3B-dense foci within the diffuse nucleoplasmic staining were often closely associated with CENP-C foci. Although not quantitatively analyzed, more of the DNMT3B foci begin to co-localize with CENP-C foci during prophase (early stage of mitosis), and there is an increase in number of DNMT3B foci associating with CENP-C-containing foci during metaphase (where it peaks). The association of DNMT3B and CENP-C, as visualized by immunofluorescence, decreases during anaphase and declines further during telophase (Figure 4-13). Taken together, these results demonstrate that although DNMT3B has a wide distribution in the nucleus, some discrete foci of DNMT3B are closely associated with CENP-C foci. Moreover, as indicated earlier by the immunoprecipitation assays (Figure 4-8), there is some level of association of DNMT3B and CENP-C throughout the cell cycle.

**Effect of CENP-C and DNMT3B Interaction on Satellite Repeat DNA Methylation**

Several evidences suggest that DNMT3B regulates DNA methylation at both pericentromeric and centromeric regions in murine cells (Chen et al., 2004; Jaco et al., 2008; Lehnertz et al., 2003). Germline mutations in DNMT3B cause ICF syndrome wherein patients display a variable degree of DNA hypomethylation in the human centromeric and pericentromeric region (Hassan et al., 2001; Miniou et al., 1997; Xu et al., 1999), however, the mechanisms by which DNMT3B and its DNA methylation activity is targeted to the centromere remain unknown. Given that the interaction between DNMT3B and CENP-C has been
confirmed by immunoprecipitation assays (discussed above), it is possible that this novel interaction functions to regulate epigenetic modifications at the centromere. To test the effect of DNMT3B-CENP-C interaction in the context of centromeric and pericentromeric heterochromatin, a transient knockdown system using siRNA was developed. Human HCT116 colorectal carcinoma cells were transfected with siRNAs directed against CENP-C and DNMT3B separately. The efficiency of the siRNA knockdown as measured by the reduction in mRNA levels by qRT-PCR (Figure 4-14A) and protein levels by immunoblotting (Figure 4-14B), indicated a specific and significant knockdown of both CENP-C and DNMT3B.

Genomic DNA was extracted from cells treated with siRNAs and bisulfite modified. The bisulfite modified DNA was amplified by PCR using primers specific for the centromeric alpha satellite repeats and the pericentromeric satellite 2 repeats. The PCR product was cloned and sequenced (BGS) to examine the effect of the knock downs of DNMT3B or CENP-C on DNA methylation at the centromeric alpha satellite repeat (Figure 4-15) and the pericentromeric satellite 2 repeat (Figure 4-16) regions. CpG sites 1 and 2 represented in the alpha satellite amplicon (Figure 4-15) are within the consensus 17 bp CENP-B box and DNA methylation at the CENP-B box is known to inhibit the binding of CENP-B (Tanaka et al., 2005). BGS analysis revealed that HCT116 cells depleted for CENP-C showed about a 30% reduction in DNA methylation at the alpha satellite repeat region and approximately 20% reduction in DNA methylation in case of DNMT3B knockdown (Figure 4-17A). The siRNA knockdown of CENP-C also led to a reduction in methylation at the pericentromeric satellite 2 repeat region with a decrease of about 25% and a decrease of approximately 20% methylation in the DNMT3B knockdown (Figure 4-17B). While the decrease in DNA methylation levels at these regions is expected upon depletion of DNMT3B, as sat2 is known DNMT3B target sequence, the decrease
in DNA methylation on CENP-C knockdown was unanticipated and reveals that CENP-C is likely involved in recruiting DNA methylation to the centromeric region possibly due to its interaction with DNMT3B. Given that the depletion of both DNMT3B and CENP-C influence the methylation levels at the centromeric and the pericentromeric repeat regions, it is possible that the interaction might also coordinate other epigenetic marks within these regions, which will be studied subsequently.

**Effect of CENP-C and DNMT3B Interaction on Centromeric Histone Modification**

Given the known coordination between the DNA methylation machinery and the histone post-translational modification machinery, coupled with our observation that CENP-C is required for normal levels of DNA methylation in the centromeric region, it is conceivable that the CENP-C and DNMT3B interaction may also affect histone modification patterns at the centromere. Levels of histone modifications at the centromeric and the pericentromeric region were measured by chromatin immunoprecipitation (ChIP) using chromatin from HCT116 cells depleted for CENP-C or DNMT3B using siRNAs. The chromatin was prepared as described in Chapter 2 (Lefevre and Bonifer, 2006) and subject to ChIP for a set of histone marks associated with open chromatin structure or a transcriptionally permissive chromatin state (di- and trimethylated H3K4 and H3 acetylated at K9 and K18) and a closed chromatin structure or a transcriptionally repressive chromatin state (di- and trimethylated H3K9 and di- and trimethylated H3K27) as well as the binding of DNMT3B and CENP-C themselves. ChIP was followed by quantitative PCR for alpha satellite (Figure 4-18) or satellite 2 (Figure 4-19) repeat regions. Consistent with previous reports (Lam et al., 2006; Sullivan and Karpen, 2004), histone marks characteristic of both permissive and repressive chromatin were detected at the centromere (Figure 4-18A). Depletion of CENP-C lead to a loss of dimethylated H3K4 and acetylated H3 (both being marks characteristic of centromeric chromatin), at the alpha satellite repeat and gain
of the heterochromatic histone marks di- and trimethylated H3K9 and dimethylated H3K27 at the alpha-satellite repeat region (Figure 4-18A). While siRNA knockdown of DNMT3B resulted in increased di- and trimethylated H3K9 similar to the depletion of CENP-C, it also resulted in an increase in H3 acetylation compared to the loss of H3 acetylation as observed in CENP-C knockdown (Figure 4-18A). The alpha satellite region did not have any significant enrichment of trimethylated H3K27 mark. These results therefore demonstrate that CENP-C and DNMT3B regulate common as well as distinct histone modifications at the centromere and suggest that loss of either one m disturbs the integrity of the unique centromeric chromatin structure either by allowing the spreading of pericentromeric histone marks into the centromere, or decondensation of the centromeric chromatin due to a more open chromatin structure.

The pericentromeric satellite 2 repeats showed enrichment for histone marks, as measured by ChIP, characteristic of repressive chromatin consistent with prior studies (Jenuwein and Allis, 2001). Unlike the centromeric region, the pericentromeric region was relatively unaltered by the depletion of CENP-C, with little increase in dimethylated H3K9 and acetylated H3 and loss of trimethylated H3K9 and K27 marks at satellite 2 repeats (Figure 4-19A). Knockdown of DNMT3B yielded more dramatic effects in comparison to the subtle changes observed upon CENP-C knockdown. Knockdown of DNMT3B lead to reduced levels of trimethylated H3K9 and H3K27 and elevated levels of di- and trimethylated H3K4, H3 acetylation, and dimethylated H3K27 at satellite 2 repeats (Figure 4-19A), suggesting that DNMT3B primarily helps maintain the repressive histone marks in pericentromeric heterochromatin as its loss resulted in an increase in H3K4 trimethylation and H3 acetylation, both marks that are associated with open chromatin.
ChIP assays were also performed to assess binding of heterochromatin protein HP1α and both DNMT3B and CENP-C at satellite repeat regions. HP1α binds to the centromeric and pericentromeric regions and its binding is essential for proper recruitment of cohesin and consequent mitotic chromosome segregation (Bernard et al., 2001). Depletion of either CENP-C or DNMT3B using siRNAs lead to a large reduction in HP1α binding at both the alpha satellite and satellite 2 regions, indicating a loss of chromatin compaction at both the repeat regions in the absence of CENP-C or DNMT3B. Interestingly, knock down of CENP-C also lead to reduced DNMT3B binding at both the alpha satellite (Figure 4-18B) and the satellite 2 regions (Figure 4-19B). On the other hand, knock down of DNMT3B resulted in a small decrease in CENP-C binding at both alpha satellite (Figure 4-18B) and satellite 2 (Figure 4-19B). Both CENP-C and DNMT3B binding were reduced consistent with the siRNA knock down in the respective cases.

Changes in the histone modification patterns that were observed, were not due to altered nucleosomal density as the total levels of histone H3 or CENP-A were comparable in all knockdowns in both the satellite alpha and satellite 2 repeat regions that were examined. CENP-A enrichment was much less at the pericentromeric region compared to the centromeric region, consistent with the specificity of the localization of CENP-A. Due to the stochastic nature of the ChIP technique and the complexity of the centromeric and pericentromeric repeat regions, some of the changes were very subtle and not clearly indicative of the exact nature of the change in chromatin structure (open/closed) due to the knockdown. Nonetheless, the most definitive changes suggest that DNMT3B and CENP-C together have a role in delineating and maintaining the boundary between centromeric and pericentromeric chromatin as knock down of either factor resulted in an increase in di-methylated H3K9, which has been proposed to mark this boundary (Lam et al., 2006).
Histone modification patterns and protein binding were also analyzed in HCT116 cells and the HCT116 isogenic cell lines with a genetic knockout of DNMT3B (3BKO) (Rhee et al., 2002) to observe the level of changes in the satellite alpha (Figure 4-20) and the satellite 2 (Figure 4-21) regions in comparison to the transient knockdowns. ChIP results indicate that 3BKO HCT116 cells display an increase in H3K4 trimethylation as well as di- and trimethyl H3K27 and a decrease in dimethylated H3K9 in comparison with the parental cell lines at the satellite alpha repeats (Figure 4-20). Also, the 3BKO HCT116 cells show a specific reduction in the trimethylated H3K9 and increase in the acetylated H3 in comparison to the parental cell lines while all other histone modifications remain comparable at the satellite 2 repeats (Figure 4-21). The 3BKO HCT116 cells also revealed a decrease in the binding of CENP-C at both regions consistent with the results from DNMT3B siRNA knockdown, while the HP1 binding was more affected only in the satellite 2 repeats in 3BKO cells (Figure 4-20, Figure 4-21).

To test if a change in the chromatin due to loss of CENP-C or DNMT3B was specific to the centromeric region or if there was a genome-wide effect, the histone modification patterns at two single copy genes that are located in non centromeric regions were analyzed. The effect of CENP-C knock down on the histone modifications and DNMT3B binding was specific to the centromeric and pericentromeric region because none of the examined marks were altered at a transcriptionally repressed tumor suppressor gene (WIFI) (Ai et al., 2006) (Figure 4-22A) or a constitutively active housekeeping gene (GAPDH) (Figure 4-22B). CENP-C was not enriched above the IgG background at either locus (Figure 4-22C, Figure 4-22D). In DNMT3B knock down samples, DNMT3B binding was detected above background levels at the methylated WIFI promoter (Figure 4-22C), but had very minor effects at the repressed WIFI promoter region (Figure 4-22A) and no DNMT3B binding was observed at the GAPDH promoter (Figure 4-22D).
Some of the results of the ChIP data from using the knockout cell lines vary from the transient knockdown studies and could be attributed to the residual protein left in the cells as siRNA knockdowns are never 100% effective. Some of the differences could also be due to the influence of other compensatory epigenetic mechanisms that might have occurred during the long term culturing of the knockout cell lines in the laboratory setting. Nonetheless, these results suggest that CENP-C and DNMT3B are involved in mutually reinforcing each other’s binding to the centromere region. In addition, these results demonstrate that CENP-C is involved in recruiting DNMT3B to the centromere, consistent with the loss of centromeric DNA methylation upon CENP-C knock down and together their binding influences the epigenetic status of centromeric and pericentromeric chromatin.

**Role of CENP-C and DNMT3B Interaction in Regulating Centromeric Transcription**

Until recently, the centromeric region was considered devoid of transcription. Evidence for a role of transcription at the centromere comes from studies in *S. pombe* and chicken-human hybrid DT40 cells where it was shown that deletion of components of the RNAi machinery resulted in aberrant accumulation of complementary transcripts from centromeric heterochromatic repeats (Fukagawa et al., 2004; Volpe et al., 2002). In *S. pombe* the increase in transcripts was accompanied by loss of histone H3 lysine-9 methylation, and consequent loss of centromere function (Volpe et al., 2002). This study also proposed that the double-stranded RNA arising from centromeric repeats targeted formation and maintenance of heterochromatin through RNAi (Volpe et al., 2002). There is also evidence that centromeric transcription occurs in both murine and human cells in a cell cycle or stress-dependent manner (Lu and Gilbert, 2008; Maison et al., 2002) and the centromere is permissive to transcription in selective hypomethylated regions of the centromere (Wong et al., 2006). Forced over-expression of centromeric transcripts lead to defects in segregation, cohesion, and altered centromeric
epigenetic marks, indicating that maintenance of proper levels of centromeric transcription is important for mitosis and chromosomal segregation (Bouzinba-Segard et al., 2006; Frescas et al., 2008). Given the known role for DNA methylation and the histone code in regulating transcription, and the recent finding that Dnmt3b null ES cells have increased levels of sister chromatid exchange and centromere transcript length (Jaco et al., 2008), it is possible that the lack of DNMT3B and CENP-C leads to aberrant centromeric transcription. To test the effect of altered DNA methylation patterns and histone modifications on centromeric transcription, total RNA was isolated from CENP-C and DNMT3B siRNA transfected HCT116 cells, DNAase treated, and used in reverse transcriptase (RT)-PCR reactions to detect levels of alpha satellite and satellite 2 transcripts. Satellite alpha transcription was detected using quantitative RT-PCR (Figure 4-23A) and the satellite 2 transcription was analyzed by semi-quantitative RT-PCR (Figure 4-23B) due to difficulties in developing quantitative RT-PCR primers for the latter. RT-PCR analysis revealed that levels of alpha satellite transcripts and satellite 2 repeats were elevated in both knockdowns relative to the control mock knockdown. Elevated centromeric and pericentromeric transcription was also confirmed in HCT116 3BKO cells, although the change in HCT116 3BKO cells was much less compared to the transient knockdowns (Figure 4-23). Once again, the more muted effects in 3BKO cells could be due to compensatory epigenetic marks or other transcriptional regulatory mechanisms that occurred during long-term selection of the knockout lines. It is also interesting to note that both the untreated cells and the control knockdown samples show some basal level of centromeric transcription suggesting that basal levels of centromeric transcription are contributing to the maintenance of centromeric chromatin structure. Taken together, these data demonstrate that loss of CENP-C and DNMT3B results in increased centromeric and pericentromeric transcription, presumably due to DNA
hypomethylation and altered epigenetic marks at these regions in the absence of CENP-C or DNMT3B. Elevated transcript levels from these regions may therefore contribute to mitotic defects and consequent genomic instability.

**Role of CENP-C and DNMT3B Interaction on Mitotic Stability**

Previous studies have established the pivotal role for CENP-C in mitotic progression (Kalitsis et al., 1998; Kwon et al., 2007) and DNMT3B itself plays an important role in maintaining chromosomal stability (Dodge et al., 2005). Observations from this study that CENP-C recruits DNMT3B to the centromere and together, DNMT3B and CENP-C modulate both histone and DNA methylation marks at centromeric chromatin and consequent transcriptional status of the centromere, indicates that the interaction itself may aid in proper mitotic progression by ensuring a normal chromatin status at the centromere. To directly assess the function of this interaction in chromosomal stability, HCT116 cells were depleted for CENP-C or DNMT3B by siRNA knockdown and were monitored for chromosomal alignment (Figure 4-24) or segregation defects during mitosis (Figure 4-25) in comparison to the cells with a control mock knockdown. Following the siRNA transfection, a mitotic ‘enrichment’ was performed by fixing the cells after 8-9 hours recovery following a double thymidine block. Cells were then stained with anti-centromere antibody and anti-tubulin antibody (mitotic spindle) and counterstained for DNA with Hoechst 33352, and then visualized using immunofluorescence microscopy. The cells depleted for CENP-C and DNMT3B were monitored for defective alignment of chromosomes at the metaphase plate (misaligned chromosomes) (Figure 4-24) and lagging chromosomes or bridges in anaphase (anaphase bridges) (Figure 4-25). Cells exhibiting the two aforementioned defects were counted in the mock or CENP-C or DNMT3B siRNA knock down cells from three independent knock down experiments. Anaphase bridges were more common in the DNMT3B knock down cells while improperly aligned chromosomes at
metaphase were more frequent in CENP-C knock down cells. This study therefore reveals that CENP-C and DNMT3B contribute to proper mitotic progression and chromosomal segregation.

**Conclusions and Discussion**

A novel interaction between the *de novo* methyltransferase DNMT3B and the constitutive centromere protein CENP-C was identified using yeast two-hybrid screening. This interaction was confirmed in mammalian cells and the region responsible for the interaction was also mapped on each of the interacting proteins. The role of this novel interaction was studied by observing the effects of disrupting the interaction between CENP-C and DNMT3B on the chromatin at the centromere and pericentromere using siRNA mediated knockdown. The results indicate that loss of either CENP-C or DNMT3B leads to DNA hypomethylation at satellite repeat regions. In addition, both proteins play an important role in regulating the histone code at the centromere and the pericentromere, with loss of either DNMT3B or CENP-C leading to alterations in marks specific to each of the two regions. Consequently, changes in the DNA methylation and histone modification patterns leads to elevated levels of centromeric transcription. The DNA methylation analysis and histone modification patterns after knockdown also indicate that CENP-C recruits DNA methylation and DNMT3B to the centromere. Also, cells depleted for CENP-C or DNMT3B display increased mitotic defects as manifested by elevated levels of chromosome alignment and segregation errors during mitosis and this effect may at least in part be mediated by elevated centromeric transcription in the absence of either of these proteins. These results therefore reveal a novel mechanism for recruiting DNMT3B and DNA methylation to the centromere as well as demonstrate the role of DNMT3B and CENP-C in maintaining the unique chromatin status at the centromere and consequent mitotic integrity.

Although several studies have shown interactions between DNA methylation and components of the histone modification machinery, the exact mechanisms of their spatial and
temporal regulation is less understood. In the context of the centromeric region, previous work indicates that the histone H3 K9 methyltransferase SUV39H1 is required for targeting DNA methylation to the murine pericentromeric region (the major satellite repeats) while the targeting of DNA methylation to the murine centromere (minor satellite repeats) was independent of SUV39H1 (Lehnertz et al., 2003) suggesting differential regulation of DNMT3B targeting to specific genomic loci. Also, the N-terminal PWWP domain has been shown to be required for recruitment of DNMT3B to pericentromeric regions of murine cells (Chen et al., 2004). Although re-expression of wild-type Dnmt3b in highly demethylated Dnmt3a/Dnmt3b double knockout ES cells resulted in remethylation of major and minor satellite repeats, the PWWP domain of Dnmt3b was specifically responsible for remethylation at the major satellite but not the minor satellite repeat (centromeric region), suggesting that although both regions are bona fide Dnmt3b targets, distinct regions of the protein are responsible for targeting it to centromeric and pericentromeric regions, (Chen et al., 2004). The region of interaction between DNMT3B and CENP-C mapped in this study is N-terminal to the PWWP domain (aa 1-140). This result is therefore consistent with the results of Chen et al. and possibly the SUV39H1-independent mechanism of DNA methylation targeting to the centromere as reported by Lehnertz et al and suggests that CENP-C may independently target DNMT3B to the centromere.

One of the factors responsible for affecting the assembly and inheritance of centromeric chromatin is histone H3 lysine K9 trimethylation and its interaction with the heterochromatin protein HP1 (Grewal and Moazed, 2003). H3-K9 lysine trimethylation provides a binding site for the chromodomain of HP1. HP1 is enriched at centromeres and recruits cohesin complexes to the centromere, which are required to bind sister chromatids prior to mitotic segregation (Bernard and Allshire, 2002; Bernard et al., 2001). Loss of the histone methyltransferase
Su(var)3-9 or its homologs leads to disruption of pericentromeric heterochromatin and loss of HP1 association leading to defects in chromosome cohesion and segregation (Melcher et al., 2000; Nonaka et al., 2002). HP1 also associates with shugosin (Yamagishi et al., 2008) and INCENP (Ainsztein et al., 1998), both of which are required for protecting centromeric structure. On the other hand, centromeric proteins also interact with components of pericentromeric heterochromatin. For example, HMis12, a conserved and essential kinetochore protein co-localizing with CENP-A at the centromere, interacts with HP1α and HP1α is essential for hMis12 localization to the centromere and normal mitotic chromosome segregation (Obuse et al., 2004). Furthermore, DNMT3B itself interacts with both HP1 and SUV(39)H1 (Geiman et al., 2004a; Geiman et al., 2004b; Lehnertz et al., 2003) and the results from this study demonstrate a marked loss of HP1 from the satellite alpha and satellite 2 repeats in the absence of DNMT3B, suggesting that DNMT3B is involved in maintaining condensed chromatin at the centromere by recruiting or maintaining H3K9 trimethylation and consequent HP1 association.

The findings from this study also tie in well with a recent study demonstrating that CENP-B recruits repressive histone marks and DNA methylation to ectopically integrated alpha-satellite repeats (Okada et al., 2007). CENP-B is present in a chromatin complex with CENP-A and CENP-C (Ando et al., 2002), and binds alpha-satellite DNA in a sequence-specific manner at the CENP-B box. Interestingly, DNA methylation at CpG sites in the consensus 17 bp CENP-B box inhibits CENP-B binding (Tanaka et al., 2005). Using an artificial alpha satellite array as a model system, Okada et al. demonstrated that CENP-B promotes de novo DNA methylation and SUV39H1 mediated assembly of trimethylated H3K9-containing repressive heterochromatin on alpha satellite DNA when integrated into a chromosome with an already functional centromere, therefore preventing the formation of a new centromere. On the other hand, CENP-
B promoted and was essential for de novo centromere formation of extrachromosomal alphoid DNA arrays (Okada et al., 2007). Although a direct interaction between DNA methyltransferase and CENP-B was not demonstrated in the prior study (Okada et al., 2007), DNMT3B may be present in a complex with CENP-B as both proteins also interact with SUV39H1 (Lehnertz et al., 2003; Okada et al., 2007), and therefore DNMT3B may be involved in recruiting or maintaining H3K9 trimethylation at both the centromeric and pericentromeric regions, consistent with the results of the Okada study. Although the binding of CENP-B itself is methylation sensitive, it is possible that CENP-B promotes DNA methylation at adjacent CpG sites via its association with SUV39H1. CENP-B’s interaction with CENP-C may also provide a means to recruit DNA methylation to centromeric sites, as both CENP-C and CENP-B are present in a nucleosomal complex along with CENP-A (Ando et al., 2002). Alternatively, the presence or absence of DNMT3B may be a factor in determining CENP-B occupancy, because CENP-C can bind to centromeres independent of CENP-B (Kwon et al., 2007) and CENP-B and CENP-C, despite recognizing the same sets of alpha–satellite DNA, occupy separate alpha satellite domains (Politi et al., 2002). The inhibition of CENP-B binding at the methylated CENP-B box also raises the possibility of a dynamic methylation status at the centromeric region, perhaps in a cell cycle dependent manner, similar to cyclical changes in the transcription factor recruitment and methylation of promoter DNA in certain genes (Kangaspeska et al., 2008). Whether such a scenario exists at the centromeric region, remains a question to be addressed in the future.

The coming of age of centromeric transcription has established that the centromere is not a transcriptionally inactive domain of the chromosome as previously viewed. Recent data suggest that transcription across centromeric repeats contributes to centromere formation and transcription and/or remodeling of nucleosomes at the centromeres may also be important for the
deposition of CENP-A (Chen et al., 2008; Folco et al., 2008; Fukagawa et al., 2004; Volpe et al., 2002). This also suggests that regulation of centromeric heterochromatin could be mediated by the epigenetic transcriptional regulatory mechanisms such as DNA methylation and histone modifications in mammalian cells. Interestingly, both condensin and HP1 proteins, that interact with DNMT3B (Geiman et al., 2004a), are also involved in centromeric transcription. Condensin binds to chromatin during mitosis and is involved in repressing pericentromeric transcription during mitosis in yeast (Chen et al., 2008). HP1 is displaced from chromosomes at the beginning of mitosis, and rebinds at the metaphase to anaphase transition, coinciding with elevated condensin binding and repression of pericentromeric transcription (Gerlich et al., 2006; Wu et al., 2006). Therefore, DNMT3B may be recruited to centromeric and pericentromeric regions to repress transcription by inducing DNA methylation and/or to facilitate recruitment of repressive histone marks or proteins like HP1 and condensins to induce/maintain chromosome condensation. Interestingly, in S. pombe the transcription of pericentromeric heterochromatin and subsequent incorporation of these transcripts into the RNAi machinery are required for initial incorporation of the yeast homolog of CENPA (CENH3) at centromeres (Folco et al., 2008). In S. pombe, loss of the normal centromere promotes the formation of a neocentromere at telomeric sites in an RNA interference-dependent manner (Ishii et al., 2008). The existence of RNAi mediated centromeric maintenance system has not yet been demonstrated in mammalian cells. Also it is not known if DNMTs themselves interact with components of RNAi mediated machinery. If that were true, it could emphasize a transcription mediated silencing mechanism by recruitment of DNMTs to the centromeric region by components of RNAi. However, given that CENP-C is a RNA binding protein (Wong et al., 2007); it is also possible that the CENP-C and DNMT3B interaction is mediated by centromeric transcripts themselves. By analogy,
noncoding centromeric RNAs may nucleate or serve as a scaffold for complexes containing chromatin remodeling/modifying factors at the centromere. It is then possible that accumulation of centromeric transcripts, as observed in this study, could prevent binding of centromere-associated proteins by causing their mislocalization away from their default target site leading to centromere dysfunction. Nevertheless, it is also possible that accumulation of large centromeric RNAs similar to the observation in Jaco et al., (but not investigated in this study) can inhibit the production of siRNAs by the RNAi machinery. In either scenario, accumulation of these transcripts may be responsible for centromere dysfunction, aneuploidy, and may be eventual cell death. However, whether these transcripts are required for normal centromere function and whether they participate in an epigenetic structure at the centromere in mammalian cells remains to be seen.

The results from this study also suggest that DNMT3B and CENP-C influence each others binding at satellite repeat regions suggesting that both of them affect the DNA binding properties of the other interacting partner. While absence of DNMT3B binding at the satellite repeat regions itself could be causal in DNA hypomethylation at these regions, it is possible that CENP-C enhances the DNA methylation activity of DNMT3B. Results from these studies may also help better understand the abnormal phenotypes observed in ICF syndrome. For example, in vitro experiments using recombinant wild-type DNMT3B and DNMT3B with mutations found in patients with ICF syndrome could be analyzed for the DNA binding properties as well as DNA methyltransferase activity in the presence and absence of CENP-C in the future to further examine the role of CENP-C in modulating DNMT3B’s enzyme activity and its causal role in the DNA hypomethylation. Double chromatin immunoprecipitation for CENP-C and DNMT3B could be performed in the future ascertain the simultaneous binding of both the proteins at the
same target sequence. This would also add further evidence on the reasons why CENP-C and DNMT3B affect each other’s binding at the satellite alpha and satellite 2 repeats (as observed in ChIP in this study). This again will emphasize the strong association of the two proteins and that CENP-C is required for DNMT3B targeting to the centromere.

Also, altered expression levels of DNMT3B due to promoter regulation or alternative transcription, or altered properties of $DNMT3B$ due to mutations in ICF syndrome cells, may affect CENP-C–DNMT3B binding. Cancer cells express elevated levels of DNMT3B, as well as many alternatively spliced forms of DNMT3B (Ostler et al., 2007; Robertson et al., 1999; Wang et al., 2006b). Some of these alternative transcripts are predicted to lack part, or all, of the CENP-C interaction domain (aa 1-140) indicating that the expression of these variants may act as a dominant negative of the wild type Dnmt3b by uncoupling the DNMT3B-CENP-C interaction and reducing CENP-C binding at the centromere. This may also lead to altered epigenetic marks within the centromeric region and in turn, enhance genomic instability. Consistent with the above hypothesis that aberrant expression of DNMT3B or DNMT3B splice variants disrupts the centromeric function, it was shown that forced expression of a transcriptional repressor or a transcriptional activator disrupted centromeric chromatin (Nakano et al., 2008). The essential need for a dynamic balance between centromeric chromatin and heterochromatin for proper centromere function was demonstrated when it was observed that targeting a transcriptional repressor to the centromere of a human artificial chromosome had a more severe effect on centromeric integrity compared to a transcriptional activator. The presence of a transcriptional repressor at the artificial centromere resulted in loss of CENP-A, CENP-B, CENP-C, and a decrease in di methylated H3K4 from the centromere accompanied by an accumulation of
histone H3K9 trimethylation, indicating a possible induction of a more heterochromatic structure similar to that in adjacent pericentromeric regions (Nakano et al., 2008).

Given the tight correlation between CENP-C binding and function at the centromere, the finding from this study that CENP-C binds at the pericentromeric satellite 2 repeat as identified by ChIP is unanticipated. This again could be due to the stochastic nature of the chromatin immunoprecipitation technique itself. The complex nature of the tandem repeats on the centromeric region may also cause CENP-C binding at the interface regions between alpha satellite (centromeric chromatin) and satellite 2 (pericentromeric chromatin), likely due to the continuous nature of centromeric and pericentromeric regions whose boundaries are epigenetically determined. Interestingly, loss of CENP-C or DNMT3B led to elevated H3K9 dimethylation at the alpha satellite and an increase in the repressive pericentromeric heterochromatin mark H3K9 trimethylation at the centromere and a reduction of this mark in pericentromeric regions. This result suggests that one of the functions of the DNMT3B-CENP-C interaction is to regulate the extent or the boundaries of each region. Also of interest, in future studies would be to visualize the effect of spatial regulation of changes in the dynamics of the histone modifications as a result of disruption of this interaction using immunofluorescence and see how they compare with the ChIP results.

Several other lines of evidences indicate that DNA methylation influences centromeric and pericentromeric integrity, although one particular DNMT has not been specifically linked to these effects. For example, neocentromeres, which are new centromeres that form at euchromatic sites and are functionally similar to normal human centromeres, have significantly elevated DNA methylation compared to the same region on the chromosome with the centromere at its normal position (Wong et al., 2006). Both CENP-A and CENP-C bind to neocentromeres
and inhibition of DNA methylation by the DNA methyltransferase inhibitor 5-azadC leads to neocentromere hypomethylation and elevated mitotic instability (Wong et al., 2006). It is also known that the transient modification of histone H3 by phosphorylation of serine 10 by aurora B kinase next to the more stable H3K9 methylation mark is necessary for dissociation of HP1 proteins from chromatin in M phase of the cell cycle (Fischle et al., 2005). The generation of serine 10 phosphorylation adjacent to H3K9 trimethylation (H3K9me3S10ph) mediated by aurora B kinase occurs at large pericentromeric heterochromatin regions during mitosis and appears to be dependent on Suv39h (Hirota et al., 2005; Monier et al., 2007). Interestingly, disruption of global DNA methylation by 5-azadC treatment, (or antisense-mediated depletion of DNMT1), inhibits recruitment of aurora B to pericentromeric regions (Monier et al., 2007). On the other hand, phosphorylation of CENP-A by aurora A and B kinases is essential for kinetochore function and aurora B kinase regulates the association of condensin I with mitotic chromosomes (Lipp et al., 2007). Therefore, defects in DNA methylation can lead to defects in Aurora-B targeting and consequent loss of pericentromeric histone H3 or CENP-A phosphorylation, indicating an essential role for DNA methylation in promoting mitotic regulation and centromere function by promoting aurora B induced histone H3/CENP-A phosphorylation. Although it is possible that DNA methylation and histone modifications synergistically regulate histone H3 phosphorylation at pericentromeres, the precise mechanism by which DNA methylation influences aurora-B recruitment to heavily methylated pericentromeres is yet to be determined. Given the observations from this study and previous studies, de novo methyltransferase DNMT3B has been demonstrated to specifically target the centromeric and pericentromeric region. Therefore, the role of DNMT3B in aurora-B targeting could be examined in the future by co-immunoprecipitation assay for a possible DNMT3B-
auroraB interaction. Alternatively, immunostaining for serine 10 phosphorylation of H3 during mitosis following DNMT3B siRNA knockdown might indicate if aurora B kinase function is dependent on DNMT3B or DNMT3B mediated DNA methylation.

Also of interest would be to test the interaction of DNMT3B and CENP-C and its implications in the mouse system. In a recent study that identified the interaction between CENP-C and PTEN, it was observed that loss of PTEN caused lesser chromosomal instability in mouse ES cells when compared to MEFs (Shen et al., 2007). This suggests that the contribution of PTEN-CENP-C interaction to genomic stability may vary between cell types. Therefore, it would also be interesting to test if the effect of DNMT3B-CENP-C interaction also displayed variability between cell types. The mouse Cenpc and human CENP-C have 50% similarity in protein sequence by pairwise alignment. It would be interesting to test if the interaction of DNMT3B and CENP-C is conserved in the mouse as well. Also of interest would be to determine how this interaction functions and if at all differs with respect to the murine ES cells and fibroblasts. The availability of several Dnmt single and double knockout mouse ES cell lines make it an excellent system to delineate the precise role of the interaction of CENP-C with the de novo methylation machinery and their coordinated regulation in maintaining the DNA methylation and histone modifications at the centromeric and pericentromeric region.

In summary, data from these studies demonstrate the physical interaction and a functional connection between constitutive centromeric protein CENP-C and DNA methyltransferase DNMT3B. Results from these studies therefore contribute to a better understanding of the regulation of centromeric chromatin as well as a possible mechanism by which DNMT3B is targeted specifically to centromeric regions in the genome. The results also shed light on various epigenetic mechanisms that coordinate with each other to maintain heterochromatin at
centromeres. The detailed mechanisms by which CENP-C influences DNMT3B mediated DNA methylation and whether they are involved in RNAi mediated heterochromatin formation are intriguing possibilities that could be examined in the future. Answers to how these mechanisms are altered in transformed cells are expected to important insights into cellular regulation of chromosome function in mitosis.
Figure 4-1. Schematic representation of the centromeric region. A) The human centromeric region is composed of tandem repeats of 170 bp DNA sequences called satellite alpha repeats. Alpha satellite repeats also contain a 17 bp consensus CENP-B box, which is the binding site for the constitutive centromeric protein CENP-B. The pericentromeric region that flanks the centromeres is composed of satellite 2 repeats, B) Representation of the unique organization of centromere chromatin in humans. On linear, two-dimensional chromatin fibers, subdomains of nucleosomes containing centromeric histone CENP-A (gray circles) are interspersed with H3 dimethylated at lysine 4 (H3K4me2) (white) to form a domain of CEN chromatin on a fraction of the megabase regions of human α-satellite DNA. The remainder of the α-satellite DNA is assembled into heterochromatin (black circles) that flanks one or both sides of CEN chromatin domain.
Figure 4-2. Confirmation of CENP-C and DNMT3B interaction in yeast using a β-galactosidase activity assay. The Y190 yeast strain was co-transformed with either the yeast recovered partial cDNA (pACT2) of CENP-C or the full-length cDNA (pGADT7) of CENP-C and full-length mouse Dnmt3b1 or human DNMT3B1. The interactions were tested in comparison to known control interactions. Positive controls (p53+TAg and Dnmt3b1 with DNMT3B3) and negative controls (p53+Lamin and Dnmt3b1+empty GAL4-AD vector) for interaction are also shown.
Figure 4-3. Mapping Dnmt3b regions involved in the interaction with CENP-C. A) Y190 yeast strain was co-transformed with the murine Dnmt3b1 deletion constructs fused to the GAL4-DBD and a partial cDNA of CENP-C fused to GAL4-AD (pACT2), B) Y190 yeast strain was co-transformed with the murine Dnmt3b1 deletion constructs fused to the GAL4-DBD and a full-length GAL4-AD-CENP-C (pGADT7) and the interaction assessed by β-galactosidase activity. Positive controls (p53+TAg and Dnmt3b1 with DNMT3B3) and negative controls (p53+Lamin and Dnmt3b1+empty GAL4-AD vector) for interaction are also shown. Numbering refers to the amino acids of full-length murine Dnmt3b1. Boxed region in panels A and B indicates minimal interaction domains and the roman numerals represent conserved catalytic DNMT motifs.
Figure 4-4. Mapping the region in CENP-C involved in the interaction with Dnmt3b. The Y190 yeast strain was co-transformed with the CENP-C deletion constructs fused to the GAL4-AD and a full-length Dnmt3b1 fused to GAL4-DBD and the interaction assessed by β-galactosidase activity. Positive controls (p53+TAg and Dnmt3b1 with DNMT3B3) and negative controls (p53+Lamin and Dnmt3b1+empty GAL4-AD vector) for interaction are also shown. Numbering refers to the amino acids of full-length CENP-C. Boxed region indicates minimal interaction domains.
Figure 4-5. Ectopically expressed DNMT3B and CENP-C interact in mammalian cells. A) pCDNA3.1-HA-CENP-C and pCMVTag2B-FLAG DNMT3B1 constructs were transiently transfected into 293T cells and whole cell extracts from transfected cells were used for co-immunoprecipitating with antibodies against the epitope tags and a control non-specific species matched normal IgG. B) pCDNA3.1-HA-CENP-C and pCMVTag2B-FLAG DNMT3B1 constructs were transiently transfected into 293T cells and the whole cell extracts from the transfected cells were co-immunoprecipitated with antibodies against the proteins and a control non-specific species matched normal IgG. Input – whole cell extract prior to co-IP, IgG – negative control for co-IP. The immunoprecipitating antibody (IP Ab) is indicated along the top of the western panel and the antibody used in western blotting (WB) is shown below it. Reciprocal co-IPs were performed in the left panels.
Figure 4-6. Immunoprecipitation with single epitope tagged constructs of CENP-C or DNMT3B. pCDNA3.1-FLAG-CENP-C or pCMVTag2B-FLAG DNMT3B1 constructs were transiently transfected into 293T cells and the whole cell extracts from the transfected cells were immunoprecipitated with antibodies against the epitope tag or the DNMT3B or CENP-C and a control species matched non-specific normal IgG. Input – whole cell prior to co-IP, IgG – negative control co-IP. The immunoprecipitating antibody (IP Ab) is indicated along the top of the western panel and the antibody used in western blotting (WB) is shown below it. Reciprocal co-IPs were performed in the left panel.
Figure 4-7. Co-immunoprecipitation of endogenous CENP-C and DNMT3B in mammalian cells. A) Immunoprecipitation of endogenous proteins CENP-C and DNMT3B using nuclear extracts from untransfected HeLa cells with antibodies against the endogenous DNMT3B or CENP-C and a control non-specific normal IgG. Two different CENP-C antibodies are used in the co-immunoprecipitation (labeled ‘1’ and ‘2’) and the positive control of CENP-C interacting with CENP-A is shown, B) Immunoprecipitation of endogenous proteins CENP-C and DNMT3B using soluble chromatin prepared from HeLa cells with antibodies against the endogenous proteins (DNMT3B or CENP-C) and a control non-specific normal IgG. Input – nuclear extract or soluble chromatin prior to co-IP, IgG – negative control for co-IP. The immunoprecipitating antibody (IP Ab) is indicated along the top of the western panel and the antibody used in western blotting (WB) is shown below it. Reciprocal co-IPs were performed in the left panel.
Figure 4-8. CENP-C and DNMT3B interact throughout the cell cycle. Immunoprecipitation of endogenous proteins CENP-C and DNMT3B using micronuclear extracts from HeLa cells enriched in the G1/S phase cells by double thymidine block (left) and the G2/M phase by 8-9 hours release after double thymidine block (right) with antibodies against the endogenous DNMT3B or CENP-C and a control non-specific normal IgG. Input – micronuclear extract prior to co-IP, IgG – negative control for co-IP. The immunoprecipitating antibody (IP Ab) is indicated along the top of the western panel and the antibody used in western blotting (WB) is shown below it.
Figure 4-9. Interaction of DNMT3B with other centromeric proteins. A) Confirmation that DNMT3B is a centromere-associated protein as shown by its ability to co-immunoprecipitate with the centromere-specific H3 variant CENP-A. pCDNA3.1 FLAG CENPA and pCDNA3.1(-) HA-Dnmt3b1 constructs were co-transfected into 293T cells and the whole cell extracts used for immunoprecipitation. B) Co-immunoprecipitation of ectopically expressed tagged constructs of CENP-A and CENP-C as a positive control interaction. pCDNA3.1 FLAG CENPA and pCDNA3.1 HA-CENP-C constructs were co-transfected into 293T cells and the whole cell extracts used for immunoprecipitation. Input – whole cell extract prior to co-IP, IgG – negative control for co-IP. The immunoprecipitating antibody (IP Ab) is indicated along the top of the western panel and the antibody used in western blotting (WB) is shown below it.
Figure 4-10. Interaction of CENP-C with other DNA methyltransferases. A) Co-immunoprecipitation of ectopically expressed tagged constructs of CENP-C and DNMT3A. 293T cells were co-transfected with pCDNA3.1 FLAG CENP-C and pCDNA3.1(-) HA-Dnmt3a and the whole cell extracts subject to co-immunoprecipitation, B) Co-immunoprecipitation of ectopically expressed tagged constructs of CENP-C and DNMT1. 293T cells were co-transfected with pCDNA3.1 FLAG CENP-C and pCDNA3.1(-) HA-DNMT1 and the whole cell extracts subject to co-immunoprecipitation. Input – whole cell extract prior to co-IP, IgG – negative control for co-IP. The immunoprecipitating antibody (IP Ab) is indicated along the top of the western panel and the antibody used in western blotting (WB) is shown below it.
Figure 4-11. DNMT3B interacts with two regions of CENP-C.  A) Schematic representation of CENP-C deletion constructs used in the mapping studies. Numbering corresponds to amino acids of human CENP-C. Regions 1 and 2 are the minimal DNMT3B interacting regions, B) CENP-C constructs indicated in Panel A, fused to the HA epitope, were co-transfected with full-length FLAG-tagged DNMT3B1 into 293T cells. Expression of CENP-C constructs and the near full-length CENP-C (23-943) (separate gel) was detected in whole cell extract of transfected 293T cells with HA antibody, C). Whole cell extracts from transfected cells were subject to co-immunoprecipitation with FLAG-agarose beads. CENP-C and DNMT3B do not co-immunoprecipitate in the absence of CENP-C regions 426-537 (region 1) and 638-760 (region 2).
Figure 4-12. Interaction between CENP-C and DNMT3B is independent of DNA. Immunoprecipitation of endogenous CENP-C and DNMT3B using micronuclear extracts from HeLa cells. Any DNA in the micronuclear extract was precipitated by incubating the extract with 50 μg/ml of ethidium bromide for 30 minutes at 4°C. The DNA-free extract was subject to immunoprecipitation with antibodies against the endogenous proteins and a control non-specific IgG. Input – micronuclear extract prior to co-IP, IgG – negative control for co-IP. The immunoprecipitating antibody (IP Ab) is indicated along the top of the western panel and the antibody used in western blotting (WB) is shown below it.
Localization of CENP-C and DNMT3B during various stages of cell cycle. HeLa cells were transfected with GFP-tagged DNMT3B1 (green) and enriched for mitotic cells by a double thymidine block followed by ~9 hrs recovery. Transfected cells were then fixed and stained with anti-CENP-C (red) antibody and counterstained for DNA with DAPI (blue). Representative images of cells in interphase or the different phases of mitosis are shown. An overlay of the red and green channels is shown in the right-most panels. Select foci are enlarged in red-boxed regions to highlight the closely associated DNMT3B and CENP-C foci. Co-localization is highest during metaphase. DNMT3B is also widely distributed throughout the nucleus in regions that do not contain CENP-C. Scale bar – 10 μM.
Figure 4-14. Efficiency and specificity of depletion of CENP-C and DNMT3B by siRNA knock down. HCT116 cells were transfected with either CENP-C or DNMT3B siRNA smartpools from Dharmacon as described in the Materials and Methods section. RNA and whole cell extract was prepared from each knock down or mock transfected cells. A) Quantitative RT-PCR analysis of total mRNA expression of CENP-C (light gray bars) and DNMT3B transcripts (dark gray bars). Values are the average of three PCR reactions from two independent knock down transfections, relative to amplification of GAPDH as a loading control. B) Immunoblotting analysis of protein expression of CENP-C and DNMT3B to determine levels of CENP-C and DNMT3B protein following siRNA knock down by western blotting. Results of a GAPDH siRNA knock down are also shown as a control. KD – knock down.
Figure 4-15. Bisulfite genomic sequencing (BGS) analysis for DNA methylation levels at the satellite alpha region. Representative CpG plot of satellite alpha repeat in HCT116 cells in mock transfected or cells transfected with CENP-C siRNA, or DNMT3B siRNA. Following knock down, genomic DNA was used for BGS analysis. The location of the CpG sites present in the CENP-B box (binding site) are also shown. Open circle – unmethylated CpG, closed circle – methylated CpG. The percent methylation and the number of clones is shown.
Figure 4-16. BGS analysis for DNA methylation levels at the satellite 2 region. Representative CpG plot of the satellite 2 repeat in HCT116 cells in mock transfected or cells transfected with CENP-C siRNA, or DNMT3B siRNA. Following knock down, genomic DNA was used for BGS analysis. Open circle – unmethylated CpG, closed circle – methylated CpG. The percent methylation and the number of clones is shown.
Figure 4-17. Quantitative analysis of total methylation at the satellite repeat regions. A) Quantitative representation of total methylation at the alpha satellite region in mock transfected or HCT116 cells transfected with siRNA directed against CENP-C or DNMT3B, B) Quantitative representation of total methylation at the satellite 2 region in mock transfected or HCT116 cells transfected with siRNA directed against CENP-C or DNMT3B. Statistical significance was determined using the Chi-Square test.
Figure 4-18. CENP-C and DNMT3B modulate epigenetic marks at centromeric regions. Chromatin immunoprecipitation (ChIP) followed by quantitative PCR was used to evaluate the effect of either CENP-C (light gray bars) or DNMT3B (dark gray bars) depletion by siRNA knockdown in HCT116 cells, relative to a mock transfection (black bars). A) ChIP for the indicated histone marks, HP1α, and histone H3/CENP-A, B) DNMT3B and CENP-C (right panel) binding as measured by ChIP followed by quantitative PCR detection at alpha satellite DNA. Results are presented as the average fold-enrichment relative to the input (10% of the supernatant from the IgG ChIP reaction). All reactions were repeated in triplicate from two independent siRNA knock downs. The error bar denotes the standard deviation from the mean. The scale in the right panels was expanded as the signal for CENP-C and DNMT3B was generally lower than that for the histone marks. IgG – negative control ChIP with rabbit normal IgG, 2XMe, 3XMe – di- and trimethylated forms, Ac – acetylation.
Figure 4-19. CENP-C and DNMT3B modulate epigenetic marks at the pericentromeric regions. Chromatin immunoprecipitation (ChIP) followed by quantitative PCR to evaluate the effect of either CENP-C (light gray bars) or DNMT3B (dark gray bars) depletion by siRNA knockdown in HCT116 cells, relative to a mock transfection (black bars). A) ChIP for the indicated histone marks, HP1α, and histone H3/CENP-A, B) DNMT3B and CENP-C (right panel) binding as measured by ChIP followed by quantitative PCR detection at satellite 2 DNA. Results are presented as the average fold-enrichment relative to the input (10% of the supernatant from the IgG ChIP reaction). All reactions were repeated in triplicate from two independent siRNA knock downs. The error bar denotes the standard deviation from the mean. The scale in the right panels was expanded as the signal for CENP-C and DNMT3B was generally lower than that for the histone marks. IgG – negative control ChIP with rabbit normal, 2XMe, 3XMe – di- and trimethylated forms, Ac – acetylation.
CENP-C and DNMT3B modulate epigenetic marks at the centromeric regions. Chromatin immunoprecipitation (ChIP) followed by quantitative PCR was used to evaluate the effect of DNMT3B knockout (gray bars) in isogenic HCT116 cells (3BKO), relative to parental HCT116 cells (black bars). A) ChIP for the indicated histone marks, HP1α, and histone H3/CENP-A. B) DNMT3B and CENP-C (right panel) binding as measured by ChIP followed by quantitative PCR detection at satellite alpha DNA. Results are presented as the average fold-enrichment relative to the input (10% of the supernatant from the IgG ChIP reaction). All reactions were repeated in triplicate from two independent chromatin preparations. The error bar denotes the standard deviation from the mean. The scale in the right panels was expanded as the signal for CENP-C and DNMT3B was generally lower than that for the histone marks. IgG – negative control ChIP with rabbit normal IgG, 2XMe, 3XMe – di- and trimethylated forms, Ac – acetylation.
Figure 4-21. CENP-C and DNMT3B modulate epigenetic marks at the pericentromeric regions. Chromatin immunoprecipitation (ChIP) followed by quantitative PCR was used to evaluate the effect of DNMT3B knockout (gray bars) in isogenic HCT116 cells, relative to parental HCT116 cells (black bars). A) ChIP for the indicated histone marks, HP1α, and histone H3/CENP-A, B) DNMT3B and CENP-C (right panel) binding as measured by ChIP followed by quantitative PCR detection at satellite 2 DNA. Results are presented as the average fold-enrichment relative to the input (10% of the supernatant from the IgG ChIP reaction). All reactions were repeated in triplicate from two independent chromatin preparations. The error bar denotes the standard deviation from the mean. The scale in the right panels was expanded as the signal for CENP-C and DNMT3B was generally lower than that for the histone marks. IgG – negative control ChIP with rabbit normal IgG, 2XMe, 3XMe – di- and trimethylated forms, Ac – acetylation.
Figure 4-22. SiRNA knock down of CENP-C has no effect on epigenetic marks in non-centromeric genomic loci. Chromatin was prepared from mock or CENP-C/DNMT3B siRNA-transfected HCT116 cells and used for ChIP with the indicated antibodies. A. ChIP analysis for the WIF1 gene at the promoter region (-198 to -115 relative to transcription start site), which is known to hypermethylated and transcriptionally silenced in HCT116 cells. B. ChIP analysis of the promoter (-310 to -180) of GAPDH gene, a constitutively active housekeeping gene. Results are shown as the average of triplicate PCR reactions from two independent siRNA knock downs and the error bar is the standard deviation. CENP-C and DNMT3B ChIP binding at WIF1 (Panel C) and GAPDH (Panel D) are also shown. DNMT3B knock down results in altered levels of some of the epigenetic marks and is bound above background level to the hypermethylated WIF1 promoter, but not the unmethylated GAPDH promoter.
Figure 4-23. Loss of CENP-C and DNMT3B-mediated epigenetic marks at the centromere region results in elevated levels of repeat transcription. HCT116 cells either mock transfected, or transfected with siRNA targeting CENP-C or DNMT3B. HCT116 cells with a genetic knockout of DNMT3B (3BKO) are also shown as a comparison.

A) Quantitative RT-PCR analysis of alpha satellite repeat transcripts. Values are the average of triplicate RT-PCR reactions relative to GAPDH. The error bar is the standard deviation from the mean, B) Semi-quantitative RT-PCR analysis of satellite 2 repeat transcription from the same cell line panel as in panel A. Representative ethidium bromide stained agarose gel photo of RT-PCR reactions is shown. Bands from reactions performed in triplicate were quantified using BioRad Quantity One software and set relative to an independent amplification for GAPDH as a loading control (lower panel). A negative RT (-RT) control was included to ensure that absence of contaminating satellite DNA in the RNA preparation.
Figure 4-24. Disruption of the CENP-C-DNMT3B interaction results in defects in chromosome alignment. HCT116 cells were transfected with siRNA against CENP-C or DNMT3B, or were mock transfected. Following transfection, cells were synchronized with a double thymidine block, released, and then fixed during M phase. Cells were then stained for DNA (blue), the centromere (anti-centromere antibody, green), and the mitotic spindle with anti-tubulin antibody (red). A) Representative images of cells undergoing normal or defective mitosis, B) Quantification of cells showing misaligned chromosomes in mock, CENP-C or DNMT3B knock down cells. Mock transfected cells showed fewer mitotic defects while cells with reduced levels of CENP-C or DNMT3B demonstrated elevated levels of misaligned metaphase. At least 50 mitotic cells were counted from three independent siRNA knock downs. Statistical significance was evaluated using the Students-t Test. Scale bar – 10 μM.
Figure 4-25. Disruption of the CENP-C-DNMT3B interaction results in defective chromosome segregation. HCT116 cells were transfected with siRNA against CENP-C or DNMT3B, or were mock transfected. Following transfection, cells were synchronized with a double thymidine block, released, and then fixed during M phase. Cells were then stained for DNA (blue), the centromere (anti-centromere antibody, green), and the mitotic spindle with anti-tubulin antibody (red). A) Representative images of cells undergoing normal or defective mitosis, B) Quantification of cells showing anaphase bridges in mock or siRNA knock down cells. Mock transfected cells showed fewer mitotic defects while cells with reduced levels of CENP-C or DNMT3B demonstrated elevated levels of aberrant chromosomal segregation during anaphase (anaphase bridges). At least 50 mitotic cells were counted from three independent siRNA knock downs. Statistical significance was evaluated using the Students-t Test. Scale bar – 10 µM.
CHAPTER 5
IDENTIFICATION AND CHARACTERIZATION OF A NOVEL SPLICE VARIANT OF DNMT3B LACKING EXON FIVE

Introduction

Known Splice Variants of DNMT3B

Alternative splicing is an important mechanism that generates a large number of mRNA and protein isoforms from smaller number of functional genes in the genome. Unlike promoter activity, which primarily regulates the amount of transcript expression, alternative splicing changes the structure of transcripts and their encoded proteins. Based on EST-database analysis, about 40–60% of all human gene products are alternatively spliced and are often regulated in a tissue and/or developmental specific manner, resulting in effects sometimes seen only in certain cells or developmental stages (Modrek and Lee, 2002; Modrek et al., 2001). Changes in alternative splicing can modulate transcript expression levels by subjecting mRNAs to nonsense-mediated decay (NMD) and alter the structure of the gene product by inserting or deleting regions of the protein resulting in a range of effects from complete loss of function to subtle effects that are difficult to detect (Stamm et al., 2005; Tazi et al., 2009). Mechanisms of alternative splicing fall into four main categories, 1) exon skipping, 2) alternative splice donor and acceptor sites, 3) mutually exclusive exon usage, and 4) intron retention. Alternative splicing is frequently disrupted in cancer and usually occurs through intron retention, leading to frame shifts and premature termination (Kim et al., 2008a). Exon skipping is a more common mechanism of alternative splicing in normal tissue (Kim et al., 2008a).

Interestingly, all three catalytically active DNA methyltransferases are subject to tissue and/or developmental-stage specific alternative splicing. DNMT3B, however, displays the greatest number of alternatively spliced isoforms by far. Several of the currently known DNMT3B isoforms have been identified by cDNA sequencing and are reported to be generated
due to alternative splicing and/or alternative promoter usage (Ostler et al., 2007; Robertson et al., 1999; Wang et al., 2006b; Xie et al., 1999) (Figure 5-1). Unlike DNMT1 and 3a, DNMT3B is the only DNA methyltransferase that is expressed as alternatively spliced variants that affect the integrity of the catalytic domain (Chen et al., 2002; Robertson et al., 1999; Weisenberger et al., 2004; Xie et al., 1999). DNMT3B1 and 3B2 contain all of the highly conserved catalytic motifs (I, IV, VI, VIII, IX, and X) and have been shown to have DNA methyltransferase activity (Okano et al., 1998a; Xie et al., 1999). Human DNMT3B1 consists of 23 exons while DNMT3B2 lacks exon 10, and DNMT3B3 lacks exons 10, 21, and 22. DNMT3B3 is the most ubiquitous and highly expressed splice variant present in most human tissues (Robertson et al., 1999). Both DNMT3B4 and DNMT3B5 lack conserved methyltransferase motifs IX and X, which, due to alternative splicing, result in truncated and presumably inactive proteins (Robertson et al., 1999). DNMT3B4 is 108 amino acids shorter than DNMT3B1 due to truncation immediately after a splice junction resulting from a frameshift and DNMT3B5 introduces 45 novel amino acids in the protein sequence (Robertson et al., 1999). DNMT3B5 was weakly expressed in brain and prostate and highly expressed only in testis and therefore not as widely expressed as the other DNMT3B variants. Many of the DNMT3B splice variants were expressed in a tissue-specific manner and testis showed the most number of splice variant expression (Robertson et al., 1999). Novel DNMT3B transcripts with alternative start sites originating from within intron 4 that retained a portion of exon 5 were detected in non-small cell lung cancer (NSCLC) cells and were termed as the ΔDNMT3B family (Wang et al., 2006b). Some of these transcripts extended through the end of the gene while others displayed variable inclusion of exons 7-9 and/or premature termination at exon 11 or 17 due to frameshifts (Wang et al., 2006b). In humans, several cancer cell lines displayed aberrant splicing at the 5’ end of
**DNMT3B** generating over 20 novel **DNMT3B** transcripts, which encode truncated proteins lacking the C-terminal catalytic domain (Ostler et al., 2007). Many of these aberrant transcripts retained some intron sequences and the most frequently identified aberrant **DNMT3B** transcript in this study was termed **DNMT3B7** (Ostler et al., 2007).

Differential expression of Dnmt3b splice variants is also observed in mouse tissues. Dnmt3b2 and Dnmt3b3 are expressed in testis, spleen, thymus, and liver, although Dnmt3b3 generally has ubiquitous but low level expression (Chen et al., 2003). The expression of Dnmt3b1 and Dnmt3b6 (lacks exons 21 and 22) was limited to ES cells (Chen et al., 2003). Both murine Dnmt3b1 and Dnmb3b2 exhibit *de novo* methyltransferase activity *in vitro*, but Dnmt3b3 does not exhibit detectable methyltransferase activity, likely due to the absence of part of motif IX within the catalytic domain (Aoki et al., 2001). Some of the murine Dnmt3b variants contain an alternate 5’-UTR (called U) although there is no change in the protein coding sequence (Weisenberger et al., 2004). Two variants similar to Dnmt3b4 and Dnmt3b5 that contain exons 10 and 11 were identified in mouse ES cells and termed Dnmt3b7 and Dnmt3b8 (Weisenberger et al., 2004). Mouse embryonic stem cells express Dnmt3b1, 3b6, 3b7, and their U isoforms along with Dnmt3b8U whereas somatic cells express Dnmt3b2, 3b3, 3b4, their U isoforms, and Dnmt3b5U (Weisenberger et al., 2004). In mice, the presence or absence of exons 10 and 11 correlated with expression in embryonic or somatic cells respectively, which was also observed in human cells (Weisenberger et al., 2004). Dnmt3b1 and 3b2 localize to small foci in the nucleoplasm and at pericentromeric heterochromatin whereas localization of Dnmt3b3 was more diffuse (Chen et al., 2004). Despite the identification of over 40 DNMT3B splice variants, the role of most of them has not been fully characterized.
Biological Role of DNMT3B Splice Variants

Although many DNMT3B splice variants have been identified, the biological roles of these variants remain largely uncharacterized, probably owing to their cell or developmental specific presence. Dnmt3b1 is required for de novo methylation after embryonic implantation as well as the de novo methylation of newly integrated retroviral sequences (Chen et al., 2003; Okano et al., 1999). Several DNMT3B isoforms are overexpressed in a variety of human cancers (Robertson et al., 1999; Saito et al., 2002). DNMT3B4, a variant highly expressed in human testis, is overexpressed in hepatocellular carcinoma and human epithelial 293 cells, resulting in pericentromeric satellite DNA hypomethylation, which is associated with genomic instability and increased cell proliferation (Saito et al., 2002). Overexpression of the novel DNMT3B splice variant with aberrant splicing at the 5' end, $DNMT3B7$ in normal cells causes hypomethylation of genes normally targeted by DNA methylation leading to aberrant hypomethylation similar to what has been observed in cancer cells (Ostler et al., 2007). This suggests that some splice variants of Dnmt3b function as a negative regulator of DNA methylation despite the lack of catalytic activity. On the other hand, although DNMT3B3 is catalytically inactive, it is known to be required for methylation of the $D4Z4$ repeats in T24 bladder cancer cells (Weisenberger et al., 2004). This was attributed to a stronger DNA binding capacity of DNMT3B3 because, on treatment of cells with the DNA methyltransferase mechanism-based inhibitor 5-aza-2'-deoxycytidine DNMT3B3 was depleted less efficiently. Expression of $\Delta$DNMT3B transcripts was associated with hypermethylation of the $RASSF1A$ tumor suppressor gene in NSCLC patients (Wang et al., 2007; Wang et al., 2006a).

In the present study, a novel DNMT3B3-like splice variant lacking exon 5 was identified and termed DNMT3B3$\Delta$5. Using a combination of biochemical and molecular analysis, the expression patterns and the biological properties of novel splice variant were studied in
comparison to the ‘parental’ form DNMT3B3. Characterization of the many splice variants of DNMT3B will be important in the understanding the many regulatory functions connected with de novo DNA methylation by DNMT3B.

**Results**

**Identification of a Novel Splice Variant of DNMT3B Lacking Exon Five (DNMT3BΔ5) Highly Expressed in Pluripotent Cells**

A novel DNMT3B isoform was detected in human pluripotent cell lines during attempts to clone the full-length DNMT3B1 and its known splice variants from human embryonic carcinoma cell lines by RT-PCR. The novel product was subsequently cloned, sequenced and identified as an isoform related to DNMT3B3 (a commonly expressed splice variant lacking exons 21 and 22), which lacked the exon 5 upon alignment to the sequence of full-length DNMT3B (Figure 5-2). Therefore, this variant was termed as DNMT3B3Δ5. Because there is a possibility that alternative splicing of exon 5 can also occur in the context of other DNMT3B isoforms, and the term ‘DNMT3BΔ5’ is used in situations where the splicing in other regions of DNMT3B was not determined. Exon 5 is immediately upstream to the PWWP domain, which is encoded by protein sequence in exons 6 and 7 and does not have any previously documented region of interest in DNMT3B. The N-terminal region of DNMT3B, upstream to the PWWP region also has very less homology to the DNMT3A.

Using semi-quantitative and quantitative RT-PCR primers (Figure 5-3B) expression of the novel splice variant was examined in greater detail. Preliminary semi-quantitative RT-PCR using primers located in exon 3 and exon 6 was performed in a panel of human cell lines comprising pluripotent and cancer cell lines. The RT-PCR result confirms the presence of DNMT3BΔ5 in many pluripotent cell lines (Figure 5-3C). Because DNMT3B3Δ5 was first detected and identified in pluripotent EC cell lines, the presence of other known DNMT3B splice
variants that are associated with pluripotent cell lines (Weisenberger et al., 2004) was also confirmed by semi-quantitative RT-PCR. The other DNMT3B isoforms associated with pluripotency, DNMT3B1 and DNMT3B6 (Weisenberger et al., 2004), were confirmed to be co-expressed in cells expressing DNMT3BΔ5 (Figure 3-D, Figure 3-E). Quantitative real-time RT-PCR primers specifically recognizing DNMT3BΔ5 (using a primer spanning the unique exon 4-exon 6 junction) were used to detect the expression of DNMT3BΔ5 in human pluripotent cell lines (Figure 5-4), which was compared the level of expression of DNMT3B transcripts with exon 5 (including DNMT3B1-6 isoforms) to estimate their relative abundance. Examination of the relative abundance ratio (Figure 5-4B) confirmed that DNMT3BΔ5 represented a moderate fraction of DNMT3B transcripts in pluripotent cell lines. The murine and human DNMT3B genes are highly conserved with 88% homology at the protein level. The murine Dnmt3b possesses one extra non-coding exon at its 5’-end. By pairwise alignment it was identified that human DNMT3B exon 5 is homologous to the murine Dnmt3b exon 6 (Figure 5-5). To examine the possible conservation of DNMT3BΔ5 expression in murine cells, semi-quantitative and quantitative RT-PCR primers (located in exon 5 and exon 7) (Figure 5-6B) were used to detect the presence of a possible Dnmt3bΔ6 in mouse cells. Indeed, a product corresponding to Dnmt3bΔ6 (homologous to DNMT3BΔ5 in human) was detected in murine cells (Figure5-6C). Consistent with human EC cells, the murine P19 EC cells expressed moderate levels of Dnmt3bΔ6 as well as Dnmt3b transcripts that include exon 6, while Dnmt3bΔ6 expression was nearly undetectable in the immortalized fibroblast line NIH3T3 (Figure 5-6C). Taken together, these results demonstrate the existence of a novel DNMT3B isoform DNMT3BΔ5, which is expressed at moderate levels in pluripotent cells and is conserved in the mouse genome.

**Expression of Novel Splice Variant in Normal and Tumor Cell lines**

The expression of DNMT3BΔ5 was first examined in several normal human tissues
DNMT3B Δ5 was detectable in a number of human tissues, including brain (adult and fetal), spinal cord, testis, and thymus, however another novel isoform corresponding to the exclusion of both exons 4 and 5 was identified in human fetal brain and termed as DNMT3BΔ4+5 (Figure 5-7B). Quantitative real-time RT-PCR primers specifically recognizing DNMT3BΔ5 (primer spanning the unique exon 4-exon 6 junction) were also used to examine the relative levels of splice variant expression in normal tissues (Figure 5-8A). Expression of DNMT3BΔ5, as assessed by qRT-PCR, was limited in the panel of normal human tissue. The expression of DNMT3BΔ5 was compared the level of expression of DNMT3B transcripts with exon 5 (including DNMT3B1-6 isoforms) to DNMT3BΔ5 expression to estimate their relative abundance. Examination of the ratio of the two isoforms confirmed that DNMT3BΔ5 represented a major fraction of DNMT3B transcripts in neural tissues, and several other tissues, including colon and heart, expressed significant levels of DNMT3BΔ5 relative to transcripts that included exon 5 (Figure 5-8B). Using quantitative RT-PCR primers for Dnmt3bΔ6 (spanning the unique exon 5-exon 7 junction), the expression of the DNMT3BΔ5 homolog in mice was examined in a panel of murine adult and fetal tissues and cell lines (Figure 5-9A). Consistent with semi-quantitative PCR, the murine P19 EC cells expressed moderate levels of Dnmt3bΔ6. In fetal tissues, Dnmt3bΔ6 represented a small but significant fraction of the total Dnmt3b in fetal brain, liver, and whole embryo and adult brain. Also consistent with human data, Dnmt3bΔ6 was highly expressed in murine testis (Figure 9B).

Given that splicing is often altered in transformed cells (Kim et al., 2008a) and that several alternatively spliced DNMT3B isoforms are highly expressed in tumor cells (Ostler et al., 2007; Robertson et al., 1999; Wang et al., 2007; Wang et al., 2006a), the expression of DNMT3B transcripts with and without exon 5 was examined by qRT-PCR in a panel of 58
tumor cell lines (Figure 5-10). A representative semi-quantitative RT-PCR analysis for the expression of DNMT3BΔ5 in a subset of the 58 cell lines is shown (Figure 5-11A). The average expression in tumor cells compared to the respective normal tissue from which they were derived showed several changes (Figure 5-11B). For example, tumor cell lines derived from colon, breast, and brain expressed moderate levels of DNMT3B transcripts with exon 5 but very low levels of DNMT3BΔ5 compared to normal cells. In contrast, expression of DNMT3BΔ5 was elevated, relative to transcripts containing exon 5, in tumor cell lines derived from liver, skin (melanoma), and lung. Cell lines like MOLT-4, HEK293, and G-401 expressed high levels of both DNMT3B isoforms. Taken together, this analysis demonstrates that the ratio of exon 5-containing to exon 5-lacking transcripts is altered in some tumor cells, which could contribute to the aberrant DNA methylation characteristics of transformed cells.

Altered Expression of DNMT3BΔ5 During Differentiation

Given the relative abundance of DNMT3B3Δ5 in human EC cells and human ES cells, expression of DNMT3BΔ5 was examined in two human neural stem cell lines and the glioma tumor initiating cell line (‘tumor stem cell’) H1228 (Figure 5-12A). H1228 cell line is a well characterized and highly invasive glioma tumor initiating cell line derived from glioma cells cultured in non-differentiating conditions. Both the neural stem cell cells and the H1228 glioma tumor initiating cell line were grown in non-differentiating conditions (NBE-serum-free Neurobasal media supplemented with basic fibroblast growth factor and epithelial growth factor). The human neural stem cell lines and the H1228 glioma tumor initiating cell line were used as a model for the analysis of DNMT3BΔ5 expression during differentiation. DNMT3BΔ5 was expressed at moderate levels in the two human neural stem cell lines cultured under non-differentiating conditions. Upon differentiation with retinoic acid there was a marked decrease in expression of DNMT3BΔ5 relative to exon 5-containing DNMT3B transcripts (Figure 5-12B).
In contrast, the tumor stem cell line, H1228 displayed the opposite trend, where DNMT3BΔ5 increased upon differentiation with retinoic acid.

Murine J1 ES cells were also used as a model for differentiation and the expression of Dnmt3bΔ6 was examined. Expression of Dnmt3bΔ6 was initially high but declined up to day four of differentiation, after which it began to increase again out to 16 days (Figure 5-13). This pattern is consistent with a number of other differentiation-associated genes such as osteocalcin and osteopontin (Candeliere et al., 1999). The progress of differentiation of J1 ES cells was also confirmed by the decline in Oct4 mRNA levels, a gene required for maintenance of pluripotency (Figure 13-C). Finally, it has recently been demonstrated that pluripotent ES-cell like cells can be derived from differentiated somatic cells by the ectopic expression of four pluripotency-associated transcription factors, Oct4, Sox2, Klf4, and c-Myc (Takahashi et al., 2007). As Dnmt3bΔ6 is highly expressed in ES cells and downregulated during differentiation, it is possible that iPS line would show elevated Dnmt3bΔ6 expression compared to the parental culture. Consistent with this idea, MEFs expressed very little Dnmt3bΔ6 compared to Dnmt3b transcripts containing exon 6, while the iPS line expressed significantly elevated levels of Dnmt3bΔ6 that far exceeded even the established J1 ES cells (Figure 5-14). These results therefore demonstrate that expression of DNMT3BΔ5 and the homologous Dnmt3bΔ6 splice variant is dynamically regulated during differentiation and is strongly associated with pluripotent cell lines or ‘stem-cell’ like properties and may therefore have functions in maintaining stem cell phenotype or regulating differentiation.

**In Vitro and In Vivo Properties of DNMT3B3A5**

The fusion of exon 4 to exon 6 preserves the normal DNMT3B open reading frame. Ectopic expression of DNMT3B3Δ5 as an epitope-tagged construct in a mammalian expression vector (pCDNA4 His Max C) by transient transfection of HCT116 cells confirmed that
alternative splicing yields an in-frame product of the expected size that appeared to be stable (Figure 5-15). The exon 5 region of DNMT3B does not contain any known functional motifs, but is immediately adjacent to the PWWP domain. Exclusion of exon 5 may alter DNMT3B’s properties due to a change in the amino acid context of the PWWP domain, or alter an as yet unknown function of this region in DNMT3B. Electrophoretic mobility shift assays (EMSA) performed with recombinant his-tagged DNMT3B3 and DNMT3B3Δ5 (generated by infection of Sf9 insect cells with recombinant baculovirus) and a fragment of human satellite 2 (Sat2) derived from chromosome 1 as the DNA probe indicate that DNMT3B3Δ5 has a higher DNA binding affinity when compared to DNMT3B3 (VanEmburgh et al., unpublished). It is known from previous studies that the PWWP of Dnmt3b is required to target the protein to murine pericentromeric regions which are composed of the major satellite repeats (equivalent to Sat2 repeats in humans) (Chen et al., 2004). The PWWP region is also implicated in Dnmt3b-induced DNA methylation at these regions (Chen et al., 2004). These results therefore indicate that sequences encoded by exon 5 influence the DNA binding properties of DNMT3B, perhaps by affecting the three dimensional structure of the adjacent PWWP domain.

The subcellular location of DNMT3B3Δ5 was compared to DNMT3B3 by transiently co-transfecting epitope-tagged versions of each, followed by immunofluorescence microscopy. DNMT3B3 displayed two exclusively nuclear patterns of localization: (1) diffuse with no enrichment in particular regions, and (2) punctate with accumulation in discrete foci that did not correspond to DAPI-dense heterochromatin. DNMT3B3Δ5 also showed these two staining patterns, however the frequency of each pattern differed markedly between the two splice variants. A representative image for each pattern is shown (Figure 5-16A). DNMT3B3 displayed a punctuate distribution more often than DNMT3B3Δ5 (Figure 5-16B). In addition,
DNMT3B3Δ5 appeared to accumulate less at heterochromatic regions in the diffuse staining pattern. Taken together, the EMSA and immunofluorescence studies reveal that DNMT3B3Δ5 has properties distinct from DNMT3B3 and may therefore regulate genomic methylation patterns differently from other DNMT3B isoforms.

Effect of Over-expression of DNMT3B3Δ5 on DNA Methylation Levels at Satellite Repeat Regions

Given the observations that DNMT3B3Δ5 displays altered DNA binding and sub-cellular localization, it is possible that these altered properties may contribute to changes in global DNA methylation upon increased DNMT3B3Δ5 expression. To examine the changes in DNA methylation levels by over-expressing DNMT3B3Δ5, HCT116 colon cancer cells stably expressing DNMT3B3Δ5 were created using lentiviral transduction as described in the Chapter 2. This line expressed DNMT3B3Δ5 from the endogenous locus at extremely low levels. Over-expression of DNMT3BΔ5 in selected stable clones was confirmed by semi-quantitative and quantitative RT-PCR followed by western blotting (Figure 5-17). Interestingly, the level of exon 5-containing DNMT3B transcripts was low in HCT116 clones stably expressing DNMT3B3Δ5 compared to the parental HCT116 cells although the protein levels remained almost equal (Figure 5-17B). Using these stable cell lines, two repetitive regions, the pericentromeric Sat2 repeat (also analyzed by EMSA above) and the centromeric alpha satellite repeats (both regions known to be DNMT3B targets) were examined for changes in DNA methylation. While hypomethylation at the Sat2 repeats is characteristic of ICF syndrome patients, a subset of ICF patients also lose methylation from the alpha satellite repeats (Ehrlich et al., 2006b; Miniou et al., 1997). The equivalent repetitive regions in mouse (major and minor satellite) also become hypomethylated in murine Dnmt3b knockouts and an ICF mouse model (Ueda et al., 2006). Using bisulfite genomic sequencing (BGS) it was determined that both Sat2 and alpha satellite
repeats were densely methylated in parental HCT116 cells (~84% and 60 %) (Figure 5-18). The DNMT3B3Δ5-expressing clones, however, demonstrated almost 10% reductions in both the alpha satellite repeats (Figure 5-18A) and the Sat2 repeats (Figure 5-18B). Chi-square analysis of the repetitive regions indicated that the Sat2 repeats were 2.07-times more likely to be hypomethylated in HCT116 cells overexpressing DNMT3B3Δ5 (95% C.I., p=0.0001) and the alpha satellite region in HCT116 cells overexpressing DNMT3B3Δ5 was 1.24-times more likely to be hypomethylated (95% C.I., p=0.1687). These results therefore suggest that aberrant expression of DNMT3B3Δ5 during tumorigenesis may contribute to genomic DNA hypomethylation at repetitive regions that are a hallmark of transformed cells.

Conclusions and Discussion

In summary, a novel conserved splice variant of DNMT3B similar to the widely expressed DNMT3B3 variant but lacking exon 5 in human DNMT3B or exon 6 in murine Dnmt3b was identified and characterized in this study. Although not yet tested, both DNMT3B3Δ5 and Dnmt3b3Δ6 are expected to be catalytically inactive as DNMT3B3 is also inactive (Okano et al., 1998a) but it is worth noting that exon 5 splicing may occur in the context of other DNMT3B isoforms which are active. Aside from brain and testis, DNMT3BΔ5 expression is limited in normal adult tissues and is altered in tumor cell lines. Comparison of average levels of expression of DNMT3BΔ5 indicates that tumor cell lines derived from cells normally expressing low levels of DNMT3BΔ5 show elevated DNMT3BΔ5 expression, while those derived from moderate/high-expressing tissues display reduced DNMT3BΔ5 expression. DNMT3BΔ5 and Dnmt3bΔ6 are dynamically regulated during differentiation of ES, EC, and neural stem cells. Conversion of differentiated, low Dnmt3bΔ6-expressing fibroblasts to iPS cells results in marked upregulation of Dnmt3bΔ6. Finally, DNMT3B3Δ5 displays distinct biochemical properties in comparison to its ‘parent’ isoform, DNMT3B3 in terms of its DNA
binding properties and subcellular localization. Also, elevated expression of DNMT3B3Δ5 results in pericentromeric hypomethylation, suggesting that it may act as a negative regulator of DNA methylation. This result also indicates that some DNMT3B splice variants can therefore alter the chromatin structure in stem cells or tumor cells due to DNA methylation defects if inappropriately expressed. Several previous studies support this finding. For example, cDNA analysis from tumor cell lines led to the identification of several alternatively spliced DNMT3B isoforms, some of which contribute to DNA hypomethylation of certain single copy genes upon over-expression (Ostler et al., 2007; Robertson et al., 1999; Saito et al., 2002; Wang et al., 2006b). A recent study also reported the identification of a transcript with a similar structure to Dnmt3bΔ6 in murine ES cells, further supporting our findings (Gowher et al., 2008).

DNMT3B4, a variant highly expressed in human testis, is over-expressed in hepatocellular cancers. Elevated DNMT3B4 expression resulted in pericentromeric satellite DNA hypomethylation, which is associated with genomic instability and increased cell proliferation (Saito et al., 2002). Consistent with this result, the over-expression of DNMT3B3Δ5 in HCT116 cells that normally express low levels of DNMT3B3Δ5 resulted in hypomethylation of sequences at and adjacent to the centromere. Expression of ΔDNMT3B transcripts was associated with poorer clinical outcome and with hypermethylation of the RASSF1A tumor suppressor gene in NSCLC patients (Wang et al., 2007; Wang et al., 2006a). In the most extensive study to date, Ostler et al. identified over 20 new DNMT3B isoforms in cancer cells. Most of the splice variants identified contained premature translation stop signals that truncate DNMT3B prior to the catalytic domain, while other transcripts involved alternative usage of exons 4-6, indicating that this area of DNMT3B, like the exon 21-22 region, is an alternative splicing hot spot. The most abundant splice variant identified, termed DNMT3B7, when ectopically overexpressed in
HEK293 cells resulted in altered gene expression and promoter CpG island hypermethylation (Ostler et al., 2007). Gene expression profiling studies indicated that elevated expression of truncated catalytically inactive splice variants of DNMT3B was one of the highly predictive trends in ovarian cancer compared to normal ovarian epithelium (Klinck et al., 2008). These studies therefore indicate that expression of certain DNMT3B splice variants in tumor cells results in alterations in gene-specific and repetitive region DNA methylation patterns.

The biological functions of the many alternatively spliced forms of DNMT3B including DNMT3B3Δ5 are not clearly understood. It is possible that many of these isoforms have altered functions. Many DNMT3B variants lack exons 20 and 21 or lack the catalytic domain entirely, and therefore are expected to be catalytically inactive (Ostler et al., 2007). Expression of inactive variants in differentiated somatic cells may help keep unauthorized de novo methylation in check by titrating DNMT3B away from potential DNA methylation targets, thereby preventing inappropriate gene silencing. As many of the DNMT3B transcripts, including DNMT3B3Δ5, are differentially expressed during development and differentiation, it is possible that their altered biochemical properties (e.g. altered DNA binding or altered DNA target site preference) are important for modulating methylation patterns during theses processes. For example, Oct4 is required to maintain the pluripotent state of ES cells. During differentiation, Oct4 levels decline due to promoter methylation by de novo DNA methylation (Li et al., 2007b). High levels DNMT3B3Δ5 in ES cells may prevent methylation of Oct4 thereby contributing to maintenance of the pluripotent state, while the reduction in DNMT3B3Δ5 levels during differentiation may restore the normal wild-type Dnmt3b function. It would be interesting to test this mechanism of regulation of pluripotency-associated genes such as Oct4 by using a siRNA specifically designed against DNMT3B3Δ5 or DNMT3BΔ5 in the future.
Alternatively spliced isoforms of DNMT3B may also exhibit differential interaction with known DNMT3B interacting proteins including other members of the DNA methylation machinery. For example, DNMT3B interacts with both DNMT3A and DNMT3L, and DNMT3L stimulates DNMT3 activity (Chedin et al., 2002; Chen et al., 2005; Jia et al., 2007). Alternative splicing that eliminates the interface regions required for interactions with Dnmt3a or Dnmt3L might cause reduced catalytic activity. On the other hand, catalytically inactive DNMT3B variants that still retain their ability to interact with DNMT3A may reduce DNMT3A’s catalytic activity or alter its target site preference. Such a mechanism may account for the hypomethylation observed upon DNMT3B3Δ5 overexpression. Alternatively, if splice variants like DNMT3B3Δ5 still interact with DNMT3L, they may uncouple DNMT3L away from active isoforms of DNMT3A and DNMT3B, thus indirectly modulating genomic DNA methylation levels. DNMT3B also interacts with several other chromatin-associated proteins such as SUV39H1, HP1 and HDACs (Geiman et al., 2004a; Geiman et al., 2004b; Lehnertz et al., 2003) as well as MCAF, CHD3 and CENP-C (our studies). It is possible that some of the functions of these interactions are also affected due to DNMT3B splice variant levels. It would also be interesting to test if DNMT3B3Δ5 possesses DNA methylation activity, thereby testing the role of exon 5 in the enzyme’s activity. Co-immunoprecipitation assays with tagged DNMT3B3Δ5 could be performed to assess if the lack of exon 5 disrupts any of the known DNMT3B interactions mediated by the PWWP domain. Future research testing the activities and protein interactions of DNMT3B splice variants will provide important insights into the regulation and maintenance of genomic DNA methylation patterns in mammalian cells.
Figure 5-1. Schematic representations of known DNMT3B splice variants. DNMT3B splice variants showing the conserved catalytic motifs I, IV, VI, IX and X and protein-protein interaction regions PWWP and ATRX domains. Numbers indicate the amino acids of DNMT3B. The light grey box in DNMT3B4 is a novel 45 amino acid protein sequence arising out of the alternative splicing. The patterned box in DNMT3B7 is a translational stop codon arising at exon 10 giving rise to a truncated DNMT3B protein. The alternative splice variants arising from exon 4, ΔDNMT3B family, are also represented.
Figure 5-2. Alignment of the novel DNMT3B splice variants. Pairwise alignment of first 800 bp sequence of the full-length DNMT3B with the novel DNMT3B splice variants identified in this study DNMT3BΔ5 and DNMT3BΔ4+5.
Figure 5-3. A novel alternative splice variant in the human and murine DNMT3B. A). Schematic showing the splicing structure of the human DNMT3B gene with 23 exons. Translation of human DNMT3B begins in exon 2. Alternatively spliced regions examined in this study are denoted with bold arrows, B) Blowup of the exon 2-7 region of DNMT3B shows the location of the PWWP domain, the primers used in semi-quantitative RT-PCR (top bold arrows) and quantitative RT-PCR (bold arrows below the exons) to detect DNMT3BΔ5, C) Representative ethidium bromide stained agarose gel photos showing expression of the newly identified splice variant DNMT3BΔ5 in a panel of human cell lines, D) RT-PCR gel photos showing splice variants associated with splicing in exons 10-11, E) RT-PCR gel photos showing splice variants associated with splicing in exons 20,21. Expression of the known pluripotency-associated transcript DNMT3B1, and the commonly spliced region within the catalytic domain (DNMT3B3) are confirmed. The identity of each product is indicated at the right of the gel photos, F) RT-PCR gel photo for the constitutively expressed gene GAPDH as a loading control.
Figure 5-4. Relative expression of DNMT3BΔ5 in pluripotent cells. A) Quantitative RT-PCR analysis of DNMT3B1-6 and DNMT3BΔ5, relative to GAPDH, in human pluripotent cell lines, B) Relative abundance of DNMT3BΔ5 in comparison to DNMT3B1-6 was determined by the ratio of relative expression DNMT3BΔ5 with respect to GAPDH to that of relative expression DNMT3B (+5) with respect to GAPDH for each of the cell lines. Values are the average of at least three independent PCR amplifications and the bar represents the standard deviation from the mean.
Figure 5-5. Alignment of the mouse Dnmt3b with human DNMT3B. Pairwise alignment of first 800 bp sequence of the full-length human and mouse DNMT3B to represent the homology between the human DNMT3B exons (denoted ‘h’) to the mouse Dnmt3b exons (denoted ‘m’).
Figure 5-6. The DNMT3BΔ5 isoform is conserved in mouse. A) Splicing structure of the murine $Dnmt3b$ gene with 24 exons. Translation starts at exon 3, B) Blowup of exons 3-8 showing locations of the quantitative RT-PCR primers (top bold arrows) and the semi-quantitative RT-PCR primers (bottom bold arrows) used in this study, C) RT-PCR gel photos showing expression of Dnmt3bΔ6 in the murine P19 EC cell line.
Figure 5-7. Expression of DNMT3BΔ5 in normal human tissues. A) Representative semi-quantitative RT-PCR amplification of DNMT3B transcripts containing exon 5 (DNMT3B1, 3B2, 3B3, top-most band) and those lacking exon 5 (DNMT3BΔ5, bottom-most band) for select human tissues, B) Blow-up of the RT-PCR reaction from fetal brain cDNA, showing another novel splice variant of DNMT3B, DNMT3BΔ4+5, as identified by sequencing. ‘*’ non-specific amplification product confirmed by DNA sequencing.
Figure 5-8. Quantitative RT-PCR expression analysis of DNMT3BΔ5 in normal human tissues. A) Expression of DNMT3B transcripts with (black bars) and without (gray bars) exon 5 by quantitative real-time RT-PCR for each of the human tissues shown. Values are presented relative to amplification of GAPDH as a loading control, B) Relative abundance of DNMT3BΔ5 as represented by the ratio of relative expression DNMT3BΔ5 with respect to GAPDH to that of relative expression DNMT3B(+5) with respect to GAPDH for each of the tissues. Values are the average of at least three independent PCR amplifications and the bar represents the standard deviation from the mean.
Figure 5-9. Expression of Dnmt3bΔ6 in murine tissues. A) Expression analysis of Dnmt3b transcripts containing exon 6 (black bars) compared to transcripts lacking exon 6 (gray bars) in each of the murine tissues and cell lines indicated relative to Gapdh. B). The relative abundance of Dnmt3bΔ6 as shown by the ratio of relative expression Dnmt3bΔ6 transcripts with respect to Gapdh to that of relative expression Dnmt3b(+6) with respect to GAPDH for each of the cell lines/tissues. Values are the average of at least three independent PCR amplifications and the bar represents the standard deviation from the mean. ‘DPC’ days post-coitum.
Figure 5-10. Expression of DNMT3BΔ5 in human tumor cell lines. Expression of DNMT3B transcripts containing exon 5 (black bars) compared to transcripts lacking exon 5 (gray bars) by quantitative RT-PCR for each of the 58 human cell lines shown, relative to GAPDH (upper portion of each panel). Relative abundance of DNMT3BΔ5 as represented by the ratio of relative expression DNMT3BΔ5 with respect to GAPDH to that of relative expression DNMT3B(+5) with respect to GAPDH for each of the cell lines (lower portion of each panel). Values are the average of at least three independent PCR amplifications and the bar represents the standard deviation from the mean. U2-OS is derived from an osteosarcoma, HEC59 from endometrial cancer, MOLT-4 from acute lymphoblastic leukemia, and HEK293 and G-401 from kidney cancer.
Figure 5-11. Altered expression of DNMT3BΔ5 in human tumor cell lines compared to normal tissues. A) Representative semi-quantitative RT-PCR amplification of DNMT3B transcripts with and without exon 5 for select tumor cell lines, B) Altered expression of DNMT3BΔ5 in human cancer cell lines compared to normal tissues. Relative abundance of DNMT3BΔ5 as represented by the ratio of relative expression DNMT3BΔ5 with respect to GAPDH to that of relative expression DNMT3B(+5) with respect to GAPDH in tumor cell lines derived from each tissue (having at least four cell lines) from Figure 5-10 was averaged (gray bars) and graphed relative to the same ratio from corresponding normal tissues (two independent tissue preparations, black bars (Figure 5-8).
Figure 5-12. DNMT3BΔ5 expression is regulated during differentiation. A) Quantitative RT-PCR analysis showing the expression of DNMT3B1-6 (black bars) and DNMT3BΔ5 (grey bars) expression, relative to GAPDH, in two neural stem cell lines (SCP’s) and one glioma tumor initiating cell line (H1228) in undifferentiated (-) and differentiating conditions (+). Expression is relative to GAPDH, B) Relative abundance of DNMT3BΔ5 as represented by the ratio of relative expression DNMT3BΔ5 with respect to GAPDH to that of relative expression DNMT3B(+5) with respect to GAPDH for each of the cell lines. Values are the average of at least three independent PCR amplifications and the bar represents the standard deviation from the mean.
Figure 5-13. Dnmt3bΔ6 expression is regulated during differentiation. A) Quantitative RT-PCR analysis showing the expression of Dnmt3b (+6) (black bars) and Dnmt3bΔ6 expression (grey bars), relative to Gapdh in mouse J1 ES cells during in vitro differentiation of ES cells by LIF withdrawal and embryoid body formation over a period of 16 days, B) Relative abundance of Dnmt3bΔ6 as represented as the ratio of relative expression Dnmt3bΔ6 with respect to Gapdh to that of relative expression Dnmt3b (+6) with respect to Gapdh for each of the cell lines, C) Relative abundance of Dnmt3bΔ6 as represented as the ratio of relative expression Dnmt3bΔ6 with respect to Gapdh to that of relative expression Dnmt3b (+6) with respect to Gapdh for each of the cell lines (black bars) and relative expression of Oct4 with respect to Gapdh (gray bars) during ES cell differentiation. Values are the average of at least three independent PCR amplifications and the bar represents the standard deviation from the mean.
Figure 5-14. Dnmt3bΔ6 is expressed in iPS cells. A) Quantitative RT-PCR analysis showing the expression of Dnmt3b (+6) (black bars) and Dnmt3bΔ6 expression (grey bars), relative to Gapdh in mouse ES cells, mouse embryonic fibroblasts (mEFs) and induced pluripotent cells (iPS) derived from mEFs, B) Relative abundance of Dnmt3bΔ6 as represented as the ratio of relative expression Dnmt3bΔ6 with respect to Gapdh to that of relative expression Dnmt3b (+6) with respect to Gapdh for each of the cell lines. Values are the average of at least three independent PCR amplifications and the bar represents the standard deviation from the mean.
Figure 5-15. Ectopic expression of DNMT3BΔ5 in HCT116 cells yields a stable protein product of the expected molecular weight. DNMT3B1, DNMT3B3, and DNMT3BΔ5 were cloned in frame in the mammalian expression vector pCDNA4 His Max C and expressed in HCT116 cells following transient transfection. The protein was detected with the antibody against the epitope tag (Anti-Express).
Figure 5-16. Immunofluorescence localization of DNMT3B3 and DNMT3B3Δ5 in human cells. GFP-DNMT3B3 or GFP-DNMT3B3Δ5 expression plasmids in pEGFPC2 were transfected into HeLa cell lines. Forty-eight hours later cells were fixed, stained for DNA (DAPI), and visualized with a fluorescence microscope and photographed. All images were deconvolved. A) Representative images of transfected cells. Blue-DNA, green-GFP tagged protein (DNMT3B3 or DNMT3B3Δ5) showing two different patterns of subcellular localization, B) Quantitation of the percent of transfected cells displaying either the diffuse or the punctuate localization patterns of DNMT3B3 or DNMT3B3Δ5 as indicated. Bar = 10 microns.
Figure 5-17. Creation of stable cell lines expressing DNMT3B3Δ5. HCT116 cells were transduced with lentivirus expressing DNMT3B3Δ5 (pLVX lentiviral constructs) and subjected to puromycin selection. The stable colonies after selection were screened for expression of DNMT3B3Δ5. A) Semi-quantitative RT-PCR showing the expression of DNMT3B and DNMT3B3Δ5, B) Expression of DNMT3B (black bars) and DNMT3B3Δ5 (grey bars) relative to GAPDH as analyzed by quantitative RT-PCR, C) Cell extracts were prepared from select clones and DNMT3B3Δ5 protein levels were assessed by immunoblotting using antibodies against DNMT3B. The isoforms expressed are indicated. The numbers following the cell line denote clone number.
Figure 5-18. Expression of DNMT3B3Δ5 alters DNA methylation in vivo. Bisulfite genomic sequencing (BGS) analysis of A) centromeric alpha satellite, and B) pericentromeric satellite 2 (Sat2) repeats in parental HCT116 cells and two HCT116 clones stably overexpressing DNMT3B3Δ5 (clones 2 and 3). Each row of circles represents the DNA methylation pattern of an individually cloned and sequenced DNA molecule. The total percent methylation for all analyzed clones is shown at the right. Open circle – unmethylated, black circle – methylated CpG.
Chapter 6
Conclusions and Future Directions

Conclusions

Dnmt3b is required for de novo methylation of repetitive elements and proviral elements and is essential for mammalian development (Okano et al., 1999; Okano et al., 1998a). Cells with Dnmt3b knockout display loss of methylation at pericentromeric repeat regions (Okano et al., 1999; Okano et al., 1998a). An autosomal recessive genetic disorder known as immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome is caused by germline mutations in DNMT3B. Patients with ICF syndrome exhibit decreased methylation at the satellite 2 repeat making aberrant DNMT3B function the causative agent of hypomethylation. Many chromatin associated factors such as HDACs, HP1, SUV39H, hSNF2H and polycomb proteins have been identified as DNMT3B interacting proteins (Geiman et al., 2004a; Geiman et al., 2004b; Lehnertz et al., 2003) and some of these interactions are mediated via the PHD or the PWWP domains (Bachman et al., 2001; Xie et al., 1999). Many of these protein associations have stimulatory roles for the transcriptional repressor function of DNMT3B (Bachman et al., 2003; Chen et al., 2005; Zhu et al., 2006), although several other protein interactions have not defined any regulatory role and therefore remain mere associations. Moreover, several DNMT3B interacting proteins have not been identified in a stable complex with DNMT3B (example- Lsh, Polycomb proteins, SUMO) indicating specific regional and temporal roles for these interactions, some of which remain to be characterized (Kang et al., 2001; Myant and Stancheva, 2008; Vire et al., 2006). The function of DNMT3B also seems to be regulated by the generation of several alternative splice variants. Although the function of several of the isoforms identified thus far remain to be characterized, their underlying role in the regulation of DNMT3B function and consequent DNA methylation cannot be undermined. Despite the wealth of
information on global DNA hypomethylation caused by mutations or knockouts of Dnmt3b, the mechanisms that target DNMT3B to specific genomic regions and the reasons for the defects in genome stability that arise due to DNA hypomethylation are poorly understood. It is likely that a full understanding of existing interactions of DNMT3B as well as the functions of DNMT3B isoforms will help unveil cross talk among the various epigenetic regulatory mechanisms. Nevertheless, the identification of novel DNMT3B interacting partners will also provide insights into the possible mechanisms by which DNMT3B is targeted to select genomic regions. The sustained association or lack of DNMT3B interactions with DNMT3B splice variants may also play a regulatory role in modulating DNMT3B function. In this study, three novel DNMT3B interacting proteins were identified using yeast two-hybrid screening. The interaction between DNMT3B and the constitutive centromeric protein CENP-C was extensively characterized while the interaction of DNMT3B with MCAF and CHD3 were limited to preliminary studies. In addition to these novel protein interactions, a novel DNMT3B splice variant DNMT3B3Δ5 was also identified. The expression of the splice variant was examined in the context of DNMT3BΔ5 (absence of exon 5 only) and the biochemical properties were studied for DNMT3B3Δ5.

Together, the results from this study contribute to a better understanding of the mechanisms by which DNA methylation mediated by DNMT3B may be regulated and provides possible insights into DNMT3B targeting and its role in the maintenance of genomic stability.

Preliminary studies following yeast two-hybrid screening confirmed the interaction of DNMT3B with MCAF and CHD3. The confirmation of interaction of DNMT3B with MCAF and CHD3 was performed by ectopic expression of tagged constructs of both proteins. MCAF and CHD3 are both associated with larger macromolecular chromatin complexes. MCAF is associated with a complex containing the histone H3 K9 methyltransferase complex SETDB1
and MBD1 and associated repressor activity (Fujita et al., 2003b; Wang et al., 2003). CHD3 is an integral part of the nucleosome remodeling complex NuRD and forms the Mi2 alpha-NuRD repressor complex that is associated with HDAC activity (Denslow and Wade, 2007). Interestingly, CHD3 also associates with SETDB1 (Ivanov et al., 2007), thereby connecting DNMT3B to histone lysine methylation independent of DNMT3B’s association with the histone H3 K9 methyltransferase SUV39H1. This also suggests that despite the common function of repressing gene expression, epigenetic silencing mechanisms can act together or differentially depending on the genomic loci, cell type, or cellular cues. The known associations of MCAF and CHD3 with a wide variety of proteins including transcription factors (De Graeve et al., 2000; Fujita et al., 2003b; Ichimura et al., 2005; Ivanov et al., 2007; Saether et al., 2007; Uchimura et al., 2006; Wang et al., 2003) make them good scaffolding agents. Also, given their dual roles as a co-activator or a co-repressor (Fujita et al., 2003b; Ichimura et al., 2005; Saether et al., 2007), it is possible that MCAF or CHD3 act as adaptors for other proteins and result in modulation of gene expression and chromatin structure in a manner specific to cell type, genomic context, or the interacting protein. Given the evidence for SUMO-mediated regulation of MCAF and CHD3 functions (Ivanov et al., 2007; Saether et al., 2007; Uchimura et al., 2006) and the association of SUMO with DNMT3B itself (Kang et al., 2001), it is also possible that the SUMO pathway modulates the interaction of DNMT3B with MCAF and CHD3 or alter the biochemical properties of the proteins themselves thereby playing a causal role in their consequent function.

Unlike MCAF and CHD3, CENP-C was known as a structural protein with no known associations with chromatin regulatory components and therefore its identification in the yeast two-hybrid screening with Dnmt3b was unanticipated. Nevertheless, due to the important role of CENP-C in centromeric integrity and the regulation of centromeric and pericentromeric DNA
methylation by DNMT3B; this interaction appeared the most novel and intriguing. Results from yeast two-hybrid screening were further confirmed in mammalian cells using co-immunoprecipitation assays. The mapping of the first 140 amino acids of Dnmt3b as the interface for Dnmt3b –CENP-C interaction is also consistent with previous studies that implicate a PWWP independent mechanism of Dnmt3b targeting at the centromeric region (Chen et al., 2004; Lehnertz et al., 2003) suggesting that CENP-C may independently target DNMT3B to the centromere. Disruption of the interaction between CENP-C and DNMT3B using siRNA-mediated knockdown of either proteins causes DNA hypomethylation at the centromeric and pericentromeric satellite repeat regions. CENP-C and DNMT3B, both regulate distinct as well as a common set of histone marks at the centromere and the pericentromere, with loss of either DNMT3B or CENP-C leading to alterations in marks specific to each of the two regions. ChIP results from this study demonstrate a loss of HP1 at the satellite alpha and satellite 2 repeats in the absence of DNMT3B, suggesting that DNMT3B helps recruit or maintain H3K9 trimethylation and HP1 association at centromeric and pericentromeric regions thereby contributing to the formation and/or maintenance of condensed chromatin at the centromere. The observation that CENP-C knockdown causes DNA hypomethylation and changes in histone modifications indicates a possible role for CENP-C in recruiting DNA methylation and DNMT3B to the centromere. Given that DNA methylation and SUV39H1-mediated histone H3K9 trimethylation are in CENP-B mediated repression of de novo centromere formation (Okada et al., 2007), the evidence of a DNMT3B –CENP-C interaction further supports the necessary involvement of DNMT3B in mediating centromeric silencing. Changes in the DNA methylation levels and histone modification patterns also leads to elevated levels of centromeric transcription and increased mitotic defects in cells depleted for CENP-C or DNMT3B. Elevated levels of
centromeric transcription in the absence of CENP-C and DNMT3B suggests that DNMT3B and consequent DNA methylation are required to repress centromeric transcription and/or facilitate recruitment of repressive histone marks or proteins like HP1 and condensins to maintain closed chromatin at the centromere. Results from the ChIP analysis also suggest that DNMT3B and CENP-C influence each others binding at the satellite repeat regions. These results therefore reveal a novel mechanism of DNMT3B recruitment to the centromere and demonstrate the role of DNMT3B and CENP-C in maintaining the chromatin status at the centromere.

It also remains to be seen if MCAF, CHD3 or CENP-C is present in a stable complex with DNMT3B. Like many other known protein associations of DNMT3B, the interaction might be dependent on specific spatio-temporal cellular cues, or may be developmental or cell type/cell cycle stage specific. Given the abundance of DNMT3B as visualized by its wide nuclear localization compared to the specific centromeric location of CENP-C, it is possible that, even if CENP-C were in a stable complex with DNMT3B, it might be difficult to isolate, as only a small fraction of DNMT3B may be associated with CENP-C. But, both CHD3 and MCAF are likely to have a wide nuclear presence as they are parts of abundant chromatin macromolecular complexes, therefore making their association with DNMT3B easier to detect if present as a stable complex. If these interactions are temporal, it also raises questions on the mechanism of their modulation.

The altered expression levels of DNMT3B due to alternative transcription is thought to be one of the possible mechanisms of regulation of DNMT3B function. Many of the splice variants of DNMT3B, including the novel DNMT3B splice variant identified in this study DNMT3B3A5, have been demonstrated to act as negative regulators of DNA methylation. Intriguingly, DNMT3BΔ5 is highly expressed in pluripotent cells and dynamically regulated
during differentiation of ES, EC, and neural stem cells and its mouse homolog Dnmt3bΔ6 is also expressed at high levels in ES cells and pluripotent mouse iPS cells generated from differentiated mEFs. DNMT3B3Δ5 also displays altered biochemical properties such as higher DNA binding affinity and a diffuse nuclear localization compared to the parent DNMT3B3 isoform. Such changes can affect the three dimensional structure of DNMT3B, which may also affect protein-protein interactions in a positive or negative manner. The interactions of DNMT3B splice variants with bonafide DNMT3B interacting proteins, if preserved, may titrate away the interactions of wild-type DNMT3B with these proteins, thereby interfering with the function of the interaction. On the other hand, levels of the wild-type or splice variant DNMT3B may determine the level at which these interactions occur or only at a specific time. Many of the DNMT3B splice variants including DNMT3B3Δ5 have alternative splicing at the catalytic region. If any of the protein interactions of DNMT3B including MCAF, CHD3 or CENP-C have stimulatory roles in DNA methylation activity of DNMT3B, then the excessive presence of these splice variants might lead to hypomethylation. It is conceivable DNMT3B acts as a transcriptional repressor independent of its catalytic activity and can become activated later by other regulatory signals brought about by their protein interactions or by post-translational modifications. If DNMT3B splice variants lack the ability to make these interactions or have the regions responsible for sensing regulatory signals are spliced out, their presence could be detrimental to the cell. This is consistent with the observation that the forced targeting of transcriptional repressors to the centromeric regions results in centromeric dysfunction (Nakano et al., 2008), supporting the idea that elevated levels of potentially inactive DNMT3B splice variants or mutated forms of DNMT3B (ICF patients) may lead to centromeric hypomethylation and consequent genomic instability. Taken together, these results suggest that chromatin-associated
factors such as MCAF and CHD3 may modulate DNMT3B functions in a spatio-temporal manner and a possible CENP-C mediated recruitment of DNMT3B to the centromere. Results from these studies also shed light on the various epigenetic mechanisms that coordinate with each other to maintain the heterochromatin structure.

**Future Directions**

The yeast two-hybrid screening has generated a list of potential DNMT3B interactions some of which have been characterized in detail in this study. The preliminary confirmation of the interaction of DNMT3B with MCAF and CHD3 could be pursued in detail by examining their association in a physiological setting by immunoprecipitation of endogenous interacting proteins and ChIP at potential targets. Further experiments are required to delineate the exact role of the interactions of DNMT3B with MCAF and CHD3 in the function and/or targeting of DNMT3B. Some of these target sites could be repetitive regions given the association of CHD3 at the pericentromeric region. Genes that are regulated by the transcription factors that associate with CHD3 or MCAF or genes regulated by SETDB1, could also be targeted by this interaction as both CHD3 and MCAF interact with SETDB1. It would also be interesting to test whether MCAF or CHD3 modulate the DNA binding properties or DNA methyltransferase activity of DNMT3B itself. Given the proven association of DNMT3B, MCAF and CHD3 with the SUMO machinery, a study of effects of sumoylation of DNMT3B could be pursued. *In vitro* and *in vivo* sumoylation assays or depletion of the sumoylation pathway could provide insights into the involvement of this pathway in mediating the interaction between DNMT3B and MCAF or CHD3, and possibly its role in regulating its subcellular localization, DNA binding properties, or DNA methyltransferase activity.

Similarly, although the absence of DNMT3B binding at the satellite repeat regions could be causal in observed DNA hypomethylation at these regions, CENP-C may stimulate
DNMT3B’s enzyme activity. However, because the absence of CENP-C itself can cause mitotic instability (Kalitsis et al., 1998; Kwon et al., 2007) it is possible that mitotic disruption can prevent access to DNA methylation by inducing cell quiescence. Therefore, passive demethylation could be a reason for the observed DNA hypomethylation in the absence of CENP-C. Given this possibility, future studies are required to delineate the role of the interaction between CENP-C and DNMT3B in the DNA methylation at the centromeric region. Toward this end, in vitro experiments studying the DNA binding properties and DNA methyltransferase activity in the presence and absence of CENP-C could be tested. Evidence that CENP-C can modulate DNMT3B enzyme activity will emphasize causal role of this interaction in DNA hypomethylation in the absence of CENP-C as well as the hypomethylation caused due to mutations in DNMT3B as observed ICF syndrome. Double chromatin immunoprecipitation assays confirming the presence of the two proteins at the same region simultaneously would also support the idea that CENP-C and DNMT3B act together to coordinate the chromatin status at the centromere as indicated by the ChIP and BGS analysis.

The regulation of centromeric heterochromatin by the RNAi machinery in the mammalian system and the role of RNA mediated gene silencing in DNMT3B mediated DNA methylation could also be pursued in the future. A better understanding of the function of DNMT3B-CENP-C interaction might provide clues to the causes of aneuploidy. Another exciting avenue for future studies would be in the investigation of how these interactions and consequent functions are affected by DNMT3B splice variants and characterization of the specific role of exon 5 in the context of DNMT3BΔ5. Understanding how the coordinated regulation of these interactions results in chromatin reorganization at specific genomic loci is important not only for understanding the underpinnings of cellular development and cancer, but also for the design of
clinically relevant and efficient therapeutics such as gene therapy using human artificial chromosomes or using stem cells and anticancer drugs.
Figure A-1. Quantitative RT-PCR for expression of DNMT3B in stable clones. HCT116 cells and isogenic HCT116 DNMT3B knockout (3BKO) cells were transduced with lentivirus expressing full-length DNMT3B1 or DNMT3B3 (as indicated by the pLVX lentiviral construct transfected). The transduced cells were subjected to puromycin selection and stable clones were isolated. RNA was extracted from stable clones and the overexpression of DNMT3B1 or DNMT3B3 was confirmed by q-RT-PCR. A) Expression of DNMT3B (DNMT3B1 or DNMT3B3) in HCT116 cells or 3BKO stable clones as estimated by q-RT-PCR relative to GAPDH, B) Expression of DNMT3B1 in 3BKO stable clones as estimated by q-RT-PCR relative to GAPDH. Additional cell line controls for expression of DNMT3B include HCT116 cells with isogenic DNMT1 knockout (DNMT1 KO), DNMT1 and DNMT3B double knockout (DKO).
Figure A-2. Protein expression analysis for DNMT3B1 and DNMT3B3 in stable cell lines. HCT116 cells and isogenic HCT116 cells with DNMT3B knockout (3BKO) were transduced with lentivirus expressing full-length DNMT3B1 or DNMT3B3 (as indicated by the pLVX lentiviral construct transfected). Cells from stable clones were lysed and whole cell extract was prepared. The protein sample was run on 10% SDS PAGE and subject to western transfer. Immunoblotting was performed with Anti-DNMT3B antibody for detecting overexpressed DNMT3B in stable clones.
Figure A-3. Creation of HCT116 cells and 3BKO cells stably expressing mouse Dnmt3b1. HCT116 cells and isogenic HCT116 cells with DNMT3B knockout (3BKO) were transduced with lentivirus expressing full-length mouse Dnmt3b1 (FLAG-HA-Dnmt3b1 (plasmid construct TG3B21, as indicated by the pLVX lentiviral construct transfected). Cells from stable clones were lysed and RNA extracted. A). The cDNA from the RNA sample was used in the semi-quantitative PCR with primers specific for mouse Dnmt3b1 and the expression detected on a agarose gel, B) Expression of the mouse Dnmt3b1 in human HCT116 and 3BKO cells was also analyzed by q-RT-PCR relative to human GAPDH.
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BIOGRAPHICAL SKETCH

Suhasni Gopalakrishnan was born in 1982 in Madurai, Tamil Nadu, India as the first child of Gopalakrishnan Sankarasubramanian and Gomathi Gopalakrishnan. In 2000, she graduated from high school, and joined the PSG College of Technology and enrolled in the Biotechnology program. She graduated in 2004 with a Bachelor of Engineering degree. In the fall of 2004, she joined the Interdisciplinary Program in Biomedical Sciences at the University of Florida. She joined the laboratory of Dr. Keith Robertson in May 2005 and became an official PhD candidate in September 2006. She successfully defended her dissertation in June 2009 and graduated from the program in Summer 2009.