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Publication Date

2013

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UNIVERSITY OF CALIFORNIA

Los Angeles

The Regulation of DNA Methylation and Hydroxymethylation
in Postmitotic Neurons and Other Mammalian Cells

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Neuroscience

by

Thuc Le

2013

ABSTRACT OF THE DISSERTATION

Regulation of DNA Methylation and Hydroxymethylation
in Postmitotic Neurons and Other Mammalian Cells

by

Thuc Le

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2013

Professor Guoping Fan, Co-Chair

Professor Kym Faull, Co-Chair

DNA methylation and hydroxymethylation are two major covalent modifications that occur to the cytosine bases in the mammalian genome. A family of DNA methyltransferase (Dnmt), including maintenance Dnmt1 and *de novo* Dnmt3a and Dnmt3b, transfers a methyl group onto the 5th carbon of the cytosine base to form 5-methyl-cytosine (5mC). Further modification on the 5mC is catalyzed by ten-eleven translocation enzymes in which a hydroxyl group is added onto the methyl group of the 5mC to form 5-hydroxymethyl-cytosine (5hmC). During development both Dnmt and Tet act in concert to regulate the pattern and extent of DNA methylation and hydroxymethylation, respectively. By understanding how the two DNA modifications are related in their levels and the kinetics of these processes, factors that have direct impact

on development and disease can be ascertained. Methods using combined liquid chromatography and tandem mass spectrometry were established to measure both the global levels and kinetics of 5mC and 5hmC in mouse embryonic stem cells (ESCs) and neurons. Using mouse ESCs lines that encompass all eight possible combinations from the three major Dnmts, the global levels DNA methylation and hydroxymethylation are robustly related. This finding indicates that 5mC is a rate limiting factor in 5hmC formation. In addition, the kinetic rate of DNA methylation determined by the genetic combination of the Dnmts was positively correlated to its DNA methylation level. The combination of the *de novo* and maintenance Dnmts has the fastest kinetic rate than either *de novo* or maintenance Dnmts combination alone.

For many years, DNA methylation studies were done on cancer and development because it was known that the DNA methylation is very active and that the DNA methylation activity is diminished in terminally differentiated cells. Using the mass spectrometric methods, I measured levels of DNA methylation in control and Dnmt mutant mice. The forebrain excitatory neurons of the conditional *Dnmt1* and *Dnmt3a* knockout (DKO) mutant mice had a loss of about 15% of the DNA methylation level when compared to age-matched control littermate. This loss was accompanied by abnormal synaptic plasticity in the hippocampus and behavioral deficits in learning and memory tasks.

The dissertation of Thuc Le is approved.

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University of California, Los Angeles

2013

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ACKNOWLEDGMENTS

This dissertation holds far more than the culmination of years of study under Professor Guoping Fan and Kym Faull. The work done here reflects the relationships with many generous and inspiring people I have encountered since the beginning of my graduate work in the Fall of 2006. The list is extensive, but I appreciate each contribution as I become the next generation of scientist:

To my advisors Professor Guoping Fan and Kym Faull for demonstrating the value of hard work in science and to utter the mantra when doing experiments- control, control and control.

To my committee members Professor William Yang, Michael Teitell for their thoughtful criticisms and encouragement. Also to Michael Levine, the Chair of the Neuroscience IDP at UCLA, for his support.

To my colleagues for sharing the experience in doing science research: Anyou Wang Ph.D., Jerry Wu Ph.D., Jian Feng M.D., Ph.D., Jing Liu, Joling Liu M.Sc., Juehua Yu Ph.D., Kee-Pyo Kim Ph.D., Leah Hutnick Ph.D., Leeron Morad M.Sc., Masa Namihira Ph.D., Nori Namihara Ph.D., Shaun Fouse Ph.D., Tami Dvash Ph.D., Yin Shen Ph.D., Ying Liu Ph.D., Zhicheng Ma M.S, Doris Xu Ph.D., Jingdong Liu Ph.D., Kevin Huang Ph.D., Kumi Sakurai Ph.D., Fangfei Shaw, Mahdi Tondar, Zhenshan Liu Ph.D., Andy Norris Ph.D., Julian Whitelegge Ph.D., Craig Lund, Farbod Fazlollahi, Joe Capri, Alex Yoon, Chris Ryan Ph.D., Pete Souda M.S., and Sarah Bassilian M.Sc.

To my mentorees who mentored me to become a better mentor: Christine Tran, Cindy Tran, Daria Osipchuk, Edgar Ureno, Josh Lee, Katherine Sheu, Lisa Moore, Nadia Sellami, and Shaoming Tang.

To my collaborators: Andy Gieschen, James Yen Ph.D., Adam Peterson, Meelad Dawlaty Ph.D., John Vincent Ph.D., Joyel Almajando, Anatol Bragin Ph.D., Andrei Rajkovic, Michael Ibba Ph.D., and Pasqual Liauw M.Sc.

To my research fund that have taught me the value of money in research and the value of being frugal as a graduate student living in Los Angeles, CA: 2012-2013 UCLA Dissertation Fellowship, 2009-2011 Molecular Cellular Neurobiology (NIH 5 T32 MH 19384-14), 2008-2009 Intellectual and Developmental Disabilities Research Center (T32-HD007032), 2007 and 2009 Cota-Roble Fellowship.

To my college professors and colleague at Santa Clara Universty for their inspiration to do science research: Patrick Hoggard Ph.D., John Birmingham Ph.D., and Derek Verley soon-to-be Ph.D.

To my parents, Dung Ha and Minh Le, and my brother, The Le, and my two sisters, Thi and Tho, for their love, support and understanding during the long years pursuing the first Ph.D. in the family.

And finally, to my long-loving wife, Bada Kang, and my rambunctious son, Dylan Le, for making a man out of me. Thank you for always coming to my defense.

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Curriculum Vitae

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Prepared manuscript

1. **Le T**, Sakurai K, Sheu K, Osipchuck D, Faull K, and Fan G. Active turnover of DNA methylation and hydroxymethylation measured by LC-ESI-MS/MS-MRM. (manuscript preparation)
2. Huang K, Yu J, **Le T**, Pellegrini M, Fan G. Gene body methylation and H3K27me3 redistribution contributes to transcriptome deregulation in mouse ESCs null of DNA methylation. (under submission 2013)

Past research at Santa Clara University

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3. Messinger DI, Kutz KK, **Le T**, Verley DR, Hsu YW, Ngo CT, Cain SD, Birmingham JT, Li L, Christie AE. Identification and characterization of a tachykinin-containing neuroendocrine organ in the commissural ganglion of the crab *Cancer productus*. *J Exp Biol* (2005) 208:3303-3319.

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Presentation

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2. **UCLA Neuroscience Graduate Forum**, Los Angeles, CA, June 2009. **Le T**, Hutnik L, Faull K, Fan G. Phosphoproteomic analysis of Dnmt1 deficient mouse cells.
3. **Society for Neuroscience**, San Diego, CA, Nov 2004. **Le T**, Verley DR, Goillard JM, Messinger DI, Christie AE, Birmingham JT. Bistable behavior originating in the axon of a crustacean motor neuron.
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3. **Society for Neuroscience**, Washington, DC, Nov 2005. Verley DR, **Le T**, Goillard JM, Christie AE, Birmingham JT. Bistable behavior originating in the axon of a crustacean motor neuron.

INTRODUCTION

(A portion of this chapter is published Moore, L.D., Le, T., and Fan, G. (2012). DNA methylation and its basic function. *Neuropsychopharmacology* 38, 23-38. PMC3521964)

DNA is well-suited for storing biological information. The sugar-phosphate backbone resists random cleavage and the complementary double-stranded structure ensures the integrity of the sequence information. Since the DNA structure discovery in 1953, a major milestone was accomplished by thousands of scientists from all over the world to sequence the whole human genome in terms of just the four nucleobases-adenosine, cytosine, thymine and guanosine (Lander et al., 2001; Venter et al., 2001). Here genetics refers to the study of heritable changes in gene activity or function due to the direct alteration of the DNA sequence. Such alterations include point mutations, deletions, insertions and translocation.

The DNA sequence alone contains a deluge of information about its genetic but it fall short in explaining why an organism has diverse kinds of cells when all of their cells, generally speaking, contain the exact genetic information. Although virtually all cells in an organism contain the same DNA sequence, the unique cell identity lies in its gene expression pattern mediated by some mechanisms. As far back as 1942, CH Waddington coined the term “epigenetics” and is commonly agreed on as a study of “changes in gene expression caused by mechanisms other than changes in the DNA sequence, some of which can be inherited.” There are numerous epigenetic mechanisms that mediate gene expression pattern for the variety of cells in multicellular

organisms including histone modifications and RNA interference. In this thesis, I will focus on a major epigenetic mechanism of the DNA modifications involving the direct chemical modification of the cytosine nucleobase which some would say gives the DNA additional 5th and 6th nucleobases- DNA methylation and DNA hydroxymethylation.

Historical discovery

DNA methylation, referring to 5-methyl-cytosine (5mC), was discovered in mammals as early as DNA was identified as the genetic materials (Avery et al., 1944; McCarty and Avery, 1946). In 1948 Rollin Hotchkiss first discovered modified cytosine in a preparation of calf thymus using paper chromatography (Hotchkiss, 1948). Hotchkiss hypothesized that this fraction was 5-methylcytosine because it separated from cytosine in a manner that was similar to the way that thymine (also known as methyluracil) separated from uracil, and he further suggested that this modified cytosine existed normally in DNA. While many researchers proposed that DNA methylation might regulate gene expression, it was not until the 1980s that several studies demonstrated the impact of DNA methylation has on gene regulation and cell differentiation. It is now well recognized that DNA methylation, in concert with other regulators, is a major epigenetic factor influencing gene activities.

In the same year as the double-helix discovery, another DNA modification was discovered in the bacteriophages- the DNA hydroxymethylation or the 5-hydroxymethyl-cytosine (5hmC). It has been established that DNA hydroxymethylation adds protection to the bacteriophage genome from host-controller nucleases (Wyatt and Cohen, 1953).

Few decades later, the DNA hydroxymethylation was discovered in mammalian tissues, including the rat brain, using crude chromatographic method (Kriaucionis and Heintz, 2009; Penn, 1976; Penn et al., 1972). A recent discovery of the Ten-Eleven Translocation (TET) family of enzymes that is responsible for generating 5hmC from 5mC has motivated researchers to investigate the role of DNA hydroxymethylation in mammalian systems (Tahiliani et al., 2009) (Figure I-1).

DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmts) that transfer a methyl group from S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to form 5-methylcytosine (5mC) (Figure I-1). Dnmt3a and Dnmt3b can establish a new methylation pattern to unmodified DNA and are thus known as *de novo* DNA methyltransferases. On the other hand, Dnmt1 functions during DNA replication to copy the DNA methylation pattern from the parental DNA strand onto the newly synthesized daughter strand (Figure I-2). All three Dnmts are extensively involved in the development of an embryo. By the time cells reach terminal differentiation, *Dnmts* expression is much reduced. This would seem to suggest that the DNA methylation pattern in postmitotic cells is stable. However, postmitotic neurons in the developed mammalian brain still express substantial levels of *Dnmts*, raising the possibility that Dnmts and DNA methylation play a novel role in the brain (Feng et al., 2005; Goto et al., 1994).

Neurons react to the environment through patterns of depolarization that both relay information and encode a response. In recent years it has become increasingly apparent that following depolarization, alterations in gene expression are accompanied by modifications of the epigenetic landscape which include alterations in the pattern of

DNA methylation (Chen et al., 2003; Guo et al., 2011a; Martinowich et al., 2003). In order for the DNA methylation pattern to be altered there must be both active DNA methylation and demethylation in the neuronal genome. However, no enzymes are known to cleave the methyl group directly from 5mC. As discussed below, the recent identification of 5hmC in postmitotic neurons suggests that 5hmC serves as an intermediate in the DNA demethylation pathway. In this introduction, I will discuss the basic function of DNA methylation in epigenetic gene regulation, and further highlight its role in neural development and neurological disease.

Location of DNA methylation

Although the brain contains some of the highest levels of DNA methylation of any tissue in the body, 5mC only accounts for about 1% of nucleic acids in the human genome (Ehrlich et al., 1982). The majority of DNA methylation occurs on cytosines that precede a guanine nucleotide or CpG sites. Although there is evidence of non-CpG methylation in mouse and human embryonic stem cells, non-CpG methylation is lost in mature tissues (Lister et al., 2009; Ramsahoye et al., 2000). Overall mammalian genomes are depleted of CpG sites which may result from the mutagenic potential of 5mC which can be deaminated to become thymine (Bird, 1980; Coulondre et al., 1978). The remaining CpG sites are spread out across the genome where they are heavily methylated with the exception of CpG islands (Bird et al., 1985).

DNA methylation is essential for silencing retroviral elements, regulating tissue-specific gene expression, genomic imprinting, and X chromosome inactivation.

Importantly, DNA methylation in different genomic regions may exert different influences on gene activities based on the underlying genetic sequence. So while DNA methylation of intergenic regions and CpG islands is associated with repressed expression, methylation of gene bodies is associated with an increase in gene expression. In the following sections the role of DNA methylation in different genomic regions will be further elaborated in intergenic regions, CpG islands, and gene body.

Intergenic Regions

About 45% of the mammalian genome consists of transposable and viral elements that are silenced by bulk methylation (Schulz et al., 2006). The vast majority of these elements are inactivated by DNA methylation or by mutations acquired over time as the result of the deamination of 5mC (Walsh et al., 1998). If expressed these elements are potentially harmful as their replication and insertion can lead to gene disruption and DNA mutation (Gwynn et al., 1998; Kuster et al., 1997; Michaud et al., 1994; Ukai et al., 2003; Wu et al., 1997). The intracisternal A particle (IAP) is one of most aggressive retroviruses in the mouse genome (Walsh et al., 1998). IAP is heavily methylated throughout life in gametogenesis, development, and adulthood (Gaudet et al., 2004; Walsh et al., 1998). Even within the embryo when the rest of the genome is hypomethylated, Dnmt1 maintains the repression of IAP elements (Gaudet et al., 2004; Walsh et al., 1998). When Dnmt1 is lost during development leading to extensive hypomethylation of mutated cells IAP elements are expressed (Hutnick et al., 2010; Walsh et al., 1998). This demonstrates that within intergenic regions one of the main

roles of DNA methylation is to repress the expression of potentially harmful genetic elements.

CpG Islands

CpG islands are stretches of DNA roughly 1000 base pairs long that have a higher CpG density than the rest of the genome but often are not methylated (Bird et al., 1985). The majority of gene promoters, roughly 70%, reside within CpG islands (Saxonov et al., 2006). In particular the promoters for housekeeping genes are often imbedded in CpG islands (Gardiner-Garden and Frommer, 1987). CpG islands, especially those associated with promoters, are highly conserved between mice and humans (Illingworth et al., 2010). The location and preservation of CpG islands throughout evolution implies that these regions possess a functional importance.

It appears that CpG islands have been evolutionarily conserved to promote gene expression by regulating the chromatin structure and transcription factor binding. DNA is regularly wrapped around histone proteins forming small, packaged section called nucleosomes. The more tightly associated with histone proteins the DNA is, the less permissive it is for gene expression to occur. One of the common features of CpG islands is that they contain less nucleosomes than any other stretches of DNA (Choi, 2010; Ramirez-Carrozzi et al., 2009; Tazi and Bird, 1990). The few nucleosomes with which CpG islands are associated often contain histones with modifications involved in enhancing gene expression (Mikkelsen et al., 2007; Tazi and Bird, 1990). Although about 50% of CpG islands contain known transcription start sites, CpG islands are often devoid of common promoter elements such as TATA boxes (Carninci et al., 2006).

Since many transcription factor binding sites are GC rich, CpG islands are likely to enhance binding to transcriptional start sites. Despite their lack of common promoter elements CpG islands enhance the accessibility of DNA and promote transcription factor binding.

The methylation of CpG islands results in stable silencing of gene expression (Mohn et al., 2008). The ability of methylation to regulate gene expression through CpG islands is particularly important for establishing imprinting (Caspary et al., 1998; Choi et al., 2005; Wutz et al., 1997; Zwart et al., 2001). Imprinted genes are expressed from only one of the two inherited parental chromosomes and their expression is determined by the parent of inheritance. During gametogenesis and early embryonic development CpG islands undergo differential methylation (Caspary et al., 1998; Kantor et al., 2004; Wutz et al., 1997; Zwart et al., 2001). Beyond imprinted genes, DNA methylation of CpG islands regulates gene expression during development and differentiation (Fouse et al., 2008; Meissner et al., 2008; Mohn et al., 2008; Shen et al., 2007; Weber et al., 2007). Since CpG islands are associated with the control of gene expression, it would be expected that CpG islands might display tissue specific patterns of DNA methylation. Although CpG islands in intragenic and gene body regions can have tissue specific patterns of methylation, CpG islands associated with transcription start sites rarely show tissue specific methylation patterns (Eckhardt et al., 2006; Illingworth et al., 2010; Maunakea et al., 2010; Meissner et al., 2008; Rakyan et al., 2004). In summary, methylation of CpG islands is highly correlated with gene silencing, however reduced methylation is not necessarily correlated with gene expression.

The role of CpG islands in regulating gene expression is still being uncovered. Methylation of CpG islands can impair transcription factor binding, recruit repressive methyl-binding proteins, and stably silence gene expression. However CpG islands, especially those associated with gene promoters, are rarely methylated. Further studies are needed to determine to what degree DNA methylation of CpG islands regulates gene expression.

Gene Body

Since the majority of CpG sites within the mammalian genome are methylated, then genes themselves must also contain methylation. Mounting evidence suggests that DNA methylation of the gene body is associated with a higher level of gene expression (Aran et al., 2011). In this case the gene body is considered the region of the gene past the first exon because methylation of the first exon, like promoter methylation, leads to gene silencing (Brenet et al., 2011). In studies of the female X chromosomes, the active X chromosome has a higher level of gene body methylation compared to the inactive X chromosome (Hellman and Chess, 2007). Interestingly in the X chromosome only methylation of introns within the gene body is associated with a higher pattern of gene expression (Cotton et al., 2011). How DNA methylation of the gene body contributes to gene regulation is still unclear.

Basic mechanism of DNA methylation

The enzymes that establish, recognize, and remove DNA methylation are broken into three classes: writers, erasers, and readers. Writers are the enzymes that catalyze the addition of methyl groups onto cytosine residues. Erasers modify and remove the methyl group. Readers recognize and bind to methyl groups to ultimately influence gene expression. Thanks to the many years of research devoted to understanding how the epigenetic landscape is erased and reshaped during embryonic development, many of the proteins and mechanisms involved in DNA methylation have already been identified.

Writing DNA methylation: the DNA methyltransferases

Three members of the DNA methyltransferase family directly catalyze the addition of methyl groups onto DNA, Dnmt1, Dnmt3a and Dnmt3b. While these enzymes share a similar structure with a large N-terminal regulatory domain and a C-terminal catalytic domain, they have unique functions and expression patterns (Xie et al., 1999; Yen et al., 1992). Probably the best studied DNA methyltransferase, especially in the nervous system, is *Dnmt1* which is highly expressed in mammalian tissues including the brain (Goto et al., 1994). Unlike the other DNA methyltransferases, Dnmt1 preferentially methylates hemimethylated DNA (Pradhan and Esteve, 2003; Ramsahoye et al., 2000). During DNA replication Dnmt1 localizes to the replication fork where newly synthesized hemimethylated is formed (Leonhardt et al., 1992). Dnmt1 binds to the newly synthesized DNA and methylates it to precisely mimic the original methylation pattern present prior to DNA replication (Hermann et al., 2004) (Figure I-2).

Additionally Dnmt1 also has the ability to repair DNA methylation (Mortusewicz et al., 2005). For this reason Dnmt1 is called the maintenance DNA methyltransferase because it maintains the original pattern of DNA methylation in a cell lineage. Knockout of *Dnmt1* in mice results in embryonic lethality between E8.0 and E10.5 (Li et al., 1992). At this time knockout embryos exhibit a two thirds loss of DNA methylation in addition to numerous apoptotic cells in a variety of developing tissues including the brain. Interestingly mouse embryonic stem cells lacking *Dnmt1* remain viable (Chen et al., 1998). However, *in vitro* differentiation results in massive cell death, recapitulating the phenotype observed in knockout embryos (Jackson-Grusby et al., 2001). These findings firmly establish that Dnmt1 plays a critical role in cellular differentiation as well as in dividing cells.

Dnmt3a and Dnmt3b are extremely similar in structure and function. Unlike Dnmt1, both Dnmt3a and Dnmt3b when overexpressed are capable of methylating both native and synthetic DNA with no preference for hemimethylated DNA (Okano et al., 1998). For this reason Dnmt3a and Dnmt3b are referred to as *de novo* methyltransferases because they can introduce methylation into naked DNA (Figure I-2). What primarily distinguishes Dnmt3a from Dnmt3b is their gene expression pattern. While *Dnmt3a* is expressed relatively ubiquitously, *Dnmt3b* is poorly expressed by the majority of differentiated tissues with the exception of the thyroid, testes, and bone marrow (Xie et al., 1999). Similar to *Dnmt1* the knockout of *Dnmt3b* in mice is embryonic lethal (Okano et al., 1999). On the other hand *Dnmt3a* knockout mice are runted but survive to about 4 weeks after birth. From these results it appears that

Dnmt3b is required during early development while *Dnmt3a* is required for normal cellular differentiation.

Writing DNA methylation: Targeting de novo DNA methylation

How the *de novo* DNA methyltransferases target specific genetic regions is still unclear. However several mechanisms have been proposed. The first theory suggests that Dnmt3a and Dnmt3b bind to DNA via a conserved PWWP domain (Ge et al., 2004). The second theory suggests that RNA interference (RNAi) mechanisms target DNA methyltransferases to silence specific sequences of DNA (Morris et al., 2004). Although RNAi is clearly involved in DNA methylation in plant cells, the existing evidence is still very weak for a role of RNAi in DNA methylation in mammalian cells. The third theory is that transcription factors regulate *de novo* DNA methylation.

Transcription factors can regulate DNA methylation by both directly recruiting DNA methyltransferases and by binding to DNA sequences and protecting it from gene silencing. In some cases DNA methyltransferases bind to transcription factors or components of repressor complexes to target methylation to DNA (Brenner et al., 2005). In other cases it appears that regardless of whether the gene is expressed, the ability of transcription factors to directly bind to promoter elements protects CpG sites from *de novo* methylation (Gebhard et al., 2010; Lienert et al., 2011; Straussman et al., 2009). CpG islands appear to primarily be protected from methylation by transcription factor binding (Brandeis et al., 1994; Gebhard et al., 2010; Macleod et al., 1994; Straussman et al., 2009). When transcription factor binding sites are mutated CpG islands are unable to retain their unmethylated state (Brandeis et al., 1994; Macleod et al., 1994).

Similarly as differentiation induces the down regulation of transcription factors that bind to specific gene promoters, the now exposed CpG sites can be targeted for DNA methylation (Lienert et al., 2011). These studies describe two mechanisms that likely function together to establish *de novo* DNA methylation. Dnmt3a and Dnmt3b can either be recruited to promoters by specific transcription factors or the *de novo* methyltransferases may simply methylate all CpG sites across the genome that are not protected by a bound transcription factor.

Erasing DNA methylation

DNA demethylation is characterized as either passive or active. Passive DNA demethylation occurs in dividing cells. Since Dnmt1 actively maintains DNA methylation during cell replication, its inhibition or dysfunction allows newly incorporated cytosine to remain unmethylated and consequently reduces the overall methylation level following each cell division. Active DNA demethylation can occur in both dividing and non-dividing cells but the process requires enzymatic reactions to process the 5mC in order to revert it back to a naked cytosine (Mayer et al., 2000; Oswald et al., 2000; Paroush et al., 1990; Zhang et al., 2007). As of yet, there is no known mechanism in mammalian cells that can cleave the strong covalent carbon-to-carbon bond that connects cytosine to a methyl group. Instead demethylation occurs through a series of chemical reactions that further modify 5mC, either by deamination and/or oxidation reactions to a product that is recognized by the base excision repair (BER) pathway which replaces the modified base with naked cytosine. Although it is generally agreed upon that the BER pathway is

the final step in DNA demethylation, the specific enzymes and the chemical intermediates that are formed during DNA demethylation are still debated.

Several mechanisms of active DNA demethylation have been proposed (Figure I-3). 5mC can be chemically modified at two sites, the amine group and the methyl group. Deamination of the amine to a carbonyl group by AID/APOBEC (activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex) effectively converts 5mC into thymine, thus creating a G:T mismatch and inducing the BER pathway to correct the base. Overexpression of AID/APOBEC promotes DNA demethylation in zebrafish (Rai et al., 2008), while knockdown or knockout inhibits the DNA demethylation of various genes necessary for cellular reprogramming and development (Bhutani et al., 2010; Muramatsu et al., 2000; Popp et al., 2010). Unlike the various *Dnmt* knockout mice, knockout *AID* mice are viable and fertile. If global DNA demethylation is as critical as DNA methylation in early development, then the knockout *AID* mice study raises the possibility that multiple mechanisms for active DNA demethylation exist and can compensate one another.

In line with the multiple mechanisms hypothesis, another active DNA demethylation mechanism is found to be mediated by the ten-eleven translocation (Tet) enzymes Tet1, Tet2, and Tet3. Tet enzymes add a hydroxyl group onto the methyl group of 5mC to form 5hmC (Buiting, 2010; Ito et al., 2010; Tahiliani et al., 2009). The developed brain contains significant 5hmC levels in multiple regions, ranging from 0.3-0.7%, which is approximately 10-fold or lower than the normal abundance of 5mC (Globisch et al., 2010; Kriaucionis and Heintz, 2009). Once 5hmC is formed two separate mechanisms can convert 5hmC back into cytosine in mammals. In the first,

iterative oxidation by Tet enzymes continues to oxidize 5hmC first to 5-formyl-cytosine and then to 5-carboxy-cytosine (He et al., 2011). In the second 5hmC is deaminated by AID/APOBEC to form 5-hydroxymethyl-uracil (Guo et al., 2011b). Consistent with the role of Tet in converting 5mC into 5hmC *in vivo*, Tet1 knockout mouse embryonic stem cells have reduced levels of 5hmC that is accompanied by a subtle increase in 5mC at a global level (Dawlaty et al., 2011). Once again, the viability of *Tet1* knockout mice is not compromised and further supporting the notion that other remaining enzymes play a compensatory role in active DNA demethylation.

Whether 5hmC functions only as an intermediate in DNA demethylation is still unclear. Like methylation, 5hmC may regulate gene expression. In support of this theory the conversion of 5mC to 5hmC impairs the binding of the repressive methyl-binding protein MeCP2 (Valinluck et al., 2004). But what is clear at this time is that 5hmC is found *in vivo* in mammalian tissue and may play an important role in regulating DNA demethylation and gene expression.

In all the mentioned mechanisms of active DNA demethylation, the BER pathway uses thymine DNA glycosylase (TDG) to cleave off the modified residue, including thymine, 5-hydroxymethyl-uracil and 5-carboxy-cytosine, and replace it with a naked cytosine (Cortellino et al., 2011). TDG is essential for DNA demethylation and is required for normal development. Knockout or inactivation of *TDG* leads to embryonic lethality in mice. Moreover these mutant embryos exhibit hypermethylation particularly in imprinting genes such as *Igf2* and *H19*, suggesting that active demethylation by TDG protects imprinted genes from spontaneous *de novo* methylation (Cortellino et al., 2011). Single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1),

another BER enzyme from the same uracil DNA glycosylase family as TDG, is also found to be involved in DNA demethylation, particularly for the recognition and cleavage of 5hmU (Cortellino et al., 2011; Guo et al., 2011b). In summary, active DNA demethylation arises from multiple pathways involving multiple enzymes and this complexity has likely contributed much of the current scientific debate.

Reading DNA methylation

While DNA methylation may itself reduce gene expression by impairing the binding of transcriptional activators, a second class of proteins with a high affinity for 5mC inhibits transcription factor binding. DNA methylation is recognized by three separate families of proteins the MBD proteins, the UHRF proteins, and the zinc finger proteins. Of these families the MBD was the first to be identified. MBD proteins contain a conserved methyl CpG binding domain (MBD) that confers a higher affinity for single methylated CpG sites (Nan et al., 1993). This family includes MeCP2, the first identified methyl-binding protein, along with MBD1, MBD2, MBD3, and MBD4 (Hendrich and Bird, 1998; Lewis et al., 1992; Meehan et al., 1989). MBDs are more highly expressed in the brain than in any other tissue and many MBDs are important for normal neuronal development and function (Amir et al., 1999). Of the MBD family MBD3 and MBD4 are unusual. For example, MBD3 due to a mutation in its MBD domain is incapable of directly binding to DNA (Hendrich and Bird, 1998). While MBD4 binds to DNA normally, it preferentially recognizes when guanine is mismatched with a thymine, uracil, or 5-fluorouracil and associates with proteins involved in DNA mismatch repair (Bellacosa et al., 1999; Hendrich et al., 1999; Millar et al., 2002; Petronzelli et al., 2000; Wong et al.,

2002). The remaining members of the MBD family have the ability to directly bind to methylated DNA and contain a transcriptional repression domain (TRD) which allows MBD proteins to bind to a variety of repressor complexes (Nan et al., 1998; Ng et al., 1999; Sarraf and Stancheva, 2004). In addition to its role as a transcriptional repressor MeCP2 appears to have a unique role in the maintenance of DNA methylation. MeCP2 binds to Dnmt1 via its TRD and can recruit Dnmt1 to hemimethylated DNA to perform maintenance methylation (Kimura and Shiota, 2003). Although MBDs are the best studied class of methyl-binding proteins, they are not the only one.

The ubiquitin-like, containing PHD and RING finger domain (UHRF) proteins, including UHRF1 and UHRF2, are multi-domain proteins that flip out and bind methylated cytosines via a SET and RING associated DNA binding domain (Hashimoto et al., 2008; Hashimoto et al., 2009). Unlike other methyl-binding proteins the UHRF proteins primary function is not to bind to DNA and repress transcription. Instead the UHRF protein family closely associates with Dnmt1 and targets it to hemimethylated DNA to maintain DNA methylation especially during DNA replication (Achour et al., 2008; Bostick et al., 2007; Sharif et al., 2007). UHRF1 appears to interact so closely with Dnmt1 that its deletion, like the deletion of Dnmt1, leads to embryonic lethality (Muto et al., 2002).

The last family of methyl-binding proteins binds to methylated DNA by a zinc finger domain and is composed of Kaiso, ZBTB4 and ZBTB38 (Filion et al., 2006; Prokhortchouk et al., 2001). Although ZBTB4 and ZBTB38 have distinct tissue expression patterns, both are highly expressed in the brain and can bind to a single methylated CpG. The zinc finger domain proteins are unusual. Despite their ability to

recognize methyl cytosine, both Kaiso and ZBTB4 have preferential binding for a sequence motif lacking a methyl cytosine (Daniel et al., 2002; Sasai et al., 2010). Kaiso is even more unusual because, unlike other methyl-binding proteins, Kaiso preferentially binds to two consecutively methylated CpG sites (Daniel et al., 2002). Yet despite their differences, zinc finger domain proteins, similar to the MBD family, repress transcription in a DNA methylation dependent manner (Filion et al., 2006; Lopes et al., 2008; Prokhortchouk et al., 2001; Yoon et al., 2003).

Crosstalk of DNA Methylation and Other Epigenetic Mechanisms

DNA methylation works with histone modifications and microRNA (miRNA) to regulate transcription. In eukaryotes DNA is associated with histone proteins which help to package the long strands of DNA into the small nuclear compartment. Chemical modifications which include methylation, acetylation, ubiquitination, and phosphorylation are added to three specific amino acids on the N terminal histone tails. These modifications not only influence how DNA strands are packaged but also their transcriptional activity. Histone modifications that loosen DNA association with histones generally provide a permissive environment for transcription while histone modifications that tightly package DNA and histones repress gene expression. Dnmts directly interact with enzymes that regulate histone modifications typically involved in gene repression. Both Dnmt1 and Dnmt3a are known to bind to the histone methyltransferase SUV39H1 which restricts gene expression by methylation on H3K9 (Fuks et al., 2003). Furthermore Dnmt1 and Dnmt3b can both bind to histone deacetylases which remove acetylation from histones to make DNA pack more tightly and restrict access for

transcription (Fuks et al., 2000; Geiman et al., 2004). In general Dnmt cooperate with histone modifying enzymes involved in adding and/or stripping histone markers in order to impose a repressive state on a gene region.

Histone modifications can also influence the DNA methylation pattern. High levels of the active histone modification, H3K4 trimethylation, prevent DNA methylation within CpG islands (Mikkelsen et al., 2007). Cfp1 is a component of the H3K4 methyltransferase complex which targets unmethylated CpG sites often found at murine CpG islands (Lee and Skalnik, 2005). Cfp1 is often associated with murine CpG islands and may play a role in maintaining the hypomethylation of CpG islands (Lee and Skalnik, 2005; Thomson et al., 2010). While little is known regarding how the DNA demethylation machinery interacts with histone modifications there still is evidence to suggest that they cooperate. For instance elevated histone acetylation can trigger DNA demethylation (Cervoni and Szyf, 2001; D'Alessio et al., 2007). Furthermore, Tet1 contains a DNA binding motif similar to Ctf1 suggesting that both proteins target similar sites, in this case CpG islands, to maintain DNA demethylation (Tahiliani et al., 2009). Although direct connection between the two has yet to be shown, Tet1 does indeed localize to CpG islands and its depletion results in an increase in methylation within those CpG islands in mouse embryonic stem cell studies (Ficz et al., 2011; Wu and Zhang, 2011). Future studies are needed to further probe the interaction of Tet with histone modifications.

Methyl-binding proteins serve as the strongest link between DNA methylation and histone modification. Both the MBDs and the UHRF proteins interact with methylated DNA and histones to enhance gene repression (Citterio et al., 2004;

Karagianni et al., 2008; Nan et al., 1998; Ng et al., 1999; Sarraf and Stancheva, 2004). MeCP2 recruits histone deacetylases to remove active histone modifications and repress gene transcription (Fuks et al., 2003; Jones et al., 1998; Nan et al., 1998). Furthermore MeCP2 enhances the repressive chromatin state by recruiting histone methyltransferases that add repressive H3K9 methylation (Fuks et al., 2003). Overall, DNA methylation and histone modifications work closely together to regulate gene expression.

Recently, miRNAs have emerged as another important epigenetic mechanism that influences gene expression. miRNAs are noncoding double-stranded RNAs that are 20-25 nucleotides long. Once transported into the cytoplasm miRNAs are processed by Dicer to generate small RNAs which can bind to their target mRNA and repress gene expression by inhibiting translation or inducing RNA degradation. Like other sequences within the genome DNA methylation can regulate the expression of miRNAs (Han et al., 2007; Lujambio et al., 2008). The loss of both *Dnmt1* and *Dnmt3b* in a colon cancer cell line revealed that about 10% of detected miRNAs are regulated by DNA methylation (Han et al., 2007). When Dnmts are inhibited, cancer cells reactivate some miRNAs that are initially silenced by hypermethylation of their CpG islands (Lujambio et al., 2008). Understood together, these studies demonstrate that DNA methylation regulates miRNA expression.

Conversely miRNAs can also regulate histone modifications and DNA methyltransferase expression and, in so doing, regulate DNA methylation (Benetti et al., 2008; Sinkkonen et al., 2008). Knockout of *Dicer* in mouse embryonic stem cells results in depletion of miRNAs, one of which is miRNA-290 which indirectly regulates *Dnmt1*,

Dnmt3a, and *Dnmt3b* expression. This leads to a loss of DNA methylation and an increase in repressive histone methylation at H3K9. These studies provided evidence of a bidirectional influence between microRNA and DNA methylation.

DNA methylation in neurological and psychiatric disorders

The pattern of DNA methylation established during development can be modulated by neural activity in order to encode learning and memory. When the mechanisms that establish and recognize the DNA methylation pattern are dysfunctional, problems with learning and memory frequently result. One of the most common forms of mental retardation, Rett Syndrome, is frequently caused by a mutation to the methyl-binding protein MeCP2 (Amir et al., 1999). The onset of symptoms at 6-18 months of age coincides with a time in early development when sensory experience is driving dendritic pruning and shaping connections in the brain (Samaco and Neul, 2011). Although *MeCP2* is expressed by the majority of cells, it is particularly important for normal neuronal function. In mice, loss of *MeCP2* in neurons is sufficient to recapitulate the majority of Rett symptoms (Chen et al., 2001; Guy et al., 2001). As previously mentioned *MeCP2* is regulated by neuronal activity and is important for neuronal maturation.

One extremely rare neurodegenerative disease illustrates the importance of proper DNMT activity in the adult brain. Patients with hereditary sensory and autonomic neuropathy type 1 (HSAN1) develop dementia and hearing loss in adulthood that result from an autosomal dominant mutation in the N terminal regulatory domain of *DNMT1*

(Klein et al., 2011). This mutation results in misfolding, impaired nuclear localization, and early degradation of DNMT1. However, the mutation does not affect the targeting of DNMT1 to the replication foci during cellular replication, but the DNMT1 association with heterochromatin beyond S phase is disrupted. This association may affect the maintenance of DNA methylation within these regions. Although there is only a modest 8% reduction of global DNA methylation level, neurodegeneration does result. The involvement of DNMT1 in the pathogenic mechanism of HSN1 supports the necessity of DNMT1 in the adult brain.

Improper methylation of a single gene or a single allele can have drastic consequences within the brain. Fragile X Syndrome is caused by abnormal methylation of a trinucleotide repeat expansion in the *FMR1* gene on the X chromosome and is a common form of mental retardation (Turner et al., 1996; Verkerk et al., 1991). The hypermethylation in the repeat expansion of *FMR1* results in transcriptional silencing (Devys et al., 1993). Translation of the *FMR1* gene is regulated by neuronal activity (Weiler et al., 1997) and its protein product, FMRP, is involved in protein synthesis at the synapses following depolarization (for review see (Fatemi and Folsom, 2011)). Similarly, improper methylation of a single imprinted allele, found in some disorders such as Prader-Willi and Angelman Syndrome, can cause significant mental impairments (for review see (Buiting, 2010)). Since incorrect expression or loss of function of a single gene can have a dramatic effect in the brain, it is important to understand the mechanism of how DNA methylation impacts gene expression.

DNA methylation can also be altered by repeated modulation of the microenvironment of the brain. In the case of recurrent seizures this microenvironment

is repeatedly subject to unusual, synchronized neuronal activity. One way to mimic this unusual neural activity is by electric convulsive stimulation which was found to result in genome-wide changes in the DNA methylation pattern (Guo et al., 2011a; Ma et al., 2009). Similarly repeated drug usage modulates neuronal function as in the case of cocaine. Cocaine usage modulates *Dnmt3a* expression within the nucleus accumbens and enhances spine formation (LaPlant et al., 2010). Also, repeated cocaine usage increases *MeCP2* which in turn increases *Bdnf* expression (Im et al., 2010). Sometimes drug exposure, like neural activity, can add posttranslational modifications to components of the methylation machinery such as MeCP2 (Deng et al., 2010; Hutchinson et al., 2012).

While DNA methylation is clearly altered in the above disorders stemming from mutations, inappropriate methylation, or repeated modulation of the microenvironment, the role of DNA methylation in most psychiatric disorders is less clear. Yet there is mounting evidence that altered patterns of DNA methylation are associated with many psychiatric disorders. For example, early life stress in the form of maternal neglect was sufficient to alter DNA methylation in the brain of a rodent model (Weaver et al., 2004). Maternal neglect increased methylation within the promoter of the glucocorticoid receptor thus reducing its expression. Surprisingly this alteration in the DNA methylation pattern was retained into adulthood leading to a heightened stress response. Similarly in humans, childhood abuse results in increased methylation of the promoter for the glucocorticoid receptor and a decrease in its expression, recapitulating the rodent model (McGowan et al., 2009). Furthermore altered patterns of DNA methylation are observed

in psychiatric patients diagnosed with schizophrenia and bipolar disorder (Mill et al., 2008).

My thesis that will present experimental strategy that address the measurements of 5mC and 5hmC in postmitotic neurons. The first two chapters establish two mass spectrometric methods in order to measure the 5mC and 5hmC levels and DNA methylation activity in mammalian cells, particularly murine embryonic stem cells (mESCs). The last chapter utilizes the recent developed methods to measure DNA methylation changes in postmitotic neurons *in vitro* and *in vivo*.

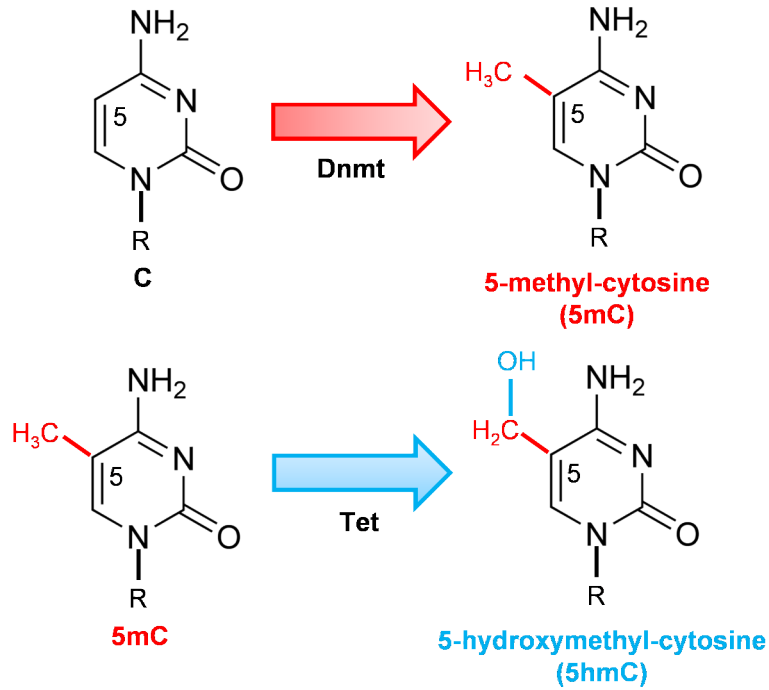


Figure I-1: DNA epigenetic chemistry. A family of DNA methyltransferases (Dnmts) catalyzes the transfer of a methyl group from S-adenosyl-methionine (SAM) to the fifth carbon of cytosine residue to form 5mC. Further modification on the 5mC is catalyzed by a family ten eleven translocation (Tet) enzymes that adds a hydroxyl group onto the methyl group of 5mC to form 5hmC.

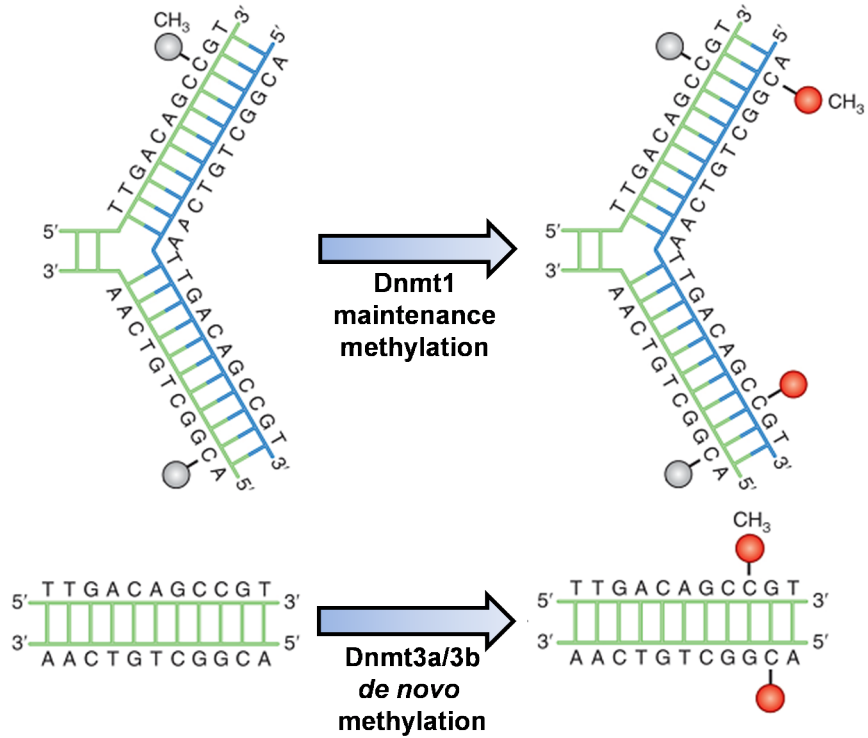


Figure I-2: Functional roles of Dnmts. During DNA replication Dnmt1 associates with the replication foci and precisely maintains the DNA methylation pattern from the parental DNA strand (green strand with gray 5mC) onto the newly synthesized DNA strand (blue strand with red 5mC). Both Dnmt3a and Dnmt3b establish new DNA methylation pattern and are known as *de novo* Dnmts.

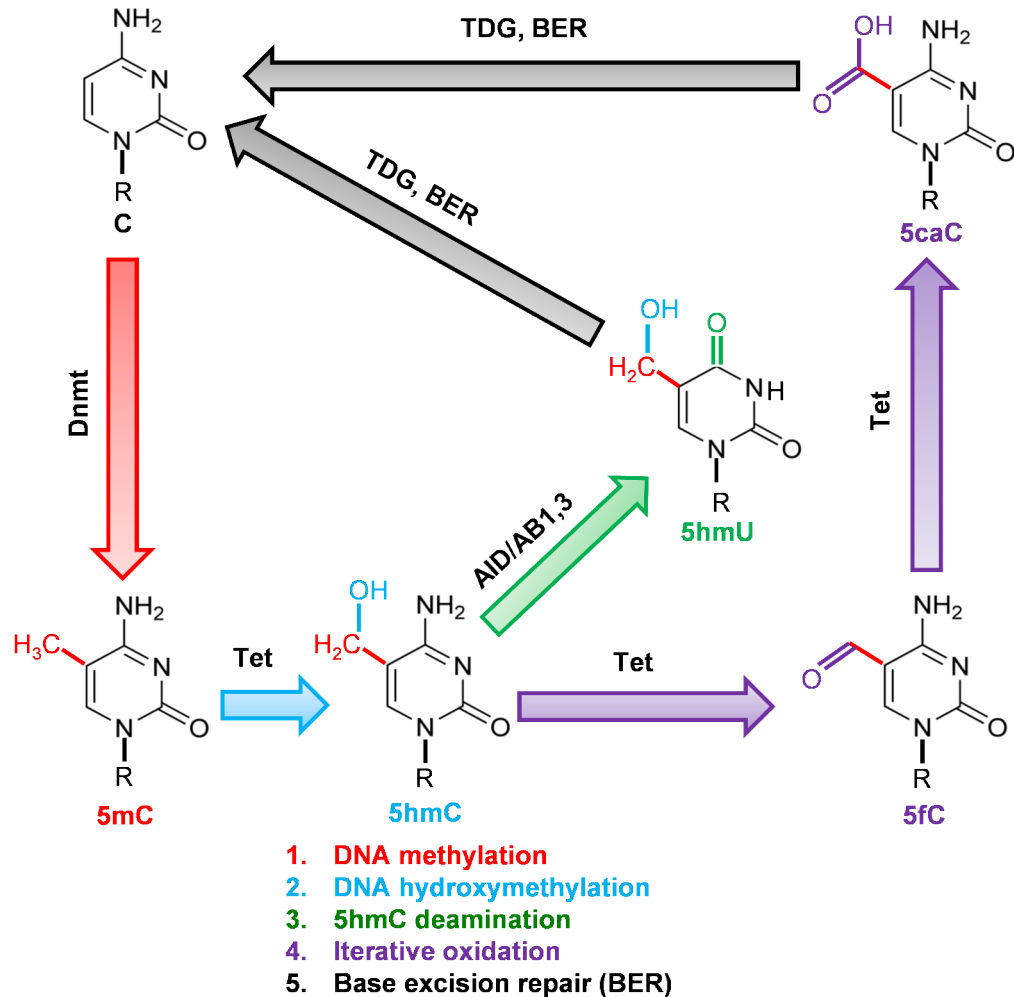


Figure I-3: DNA epigenetic pathways. Dnmts convert cytosine to 5mC which can then be further modified into 5hmC by Tet. In active DNA demethylation the 5hmC can be chemically modified at two sites and subsequently trigger base excision repair mechanism involving TDG and SMUG1 (black arrows). AID/APOBEC can deaminate (green arrow) the amine group to form 5-hydroxymethyl-uracil (5hmU). And in another pathway, Tet can further oxidize (purple arrows) 5hmC to form 5-formyl-cytosine (5fC) and then 5-carboxy-cytosine (5caC). Eventually, the final products of each pathway are recognized and replaced with a naked cytosine mediated by TDG and/or SMUG1 (black arrows).

CHAPTER 1- DNA methylation and hydroxymethylation levels in biological samples

(A portion of this thesis chapter is published in Le, T., Kim, K.P., Fan, G., and Faull, K.F. (2011). A sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels in biological samples. *Anal Biochem* 412, 203-209. 3070205)

Abstract

The recent discovery of 5-hydroxymethyl-cytosine (5hmC) in embryonic stem cells and post-mitotic neurons has triggered the need for quantitative measurements of both 5-methyl-cytosine (5mC) and 5hmC in the same sample. We have established a method using liquid chromatography electrospray ionization tandem mass spectrometry with multiple reaction monitoring (LC/MS/MS-MRM) to simultaneously measure levels of 5mC and 5hmC in digested genomic DNA. This method is fast, robust and accurate, and is more sensitive than the current 5hmC quantitation methods such as end-labeling with thin-layer chromatography and radio-labeling by glycosylation (Szwagierczak et al., 2010; Tahiliani et al., 2009). Only 50 ng of digested genomic DNA is required to measure the presence of 0.1% 5hmC in DNA from mouse embryonic stem cells. Using this procedure we show that human induced pluripotent stem cells exhibit a dramatic increase in 5mC and 5hmC levels compared to parental fibroblast cells, suggesting a

dynamic regulation of DNA methylation and hydroxymethylation during cellular reprogramming.

Introduction

The pattern of methylated cytosine residues in DNA provides an inheritable epigenetic code that regulates gene expression during development. The covalent addition of a methyl group at the 5-position of cytosine primarily occurs in the CpG dinucleotide, and is catalyzed by a family of DNA methyltransferases (Dnmts) including maintenance Dnmt1 and *de novo* Dnmt3a and Dnmt3b. DNA methylation is involved in various biological processes such as genomic imprinting, silencing of retroviral transposons, X chromosome inactivation, and cellular differentiation. Mechanistically, promoter methylation can lead to transcriptional repression directly by inhibiting transcriptional binding, or indirectly by recruiting various proteins including methyl CpG binding proteins (MBDs), co-repressors and histone modification enzymes involved in chromatin remodeling (Cross et al., 1997; Fan et al., 2005; Jones et al., 1998; Martinowich et al., 2003; Nan et al., 1997; Nan et al., 1998). Importantly, many studies have shown that DNA methylation is a dynamic process in cellular proliferation and differentiation, and is tightly regulated in normal development. Aberrant DNA methylation patterns and mechanisms are deleterious to the developing central nervous system (CNS) (Fan et al., 2001; Fan et al., 2005; Feng et al., 2010; Hutnick et al., 2009).

Recently there has been renewed interest in another, related, mammalian DNA modification, 5-hydroxymethyl-cytosine (5hmC). Significant levels of 5hmC are found in the developed murine central nervous system and in embryonic stem cells (Ito et al., 2010; Kriaucionis and Heintz, 2009; Penn et al., 1972; Tahiliani et al., 2009). *In vivo*

addition of a hydroxyl group onto 5-methyl-cytosine (5mC) is catalyzed by 2-oxoglutarate oxygenase Tet1, Tet2, and Tet3 (Ito et al., 2010; Tahiliani et al., 2009). There are also reports that 5hmC can be formed by other mechanisms beside Tet pathway, including UV irradiation of 5mC in aerated aqueous solution (Privat and Sowers, 1996) and DNA methyltransferase reaction of cytosine with formaldehyde (Liutkeviciute et al., 2009). To date, only the Tet pathway has been demonstrated to produce 5hmC in mammalian genomic DNA.

Speculation that 5hmC is involved in the DNA demethylation pathway comes from the two reported mechanisms of converting 5hmC into C. Bacterial DNA methyltransferases catalyze the removal of formaldehyde from 5hmC, thus converting 5hmC to C (Liutkeviciute et al., 2009). Another demethylation mechanism involves the photochemical hydration of 5hmC in basic solution (Privat and Sowers, 1996). However, these two possible DNA demethylation mechanisms have yet to be confirmed in mammalian models.

Some of the most commonly used methods for profiling and quantification of DNA methylation, such as bisulfite sequencing and methylation-sensitive enzyme-based assays, are unable to distinguish between 5hmC and 5mC (Huang et al., 2010; Tahiliani et al., 2009). Several methods have been used to measure the 5hmC levels in the genome: these include end-labeling followed by thin layer chromatography (Tahiliani et al., 2009), high performance liquid chromatography (HPLC) with UV detection (Liutkeviciute et al., 2009), enzymatic radioactive glycosylation labeling (Szwagierczak et al., 2010), and single molecule, real-time sequencing (Flusberg et al., 2010). The thin layer chromatography method has the advantage of being low cost and simple, but

requires the availability of radioactive substrates and the accuracy is not comparable to other available methods. The specificity of UV detection relies heavily on the chromatographic separation to avoid co-elution of other components, including other DNA and RNA nucleotides that may be present in biological samples. The glycosylation method is based on enzymatic incorporation of radio-labeled glucose into genomic 5hmC, with quantification by radioactive counting. However a complete enzymatic reaction cannot be readily assured and 5mC levels cannot be measured simultaneously. Independent measurement of 5hmC is possible with next generation sequencing, but the technology has yet to be perfected for accurate quantitation of many low abundant nucleotides including 5hmC.

Previous work demonstrated the precision, selectivity and sensitivity of liquid chromatography tandem mass spectrometry for measuring 5mC in biological samples, and as a diagnostic tool for cancer (Burke et al., 2009; Kok et al., 2007; Liu et al., 2007; Liu et al., 2009; Quinlivan and Gregory, 2008; Song et al., 2005; Yang et al., 2009). Using this technique all known DNA (excluding 5hmC) and RNA components, have been separated, distinguished and independently quantitated (Song et al., 2005). This approach allows DNA methylation to be measured both at the global (Kok et al., 2007; Liu et al., 2007; Quinlivan and Gregory, 2008; Song et al., 2005) and gene promoter regions (Liu et al., 2009). However, none of the previous reports include 5hmC. We were prompted to develop a fast, sensitive and accurate method to measure both 5mC and 5hmC levels to support ongoing work on epigenetic control of stem cells and neural development. Here we report the use of liquid chromatography electrospray ionization tandem mass spectrometry with multiple reaction monitoring (LC/MS/MS-MRM) for the

determination of genomic DNA methylation and hydroxymethylation (Figure 1-1). In this scan mode, the mass spectrometer takes advantage of the unique parent masses of each nucleoside and the facile cleavage of the base so that each parent to fragment ion transition is specific and quantitative signature (Figure 1-2). Separation of the deoxyribonucleosides is achieved within 6 minute using sub-two micron particle size reverse phase chromatography columns. In addition, mass-based detection discriminates between the three nucleoside bases of interest- 5hmC, 5mC and cytosine (C). The combination of LC and MS minimizes any possible cross-talk between the measurements of low abundant molecules (5hmC and 5mC) in the face of a chemically similar abundant species (C). Together, our data indicate that the MRM method provides unambiguous and independent quantification of 5hmC, 5mC, and C with high reproducibility and low limits of detection of around 0.5 fmol per sample. This limit of detection can be equated to 50 ng of digested genomic DNA to measure 5hmC levels at the 0.1% level. Furthermore, the method is relatively fast, requiring less than 48 hours from extracting genomic DNA (few hours to a day), to digesting genomic DNA into nucleoside components (1-2 hours), and measuring the 5hmC and 5mC levels using the MRM method (6 minutes per sample).

Material and Methods

Cell culture

mESCs of wild-type J1, *Dnmt1*^{-/-} (cc), double knockout *Dnmt3a*^{-/-};*Dnmt3b*^{-/-} (DKO), and triple knockout *Dnmt1*^{-/-}; *Dnmt3a*^{-/-};*Dnmt3b*^{-/-} (TKO), were maintained on gelatin-coated plates in mESC medium containing DMEM (Invitrogen), 10% fetal bovine serum (FBS, Gibco), 100 μ M 2-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate, 1X L-glutamine (Gibco), 1X penicillin/streptomycin (MP Biomedicals), and 100 units/mL of leukemia inhibitory factor (LIF).

Human BJ fibroblasts from neonatal foreskin were purchased from American Type Culture Collection (ATCC). These were maintained in medium containing DMEM, 1X penicillin/streptomycin, 1X glutamine, and 10% fetal bovine serum. Fibroblasts were passaged every 3 ~ 5 days using 0.05 % trypsin (Invitrogen).

Undifferentiated hESCs (HSF1) were maintained on a feeder layer of mitomycin C (Sigma)-treated mouse embryonic fibroblasts (MEF) in hESC medium containing DMEM/F12 (Invitrogen) supplemented with 20% Knockout Serum Replacement (KSR, Gibco), 1X glutamax, 1X non-essential amino acids, 0.11 mM β -mercaptoethanol (Sigma), 1X penicillin/streptomycin and 10 ng/ml of bFGF (PeproTech). hESCs were passaged every 5 ~ 7 days using Collagenase IV (Gibco) and Dispase (Gibco) at a final concentration of 1 mg/ml in hESC medium. All cells were cultured under a protocol approved by the Chancellor's Animal Research Committee (ARC) and Embryonic Stem Cell Research Oversight (ESCRO) Committee at UCLA.

Derivation of human induced pluripotent stem cells (iPSCs)

The production of human iPSCs followed a published protocol (Takahashi et al., 2007) with slight modifications. Specifically, retroviruses containing OCT4, SOX2, KLF4 and c-MYC were produced in the Platinum-E (PLAT-E) retrovirus packaging cell line (Cell Biolabs). Viral supernatants were collected 48 hours and 72 hours after transfection and filtered through a 0.45 µm PVDF filter (Millipore). The Slc7a1-expressing human BJ fibroblasts were plated at 1.5×10^5 cells per well on a 6-well plate on day 1. On day 2, each retroviral supernatant was added into the fibroblasts in the presence of 4 µg/ml of polybrene (Sigma). A second round of transduction was performed on day 3. Infection efficiency was monitored by fluorescence microscopy of cells transduced by retrovirus carrying GFP. On day 5, cells were trypsinized and re-plated in a density of 1×10^5 per 10 cm plate on mitomycin (Sigma)-treated MEFs. On day 6, the medium was changed to hESC medium. iPS cell colonies were picked 3 weeks after infection. The picked colonies were cultured and passaged according to standard culturing protocols. All plasmids including pMXs-OCT4, pMXs-SOX2, pMXs-KLF4, pMXs-c-MYC and pLenti6/UbC/mSlc7a1 used for the derivation of iPSCs were purchased from Addgene.

DNA extraction and hydrolysis

hESC and iPSC colonies were harvested and passed through a cell strainer (BD falcon) to remove the feeder cells. Colonies were washed with PBS, treated with 500 µL of DNA lysis buffer (100 mM Tris-HCl pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.2% SDS), 5

μL of proteinase K (100 mg/mL, Roche), and 5 μL of RNase A (10 mg/mL, Roche), and incubated overnight at 37 °C in a shaking incubator. Genomic DNA was purified by a standard phenol/chloroform extraction followed by precipitation with two volumes of cold 100% ethanol. Subsequently the extracted genomic DNA was redissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Genomic DNA was quantified spectrophotometrically at 260 nm (Thermo Scientific NanoDrop).

DNA hydrolysis was performed by using DNA Degradase Plus (Zymo Research). Briefly, 1 μg of genomic DNA was mixed with 2.5 μl 10X DNA Degradase Reaction buffer, 1 μl DNA Degradase Plus and water to make a total reaction volume of 25 μl . The reaction mixture was incubated in 37 °C for more than an hour. Finally, the reaction was inactivated by adding 175 μl of 0.1% formic acid to yield a final concentration of 5 ng of digested DNA/ μl .

DNA standards

Three 897bp DNA standards, each homogenous for either unmodified C, 5mC, or 5hmC, were purchased (Zymo, Irvine, CA), and used to generate a calibration curve. The standards were prepared by PCR using the appropriate nucleotides and were spin-column purified by the manufacturer to obtain 50 ng/ μL solutions. By MRM criteria these standards were all more than 99.6% pure.

Multiple Reaction Monitoring (MRM) Quantitation

DNA hydrolysis samples (10 μl typically containing 50 ng of digested DNA) were injected onto a reverse phase UPLC column (Eclipse C18 2.1 x 50 mm, 1.8 μ particle

size, Agilent) equilibrated and eluted (100 μ L/min) with water/methanol/formic acid (95/5/0.1, all by volume). The effluent from the column was directed to an electrospray ion source (Agilent Jet Stream) connected to a triple quadrupole mass spectrometer (Agilent 6460 QQQ) operating in the positive ion multiple reaction monitoring mode using previously optimized conditions, and the intensity of specific $MH^+ \rightarrow$ fragment ion transitions were recorded (5mC m/z 242.1 \rightarrow 126.1, 5hmC 258.1 \rightarrow 142.1 and dC m/z 228.1 \rightarrow 112.1). The measured percentage of 5mC and 5hmC in each experimental sample was calculated from the MRM peak area divided by the combined peak areas for 5mC plus 5hmC plus C (total cytosine pool).

With each batch of experimental samples a series of standard samples was simultaneously prepared using the 897bp DNA standards, and run. The standard samples contained increasing amounts of 5mC and 5hmC in the presence of the same amount of C (0-10% for 5mC and 0-2% for 5hmC). Calibration curves were constructed for 5mC and 5hmC from the data obtained from the standard samples (measured 5mC or 5hmC peak area/total cytosine pool plotted against actual percentage of either 5mC or 5hmC in the samples). The measured percentage of 5mC and 5hmC in each experimental sample was then converted to actual percentage 5mC and 5hmC by interpolation from the calibration curves. This provided a correction for any differences that might exist in the molar MRM responses of the various nucleosides.

TET1 cloning, virus production and infection

The Flag-tagged TET1 catalytic domain (NM_030625) was amplified from hESCs cDNA using PCR with Hotstar Taq polymerase (Qiagen). Primers used for the

amplification of TET1 are described in Supplementary Table 1. PCR products were purified and cloned into pCR4-TOPO plasmid using TOPO TA cloning kit (Invitrogen) following the manufacturer's protocol. TET1 sequences were verified by DNA sequencing. The TET1 catalytic domain was digested and ligated into BamHI and EcoRI sites of lentiviral plasmid, FUIGW (Addgene).

For lentivirus production, 293T cells were plated at 8×10^6 cells per 10 cm plate and incubated at 37 °C overnight. Cells were transfected with 9 µg of FUIGW-Flag-TET1-GFP or FUIGW) along with 4.5 µg of pMLDg/pRRE (Addgene), 1.8 µg of pRSV-Rev (Addgene), and 2.7 µg of pCMV-VSVG (Addgene) by Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol; 48 and 72 hours after transfection, viral supernatants were collected and filtered through a 0.45 µm PVDF filter (Millipore). Fresh viral supernatants were infected into 293T cells in the presence of 4 µg/ml of polybrene (Sigma) overnight. Infected cells were analyzed by immunostaining and extracted for LC/MS/MS-MRM.

Immunocytochemistry

Antibodies used for immunostaining were: OCT4 (1:20, Santa Cruz), NANOG (1:100, Abcam), TRA1-60 (1:200, Chemicon), SSEA4 (1:200, Chemicon), SOX2 (1:200, Cell Signaling) DNMT3B (1:500, a gift from Dr. En Li). Human ES and iPS cells were plated on sterile coverglasses in 6-well plates and cultured for 24 ~ 48 hr. The medium was aspirated and cells were washed once with PBS and fixed with 4% paraformaldehyde/PBS for 30 min at room temperature. Cells were washed three times with 0.2 % Tween 20/PBS, then permeabilized with 0.2% Triton X-100/PBS for 30 min

at room temperature and washed once with 0.2% Tween20/ PBS. Blocking was performed for 1 h at room temperature with 2% BSA/0.1% Tween 20/PBS. Primary antibodies diluted in blocking solution were incubated for 1 h at room temperature. Cells were washed three times with 0.2% Tween 20/PBS. Cy2- and Cy3- conjugated secondary antibodies diluted in blocking solution were incubated at room temperature for 30 min. Cells were washed three times with 0.2% Tween 20/PBS, stained with DAPI and mounted on glass slides (Fisher Scientific). Images were analyzed on a Nikon Eclipse 80i inverted microscope equipped with a CCD camera by using Spot Advance imaging software (Diagnostics Instruments).

Southern blot analysis.

Genomic DNA (5 µg) was digested with *Bst*BI (NEB) overnight at 37 °C, separated on a 1% agarose gel, and transferred to a Hybond-N⁺ membrane (Amersham) in 10X SSC. The membrane was hybridized with P³²-end-labeled oligo probes for *Sat 2* and *Sat 3* in QuikHyb solution (Stratagene) at 42 °C for 2 hours. The hybridized membrane was washed twice in 2X SSC/0.1 % SDS at room temperature and washed once in 0.1XSSC/0.1 % SDS at 60 °C. The membrane was exposed to a Kodak BioMax MS film. Oligo probes are described in Supplementary Table 1.

Bisulfite sequencing

Genomic DNA (2 µg) was subjected to bisulfite conversion using EZ DNA Methylation Kit (Zymo research) following the manufacturer's protocol. Subsequently, PCR was carried out with HotStar Taq polymerase (Qiagen). Primers (OCT4 and

NANOG) and PCR conditions are described in Supplementary Table 1. PCR products were purified by Wizard SV gel and PCR clean-up kit (Promega) and cloned into pCR4-TOPO plasmid using TOPO TA cloning kit (Invitrogen) following the manufacturer's protocol. Following transformation, 10 ~ 12 colonies were subjected to direct sequencing with the M13 reverse primer, followed by inoculations and minipreps.

Results

Mass spectrometric characterization of 5hmC, 5mC, and C

An equal molar mixture of three commercial 897bp standard DNA fragments (Zymo, Irvine, CA), each homogenous for either unmodified C, 5mC, or 5hmC, was prepared and digested into the nucleoside components. This mixture produced ions during electrospray ionization corresponding to the protonated nucleosides deoxycytidine (dC), 5-methyl-2'-deoxycytidine (5mdC) and 5-hydroxymethyl-2'-deoxycytidine (5hmdC) at m/z 228.1, 242.1 and 258.1, respectively. Collisionally induced dissociation (CID) of these protonated nucleosides produced a number of fragments, the most abundant of which correspond to the protonated bases liberated by cleavage of the glycosidic bond at m/z 112.1 (C), 126.1 (5mC) and 142.1 (5hmC). Mass-based distinction between these nucleosides is therefore possible because the parent masses are unique as are the corresponding bases that result from glycosidic cleavage. The gas phase glycosidic cleavage of nucleosides is efficient, and the intensity of transitions of the protonated nucleosides to their corresponding bases can be used in the MRM mode for independent quantification: m/z 228.1 \rightarrow 112.1, 242.1 \rightarrow 126.1 and 258.1 \rightarrow 142.1 for dC to C, 5mdC to 5mC and 5hmdC to 5hmC, respectively.

When the equivalent of 50 ng of DNA was analyzed by LC/MS/MS-MRM, the sequentially eluting symmetrical peaks corresponding to dC to C, 5hmdC to 5hmC, and 5mdC to 5mC transitions revealed no detectable cross-talk (Figure 1-3). Using commercial DNA fragments as standards, the linearity of the response was tested by preparing and analyzing samples with varying amount of 5mC and 5hmC in the

presence of a constant amount of C containing DNA. Calibration curves constructed from this data set for both 5mC and 5hmC were linear (Figure 1-4), and were used to calculate the percent DNA methylation and hydroxymethylation in experimental samples.

Validation of the MRM method

The method was then used to measure the percentage of 5mC and 5hmC in some mouse embryonic stem cell (mESC) lines. The 5mC level of *Dnmt1*^{-/-} mESC is about 25% of the wild-type 5mC level. Also, the double knockout, *Dnmt3a*^{-/-} and *Dnmt3b*^{-/-}, mESC at passage 35 (P35) has a 5mC level of about 16% of the wild-type (Figure 1-5). These results are consistent with previous studies that used nearest neighbor analysis and bisulfite next generation sequencing (BS-Seq) (Jackson et al., 2004; Popp et al., 2010).

A comparison of the 5mC and 5hmC levels in various mESC lines shows a strong correlation (Figure 1-5). This correlation is consistent with the biological conversion of 5mC to 5hmC by oxygenase TET enzyme (Ito et al., 2010; Tahiliani et al., 2009). A higher 5mC level would favor more 5hmC conversion, and thus raise the global level of 5hmC.

To confirm another previous study, the FLAG-tagged TET1 catalytic domain was over-expressed in 293T cells (Figure 1-6A). Using the MRM method a drastic increase in 5hmC level was recorded accompanied by about 50% loss of 5mC level compare to control cells (Figure 1-6B). This observation was consistent with the previous study using 5mC antibody fluorescence immunocytochemistry that showed transfected 293T

cells have 55% of the DNA methylation level found in control cells (Tahiliani et al., 2009).

Measuring 5hmC and 5mC in somatic and induced pluripotent stem cells

BJ fibroblasts were used to generate induced pluripotent stem cells (iPSCs) by retrovirally introducing *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. Two iPSC colonies (BJ iPS #7 and BJ iPS #8) were picked and expanded for further analysis. Both iPS lines showed a significant increase in 5mC after reprogramming from BJ fibroblasts (Figure 1-7). This 5mC increase was accompanied by a significant increase in the 5hmC level.

Southern blot was performed on *Sat 2* and *Sat 3* repetitive sequences of BJ fibroblasts, BJ iPS #7 and BJ iPS #8, and showed an increase in DNA methylation at BstBI sites (TTCGAA) in the repetitive regions (Figure 1-8A). These results are consistent with the MRM result. However the promoter regions of both *Nanog* and *Oct4* underwent DNA demethylation (Figure 1-8B), suggesting that the 5mC level increase occurs on selective gene regions.

Discussion

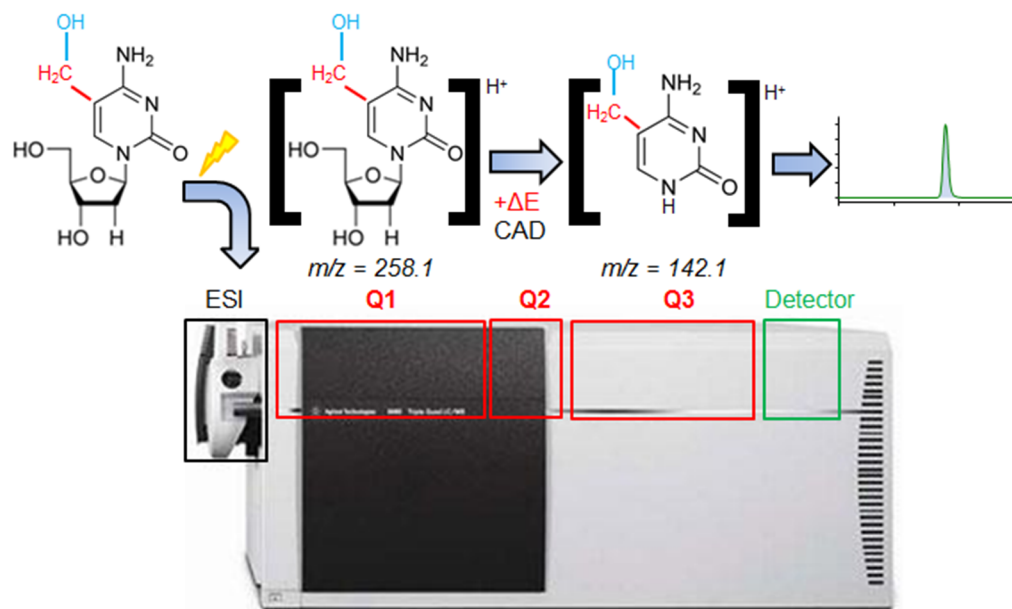
We report the details of a fast and reliable method for measuring the relative levels of 5mC and 5hmC in small samples of digested DNA. Through the use of ultra performance liquid chromatography (UPLC) with sub-micron particle size packing, the analysis time is reduced to 6 minutes per sample. Using this method the limit of detection for these two nucleosides is around 0.5 fmol injected on-column. The linearity of the response is demonstrated across one order of magnitude which is more than sufficient for biological samples, and is probably much greater, and the levels of 5mC and 5hmC have been measured in ten different cell lines. Experience has shown that batches exceeding one hundred samples can be analyzed without any noticeable change or deterioration in chromatographic performance and MRM response. The durability of the UPLC columns used in this work is such that hundreds of samples have been analyzed on the same column, although as a precaution high organic washes every 20-30 samples are used to avoid any complications that could arise from the accumulation of materials not eluted during the isocratic analyses.

Both internal (Quinlivan and Gregory, 2008) and external standards (Kok et al., 2007; Liu et al., 2007; Liu et al., 2009; Song et al., 2005) have been used for quantitative measurements of DNA methylation. External standards that mimic the processing of biological samples have been used here. This has been done by preparing pre-mixed standard DNA samples, and then processing them through the entire work-up and digestion. The resulting standard curves reflect the unavoidable errors that arise during sample work-up such as ion suppression that might arise from

components used in the reaction solutions. Consistent with the previous report (Song et al., 2005), our results reveal no evidence that small variations in the completeness of DNA hydrolysis adversely affects the linearity of the observed responses.

In this report, the DNA methylation levels in iPSCs are not similar to ESCs and fibroblasts. Various findings have already indicated that there are epigenetic differences between normal ESCs and iPSCs, particularly in DNA methylation patterns (Deng et al., 2009; Doi et al., 2009; Kim et al., 2010; Pick et al., 2009; Stadtfeld et al., 2010). Our preliminary results on the comparison between iPSCs and parental somatic cells show a significant number of genes undergo increased DNA methylation during reprogramming (Wang et al., 2011). It has been reported by others that the epigenetic mechanism of DNA methylation is a limiting factor in the reprogramming process, and that the DNA methylation pattern may not truly emulate the pattern found in ESC (Kim et al., 2010; Mikkelsen et al., 2008). For example, treatment of DNA methyltransferase inhibitor, 5-aza-cytidine, facilitated the transition of partially reprogrammed cells to iPSC (Mikkelsen et al., 2008). Interestingly, the level of 5hmC in iPSC from reprogrammed fibroblast reported here appears to be restored to the levels found in ESC.

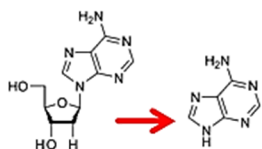
In conclusion, we have established an accurate and robust assay for the simultaneous quantification of 5hmC and 5mC levels in biological samples. LC/MS/MS-MRM is acknowledged as a gold standard in quantitation methodology, and the method described here will have widespread applicability and is sufficiently flexible for expansion to include other rare nucleosides.



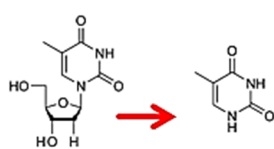
ESI- Electrospray ionization source
 CAD- collision activated dissociation

Figure 1-1: Multiple monitoring reaction. The LC/MS/MS-MRM method takes advantage of the unique parent masses of each nucleoside and the facile cleavage of the base so that each parent to fragment ion transition is specific and quantitative signature. The mass spectrometer used is a triple quadrupole and each quadrupole has a specific role in the MRM scan mode. The nucleoside, 5hmC, is first ionized and the quadrupole one selects that specific parent ion while preventing all other ions from going further down the mass spectrometer. The parent ion then enters the collision chamber, or quadrupole two, in which ion are fragmented by high inert gas molecules. Then the quadrupole three selects a specific fragment ion to go through and eventually hit the detector to get a digital readout.

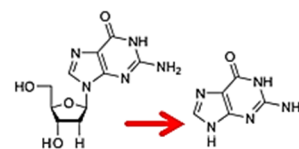
A: 252.1 to 136.1



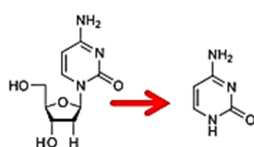
T: 243.1 to 127.1



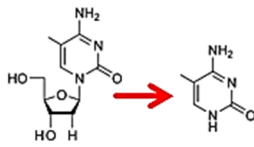
G: 268.1 to 152.1



C: 228.1 to 112.1



5mC: 242.1 to 126.1



5hmC: 258.1 to 142.1

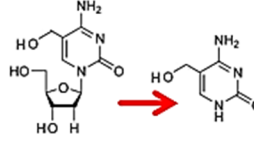


Figure 1-2: Parent to fragment ion transitions for each nucleoside found in the mammalian genome. Note that each nucleoside has a unique parent to fragment ion transition.

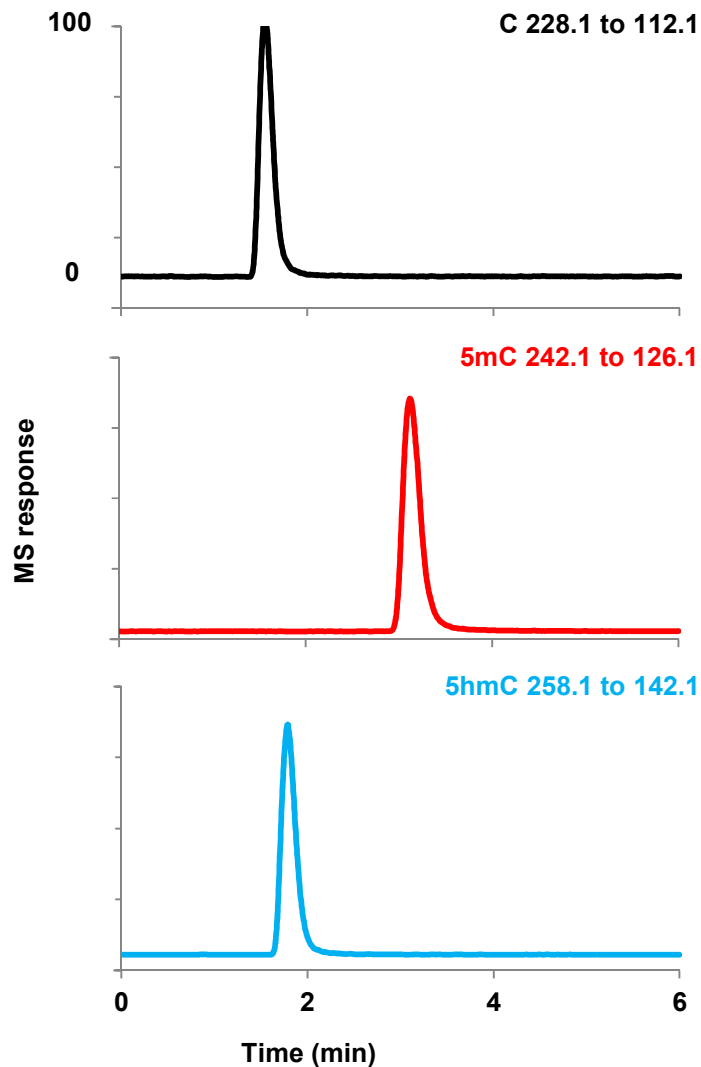


Figure 1-3: Ion chromatograms from standard DNA fragments. LC-MS/MS-MRM chromatograms of nucleosides derived from an equal molar mixture of three commercial 948bp standard DNA fragments showing peaks corresponding to the response obtained from gas phase transitions of dC to C, 5hmdC to 5hmC, and 5mdC to 5mC.

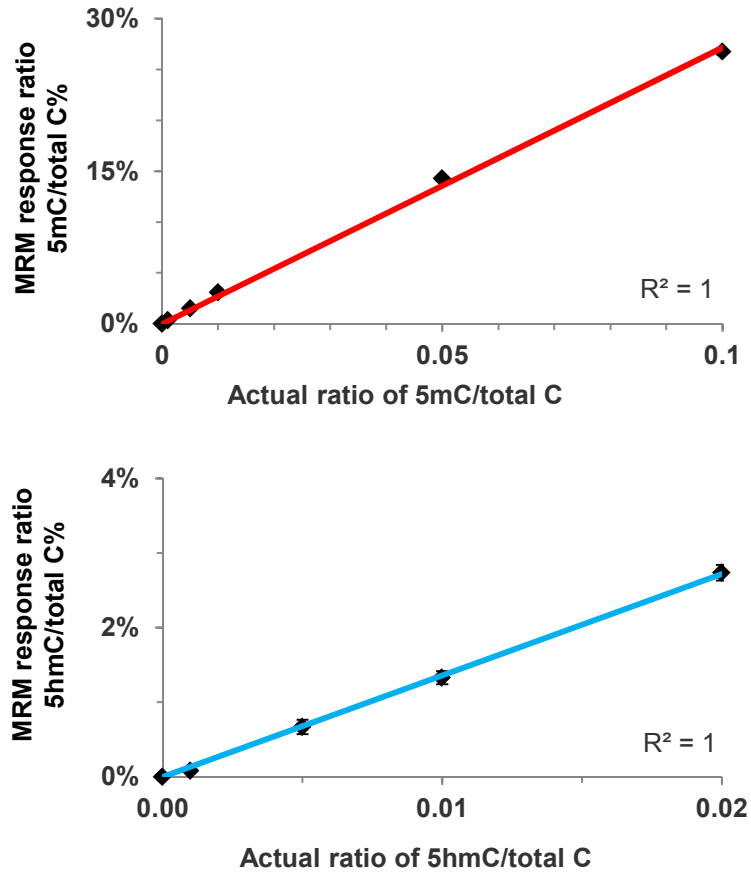


Figure 1-4: Standard curves for 5mC and 5hmC. Percent DNA methylation and hydroxymethylation is plotted against the known ratios of methylated or hydroxymethylated DNA to the total pool of cytosine in the standard sample.

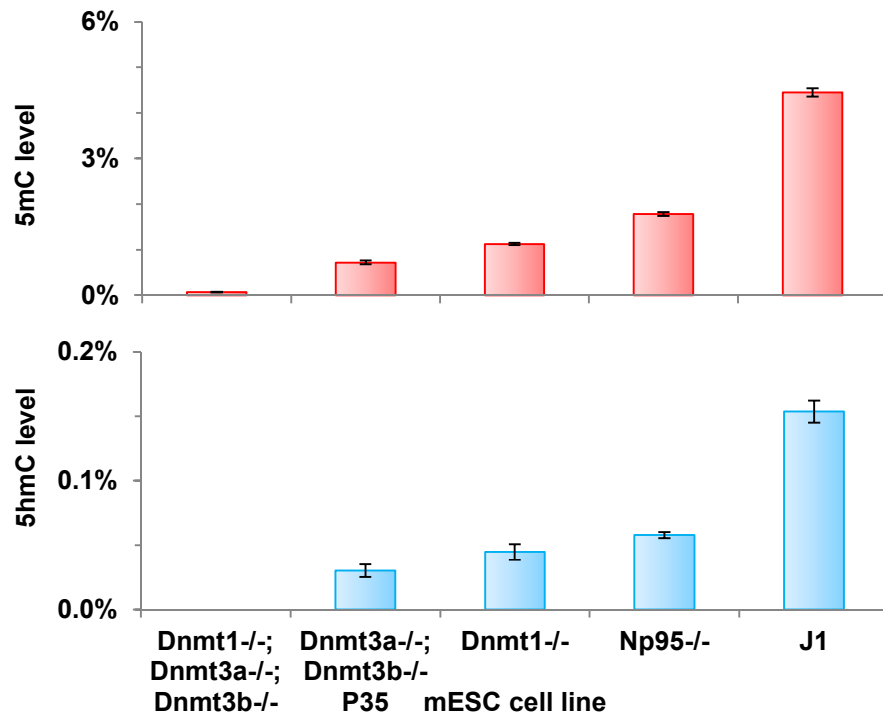


Figure 1-5: Percentage of 5hmC and 5mC in mESC DNA. 5mC and 5hmC contents are expressed as the percentage of 5mC or 5hmC in the total pool of cytosine. Data are the mean \pm s.d. from triplicate analyses.

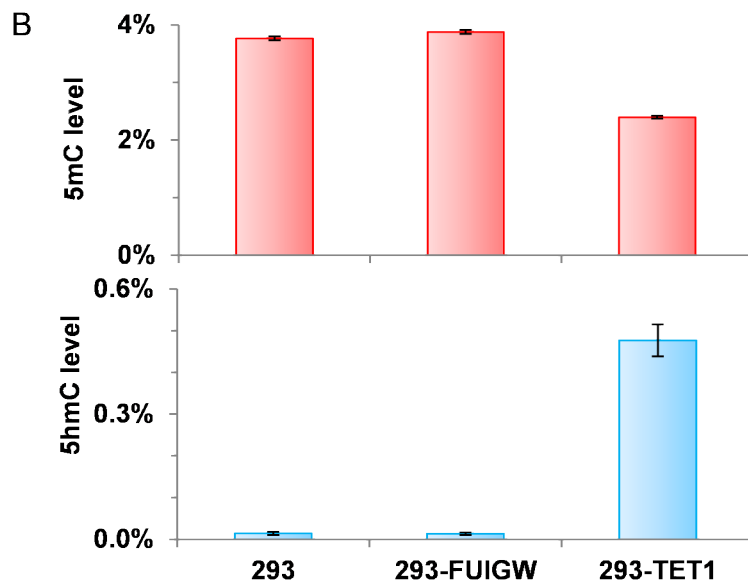
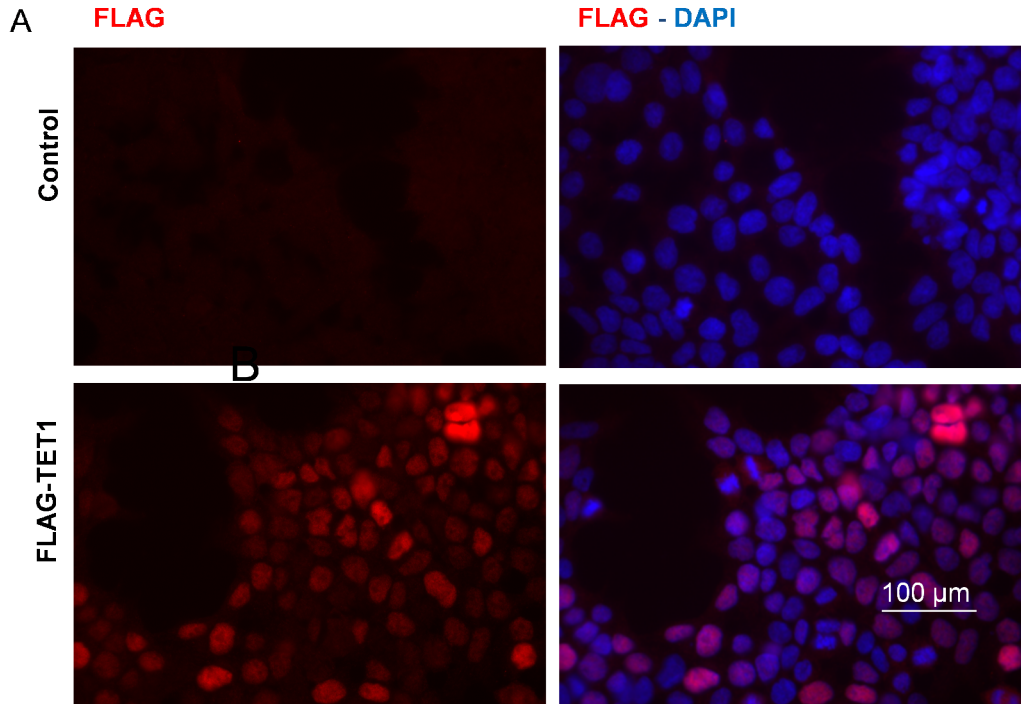


Figure 1-6: Over-expression of human TET1 catalytic domain in 293T cells. (A) 293T cells over-expressing FLAG-catalytic domain of TET1 were co-stained for FLAG antibodies and DAPI . Scale bar, 100 μ m. (B) Percentage of 5hmC and 5mC in DNA from TET1 transfected cells, mock-transfected and un-transfected cells (B). Data are the mean \pm s.d. from triplicate analyses.

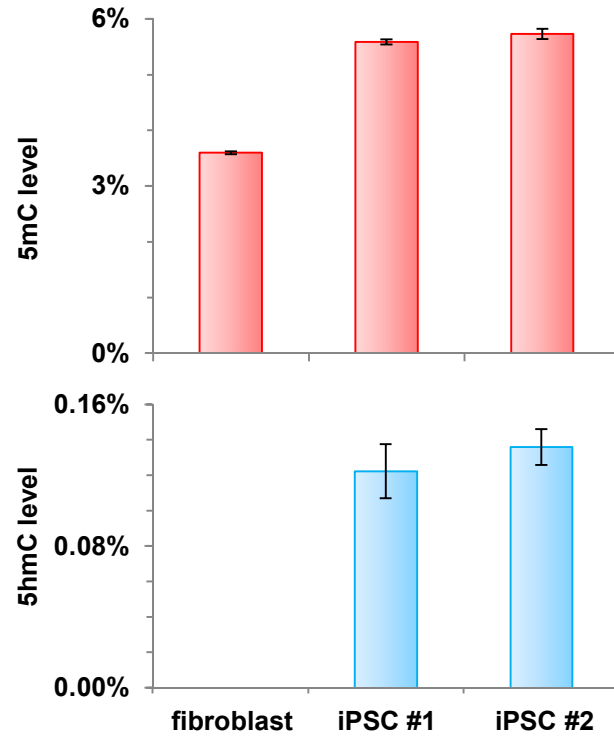


Figure 1-7: Reprogramming BJ fibroblast into iPSCs. Percentage of 5mC and 5hmC in DNA from BJ fibroblast and two BJ iPS cell lines, #1 and #2.

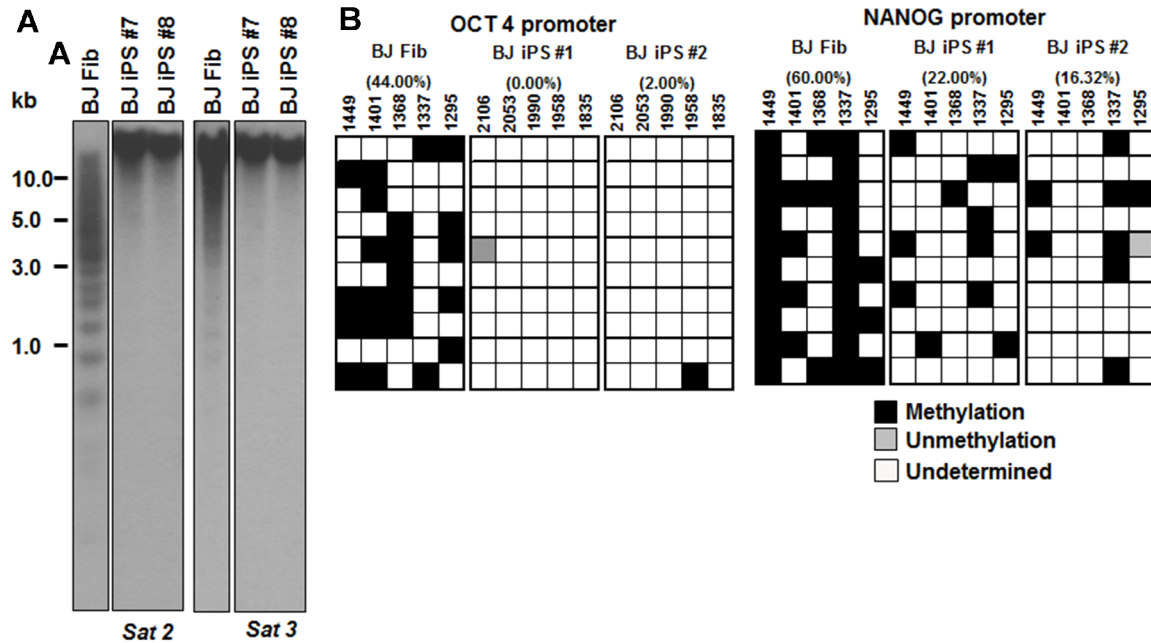


Figure 1-8: Characterizing iPSCs from reprogrammed BJ fibroblast. (A) Southern blot analysis of DNA methylation in BJ and two BJ iPS cell lines. DNA was digested with methyl-sensitive BstBI, separated on agarose gel, transferred to the membrane and hybridized to probes of the repetitive regions of Sat 2 and Sat 3. Small DNA fragments of BJ fibroblast are indicative of DNA hypomethylation in Sat 2 and Sat 3 repetitive regions. (B) Bisulfite sequencing of *Oct4* and *Nanog* promoter of BJ fibroblasts and two BJ iPS cell lines. Each row represents one clonal analysis and each box represents a CpG site where the site number is indicated above. The methylation analysis is displayed according to the key. The overall percentage methylation of the gene promoter is indicated for each sample.

CHAPTER 2- The activity of DNA methylation and hydroxymethylation

Abstract

Modifying DNA cytosine residues to form 5-methyl-cytosine (5mC) and 5-hydroxymethyl-cytosine (5hmC) are essential epigenetic code for regulating gene expression. Alteration of this epigenetic code occurs during development. However, the kinetics of 5mC and 5hmC formation leading to these altered changes are unknown. Here we present a simple, non-radioactive assay for measuring the kinetics of global DNA methylation and hydroxymethylation in mouse embryonic stem cells (mESCs). By supplying cells with stable isotope-labeled methionine ($^2\text{H}_3\text{-met}$), the *in vivo* precursor of the methyl donor S-adenosylmethionine, cytosine residues that undergo methylation and hydroxymethylation become isotopically marked. The labeled forms of 5mC and 5hmC have mass increases of 3 and 2 Da, respectively, and can be identified and quantified by combined liquid chromatography-tandem mass spectrometry using multiple reaction monitoring (LC/MS/MS-MRM, figure 2-1). Using mESCs, label incorporation into both 5mC and 5hmC was maximal at 48 hours. At this point the level of incorporation nearly matched the proportion of labeled/unlabeled methionine in the culture media. Upon label removal from the media, the amount of labeled 5mC and 5hmC asymptotically declined over the following 48 hours. Using mESC lines that encompass all possible combinations of the three major DNA methyltransferases, the contribution of each enzyme to the kinetics of DNA methylation were measured and

correlated with the global levels of 5mC and 5hmC. The method is valuable for measuring the kinetics of global DNA cytosine methylation and hydroxymethylation, and for documenting the effects of pharmacological intervention and environmental factors on the kinetics of these important processes.

Introduction

DNA cytosine methylation and hydroxymethylation are *in vivo* chemical modifications of the mammalian genome. Both methylation and hydroxymethylation processes have profound effects on various biological processes including genomic imprinting, silencing of retroviral transposons, X-chromosome inactivation, and cellular differentiation. Understanding the kinetics of DNA cytosine methylation and hydroxymethylation will illuminate factors that have consequences for these and other important phenomena such as cancer and cellular reprogramming (Suva et al., 2013).

DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmts) that transfer a methyl group onto the fifth carbon of DNA cytosine residues to form 5-methyl-cytosine (5mC). The three major Dnmts (Dnmt1, Dnmt3a and Dnmt3b) are divided into two major functional groups. Dnmt3a and Dnmt3b establish new methylation sites on unmodified genomic cytosine residues and are thus known as *de novo* Dnmts. Dnmt1, known as the maintenance Dnmt, copies the methylation pattern of the parental DNA strand onto the newly synthesized DNA strand during DNA replication, thus allowing the DNA methylation pattern to be inherited. All three Dnmts are active during mammalian development (Moore et al., 2012).

DNA hydroxymethylation is carried out by three ten-eleven translocation (Tet) deoxygenases that convert 5mC into 5-hydroxymethyl-cytosine (5hmC) (Ito et al., 2010; Tahiliani et al., 2009). 5hmC has been implicated as an intermediate in the DNA demethylation process (Cortellino et al., 2011; Guo et al., 2011b; Hajkova et al., 2010; He et al., 2011; Tahiliani et al., 2009). Two major mechanisms of DNA demethylation

have been proposed. In the first mechanism, referred to as the iterative oxidation pathway, the Tet enzymes oxidize 5hmC to 5-formyl- and then 5-carboxy-cytosine (He et al., 2011; Ito et al., 2011). In the second mechanism, 5hmC is deaminated by deaminase AID/APOBEC to form 5-hydroxymethyl-uracil (Guo et al., 2011b). In both mechanisms, the base-excision repair (BER) process involving thymine DNA glycosylase (TDG) excises the modified residues, either 5-carboxy-cytosine or 5-hydroxymethyl-uracil, and replaces it with cytosine (Cortellino et al., 2011; He et al., 2011). However, recent research indicated that 5hmC may not function solely as an intermediate in DNA demethylation because of its stable global levels and pattern (Ficz et al., 2011; Globisch et al., 2010; Jin et al., 2011; Kriaucionis and Heintz, 2009; Le et al., 2011; Pastor et al., 2011; Tahiliani et al., 2009; Wu and Zhang, 2011). Furthermore, methyl CpG binding protein 2 (MeCP2) binds to actively transcribed genes enriched with 5hmC, suggesting a role for this modified base in marking genes for activation (Mellen et al., 2012). Although this finding runs counter to a previous report showing 5hmC inhibits MeCP2 from binding to DNA in an *in vitro* assay (Valinluck et al., 2004), these apparently contradictory findings illustrate the nuances of the epigenetic roles of 5hmC.

Several assays have been described for measuring Dnmt activity. In one method, [³H]-SAM is used to form [³H]-oligonucleotides from DNA oligonucleotide substrates (Fuks et al., 2000; Kim et al., 2004; Roth and Jeltsch, 2000; Yokochi and Robertson, 2004). In another assay, a methylation-sensitive restriction enzyme is used to determine the methylation status of DNA oligonucleotide substrates at specific CpG-dinucleotides (Woo et al., 2005). In yet another approach, recombinantly tagged-methyl CpG binding

domain (MBD) protein is used to bind to methylated DNA oligonucleotide substrates. The DNA methylation level is then measured spectrophotometrically by the intensity of the tagged MBD that is bound to the DNA substrate using antibody conjugated to horseradish peroxidase. In a last method, modified DNA oligonucleotide substrates are directly identified and quantified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Humeny et al., 2003). All four of these *in vitro* assays use synthesized DNA oligonucleotide substrates to evaluate the kinetic properties of Dnmts that are derived from either nuclear extracts or by recombinant technology. The conditions used in these assays may not reflect the *in vivo* cellular environment.

A recent report describes the mass spectrometric measurement of DNA methylation kinetics in various human cancer cell lines using an isotope-labeled precursor (Herring et al., 2009). In this work stable isotope-labeled methionine ($^2\text{H}_3\text{-met}$) was provided to cells for the enzymatic production of $^2\text{H}_3\text{-S-adenosylmethionine}$ (SAM) (Figure 2-1A). Consequently, the Dnmts isotopically label 5mC by the transfer of the methyl group from labeled SAM will increase its mass by 3 Da. And any further subsequent formation of 5hmC from the addition of a hydroxyl group by the Tet enzymes will result in loss of one deuterium atom, thus labeled 5hmC will have a 2 Da mass increase (Figure 2-1B). We have adapted this approach to explore the DNA methylation kinetics of various Dnmt combinations on the genome of mouse embryonic stem cells (mESCs) by adding $^2\text{H}_3\text{-met}$ to the culture medium. Combined liquid chromatography-electrospray ionization tandem mass spectrometry with multiple reaction monitoring (LC/MS/MS-MRM) is used to determine the relative amounts of both

labeled and unlabeled 5mC and 5hmC. *Dnmt* reconstitution mESCs from a triple-knockout *Dnmt* genetic background (TKO), and *Dnmt* mutant cell lines, encompassing all eight possible *Dnmt* combinations from the three major *Dnmts* were used. The results indicate that global DNA methylation in WT mESCs has a 48-hour turnover, and that DNA methylation kinetics are correlated with global DNA methylation levels. Moreover, the combination of *de novo* and maintenance *Dnmts* gave DNA methylation kinetic rates that were faster than either *de novo* or maintenance *Dnmts* alone.

Materials and methods

Cell culture

mESCs were maintained on gelatin-coated plates in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 15% fetal bovine serum (Gibco), 100 μ M 2-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate, 1X L-glutamine (Gibco), 1X penicillin/streptomycin (MP Biomedicals), and 100 units/mL of leukemia inhibitory factor (LIF). In the time-course experiments, additional methionine or $^2\text{H}_3$ -methionine was added to bring the final concentration to 300 μ M. The culture media was changed every 24 hours.

To generate the *Dnmt* reconstitution TKO mESC lines, a rescue plasmid (pCAG-IRES-blast vector, Addgene) containing a single *Dnmt* gene and Blasticidin-resistant genes was electroporated into the TKO *Dnmts* mESCs. Blasticidine (5 μ g/mL, Invitrogen) was added to the cell media to select for drug-resistant colonies 24 hours after electroporation.

Sample preparation

Cells were washed thrice with 1mL/well PBS, aspiration to remove the wash, then treated with 500 μ L/well DNA lysis buffer containing 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, 5 μ L of proteinase K (100 mg/mL, Roche), and 5 μ L of RNase A (10 mg/mL, Roche). The mixtures were transferred to microcentrifuge tubes (1.5 mL) and incubated overnight at 37°C in a shaking incubator. Chloroform was added (500 μ L), the samples were inverted about 15 times and centrifuged (13,000 x g,

15 min, RT). The upper phase was transferred to a clean microcentrifuge tube (1.5 mL) to which was added cold ethanol (4°C, 1 mL). After centrifugation (13,000 x g, 1 min, RT), the supernatant was removed by aspiration and the pellet was re-suspended in TE buffer (typically 50 µL containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The DNA content was measure in an aliquot (1 µL) from the OD₂₆₀ nm (Thermo Scientific NanoDrop) using an average extinction coefficient for double-stranded DNA of 0.020 (µg/ml)⁻¹ cm⁻¹.

Aliquots of the TE buffer re-suspended material containing 2 µg of genomic DNA were digested with a cocktail of nuclease enzymes (DNA Degradase Plus™, Zymo Research; 2.5 µl 10X DNA Degradase Reaction buffer, 1 µl DNA Degradase Plus and water to make a total volume of 25 µl) following the manufacturers' protocol. After incubation (37 °C, >1 hour) aqueous formic acid was added (25 µl, 0.1% v/v) to yield a final concentration of 40 ng of digested DNA/µl.

DNA standards

Three 897 bp DNA standards, each homogenous for either unmodified cytosine (C), 5-methyl-cytosine (5mC), or 5-hydroxymethyl-cytosine (5hmC), were purchased (Zymo, Irvine, CA), and used to generate a calibration curve with each batch of samples. The standards had been prepared by PCR using the appropriate nucleotides and were spin-column purified by the manufacturer to obtain 50 ng/µL aqueous Tris buffered solutions. By MRM criteria these standards were all more than 99.6% pure. With each batch of experimental samples a series of standard samples was simultaneously prepared using the DNA standards. The standard samples contained

increasing amounts of 5mC and 5hmC in the presence of the same amount of C (0, 0.1, 1, 5 and 10% for 5mC and 0, 0.1, 0.5, 1, and 2% for 5hmC).

Quantitation by Multiple Reaction Monitoring (MRM)

The MRM quantitation method was slightly modified from that described previously (Le et al., 2011). DNA hydrolysis samples were injected (1 -20 μ L) onto a reverse phase UPLC column (Eclipse C18 2.1 x 50 mm, 1.8 μ particle size, Agilent) equilibrated with buffer A (0.1% aqueous formic acid) and eluted (200 μ L/min) with an increasing concentration of buffer B (methanol: min/%B; 0/0, 2/0, 4/5, 6/5, 8/0, 10/0). The injection volume for each sample was adjusted such that the C peak area was at least 1 million area counts (Agilent MassHunter Quantitative Analysis software, version B.04.00). The effluent from the column was directed to an electrospray ion source (Agilent Jet Stream) connected to a triple quadrupole mass spectrometer (Agilent 6460 QQQ) operating in the positive ion multiple reaction monitoring mode, and the intensity of specific $MH^+ \rightarrow$ fragment ion transitions were recorded (5mC 242.1 \rightarrow 126.1, 5mC+3 245.1 \rightarrow 129.1, 5hmC 258.1 \rightarrow 142.1, 5hmC+2 260.1 \rightarrow 144.1 and C 228.1 \rightarrow 112.1) using previously optimized condition for maximal MRM response from each nucleoside.

Calibration curves were constructed for 5mC and 5hmC from the data obtained from the standard samples. The measured 5mC or 5hmC peak area was divided by the total cytosine peak area (summed peak areas for C, 5mC and 5hmC) and was plotted against actual percentage of either 5mC or 5hmC in the samples. The measured percentage of 5mC and 5hmC in each experimental sample was then converted to actual percentage 5mC and 5hmC by interpolation from the calibration curves. This

provided a correction for any differences that might exist in the molar MRM responses of the various nucleosides.

Calculation of expected DNA methylation and hydroxymethylation incorporation rates

With the addition of $^2\text{H}_3\text{-met}$ to the culture medium, the expected % incorporation ($\% \text{ incorporation}_{\text{expected}}$) for both modified cytosine bases was calculated, assuming a 12-hour cell cycle (Pauklin et al., 2011), as follows:

$\% \text{ incorporation}_{\text{expected}} = R \times (1 - 2^{-t/12}) \times 100\%$; where R = the ratio of the [$^2\text{H}_3\text{-met}$]/total met in the media, which in this case is 1/3.

The $\% \text{ incorporation}_{\text{expected}}$ will decline with time (t) when the $^2\text{H}_3\text{-met}$ is removed from the media. The decline in $\% \text{ incorporation}_{\text{expected}}$ after removal of $^2\text{H}_3\text{-met}$ was calculated as follows:

$\% \text{ incorporation}_{\text{expected}} = 5 \times 2^{-t/12} \times 100\%$; where $^2\text{H}_3\text{-met}$ was removed at t=48 hours. The value 5 was computed from equation (1) where t was set at 48 hours and R = 1/3.

Immunocytochemistry

Antibodies used for immunostaining were: Dnmt1 (1:300, Cosmo Bio), Dnmt3A (1:500, Imgenex), Dnmt3B (1:1000, Novartis). mESCs were plated on sterile coverglasses in 6-well plates and cultured for 24~48 hour. The medium was aspirated and cells were washed once with PBS and fixed with 4% paraformaldehyde/PBS for 30 min at room temperature.

Cells were washed again thrice with PBS, then permeabilized with 0.5% Triton X-100/PBS for 10 min at room temperature. Blocking was performed for 1 hour at room temperature with 5% normal goat serum/0.5% Triton X-100/PBS. The blocking solution was aspirated and primary antibodies, diluted as described above in blocking solution, were added and the samples incubated overnight at 4°C. Cells were washed thrice with PBS. The secondary antibody (either Cy2- or Cy3- conjugated), diluted in blocking solution, was added (30-45 min, RT). Cells were washed twice with PBS, then stained with 4',6-damidino-2-phenylindole (DAPI; 1 µg/mL in PBS), and washed once with PBS before mounting the coverslip onto a glass slide (Fisher Scientific). Images were recorded on a Nikon Eclipse 80i inverted microscope equipped with a CCD camera using Spot Advance imaging software (Diagnostics Instruments).

Results

When WT mESCs were incubated with $^2\text{H}_3$ -met for 48 hours, both labeled 5mC and 5hmC were readily detected (Figure 2-2). In this experiment 5mC and 5hmC accounted for on average 5.46 (± 0.015) and 0.24 (± 0.005) percent, respectively, of the total C expressed as the sum of C+5mC+5hmC, consistent with what is typically observed and with previously published data using the same technique (13).

Under conditions when $^2\text{H}_3$ -met was available continuously for up to 120 hours, the temporal increase in WT mESCs percent labeled 5mC and 5hmC reached a plateau at around 48 hours, at which point the percent label in the nucleosides closely matched the proportion of $^2\text{H}_3$ -met to total methionine in the media (Figure 2-3A). In similar experiments when the $^2\text{H}_3$ -met was removed from the culture media at 48 hours, the amount of labeled 5mC and 5hmC decreased in a time-dependent manner and both labeled nucleosides were undetectable by 96 hours (Figure 2-3B).

Ten mESC lines were generated and used to study the kinetics of 5mC and 5hmC label incorporation from various combinations of the three Dnmts (Dnmt1, Dnmt3a and Dnmt3b, Table 2-1). These cell lines represent all 8 possible combinations of the three Dnmts including each individually expressed, combinations of two, and in WT cells all three Dnmts. The available cell lines include two isoforms of Dnmt3a (isoforms 1 and 2), both individually expressed. Also included are two single expressing Dnmt1 lines that were produced using different genetic methods including genetic KO of both *Dnmt3a* and *Dnmt3b* (DKO), and re-introduction of *Dnmt1* into TKO mESC (TKO mD1). Evidence of nuclear Dnmt localization was obtained from DAPI and Dnmt

immunocytochemistry, for which merged images of the *Dnmt* reconstitution TKO mESCs showed superimposition of staining (Figure 2-4).

When all 10 mECS cell lines were compared in a single experiment, the WT (J1) cells had 5mC and 5hmC levels of 4.8 and 0.15%, respectively, and the TKO cell line had values of 0.2% and undetectable for 5mC and 5hmC, respectively. The remaining 8 cell lines had global levels of 5mC and 5hmC ranging between these extreme values, and when the levels were plotted for all ten cell lines, a linear relationship emerged (Figure 2-5A). Cell lines that had at least one *de novo* and one maintenance *Dnmt* were grouped together at the high end of the correlation, cell lines with single or double *de novo* *Dnmt* were grouped in the middle of the correlation, and cell lines that had a single maintenance and no *de novo* enzyme were grouped at the low end of the correlation. The average ratio of 5mC and 5hmC global levels across all mESCs was 43.9 ± 5.9 , excluding DKO, TKO mD1 and TKO (Figure 2-5B). The ratios in the DKO and TKO mD1 mESCs are higher, with values of 226 and 89 respectively. Because 5hmC could not be detected in TKO, the ratio could not be calculated.

A time course experiment covering the first 6 hours of label administration showed a linear increase in label incorporation into 5mC for all ten cell lines, except for the TKO where there was no detectable label incorporation. Graphical representation of this data for four of the cell lines is shown in Figure 2-6. From these data, rates of label incorporations were calculated as % change/hour from the peak area ratios ($^2\text{H}_3\text{-5mC}/(^2\text{H}_3\text{-5mC} + 5\text{mC})$) over the 6-hour time interval. The calculations produced values ranging from 2.17% change/hour for WT (J1) to 0.92% change/hour for DKO and 0.00% change/hour for TKO (Table 2-2). The kinetic rates were grouped into three categories

based on cell line *Dnmt* genetic status, where the fastest rates occurred with both maintenance and *de novo* Dnmts (J1, *Dnmt3a*^{-/-} and *Dnmt3b*^{-/-}), followed by just *de novo* Dnmts (*Dnmt1*^{-/-}, TKO mD3a1, TKO mD3a2, and TKO mD3b1), and lastly just maintenance *Dnmt1* (DKO and TKO mD1). When the average rates were computed for cell lines with only *de novo* Dnmts and cell lines with only maintenance *Dnmt1* and then summed, the kinetic rate is comparable to the average kinetic rate of the cell lines with both *de novo* and maintenance Dnmts (Table 2-3). When the rates of label incorporation into 5mC for each cell line were plotted against the global level of 5mC, a linear relationship emerged (Figure 2-7).

Discussion

The incorporation of label from exogenously supplied $^2\text{H}_3$ -met into the two modified DNA cytosine residues was rapid and readily measurable by LC/MS/MS-MRM in samples derived from mESC cultures containing as few as 10^6 cells. These experiments were performed on a relatively small scale with minimal consumption of labeled precursor. While no attempt was made to microtize the protocol, because only 5-10% of the DNA was used for hydrolysis and then between 5-35% of each sample was injected, it is predicted that experiments could be performed with as few as 10^4 - 10^5 cells without compromising the accuracy of the measurements. Supplementation of the DMEM with additional methionine (labeled and unlabeled) had no significant effect on the global levels of 5mC and 5hmC of the WT (J1) cells. Because the analysis of nucleosides by LC/MS/MS-MRM has proven to be sensitive and applicable to many modified bases in addition to 5mC and 5hmC (Globisch et al., 2010; Le et al., 2011; Song et al., 2005), the extension of the methodology reported here to include other modified DNA bases should be straightforward and could provide important insights into the kinetics of these other processes.

The temporal measurements of label incorporation into 5mC and 5hmC from DNA of WT J1 cells reached near equilibrium with the media after 48 hours of culture, and the label was washed out during the ensuing 48 hours. This rate of global incorporation is slightly slower than the calculated rate of percent incorporation based on a 12-hour cell-cycle. The reasons for this difference include mESCs having a slower cell cycle time, $^2\text{H}_3$ -met concentration in the cell not matching with the cell media, and

DNA demethylation activity competing with DNA methylation during DNA replication. Nevertheless, the rate of label accumulation and loss was similar to the calculated rate based on the 12-hour cell cycle for both modified cytosine, although both label accumulation and loss may be faster for 5mC. This subtle difference will require verification in future work.

The global levels of 5mC and 5hmC varied between 0.1- 5% within the ten mESC lines. The *Dnmt* reconstitution TKO cell lines allowed for incorporation measurements on cell lines expressing single *Dnmt* to methylate a genome that was initially devoid of DNA methylation. It seems that the pluripotent ESCs are tolerable to a wide range of global levels of the 5mC and 5hmC imposed by the various combinations of Dnmts. Moreover, the two DNA modifications are correlated, confirming previous findings (Le et al., 2011; Szwagierczak et al., 2010). Consistent with the role of Tet in converting 5mC into 5hmC, *Tet1* knockout mESCs have reduced levels of 5hmC that is accompanied by a subtle increase in 5mdC at the global level (Dawlaty et al., 2013; Dawlaty et al., 2011). Because the data for wild-type falls on the regression line, these findings supports the notion that in the face of normal Tet and demethylating activities, 5mC is a rate-limiting factor in the formation of 5hmC even in wild-type cells.

It is interesting to note that both the single-expressing *Dnmt1* mESC lines (DKO and TKO mD1) have the same level of DNA methylation even though they were derived from two different genetic approaches, knockout versus reconstitution. Having internally consistent DNA methylation levels validated both approaches.

The global DNA methylation levels are positively correlated to their DNA methylation rates. The combination of the *de novo* Dnmt3a/3b and the maintenance

Dnmt1 has the greatest overall DNA methylation kinetic rate compared to the combination of the *de novo* Dnmts alone, followed by maintenance Dnmt1 alone. Moreover, the average kinetic rate of cell lines with the combination *de novo* and maintenance Dnmts is comparable to the sum of the average kinetic rate of cell line of just *de novo* Dnmts and cell lines with just maintenance Dnmt1. This would suggest that the *de novo* and maintenance Dnmts have additive effects on the overall DNA methylation kinetic rates.

The observed slow kinetic rate of Dnmt1 in DKO and TKO mD1 can be explained by the acidic linker that is positioned directly between the unmethylated DNA and the active site of the Dnmt1 (Song et al., 2011). Mutagenesis and enzymatic studies of Dnmt1 revealed that the acidic linker severely limits Dnmt1 from *de novo* methylation activity, thus Dnmt1 prefers hemimethylated DNA substrate for methylation. In the absence of *de novo* Dnmts, the acidic linker would severely limit the Dnmt1 kinetics for establishing a new DNA methylation pattern. The method reported here may prove valuable for investigating various factors including Dnmt inhibitors that affect the DNA methylation and hydroxymethylation activity.

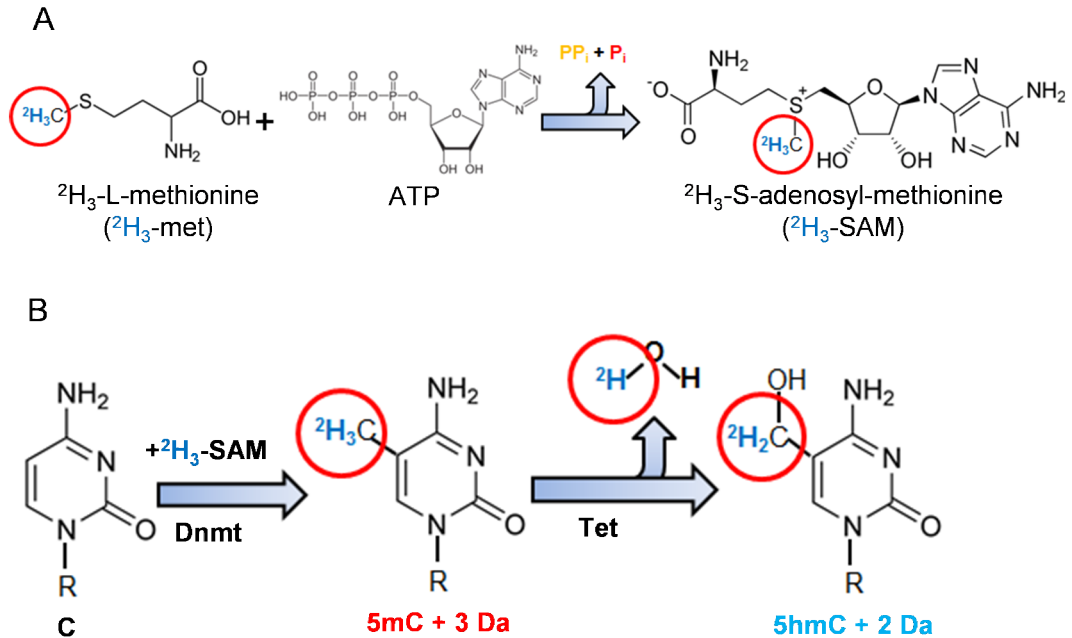


Figure 2-1: Stable isotope methionine and nucleoside isotopomers. Cells enzymatically combine L-methionine and adenosine triphosphate (ATP) by methionine adenosyltransferase to generate the methyl-donor S-adenosyl-methionine (SAM). Stable isotope methionine has all three deuterium atoms on the methyl carbon. By adding stable isotope methionine into the media, the cells can generate isotope labeled SAM (A) and use it to label newly formed DNA methylation and hydroxymethylation (B). This would allow newly formed 5mC to have an effective mass increase of 3 Da and any subsequent formation of 5hmC will result in loss of one deuterium atom, consequentially labeled 5hmdC will have a 2 Da mass increase. The use of mass spectrometry allows the identification and quantitation of newly formed 5mC and 5hmdC isotopomers.

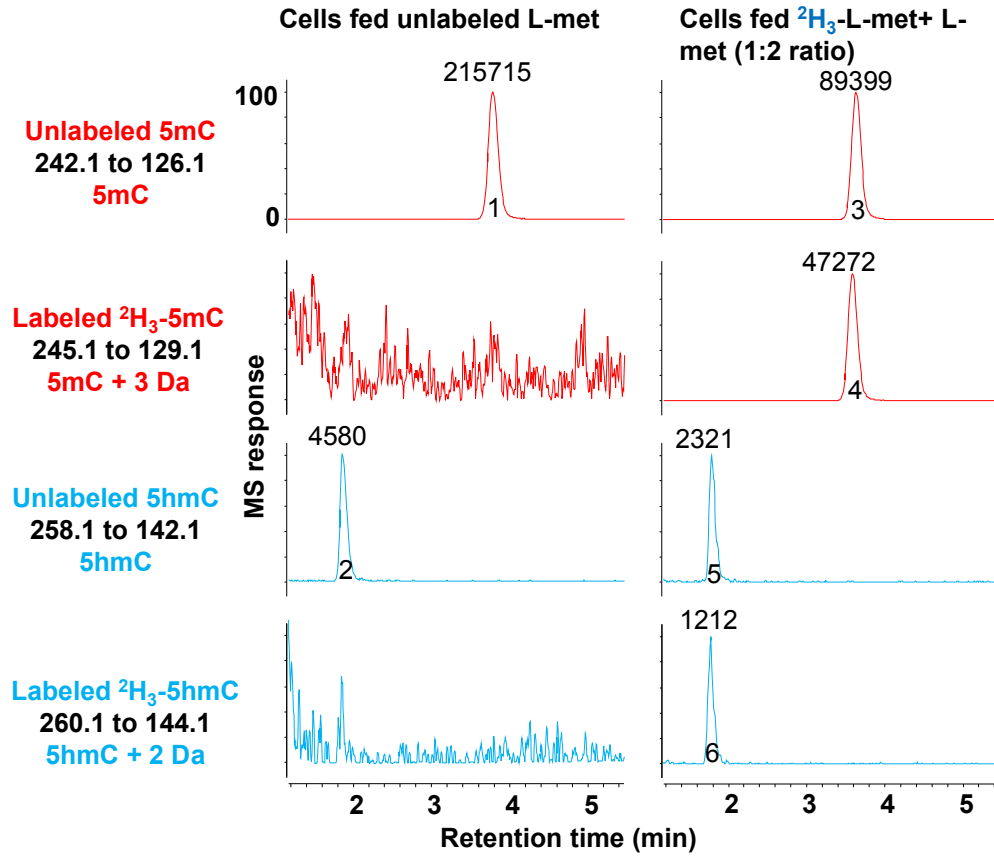


Figure 2-2: Nucleoside MRM response from WT mESCs incubated with stable isotope methionine. MRM response for 5mC (242.1→126.1), $^2\text{H}_3$ -5mC (245.1→129.1), 5hmC (258.1→142.1) and $^2\text{H}_2$ -5hmC (260.1→144.1) from wild-type mESCs incubated with unlabeled methionine (left panel) and $^2\text{H}_3$ -met (right panel) for 48 hours. For peaks labeled 1-6, the ratios of peak area/ C_{total} (summed C + 5mC + $^2\text{H}_3$ -5mC + 5hmC + $^2\text{H}_2$ -5hmC) expressed as a percentage from 3 replicate injections of each sample were: 5.55 ± 0.13 SD for peak 1; 0.24 ± 0.005 SD for peak 2; 5.32 ± 0.02 SD for peaks 3 plus 4; and 0.24 ± 0.003 SD for peaks 5 plus 6.

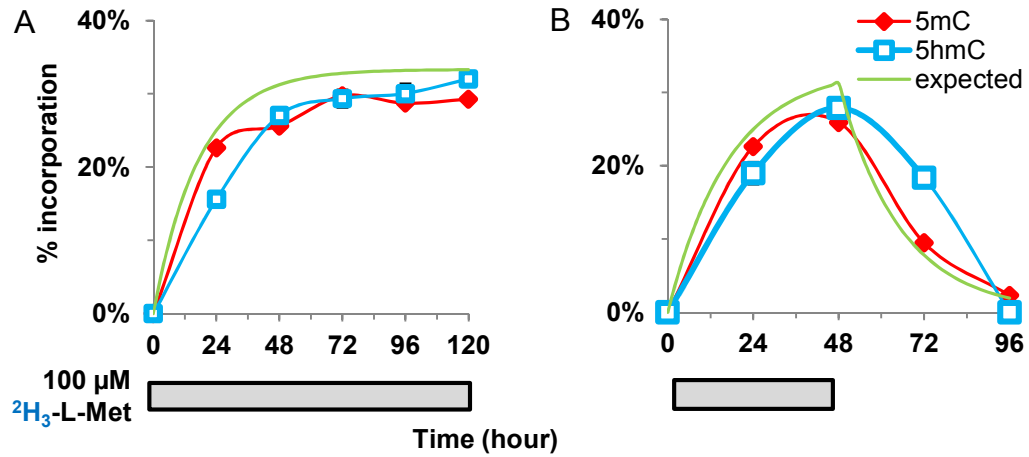


Figure 2-3: Continuous and pulse-chase experiment on the stable isotope methionine. Time course of $^2\text{H}_3$ -met incorporation into 5mC and 5hmC from wild-type mESCs with continuous availability of labeled precursor (A), and with removal of labeled precursor at 48 hours (B). The ordinate values were calculated as ratio of peak area of $^2\text{H}_3$ -5mC/summed 5mC + $^2\text{H}_3$ -5mC + 5hmC and $^2\text{H}_2$ -5hmC/summed 5hmC + $^2\text{H}_2$ -5hmC. The % incorporation expected, based on a 12-hour cell-cycle, was calculated as described in the Methods and is shown as a continuous solid green line.

Cell line #	# Dnmts expressed	Actual mESC line	Dnmt Status		
			Dnmt1	Dnmt3a	Dnmt3b
1	3	J1 (WT)	+	+	+
2	2	Dnmt3b ^{-/-}	+	+	-
3	2	Dnmt3a ^{-/-}	+	-	+
4	2	Dnmt1 ^{-/-}	-	+	+
5	1	DKO	+	-	-
6	1	TKO mD1	+	-	-
7	1	TKO mD3a1*	-	+	-
8	1	TKO mD3a2 [‡]	-	+	-
9	1	TKO mD3b1	-	-	+
10	0	TKO	-	-	-

= expressing *Dnmt*

= knockout *Dnmt*

* = Dnmt3a isoform 1

‡ = Dnmt3a isoform 2

Table 2-1: Tabulation of the genetic status of the ten mESC lines. These cell lines were used to explore the effect of all combinations of *Dnmt* gene expression on the kinetics of 5mC and 5hmC turnover.

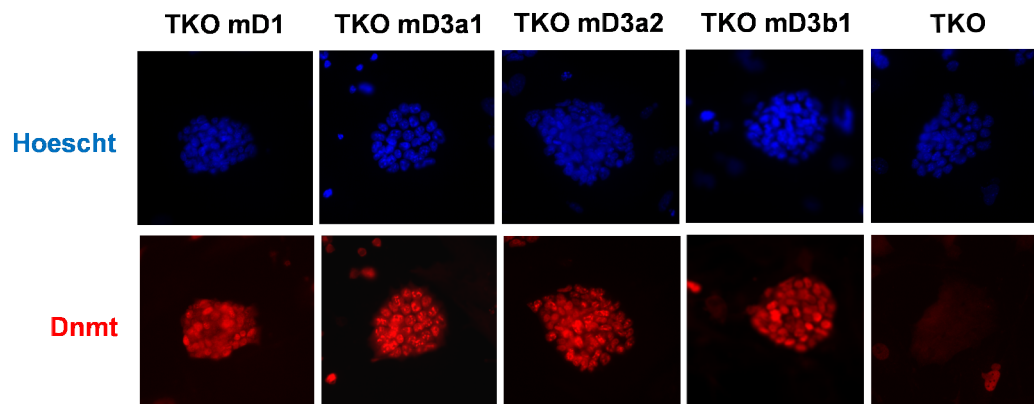


Figure 2-4: Immunocytochemistry of DAPI and Dnmt staining for the reconstitution TKO mESC lines. The *Dnmt* status of each cell lines is given in Table 2-1

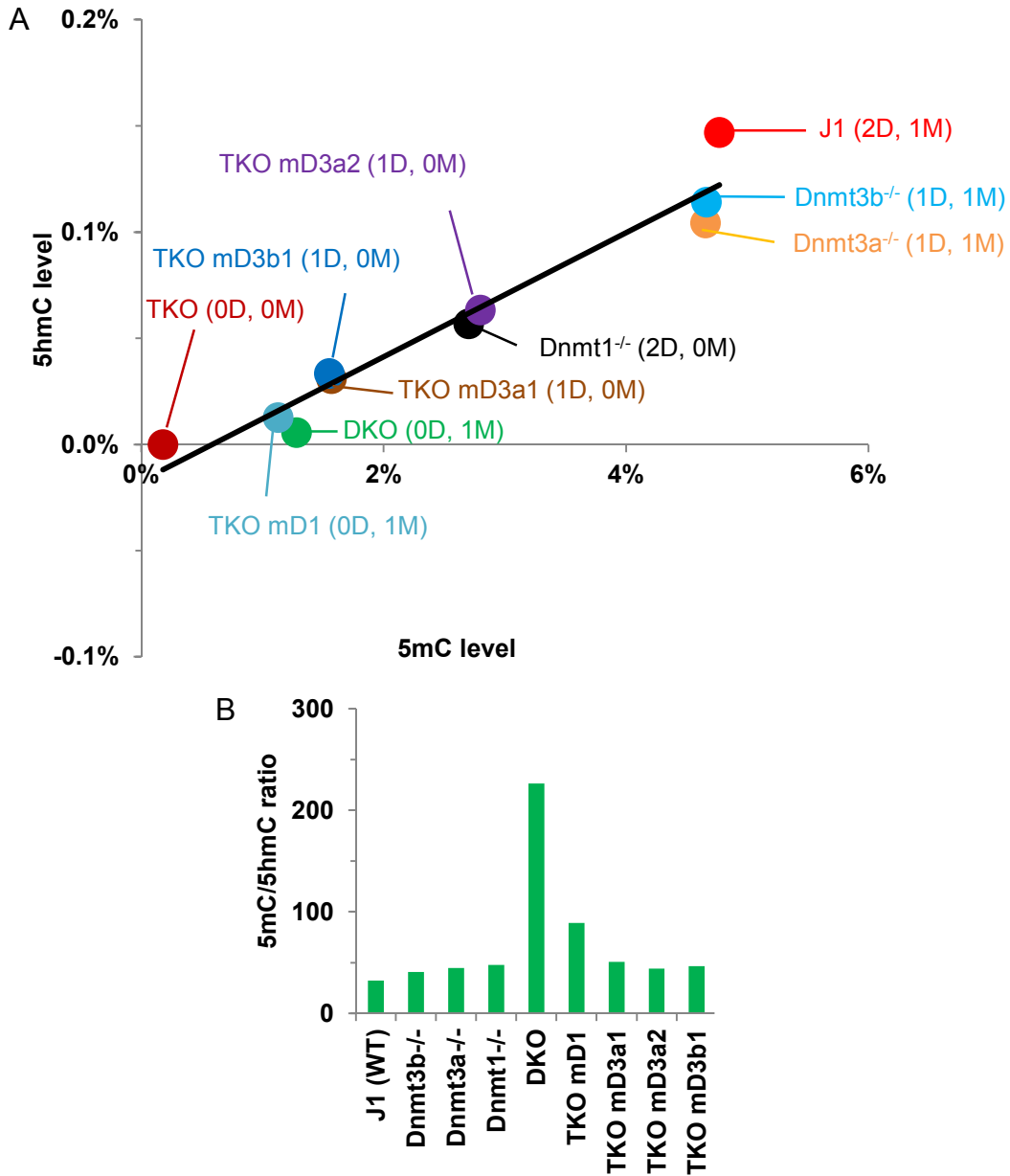


Figure 2-5: (A) Relationship between global levels of 5mC and 5hmC in the ten cell lines. The *Dnmt* status of each line is given in Table 2-1 and is also shown on the graph in which the number of *de novo* and maintenance Dnmts are represented in parenthesis by D and M, respectively (A). The Pearson and Spearman correlations were calculated to be 0.97 and 0.98, respectively. (B) The ratios of 5mC/5hmC global levels is presented as a bar graph for nine of the cell lines, with data for TKO excluded because the level of 5hmC was below the limit of detection.

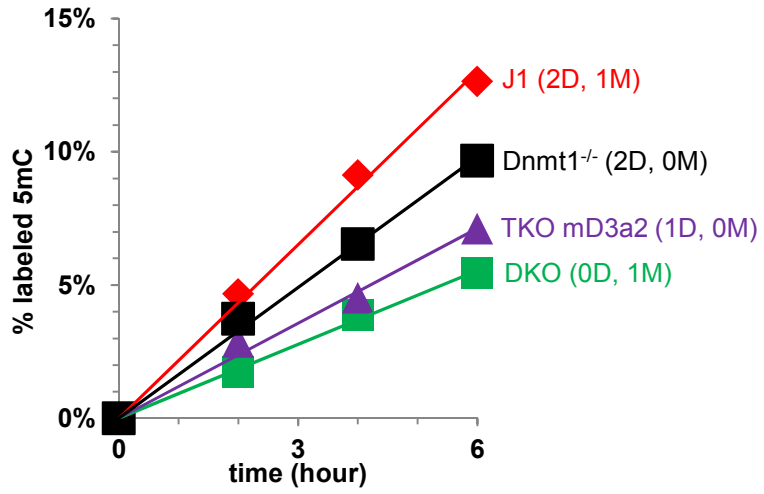


Figure 2-6: Time course of ²H₃-met incorporation for the first six hours into 5mC from four of the mESC lines with differing *Dnmt* combinations.

mESC	# of <i>de novo</i> Dnmt	# of maintenance Dnmt	% change/hr for 5mC	Pearson correlation <i>r</i>	
J1	2	1	2.17	0.998	} combination of both <i>de novo</i> and maintenance Dnmts
Dnmt3a ^{-/-}	1	1	1.98	0.998	
Dnmt3b ^{-/-}	1	1	1.71	0.999	
Dnmt1 ^{-/-}	2	0	1.63	0.998	} <i>de novo</i> Dnmt3a/3b
TKO mD3b1	1	0	1.25	0.999	
TKO mD3a2	1	0	1.21	0.929	
TKO mD3a1	1	0	1.19	0.995	} maintenance Dnmt1
TKO mD1	0	1	1.14	0.984	
DKO	0	1	0.92	0.998	
TKO	0	0	0.00	n/a	

Table 2-2: Tabulation of the labeled 5mC % change/hour and the corresponding Pearson correlations for the ten mESC lines. The rates of label incorporation were calculated as labeled 5mC % change/hour from the peak area ratios ($^2\text{H}_3\text{-5mC}/(^2\text{H}_3\text{-5mC} + 5\text{mC})$) over the 6-hour time interval.

Dnmt functional category	Mean labeled 5mC % change/hour (± stdev)	Cell lines
(1) Maintenance	0.0103 (± 0.001)	DKO and TKO mD1
(2) <i>De novo</i>	0.0132 (± 0.002)	Dnmt1 ^{-/-} , TKO mD3a1, TKO mD3a2, TKO mD3b1
(3) Maintenance + <i>de novo</i>	0.0196 (± 0.002)	J1, Dnmt3a ^{-/-} , Dnmt3b ^{-/-}

(4) Sum (1) and (2) 0.0235

(5) Difference between (4) and (3) 0.0039

Table 3. Tabulation of the calculated average labeled 5mC % change/hour for cell lines. The Dnmt functional categories are (1) maintenance, (2) de novo and (3) maintenance + de novo Dnmts. The sum of the average kinetic rates of (1) and (2) is computed in (4). The difference (5) is calculated between (3) and (4).

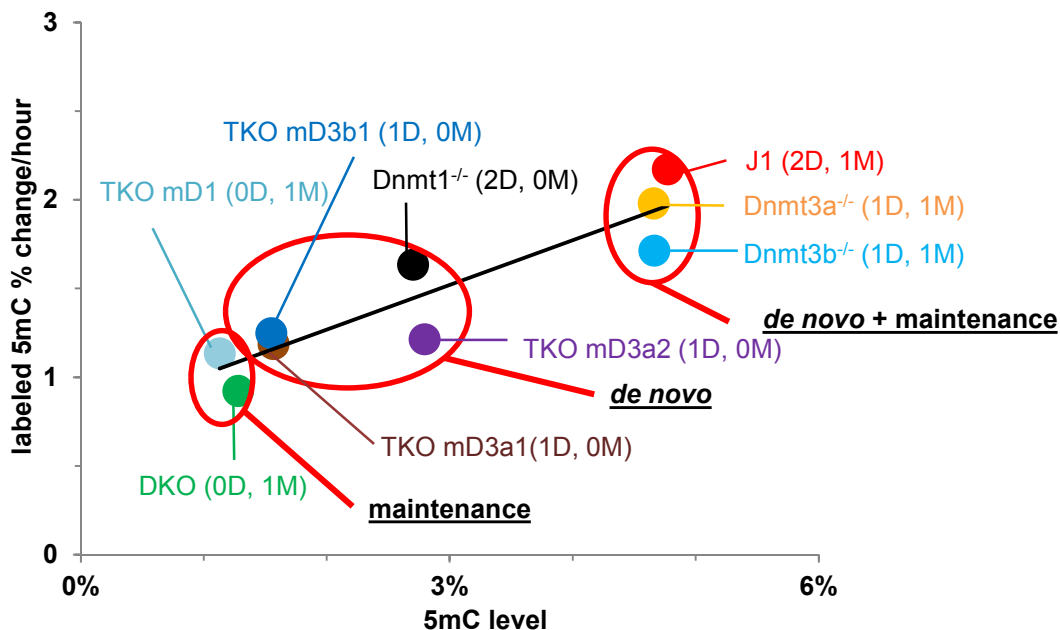


Figure 2-7: Relationship between the global level of 5mC and % change/hour. The peak area ratios ($^2\text{H}_3\text{-5mC}/(^2\text{H}_3\text{-5mC} + 5\text{mC})$) over the 6-hour time interval for 9 cell lines. TKO was excluded from this analysis because it did not show any isotope incorporation into its 5mC. The Pearson and Spearman correlations were calculated to be 0.91 and 0.86, respectively. The *Dnmt* status of each line is given in Table 2-1 and the % change/hour is given in Table 2-2. The number of *de novo* and maintenance Dnmts are represented in parenthesis by D and M, respectively.

CHAPTER 3- DNA methylation in developing and mature neurons

The pattern of modified cytosine residues, including 5mC and 5hmC, provides an inheritable epigenetic code that regulates gene expression during development. DNA methyltransferases (Dnmts) and ten-eleven translocation (Tet) enzymes act concertedly to regulate the pattern and extent of DNA methylation and hydroxymethylation, respectively. Mechanistically, these modified cytosine influence transcriptional activity by recruiting or inhibiting the binding of various proteins such as transcription factors, methyl CpG binding proteins (MBDs), and histone modification enzymes (Cross et al., 1997; Fan et al., 2005; Jones et al., 1998; Martinowich et al., 2003; Meehan et al., 1989; Nan et al., 1997; Nan et al., 1998; Ng et al., 1999; Valinluck et al., 2004). The pattern of 5mC and 5hmC formation is a dynamically regulated during cellular proliferation and differentiation (Bestor, 1992; Fouse et al., 2008; Ito et al., 2010; Koh et al., 2011; Li et al., 1992; Okano et al., 1999; Shen et al., 2007; Wu et al., 2011a; Wu et al., 2011b; Wu and Zhang, 2011).

Recently, it was found that 5hmC is an intermediate in DNA demethylation. Here 5hmC is modified in two ways: these are deamination by activation-induced deaminase/apolipoprotein B mRNA-editing enzyme complex (AID/APOBEC) or iterative oxidation by the Tet enzymes (Bhutani et al., 2011; Cortellino et al., 2011; Guo et al., 2011b; He et al., 2011; Ito et al., 2011). Because the final product of both these pathways is cytosine, both processes erase DNA methylation marks. The combined activities of a number of enzymes and pathways contribute to the precise regulation of

stage-specific pattern of methylation and hydroxymethylation required in the developing central nervous system (CNS).

The precise temporal regulation of DNA methylation and demethylation activities is particularly important for the differentiation and maturation of the mammalian CNS. In particular, the differentiation switch of multipotent neural progenitor cells (NPCs) from neurogenesis to astrogliogenesis coincides with DNA methylation and demethylation events on the glial fibrillary acidic protein (*Gfap*) gene promoter region (Qian et al., 2000; Sauvageot and Stiles, 2002; Teter et al., 1994). Early in neurogenesis at E11.5, DNA methylation of the *Gfap* promoter represses its expression (Takizawa et al., 2001; Teter et al., 1996). The continual expression of *Dnmt1* in NPCs has been found to be important for the maintenance of the methylation pattern on the *Gfap* promoter through subsequent cell divisions (Fan et al., 2005). By E14.5, a major neural development event occurs in the NPCs where the differentiation program switches from neurogenesis to astrogliogenesis. And it is around this time that the *Gfap* promoter undergoes DNA demethylation for the differentiation of the astrocytic lineage (Teter et al., 1996).

The temporal regulation of the DNA methylation pattern of the *Gfap* promoter also coincides with the gene expression of the two *de novo* Dnmts, *Dnmt3a* and *Dnmt3b*. During the neurogenic period, from E11.5 to E14.5, both *de novo* Dnmts are expressed (Feng et al., 2005). However, *Dnmt3a* is the only *de novo* Dnmts that continues to be expressed further into development while *Dnmt3b* declines to nearly undetectable levels in the CNS. The peak expression of *Dnmt3a* coincides with remethylation and reduced transcription of the *Gfap* promoter (Fan et al., 2005; Nguyen et al., 2007). Overall, the coordinated expression of Dnmts and their ability to regulate

the methylation pattern of the *Gfap* promoter organize and regulate neuronal development.

The importance of these coordinated events is highlighted by conditional *Dnmts* knockout mice models for neural development (Fan et al., 2001; Fan et al., 2005; Feng et al., 2010; Golshani et al., 2005; Hutnick et al., 2009; Nguyen et al., 2007; Takizawa et al., 2001). In the conditional *Dnmt1* knockout mice, approximately 89% of the cells found exclusively in the dorsal forebrain exhibited DNA hypomethylation from E13.5, a neurogenic time period (Hutnick et al., 2009). This overall DNA hypomethylation also included *Gfap* promoter of the NPCs. Consequently, the P7 mutant mouse cortex has significantly higher density of astrocytes compared to age-matched control mice, as judged by *Gfap* immunostaining. Furthermore, hypomethylated neurons were characterized by multiple maturation defects including dendritic arborization and impaired neuronal excitability (Fan et al., 2001; Golshani et al., 2005; Hutnick et al., 2009). These results are consistent with the conclusion that *Dnmt1* plays a critical role in the neurogenic and astrogenic switch during neural development (Fan et al., 2001; Fan et al., 2005; Feng et al., 2010; Hutnick et al., 2010) and in maintaining the methylation pattern of the *Gfap* promoter (Fan et al., 2001; Fan et al., 2005; Feng et al., 2010; Hutnick et al., 2009). Additionally, these results suggest that DNA methylation is essential for neuronal maturation.

Unlike the aforementioned *Dnmt1* studies, the majority of cortical neurons developed normally in the conditional *Dnmt3a* knockout mice model (Nguyen et al., 2007). The *Gfap* promoter remained hypomethylated in about 50% of the cortical tissue, which is normally remethylated in postnatal astrocytes. However, the effect from the

loss of Dnmt3a was more pronounced in neuromuscular function. These mutant mice have fewer motor neurons in the hypoglossal nucleus, resulting in defects of neuromuscular junctions of the diaphragm muscle.

Human genetic disorders that disrupt DNA methylation mechanism and/or pattern are associated with mental retardation (Feng et al., 2007; Moore et al., 2012; Robertson, 2005). ICF (Immunodeficiency, Centromere instability and Facial anomalies) syndrome, a rare human genetic disorder, is caused by a recessive mutation of the *DNMT3B* gene (Jin et al., 2008; Okano et al., 1999; Xu et al., 1999). One of the hallmark of the developmental disease is the hypomethylation of the pericentromeric repeat regions. A significant portion of ICF patients suffer mental retardation. Looking into the mouse model, *Dnmt3b* is found to be highly expressed in the neural tube between E7.5–9.5 (Okano et al., 1999; Xu et al., 1999), implicating the importance of *Dnmt3b* in early stages of neurogenesis.

Together the *Dnmt* knockout and the human disease studies confirm that precise regulation of DNA methylation is essential for differentiation and maturation of the CNS. While these studies provide ample evidence that DNA methylation is essential in neuronal development and function, they did not reveal the role of continuous Dnmt expression in postmitotic neurons. Thus, identifying the crucial learning and memory genes affected by DNA methylation would be beneficial to understand human disorders.

The role of the DNA methylation in developed mammalian brain

At the conclusion of neural development the brain primarily consists of postmitotic neurons and glial cells with little proliferation potential. The genetic expression of *Dnmts* normally declines in terminally differentiated cells, implying decreased flux in the methylation, hydroxymethylation and demethylation processes (Deng and Szyf, 1999; Goto et al., 1994; Singer-Sam et al., 1990; Szyf et al., 1991; Szyf et al., 1985). Thus, the DNA methylation becomes a static epigenetic mark in the somatic cells. However, the brain appears to be an exception. Reports of substantial expression and activity of *Dnmts* and *Tets* in the adult murine CNS warrant re-examination of the role of DNA methylation in the developed brain (Feng et al., 2005; Goto et al., 1994; Inano et al., 2000; Ito et al., 2010). Both *Dnmt1* and *Dnmt3a* are expressed by postmitotic neurons, while *Dnmt3b* expression is either low or undetectable (Feng et al., 2005; Goto et al., 1994). This surprising discovery argues for the *Dnmts* unique role in neuronal maturation besides the normal function in fast dividing cells to establish and/or maintain DNA methylation pattern.

Some notable reports have been made linking *Dnmts* expression and activity, and changes in the DNA methylation pattern, with synaptic plasticity (Chen et al., 2003; Martinowich et al., 2003). Cortical neurons under depolarizing conditions experienced active demethylation of the *Bdnf* gene. This resulted in release of bound MeCP2, a known DNA methyl binding repressor. Consequently, phosphorylated CREB, a transcriptional factor involved in learning and memory, bound to the unmethylated gene region to induce *Bdnf* gene expression (Martinowich et al., 2003). Independent of this

work, electroconvulsive stimulation of mice also induced DNA demethylation and increased gene expression of *Bdnf* (Ma et al., 2009). Both reports revealed DNA demethylation and increased gene expression occurred in response to neuronal stimulation.

Inhibitors

Pharmacological studies support a continuing post-development role of DNA methylation in post-mitotic neurons. Researchers suggested that DNA methylation targets specific genes involved in synaptic plasticity and learning and memory. Inhibiting Dnmts in the murine hippocampus by either 5-azacytidine or 5-aza-deoxycytidine attenuated the late-phase of the long-term potentiation (LTP) and increased *brain-derived neurotrophic factor (BDNF)* gene expression (Levenson et al., 2006; Miller and Sweatt, 2007; Nelson et al., 2008). However, it is unclear if these effects are due to specific inhibition of Dnmts or due to the toxic effects of compounds that lead to a generalized inhibition of protein synthesis. The lack of selectivity of Dnmt inhibitors makes it difficult to correlate specific neural effects with the function of Dnmts. Mechanistically, these compounds inhibit Dnmts only when they are first metabolized to become triphosphate deoxyribose form and then incorporate itself into the DNA during the S-phase (Juttermann et al., 1994).

Genetic studies

To avoid the use of Dnmt inhibitors, conditional *Dnmt* knockout mice were used to investigate the role of DNA methylation in post-mitotic neurons (Fan et al., 2001;

Feng et al., 2010; Hutnick et al., 2009). To investigate the role of Dnmts in postmitotic neurons, a postmitotic neuron specific promoter, calcium/calmodulin-dependent protein kinase II α (CamKII α)(Ouimet et al., 1984), was used to drive the expression of Cre recombinase (CamKII α -Cre93) in order to induce *Dnmt* gene deletion. Previous report on the conditional knockout of *Dnmt1* in postmitotic neurons did not have any significant DNA methylation changes, particularly the repetitive elements (Fan et al., 2001). Because Dnmt1 and Dnmt3a might have redundant roles in the CNS, a conditional double knockout mouse (DKO) was generated in which expression of both *Dnmt1* and *Dnmt3a* were abolished starting postnatal age 14 (Feng et al., 2010). The DKO mice had smaller hippocampii without significant neuronal loss. The DKO mice had abnormal learning and memory behavior based on Morris water maze and contextual fear conditioning tests. These phenotypes were accompanied by several demethylated promoters that were enriched for genes involving immune response. Consistent with the characterization of the demethylated genes, gene expression analysis by microarray experiment revealed significant enrichment of upregulated immune genes including the major histocompatibility complex class I (*MHCI*) and those in the complement system, such as *Stat1*. MHC I molecules are important for immune responses to antigens, but are also expressed in the CNS where they are thought to regulate activity-dependent synaptic plasticity (Fourgeaud et al., 2010; Glynn et al., 2011; Goddard et al., 2007; Huh et al., 2000). It has been reported that *MHCI*-deficient mice have neurophysiological abnormalities and that MHC I directly regulate synaptic density (Glynn et al., 2011; Huh et al., 2000).

Globally, the cortex of the DKO mice had a 20% loss of DNA methylation when compared to brain tissue from age-matched control littermates (Figure 3-1). Moreover, the cortex of another mutant, conditional *Dnmt* triple knockout (TKO), show similar loss of DNA methylation as found in DKO mice. Surprisingly, the single conditional knockout (SKO) mice of either *Dnmt1* or *Dnmt3a* did not show significant difference in DNA methylation and hydroxymethylation levels when compare to control littermate. Together, studies of these conditional mice studies demonstrate the redundant roles for both Dnmt1 and Dnmt3a in postmitotic neurons. The studies provided the first direct evidence that Dnmts are required for the maintenance of neuronal DNA methylation, for proper control of gene expression, and consequently for synaptic plasticity, as well as learning and memory. Moreover, this result suggests that control mice have gene regions that are pliable with regard to their DNA methylation pattern, where both active demethylation and re-methylation activities occur.

The importance of Dnmts for synaptic plasticity by continually regulating the DNA methylation pattern beyond neural development are further highlighted by two notable studies. The broader question of chemical modification of the genome in response to stimulation arises in a study of rodent behavior. Rodent maternal care reduced DNA methylation of the glucocorticoid receptor (GR) gene promoter, and simultaneously increases GR gene expression in offspring (Weaver et al., 2004). Restricted maternal care led to opposite effects. However, the extent to which other behaviors are under the control of DNA methylation and demethylation activities has yet to be investigated thoroughly. In dramatic fashion, the pervasiveness and importance of the methylation/demethylation process was highlighted in the electroconvulsive stimulation

of adult mice. Nearly 2000 CpGs sites underwent rapid *de novo* methylation and about 1000 CpG sites exhibit active demethylation in the dentate gyrus within a 24-hour window (Guo et al., 2011a). These activity-induced DNA methylation and demethylation occurred on genes related to synaptic plasticity. The long-lasting changes in synaptic plasticity induced by neural activity, including electroconvulsive and maternal care, require changes in neuronal gene expression and epigenetic mechanisms such as DNA methylation are thought to contribute to this adaptive mechanism (Chen et al., 2003; Feng et al., 2007; Martinowich et al., 2003; Miller and Sweatt, 2007; Tsankova et al., 2007).

The importance of 5hmC in the DNA methylation process was not fully appreciated until it was realized that this was the first product of Tet activity (Penn et al., 1972; Tahiliani et al., 2009). Low but significant 5hmC levels are found in various developed brain regions, ranging between 0.2-0.7% of all cytosine, about 10-fold lower than the abundance of 5mC (Globisch et al., 2010; Kriaucionis and Heintz, 2009). The importance and function of this low-abundant base is obscure. To define the role of Tet enzymes and of 5hmC, genetic knockout mice were generated for *Tet1* and *Tet2* (Dawlaty et al., 2013; Dawlaty et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011). Depletion of either of these proteins reduced 5hmC levels but was not embryonically lethal. In the *Tet1* knockout mice cortex, the reduced level of 5hmC was parallel with slight increase level of 5mC compared to control littermate (Figure 3-2, unpublished data). In the *Dnmt* DKO cortex study, both the 5hmC and 5mC levels were both found to be lower than the control littermate (Feng et al., 2010) (figure 3-1).

In vitro model

To further investigate the roles of Dnmts and Tet in regulating both DNA methylation and hydroxymethylation in postmitotic neurons, an *in vitro* neuronal model was developed using transgenic mice with all three *Dnmts* genes floxed. Three systems were generated involving the complete elimination of the DNA methylation mechanism by genetic Dnmt knockout using Cre recombinase, overexpression of the *Tet1* catalytic domain, and combining both *Dnmt* genetic knockout and overexpression of *Tet1* (Figure 3-3). Eliminating the DNA methylation mechanism altogether in the *Dnmt* triple-knockout cortical culture (TKO) can potentially unveil gene regions targeted by intact endogenous DNA demethylation mechanism. Overexpression of *Tet1* catalytic domain in cortical culture (Tet) can accelerate the DNA demethylation activity and thus provide clues to gene regions that are targeted by *Tet1*. Various studies have already reported that Tet activity induces DNA demethylation (Guo et al., 2011b; Ito et al., 2011; Le et al., 2011; Tahiliani et al., 2009). And in the third system, eliminating the DNA methylation mechanism altogether and overexpressing *Tet1* catalytic domain in cortical culture (TKO-Tet) can determine the extent in which the DNA demethylation activity regulates the overall DNA methylation. I attempted to clarify the roles of Dnmts in maintaining and regulating the DNA methylation pattern in postmitotic neurons.

Results

The cortical culture extracted from P0-1 male transgenic pups was used to generate the three systems. The lentiviral infection carrying *Cre* recombinase gene and *GFP*, which is driven by the *IRES* promoter, was used to generate the TKO cortical culture where the DNA methylation activity was completely eliminated (Figure 3-4). *Cre* recombinase efficiency, measured by quantitative PCR for the 1-lox and 2-lox P sites of the *Dnmt1* gene, was about 90% (data not shown). The Tet cortical culture was generated by lentiviral infection carrying the catalytic domain of *Tet1* gene and *GFP*, which is driven by the *IRES* promoter (Figure 3-4). And finally, TKO-Tet cortical culture was generated by infection of the two lentiviruses containing the *Cre* and *Tet1* gene. The cortical culture began its lentiviral infection at 3 days in vitro (3DIV) and was maintained for 14 days post-infection (14DPI).

There was significant 15% DNA methylation loss in the TKO cortical culture compared to GFP-control cortical culture. (Figure 3-5). Both TKO and GFP-control cortical cultures show similar level of DNA hydroxymethylation. In the Tet cortical culture, a more dramatic loss of DNA methylation was observed compared to TKO, about 19% loss (Figure 3-5). However, the loss of DNA methylation was accompanied by a significant increase in 5hmC levels to 1%; that is 10-fold more than the 5hmC level found in both the GFP-control and TKO. Interestingly, the percent loss of 5mC is greater than the percent gain of 5hmC, thus supporting the role of *Tet1* in active DNA demethylation in postmitotic neurons rather than just a simple conversion of 5mC to 5hmC. The TKO-Tet cortical culture exhibited a dramatic 27% loss of DNA methylation

level; and this is in a system where the DNA demethylation activity is accelerated while there is no DNA methylation mechanism to remethylate those sites (Figure 3-5). It is interesting to note that both Tet and TKO-Tet cortical cultures overexpress the catalytic domain of *Tet1* that is void of regulatory units that can potentially modulate its 5mC to 5hmC conversion activity. This would suggest that there is a protective endogenous mechanism for the majority of the 5mC from being all converted into 5hmC.

The redundancy amongst the Dnmts will impact on the degree of methylation loss. Dnmt redundancy is revealed by the fact that DNA methylation losses are not observed in *Dnmt1* knockout cerebellar cultures, nor in single conditional knockouts of either *Dnmt1* or *Dnmt3a* mice, while the DKO shows a 20% loss (Bestor, 1992; Fan et al., 2001; Feng et al., 2010; Kim et al., 2002). One would expect the TKO neurons to show a greater or at least similar loss compared to DKO mice study from Feng *et. al.*; however the TKO methylation loss was reduced to 15% (Figure 3-5). One explanation for this trend reversal is the difference in the systems: one used a two-week cortical culture (TKO) and the other a one-year old brain (DKO). Neural activity for example, which is likely to be vastly different in these two systems, has been demonstrated to alter the DNA methylation pattern (Guo et al., 2011a).

It was recently reported that the dramatic accumulation of non-CG methylation (mCH) and 5hmC in neurons coincided with the fetal to young adult brain development (Lister et al., 2013). Both mCH and 5hmC were found to be major components of modified cytosines in the genome of both mouse and human frontal cortex. The mCH level in adult neurons was inversely proportional to the gene expression level, suggesting mCH role in limiting transcriptional activity; this finding is opposite to the

embryonic stem cell study where mCH level is positively correlated with gene expression (Lister et al., 2009). Moreover, the increase genetic expression and protein level of Dnmt3a coincided with mCH accumulation and that the genetic regions in which Dnmt3a binds to were enriched for mCH in neurons. The 5hmC marked regulatory regions in the fetal brain that are later demethylated and exhibit active transcription in the adult brain. The Tet2^{-/-} mice study revealed that these 5hmC markers depend on Tet2 activity (Lister et al., 2013). Collectively, the dynamic changes of the DNA methylation are highly associated with neuronal-specific genes that undergo transcriptional changes during mammalian brain development.

Discussion

The complexity and heterogeneity of the mammalian brain significantly limits the power of any high-throughput results, particularly gene expression and DNA methylation pattern analysis. Because many distinct neuronal and non-neuronal cells are highly compacted in a given brain region, changes from a single cell type are potentially masked. All the major *in vivo* studies of postmitotic neuronal are marred with the problem of neuronal sample impurity (Feng et al., 2010; Guo et al., 2011a). In the electroconvulsive stimulation study the dentate gyrus sample contained about 90% NeuN+ neurons (Guo et al., 2011a). Even the most recent protocol for primary adult neuronal tissue culture only achieves an estimated 80% neuronal purity (Brewer and Torricelli, 2007). When changes in DNA methylation are identified, the results may reflect the cellular heterogeneity that originates from the use of tissue extract from the whole mouse brain that includes oligodendrocytes, astrocytes, and microglia, as well as a variety of neurons. Therefore, changes in DNA methylation measured in these samples cannot be attributed solely to neurons. In addition, the current DNA sequencing technology cannot distinguish between 5mC and 5hmC and the precision of the technique is such that changes in methylation levels below 20% are difficult to interpret as either a biological phenomenon or an inherent technical variation in the method. However, recent development have been able to sequence 5hmC at a base-resolution analysis (Lister et al., 2013; Yu et al., 2012) but require two sequencing runs for complete analysis.

Sample purity is critical when dealing with DNA methylation changes of 20% or less. Using flow cytometry (fluorescence-activated cell sorting, FACS) combined with isotopic labeling with LC/MS/MS-MRM from Chapter 2, DNA methylation and hydroxymethylation activities can be measured directly from postmitotic neurons. Primary cortical cultures incubated in $^2\text{H}_3$ -met can then be run in flow cytometry to produce homogenous neuronal samples. Thereafter, the LC/MS/MS-MRM can measure the kinetics of 5mC and 5hmC in these samples. This approach can directly address the important outstanding issue about DNA methylation activity in postmitotic neurons.

There are two approaches for flow cytometry: NeuN (Neuronal Nuclei is also known as Fox-3 (Kim et al., 2009; Mullen et al., 1992)) antibodies and transgenic Ai9 mice. The NeuN antibody has been used to enrich postmitotic neurons from brain tissues (Feng et al., 2010; Iwamoto et al., 2011; Jiang et al., 2008; Lister et al., 2013). Primary cortical cultures from P0-1 wild-type mice were established. The isotope label $^2\text{H}_3$ -met (100 μM) was added to the neuronal media after 3 days *in vitro* (3DIV) to label newly formed 5mdC and 5hmdC. At 7 DIV, nuclei was extracted from primary cortical culture, tagged by NeuN antibodies conjugated with anti-mus IgG (Alexa 488) secondary antibodies, sorted by flow cytometry, and subsequently the DNA was extracted (Figure 3-6A). The flow cytometric protocol was previously described (Feng et al., 2010; Jiang et al., 2008). A small portion of the NeuN⁺ nuclei were re-processed to validate purity (Figure 3-6B). Immunonegative (NeuN⁻) nuclei were also collected. Four days after $^2\text{H}_3$ -met addition, approximately 10% of the 5mC was labeled in NeuN⁺ nuclei (Figure 3-6C). However in the NeuN⁻ nuclei, approximately 10% of the 5mC was also labeled. This is amount of isotope incorporation after four days is considerably less than

what is found in the mESC studies. By 48 hours, wild-type mESC has 30% isotope incorporation for both 5mC and 5hmC. Overall, the neuronal NeuN experiment supports the existence of dynamic regulation of DNA methylation in post-mitotic neurons.

To examine the potential concern that non-specific staining from NeuN antibody to non-neuronal cells, a transgenic mice was used to generate fluorescently labeled postmitotic neurons which could then be later sorted by flow cytometry. Mice with Ai9 transgene contain a red fluorescent protein gene, *tdTomato*, with a floxed stop codon (Madisen et al., 2009). By crossing to a *CamKII α -Cre* mouse line only the postmitotic neurons would be able to get rid of the stop codon and allow the *tdTomato* to be expressed, thus labeling the postmitotic neurons with red fluorescent protein. The expression of *tdTomato* can be seen under the microscope (Figure 3-7A). After 3 DIV, ²H₃-met was added into the media of the primary cortical culture. After 24 hours, the primary cortical culture was fixed and later sorted by flow cytometry (Figure 3-7B). Unfortunately the DNA recovery was not enough for the LC/MS/MS-MRM analysis but portion of the samples were saved before the flow cytometry. Even though the neuronal impurities is in question, there is still isotope incorporation of the 5mC in the treated group of about 1% (Figure 3-8).

The experimental protocols established here will provide an experimental strategy for unraveling the effects 5mC and 5hmC formation in a multitude of other experimental paradigms, including the response to neural activity, environmental stimuli, and pharmacological intervention. Ultimately, this work will lead to a better understanding of the basic mechanisms of DNA methylation and its role in neurological disorders.

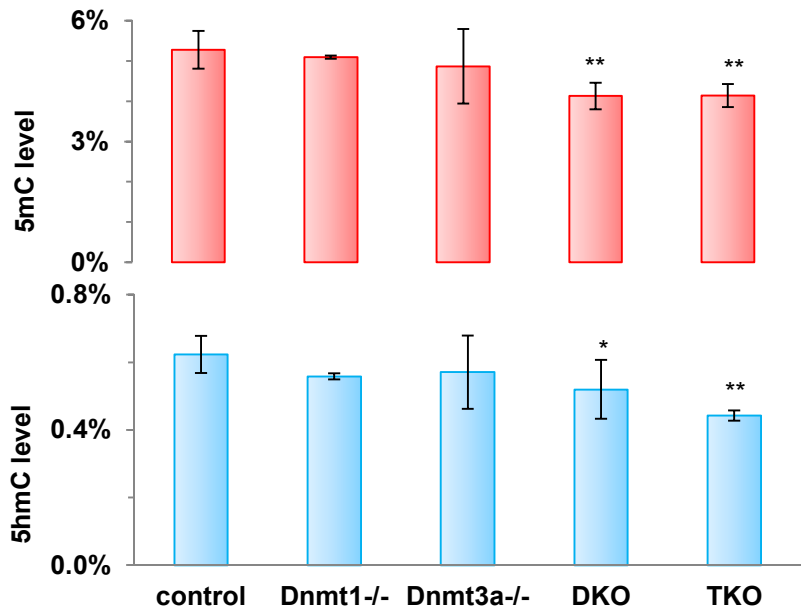


Figure 3-1: 5mC and 5hmC levels in control and DKO cortex using LC/MS/MS-MRM. DNA methylation and hydroxymethylation content are expressed as the percentage of modified cytosine in the total cytosine pool. Data are the mean \pm s.e.m. from replicates of 4 separate samples for each genetic group. Unpaired Student t-test compared to control cortex samples, * P < 0.05 and ** P < 0.005.

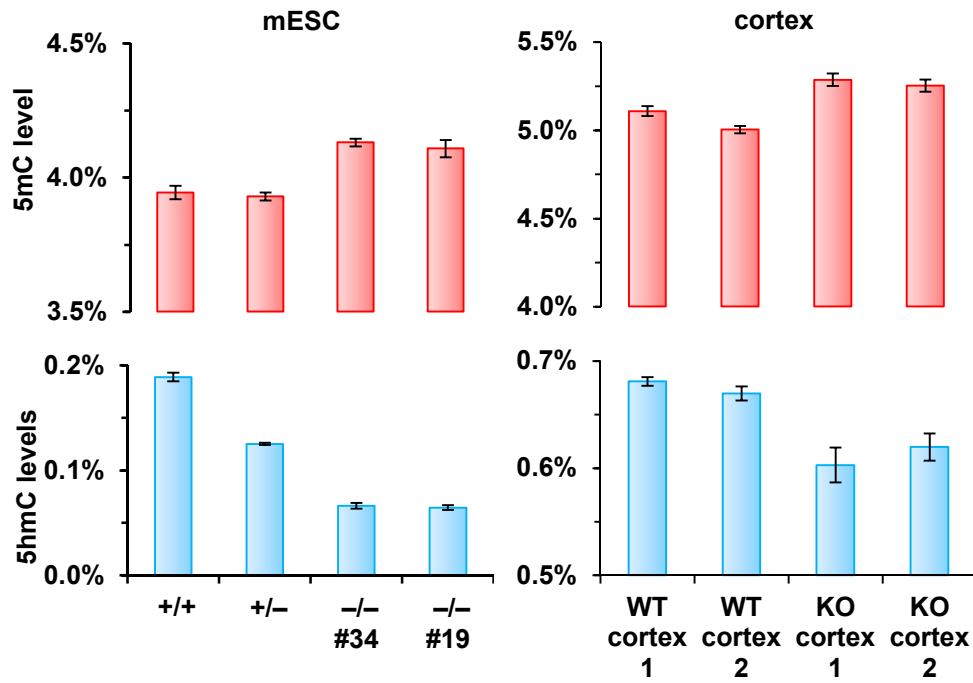
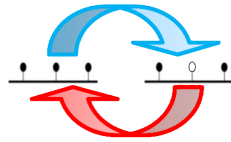


Figure 3-2: 5mC and 5hmC levels in *Tet1*^{-/-} mESC and mouse cortex samples using LC/MS/MS-MRM. Complete loss of *Tet1* reduces 5hmC levels to near half in both mESC and brain cortex compare to control. Heterozygous *Tet1* samples from mESC and cortex have reduce level of 5hmC but not as much compare to the 5hmC level in full *Tet1* knockout.



Control- constant DNA methylation and demethylation driven by Dnmt and Tet, respectively

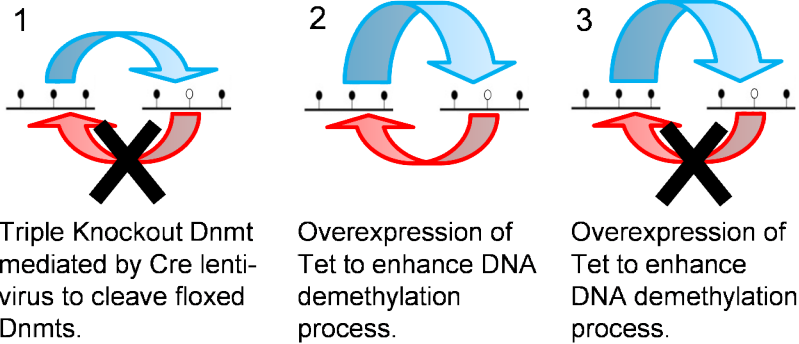


Figure 3-3: Genetic manipulation of the cortical culture to study DNA methylation turnover. The balance activity of DNA methylation and demethylation was disturbed using viral infection to manipulate the genetic condition of the primary cortical culture. The dynamic process can either be manipulated by abolishing the mechanism for DNA methylation altogether using Cre recombinase to knockout all Dnmts (1), enhancing the DNA demethylation activity by overexpression of Tet (2), or both (3).

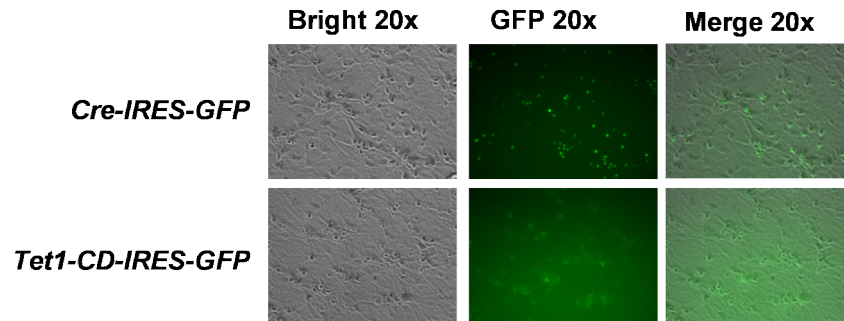


Figure 3-4: Images of cortical culture extracted from *Dnmt* triple-floxed mice pup. Brightfield and fluorescent image of the generated TKO and overexpressed *Tet1* catalytic domain cortical culture at 17 DIV, 14 days post infection.

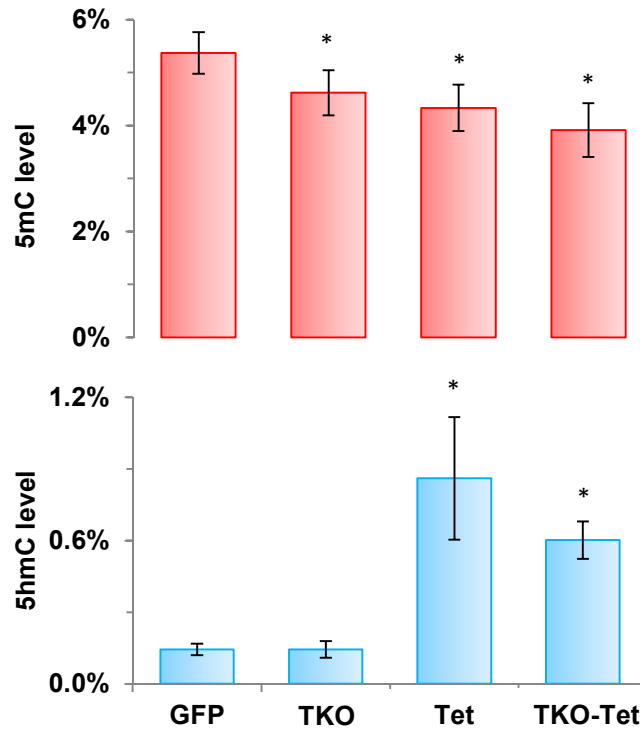


Figure 3-5: 5mC and 5hmC levels in various genetic conditions of the culture conditions. Data are the mean \pm s.e.m. from replicates of 8 separate samples for each genetic group. * Unpaired Student t-test, $P < 0.001$ compared to GFP control cortical culture.

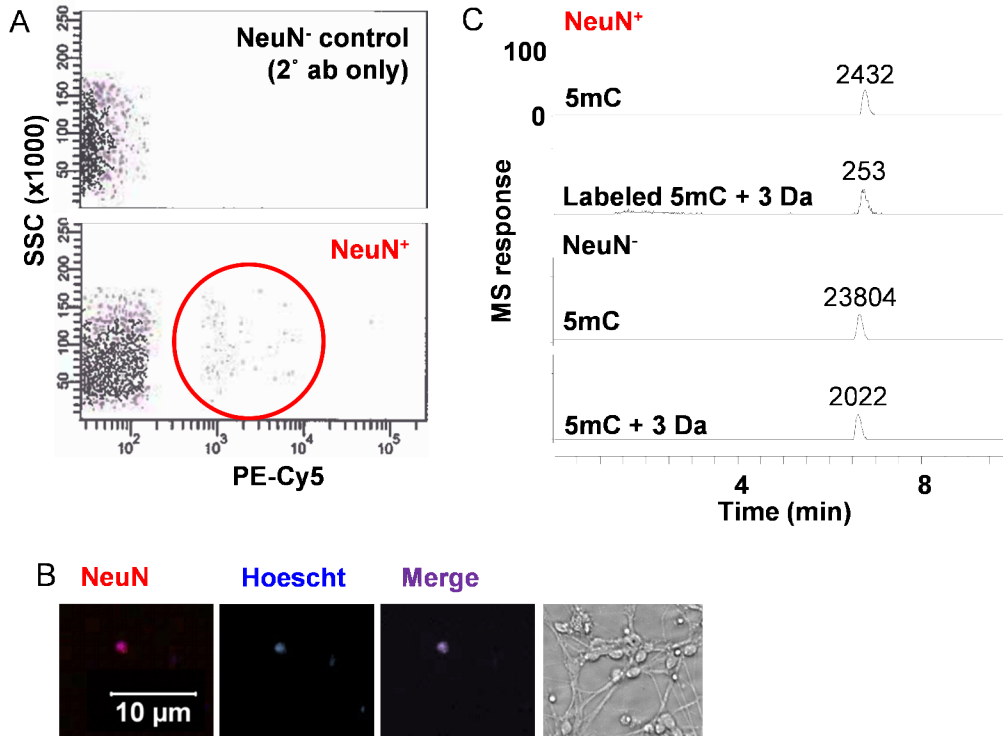


Figure 3-6: Fluorescent-activated cell (FAC) sorting was used to enrich NeuN⁺ population in primary cortical culture. (A) Scatter plot of the population is shown for negative control and NeuN-stained samples. Side scatter light is plotted against the relative log intensity of the red light (PE-Cy5). (B) A small portion of the NeuN⁺ nuclei were re-processed to validate the purity as shown here in the immunostaining. The size of the nuclei is comparable to the cell body of the neurons in brightfield image taken at the same power. (C) MRM response for 5mC (242.1→126.1) and ²H₃-5mC (245.1→129.1) from NeuN⁺ and NeuN⁻ cortical culture after 4 day treatment of ²H₃-met. It is interesting to note that the level of labeled DNA methylation level only went to 10% at 96 hours for both NeuN⁺ and NeuN⁻ population while in mESC it was about 30% in 48 hours.

Ai9 3 DIV

Control 3 DIV

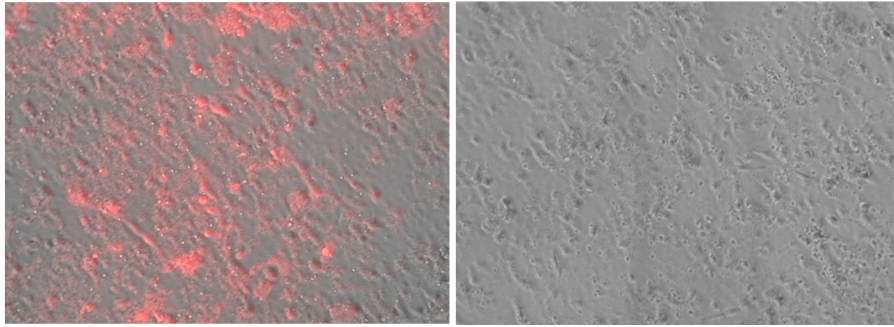


Figure 3-7: Cortical culture extracted from *CamKII α -Ai9* mice P6 pup. Neurons from *CamKII α -Ai9* mice were visualized under fluorescent microscope merged with brightfield image of the same power. In contrast, neurons from control mice that are negative for the *Cre* gene do not express the red fluorescent protein.

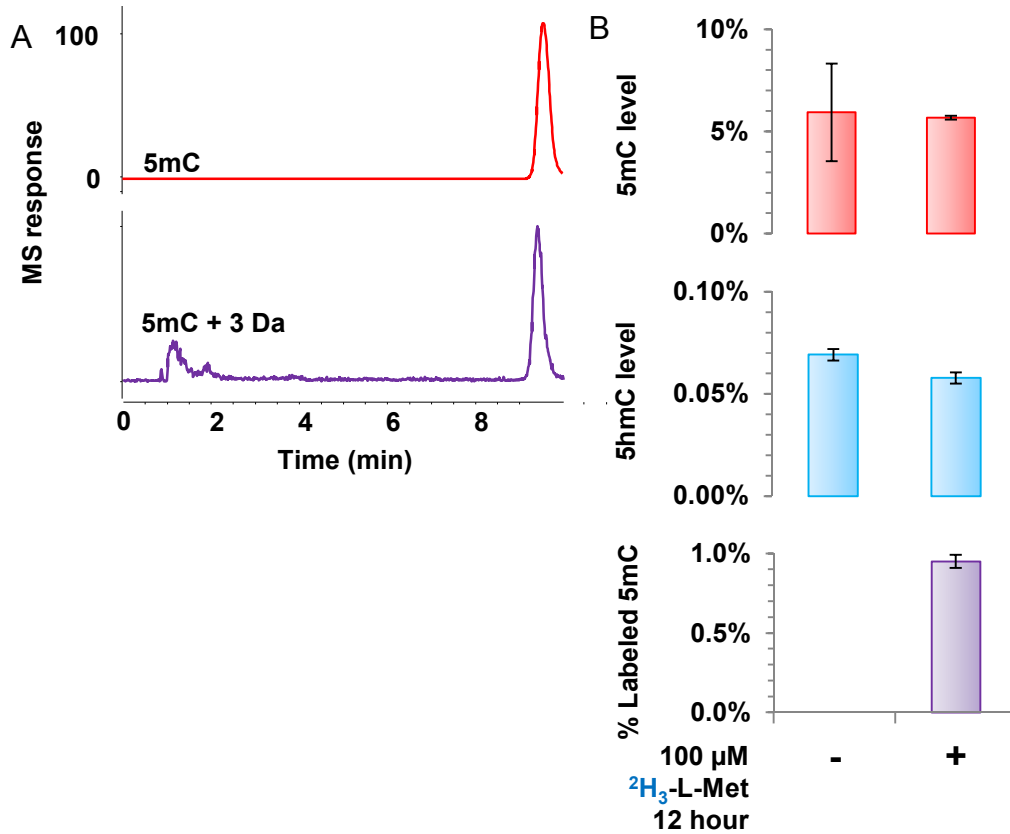


Figure 3-8: $^2\text{H}_3$ -met incorporation for the first 12 hours into 5mC from unsorted cortical culture from *CamKII α -Ai9* mice pup. (A) MRM response for 5mC (242.1 \rightarrow 126.1) and $^2\text{H}_3$ -5mC (245.1 \rightarrow 129.1) from cortical culture after 12 hour treatment of $^2\text{H}_3$ -met. (B) Measured DNA methylation, hydroxymethylation and % labeled 5mC in untreated control and $^2\text{H}_3$ -met treated. Data are the mean \pm s.e.m. from triplicate analysis of one sample for each group.

CHAPTER 4- Future direction and perspective

The advent of next generation DNA sequencing has provided powerful tools to examine the genome-wide DNA methylation pattern with single nucleotide resolution. Major progress has been made to map-out the DNA methylation pattern genome-wide in various biological samples (Lister et al., 2013; Lister et al., 2008; Lister et al., 2009; Meissner et al., 2008; Popp et al., 2010). However these analyses can only give snapshots of DNA methylation pattern and that only by comparison among the different time points or conditions can uncover changes in the DNA methylation pattern. But this kind of data interpretation does not necessary distinguish the actual biological changes of the DNA methylation from the stochastic nature of the DNA methylation pattern within each cells, particularly for those DNA methylation changes that exhibit less than 100%.

To address this issue directly, methods utilizing the combination of liquid chromatography and tandem mass spectrometry were developed to greatly facilitate research into epigenetic mechanism in postmitotic neurons and other mammalian cells. Here in this thesis, two LC/MS/MS-MRM methods were established to measure global level and kinetic rates for both DNA methylation and hydroxymethylation levels in biological samples. The kinetic activities can be determined by measuring rate of incorporation of isotope-labeled 5mC and 5hmC in a time-course experiment. Both the LC/MS/MS-MRM methods are fast and can easily be setup for high-throughput analysis. And the development of the LC/MS/MS-MRM methods has led better understanding on how the two cytosine modifications are related in their levels and the kinetics of these

processes. The global levels of DNA methylation and hydroxymethylation are robustly related in mESCs. The kinetic rate of DNA methylation determined by the genetic combination of the *Dnmts* was positively correlated to its DNA methylation level. In addition, the kinetics rate reflects the functionality of the Dnmt in mESCs. Furthermore, it was found that about 10-20% of neuronal DNA methylation sites undergo active turnover, supporting the concept of a dynamic neuronal methylome. Further works are underway to measure the individual Dnmt contribution to the overall DNA methylation kinetics in postmitotic neurons using transgenic mouse models. By investigating the kinetics of the two DNA modifications, factors with immediate impact on development and disease can be illuminated. Overall, the LC/MS/MS-MRM methods are valuable for measuring the kinetics of global DNA cytosine methylation and hydroxymethylation, and can easily be applied to demonstrate the effects of pharmacological intervention and environmental factors on the kinetics of these important processes.

Inevitably, a major shortcoming to these LC/MS/MS-MRM methods contains no DNA sequence information. One tactic is to develop DNA digestion protocol in which the genomic components are reduced to dinucleoside rather than single nucleoside form. With appropriate standards and making necessary adjustments on the mass spectrometer, it is possible to quantitate dinucleoside without much difficulty similar to nearest neighbor analysis (Gruenbaum et al., 1981; Ramsahoye et al., 2000). Changes made to the DNA digestion protocol in which the CpG and non-CpG dinucleoside remain intact could yield information on the methylation and activity status for these two DNA contexts which are heavily studied in the brain. Recent discovery uncovered a significant accumulation of methylated non-CpG level in the neuronal genome and this

coincides with synaptogenesis (Lister et al., 2013). Combined with transgenic mouse model and this propose tactic, it is possible to investigate roles of each Dnmt in non-CpG methylation and would greatly complement the works done on the next generation DNA sequencing.

The next step is to apply this method to analyze neural tissues from patients with neural disorders to gain new insights into the etiology of psychiatric illness and open new avenues of therapy. Careful sampling is necessary to yield fruitful data. Earlier works have been marred with sample heterogeneity. For example, NeuN antibody have been used to enrich for neurons in FAC sorting (Iwamoto et al., 2011; Jiang et al., 2008; Lister et al., 2013); however neurons exhibit diverse cell types to accommodate the complexity of the brain circuitry. The use of transgenic mice to generate fluorescently labeled genetically-defined neuronal cell type can further resolve the sample heterogeneity issue that have plagued the field (Lobo et al., 2006; Madisen et al., 2009).

Because DNA methylation and hydroxymethylation exhibit steady states that are reproducible across many individual samples, these epigenetic markers are likely result from regulated processes. However it remains elusive how extensive both DNA methylation and hydroxymethylation levels in neurons result from targeting mechanism involving proteins that function as a guide for the Dnmts to methylate certain genomic region. Alternatively, genome regions from accessible chromatin structure or active transcriptional activity can be exposed to Dnmts and allow DNA methylation to occur unless there are proteins that would prevent DNA methylation.

Although evidence support regulatory roles for DNA methylation and hydroxymetylation in the brain (Lister et al., 2013), the mechanisms by which these DNA modifications affect gene expression await elucidation. Notably, MeCP2 binds to both DNA methylation and hydroxymethylation in the nervous system (Mellen et al., 2012) and the disruption of the MeCP2 causes neurodevelopmental disorder Rett syndrome (Amir et al., 1999). It is essential to understand the regulatory mechanism in which the DNA methylation and hydroxymethylation pose in the developed brain by fully integrating major epigenetic studies, including histone modification and microRNA. In order to fully understand how gene expression is regulated within the nervous system future research must consider the epigenome as a whole. This type of analysis will reveal DNA binding factors that are recruited to the DNA modification sites. By dissecting the biological mechanisms that mediate crosstalk among these biological mechanisms and integrating high-throughput data, we can begin to study the epigenome as a whole. Finally for a complete understanding of how the epigenome regulates gene expression, future research will have to uncover the biological mechanisms that mediate activity-dependent changes in the epigenomic landscape of the mammalian brain.

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