EPIGENETIC EFFECTS OF DIETARY SUPPLEMENTATION AND NUTRITION

By

JASON ORR BRANT

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To my Mom

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Jason Orr Brant

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DNA methylation is associated with long-term repression of transcription, and has generally been considered a fairly stable epigenetic mark. Changes in DNA methylation patterns were thought to normally only occur during embryonic and germ cell development, however recent reports have indicated nutritional insults, exposure to environmental toxins and aging can, in fact, alter DNA methylation patterns. Two experimental approaches were utilized in order to gain a better understanding of how nutritional insults result in altered DNA methylation. The first approach focused on the effects on DNA methylation due to exposure to a low protein diet *in utero*, on both a genome-wide scale and at specific loci. The results indicate that there were subtle changes in DNA methylation occurring at differentially methylated regions within the *H19*/*Igf2* imprinted domain in animals exposed to a low protein diet during development. Additionally, it was demonstrated that the expression of *H19* and *Igf2* was also altered. These findings are important in that they indicate that exposure to nutritional insults *in utero* can induce epigenetic changes in offspring, however the genome-wide analysis indicates that that was no major changes occurring in DNA methylation in low protein compared to normal protein animals. The second approach involved examining the effects of folic acid supplementation and withdrawal in Chinese women of child bearing age on DNA methylation. Although the success

in prevention of neural tube defects has been dramatic, and folic acid is generally considered to be safe, the long-term consequences of increased folate levels have yet to be extensively studied in long term clinical trials. Folate plays a major role in one carbon metabolism, and is involved in the methylation of DNA. In order to examine the effects of increased folate levels on DNA methylation, blood samples were analyzed from a population-based, randomized trial of folic acid supplementation and withdrawal. The results indicate that folic acid supplementation and withdrawal produced changes in DNA methylation in a locus-specific manner. Methylation-Specific PCR of the promoters of tumor suppressor genes (TSGs) indicate that there was an observable increase in DNA methylation after 6 months of folic acid supplementation in two out of ten subjects analyzed, which was no longer detectable after 3 months of withdrawal. No wide-spread hypermethylation of TSGs were detected. There were also dramatic changes in DNA methylation at the maternally imprinted *SNRPN* promoter, with a near complete loss of DNA methylation after 6 months of folic acid supplementation, and a complete loss after 3 months of folic acid withdrawal. Analysis of the L1 repetitive element determined that there was no major change in DNA methylation occurring at this element. Overall, this study has demonstrated that exposure to nutritional insults *in utero* can induce both changes in DNA methylation and in gene expression levels, and that dietary supplementation and withdrawal in an adult population can induce changes in DNA methylation. These results warrant further studies into the biological significance of these observed changes.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Epigenetics

Epigenetics is a process or mechanism that affects heritable changes in gene expression independent of the underlying linear DNA sequence. Epigenetic mechanisms include modifications of chromatin that can shape the transcriptional memory of a cell. This collection of modifications complements the genetic information to determine which genes are transcribed and at what levels (Jaenisch and Bird, 2003; Wu and Morris, 2001). Epigenetic modifications can be stable and heritable through cell mitosis and meiosis. However, these epigenetic marks are also dynamic and may undergo both global and locus-specific changes, especially during development and differentiation, to establish appropriate levels of transcription in a cell-type specific manner, and throughout specific stages of development (Li, 2002; Reik et al., 2003). Once established, these epigenetic modifications can then be maintained in terminally differentiated cells.

DNA Methylation

Methylation of DNA is an important epigenetic modification in mammalian genomes. The covalent addition of a methyl group to the 5 carbon position of cytosine is catalyzed by a reaction involving of family of proteins known as the DNA methyltransferases (DNMTs), and in mammals occurs predominately in the context of CpG dinucleotides (Robertson, 2002), which are underrepresented and non-randomly distributed in the genome (Jones and Takai, 2001; Wilson et al., 2007). The mammalian genome consists mainly of CpG poor regions punctuated by CpG rich regions, termed CpG islands (Miranda and Jones, 2007), which were first defined by Jones et al. as being longer than 500 bases, having a GC content greater than 55% and an observed CpG/expected CpG ratio of 0.65 (Jones and Takai, 2001). The majority of CpG islands

are found within promoter regions, and about 40% of genes contain CpG islands within the 5' region of the gene (promoter, untranslated region (UTR), and first exon) (Jones and Baylin, 2002). The majority of CpG islands are hypomethylated, whereas CpG poor regions, such as intergenic and intronic regions tend to be hypermethylated (Miranda and Jones, 2007). The mammalian genome contains only a small amount of protein coding DNA, with the overwhelming majority of the genome consisting of introns, repetitive elements, and parasitic DNA. Repetitive and parasitic DNA contains potentially active transposable elements that need to be stably silenced to ensure genomic stability and maintain integrity of the genome (Jones and Baylin, 2002; Jones and Laird, 1999). The DNMT family of proteins can be loosely categorized into two main types, based upon their preferred DNA substrate and timing of methylation. *De novo* DNA methylation, which is catalyzed by DMNT3a and DNMT3b (Okano et al., 1999), establishes the methylation patterns during gametogenesis, post-fertilization fetal development and in the differentiation of cells. Once established, these methylation patterns need to be stably maintained throughout mitosis and are done so through the actions of the maintenance methyltransferase, DNMT1. DNMT1 preferentially recognizes hemimethylated DNA and remethylates newly synthesized daughter strands of DNA during replication, thus ensuring the methylation patterns are maintained during mitosis (Bacolla et al., 1999; Flynn et al., 1996; Glickman et al., 1997; Pradhan et al., 1997; Yokochi and Robertson, 2002).

Role of Methylation

Although DNA methylation is generally associated with transcriptional repression, the exact mechanism of how DNA methylation leads to repression is unclear. DNA methylation has been implicated in a diverse array of processes, including transcriptional regulation, X-chromosome inactivation, silencing of repetitive DNA and transposable elements, genomic imprinting, chromatin structure and genomic stability (Baylin et al., 2001; Jones and Laird, 1999;

Robertson, 2001). Although the mechanism of repression is still not clear, numerous models for how DNA methylation represses transcription have been proposed. One model proposes that DNA methylation directly interferes with the binding of regulatory proteins and transcription factors to their target sites. This inability of transcription factor binding leads to repression (Deng et al., 2001; Eden and Cedar, 1994; Rhodes et al., 1994). However, most models suggest that DNA methylation changes the interactions between DNA and proteins, thus inhibiting transcription initiation or that DNA methylation leads to conformational changes in chromatin structure which lead to chromatin condensation and to transcriptional repression (Harikrishnan et al., 2005; Jones et al., 1998; Nan et al., 1998). The methyl-binding domain (MBD) proteins (Hendrich and Bird, 1998; Sansom et al., 2007) are a family of proteins that can bind to methylated DNA and recruit other repressive complexes via association with a transcription repressor domain (TRD) (Nan et al., 1998). This allows MBPs to target chromatin remodeling proteins, such as histone deacetylases (HDAC), to methylated DNA (Feng and Zhang, 2001), as well as to form complexes with other MBD family members and DNMT1 to ensure repression is maintained after replication (Feng et al., 2002).

The role of DNA methylation in transcriptional repression of repetitive elements is of critical importance. The majority of the mammalian genome is comprised of repetitive elements, many of which contain long terminal repeats (LTRs) capable of functioning as promoters if not silenced, allowing the transcription and spreading of these parasitic DNA elements. (Yoder et al., 1997). The movement of these parasitic elements can have devastating effects on the integrity of the genome, as their expression can lead to non-allelic recombination, as well as insertion into and subsequent disruption of genes, altering their transcription and or function (Kazazian and Moran, 1998). Cells in which DNMT1 have been knocked out contain only about 30% of their

global DNA methylation levels and have a ten-fold increase in chromosomal rearrangements (Chen et al., 1998).

Repetitive Elements

Repetitive sequences make up about 45% of the human genome (Rollins et al., 2006) (Lander et al., 2001). Repetitive sequences are divided into 4 categories: DNA transposons, which are about 3% of the human genome, retrotransposons, and endogenous retroviruses, which range from 40-42% of the human genome and satellite DNA which are simple repeat sequences, with repetitive units ranging from 2-70 bases aligned in tandem. Retrotransposons and endogenous retroviruses can be further classified based on the presence or absence of a LTR. Among the non-LTR repetitive elements, there are both autonomous elements, capable of integrating copies of itself without assistance, and non-autonomous elements, which require the action of other elements in order to become integrated into the genome. Non-LTR autonomous elements are also known as Long Interspersed Nuclear Elements, or LINE 1, or simply L1 elements. The most prominent member of the non-autonomous elements is comprised of Short Interspersed Nuclear Elements, or SINEs, of which the *Alu* family of repeats is the most abundant member (Reviewed by Wilson et al.) (Wilson et al., 2007).

With the discovery that tumors show global hypomethylation of repetitive elements in relation to tumorogenesis and cancer (Riggs and Jones, 1983), analyzing the methylation status has led to important insights. For instance, both LINE's and SINE's have been found to be hypomethylated in cancer cells. Additionally, satellite DNA has also been reported to become hypomethylated in a variety of cancers (Jeanpierre, 1994). Satellite 2 (Sat2) is predominately localized in the pericentromeric heterochromatin of select human chromosomes. Additionally, Sat-α, the major component of human centromeres (Lee et al., 1997), also becomes

hypomethylated in cancers (Ehrlich, 2002). It is estimated that 35-40% of all methylation in the genome occurs in repetitive elements (Bestor, 1998; Kochanek et al., 1993; Schmid, 1998). For this reason, examining the methylation levels of various types of repeat DNA is an excellent surrogate marker for analyzing total genomic methylation levels (Yang et al., 2004).

Global DNA Methylation Levels

In healthy somatic human cells, CpG methylation ranges from 70-90% (Miranda and Jones, 2007). These methylated dinucleotides comprise approximately 0.75-1% of the total bases in the human genome (Ehrlich et al., 1982; Tuck-Muller et al., 2000). As discussed earlier, most CpG islands are unmethylated, while the remaining CpG dinucleotides found within intergenic and intronic regions are hypermethylated. Some CpG islands within gene promoters become methylated as a normal process of development and tissue specific differentiation, genomic imprinting, as well as during the process of X-chromosome inactivation in females, and as a consequence of the aging process (Hellman and Chess, 2007; Jones, 1999; Reik et al., 2001; Richardson, 2003). However, it is becoming increasingly clear that abnormal methylation, be it locus-specific hyper or hypomethylation, or global hypomethylation can lead to aberrant gene expression and contribute to disease formation. Hypermethylation induced gene silencing at the promoters of tumor suppressor genes can greatly increase the risk of initiation of cancer development. For example, methylation-mediated silencing of the DNA repair protein *MLH1* has been shown to be a factor involved in colorectal cancer and an increase in microsatellite instability (Cunningham et al., 1998) A now well recognized hallmark of cancer is global hypomethylation with locus-specific hypermethylation, prompting Feinberg and colleagues to state “Although individual genes vary in hypomethylation, all tumors examined so far, both benign and malignant, have shown global reduction of DNA methylation. This is a striking feature of neoplasia.” (Feinberg et al., 2006).

Transposable elements, which are normally silenced by DNA methylation, are DNA sequences with the ability to integrate into the genome at different sites. As discussed above, DNA transposons, retrotransposons and endogenous retroviruses account for almost half of the mammalian DNA content (Kazazian, 2004). The two most abundant types of retrotransposons are autonomous LINE family and the non-autonomous SINE family. LINE, or L1 elements, possess a strong internal promoter and contains 2 open reading frames (ORF's) which encode for an RNA binding protein and a reverse transcriptase with endonuclease activity (Feng et al., 1996; Gama-Sosa et al., 1983) which enable them to integrate anywhere in the genome. Most L1 elements exist in a state where the 5' end is truncated (Ostertag and Kazazian, 2001), the ORFs contain mutations (Skowronski et al., 1988), or both, rendering them essentially inactive. However some subfamilies can still be transcribed when activated, with an estimated 80 to 100 active L1 elements per diploid genome (Brouha et al., 2003; Deininger et al., 2003; Kazazian, 2004). LINE hypomethylation can occur early in cancer initiation, and hypomethylation has been observed in a number of cancers relative to adjacent healthy tissue (Hoffmann and Schulz, 2005; Suter et al., 2004; Takai et al., 2000). The SINE family is a non-autonomous element which relies on the action of the L1 proteins to facilitate transposition (Dewannieux et al., 2003). The most abundant SINE's in humans is the *Alu* family of repetitive elements. *Alu* does not encode any proteins, but has expanded to over 1.1 million copies and now accounts for 11-13% of the human genome (Esnault et al., 2000; Wei et al., 2001) Although *Alu* elements are methylated in somatic cells, the maintenance of methylation appears to vary greatly between individuals (Sandovici et al., 2005). *Alu* demethylation has not been extensively studied in disease and does not seem to correlate well with global hypomethylation (Weisenberger et al., 2005).

DNA Methylation and Development

Although DNA methylation is generally considered to be a fairly stable epigenetic mark, there are two developmental periods when dynamic changes in genome-wide DNA methylation patterns occur; during embryonic and germ cell development. Embryogenesis begins with fertilization to form a single-celled zygote, which progresses to form a multicellular organism with over 200 functionally distinct and diverse cell types (Mann and Bartolomei, 2002). Each differentiated cell type has its own epigenetic signature which reflects genotype, developmental history and environmental influences, which is then manifested in the cell's phenotype (Nafee et al., 2008). During normal development, these cells must undergo major epigenetic reprogramming. During germ cell development, a significant part of the genome is demethylated, and becomes remethylated in a cell or tissue-specific manner (Reik et al., 2001).

The first phase of methylation reprogramming occurs after fertilization and before the formation of the blastocyst. Post-fertilization, a rapid paternal-specific loss of methylation is observed (Dean et al., 2003; Mayer et al., 2000; Oswald et al., 2000). This putative active-demethylation is complete before replication begins in the paternal pronucleus but spares paternally imprinted genes, heterochromatin around centromeres, and some repetitive elements (Morgan et al., 2005). During subsequent replication in the embryonic tissue, there is a passive loss of methylation due to a lack of maintenance methylation during DNA replication (Bestor, 2000) Upon implantation, *de novo* methylation begins to reestablish DNA methylation patterns, thus ensuring proper patterns of gene expression within the developing embryo (Li, 2002; Reik et al., 2001; Santos and Dean, 2004) (figure 1-1).

Imprinting

In diploid organisms, the overwhelming majority of autosomal genes are expressed from or repressed on both parental copies, in a biallelic manner. There is, however, a subset of genes,

known as imprinted genes, in which the transcriptional competence of the gene is determined by parental inheritance. This results in functionally different alleles of the same gene within the same cell. As the DNA sequence of these alleles is essentially identical, the functional differences must be imparted through an epigenetic modification of one or both alleles (Reik and Walter, 1998; Tilghman, 1999). Genomic imprinting was first discovered almost 25 years ago as a result of nuclear transplantation experiments in mouse (McGrath and Solter, 1983, 1984; Surani and Barton, 1983; Surani et al., 1984, 1986). These elegant experiments demonstrated that mammalian development requires genetic information from both the paternal and maternal genomes. Both diploid androgenetic and diploid gynogenetic embryos failed to thrive, suggesting that there were genes exclusively expressed from one parental genome and the failure to thrive of uniparental embryos was due to loss of function of these genes. The first imprinted gene to be discovered was the Insulin-like growth factor 2 gene (*Igf2*), which is a fetal-specific growth factor. Targeted mutations in this gene resulted in a heterozygous fetal undergrowth phenotype, but only when the mutated gene was inherited paternally (DeChiara et al., 1991). Additionally, the extent of the growth inhibition was identical in paternal heterozygotes and homozygotes, indicating that the entire extent of *Igf2* activity was contributed from the paternal genome (DeChiara et al., 1991). In the last decade and a half, close to 200 genes have been identified as imprinted or are predicted to be imprinted. (Information on the current state of imprinted genes can be found at geneimprint.com)

Imprinted genes tend to be clustered together and organized in large chromosomal domains (Delaval and Feil, 2004; Ferguson-Smith and Surani, 2001; Reik and Walter, 2001). These imprinted domains generally contain both paternally and maternally imprinted genes, as well as both protein coding genes and non-coding RNA genes. This chromosomal organization

of imprinted genes is often conserved among mammalian species (Reik and Walter, 1998; Verona et al., 2003). Each imprinted cluster is generally under the control of a *cis*-acting element, termed the imprinting control region (ICR), which coordinately regulates gene expression throughout the imprinted domain (Reik et al., 2003). The ICR acquires differential DNA methylation patterns in a parent of origin manner in the developing germ cells, when the parental genomes are separated, thus allowing differential modification (Figure 1-2). These methylation marks are then stably maintained during development in all tissues where the imprint is recognized. This differential methylation mark must also be able to be erased and "reset" to a parent of origin specific imprint during germ cell development (Delaval and Feil, 2004).

The *H19*/*Igf2* imprinted domain is probably the most well-studied imprinted domain. The imprinted locus is located on distal mouse chromosome 7 (Figure 1-3) and has been implicated in a number of congenital growth abnormalities (Henry et al., 1991). Insulin-like growth factor 2 (*Igf2*) encodes a fetal-specific growth factor that is widely expressed during mouse fetal development from the paternal allele, and is of particular importance in placenta growth (Constancia et al., 2002). In mice, *Igf2* is highly expressed in the embryo, but expression is essentially nonexistent in the adult (DeChiara et al., 1991). In adult humans, *IGF2* is expressed biallelically from an adult-specific promoter (de Pagter-Holthuizen et al., 1987), which appears to have evolved to become inactivated in mice (Rotwein and Hall, 1990). Downstream of *Igf2* is the maternally imprinted *H19* gene which encodes a non-translated RNA (Bartolomei et al., 1991). *H19* is also highly expressed during fetal development, particularly in tissues of mesoderm and endoderm origin (Bartolomei and Tilghman, 1997; Poirier et al., 1991). Human and mouse *H19* have extensively conserved secondary structure characteristics, suggesting that

although the RNA is not translated, it may play an important biological role (Brannan et al., 1990). Located 2-4 kb upstream from the maternally imprinted *H19* gene is a differentially methylated region (DMR) which is hypermethylated on the paternally inherited allele (Brandeis et al., 1993; Tremblay et al., 1997). This DMR acts as an imprinting control region (ICR) for a rather complicated hierarchy of multiple DMRs that are responsible for maintaining proper imprinted gene expression throughout this domain. *Igf2* contains four separate promoters and three different DMRs (Lopes et al., 2003). The 5' most promoter and DMR (DMR0) of *Igf2* has been shown to be placenta specific, and is the only maternally methylated DMR in the region (Moore et al., 1997). The paternally methylated DMR1 is located upstream of the fetal *Igf2* promoters and contains a methylation-sensitive silencer (Constancia et al., 2000; Eden et al., 2001). DMR2 is located in the last exon of *Igf2* and contains a methylation-sensitive activator (Murrell et al., 2001) (Figure 1-3).

Expression of both *H19* and *Igf2* appears to be under the control of multiple downstream enhancer elements that can modulate expression of either *H19* or *Igf2*. The *H19* DMR, or ICR, contains binding sites for CCCTC binding factor (CTCF). Methylation of the DMR prevents binding of CTCF to the paternal allele, thus allowing the enhancer to act on the *Igf2* promoter. On the maternal allele, CTCF binds to the unmethylated maternal allele and acts as an insulator, blocking enhancer access to *Igf2*, and driving expression at the unmethylated maternal *H19* promoter (Figure 1-4) (Bell and Felsenfeld, 2000; Hark et al., 2000).

Nutrition and DNA Methylation

Throughout most western countries, cardiovascular and respiratory diseases and cancer account for fully three quarters of all mortalities in adults (Murray and Lopez, 1994). For this reason, chronic diseases are becoming the major focus of health care related problems. There is a growing body of evidence suggesting that chronic diseases may originate in response to

nutritional insults during *in utero* development (Roseboom et al., 2001b) (Lillycrop et al., 2005; Waterland and Jirtle, 2003). In both human and animal studies, both epidemiological and experimental, evidence suggest nutrient deprivation *in utero* can have adverse long-term effects on the metabolic and physiological states of offspring. Epidemiological studies looking at standard obstetric birth records show that low birth weight has been linked with increased incidence of hypertension (Law et al., 1991), non-insulin-dependent diabetes (Phillips et al., 1994), chronic bronchitis and coronary heart disease (CHD) (Barker et al., 1990). Cancer has been linked with a high birth weight in similar epidemiological studies (Hjalgrim et al., 2003; Michels et al., 1996; Michels and Xue, 2006). Unfortunately the intrauterine conditions that led to these results cannot be determined by these types of studies, and many factors could contribute to low birth weight. The association between small birth weight and CHD has been studied in several countries (Barker et al., 1990; Barker and Osmond, 1986; Barker et al., 1993; Barker et al., 1989; Hales et al., 1991). These studies depended on size at gestational age (SGA), rather than prematurity (Eriksson et al., 1999; Leon et al., 1998; Osmond et al., 1993). Death rates among men who were thin at birth, as recorded by a low ponderal index, but had accelerated weight gain during childhood had the highest death rates from CHD. The ponderal index determines an individual's leanness and is similar to the body mass index (BMI). These men had a 5-fold increase in mortality compared to men with high birth weights that were lean in childhood. This effect rate is the highest observed in cardiovascular epidemiology. The authors suggested that the increased death rate may have been due to poor prenatal nutrition followed by improved postnatal nutrition (Eriksson et al., 1999). The results of these epidemiological studies have led to the Barker Hypothesis, or the "fetal origins hypothesis", which states that the fetus

adapts to a limited supply of nutrients *in utero*, which in turn permanently alters its physiology and metabolism, leading to an increased risk of adult onset disease (Barker, 1995).

The Dutch famine of 1944-45 has provided a wealth of epidemiological data relating malnutrition during development to adult onset chronic degenerative diseases. During World War II, due to a ban on all food transport in the Netherlands, and an unusually harsh winter which blocked passage from the rural east to the urban west, the daily food rations fell below 1000 calories per day, and at the height of the famine, between 400 and 800 calories per day in the western cities of the Netherlands. During this disastrous famine, women were still able to conceive and give birth, and these offspring were studied throughout their lives, and have provided an enormous opportunity to study the effects of malnutrition during gestation and correlate this to health related issues in adult life (Roseboom et al., 2001a). For the study, the obstetric records of 2424 offspring were included, and 741 adults agreed to attend the clinic for extensive measurements. Three periods of 16 weeks were used to distinguish between babies exposed during early, mid and late gestation. Babies born before the famine, or those conceived after the famine were termed unexposed and used as comparison. People exposed to famine in early gestation appeared to have a higher risk of coronary heart disease (Ravelli et al., 1999), had a higher BMI (Roseboom et al., 2000a; Roseboom et al., 2000b) and a more atherogenic lipid profile (Roseboom et al., 2001a), and those exposed to famine in later stages of development showed increases in the occurrence of obstructive airways disease (Lopuhaa et al., 2000). People who were exposed to famine in early gestation were also more likely to rate their overall health as poor (Roseboom et al., 2001b). Critics have pointed out that many variables, besides extreme shortage of food, must also be taken into accountl (Huxley et al., 2002; Huxley and Neil, 2004). The famine coincided with an unusually harsh winter. This, combined with the stress of war,

absence of their spouses, and a general decline in basic services, coinciding with widespread infection, are confounding factors that need to be considered. However, the mechanism by which early nutritional insult leads to an increase in disease susceptibility and altered metabolism in adult life, remains, at best, poorly understood. One possibility is that epigenetic changes in the placenta and/or fetus due to exposure to maternal malnutrition during development may lead to stable, heritable, aberrant changes in the regulation of genes important for proper placental and fetal development. As discussed earlier, methylation of CpG dinucleotides is a well characterized epigenetic modification generally involved in silent chromatin. As DNA methylation patterns are reprogrammed in the early embryo and maintained throughout adult life, it is possible that early deprivation of nutrients could lead to alterations within the establishment of proper methylation imprints (Waterland and Garza, 1999). Many genes responsible for proper placental and fetal development are imprinted in placental mammals, (Constancia et al., 2002) and since methylation imprints are established during gametogenesis (Reik et al., 2001), it has been proposed that exposure to nutritional insults during gametogenesis and placental and fetal development will lead to alterations in the epigenetic states of imprinted genes, specifically aberrant DNA methylation at differentially methylated regions (Lillycrop et al., 2005; Waterland and Garza, 1999). This hypothesis will be discussed in greater detail below.

Developmental Origins of Health and Disease Hypothesis

As early as the time of Hippocrates, the concept that adult diseases may originate during the process of development has been proposed. Since then, epidemiological studies and experimental data have contributed to an ever changing hypothesis. As recently as 40 years ago, Rose published observations of familial patterns of coronary heart disease (Rose, 1964). Shortly thereafter, Fordsdahl showed that poor living conditions in early life were important risk factors for arteriosclerotic heart disease (Forsdahl, 1977). These hypotheses were greatly expanded by

the work of Barker et al. (Barker, 1995; Barker and Osmond, 1986), which would later lead to the concept of the fetal origins of adult disease. Barker termed this the "thrifty phenotype", which states that environmental cues during development may influence development in such a way as to prepare the fetus for a predicted environment in adult life. This concept of developmental plasticity suggested that these adaptations may alter metabolism in a way as to be detrimental in later life. At the same period as Baker and colleagues were performing their work, Trichopoulos proposed a similar hypothesis, the fetal origins of cancer, for the origination of breast cancer *in utero* (Trichopoulos, 1990). Realization that developmental plasticity extends into the postnatal period led to the term developmental origins hypothesis (Gluckman and Hanson, 2004). Waterland and Garza demonstrated that epigenetic changes can occur in a limited period of opportunity during development and that these changes persist into adulthood and this hypothesis was termed metabolic imprinting (Waterland and Garza, 1999). All of these refinements in nomenclature of these hypotheses were intended to highlight specific biological mechanisms that could be unified into a single hypothesis, now termed the developmental origins of health and disease (DOHaD) (Waterland and Michels, 2007). Thus, when conducting epidemiological studies of adult disease, it is important to recognize that both the genome and the epigenome interactively influence sensitivity to disease in adult life (Dolinoy et al., 2007).

Folic Acid and DNA Methylation

Folate is a water soluble B vitamin that is essential for the synthesis of nucleotides and plays a major role in one carbon metabolism (Mackenzie, 1984). Folate, in its various coenzyme forms, acts as both a methyl donor and acceptor in one-carbon metabolism, with its most prominent role being involved in the remethylation of homocysteine to methionine (Figure 1-5.). Methionine is a precursor of S-Adenosylmethionine (SAM), which serves as the universal one-carbon donor involved in methylation of DNA, RNA, lipids and proteins (Lamprecht and Lipkin,

2003; Lucock, 2000)(Figure 1-5). Because of this essential role, perturbations in the levels of folate can have profound effects on both nucleotide synthesis and methylation of DNA, both of which are critical in maintaining the integrity of DNA and the proper regulation of gene expression (Dolinoy and Jirtle, 2008; Robertson, 2005). Folate plays an important role in the pathogenesis of several disorders, including anemia, cardiovascular disease, and developmental abnormalities such as neural tube defects (NTD's) (Kim, 2003), and has been linked to an increased risk of developing several types of cancer, including colon, pancreas and possibly breast cancers (Kim, 2007; Larsson et al., 2006; Ulrich, 2007).

There is an overwhelming body of evidence in support of the benefits of periconceptional supplementation with folic acid, a synthetic, oxidized form of folate, in the reduction in neural tube defects, and it is recommended that women of child-bearing age consume 400 μg of folate daily. As this level of intake was generally not being achieved in the United States population, a program of mandatory, nation-wide fortification of flour and uncooked cereal grains with folic acid was implemented in 1998 in the US, and shortly thereafter in Canada. After the fortification program began, plasma folate concentration increased by 100% along with a reduction in homocysteine levels, resulting in a 20-50% decrease in the incidence of NTD's (Honein et al., 2001; Jacques et al., 1999; Ray, 2004). Interestingly, it was observed that following the implementation of this program, there was a temporal association between folic acid fortification and an increase in colorectal cancer rates in both the US and Canada (Mason et al., 2007). Although it is impossible to draw a causal link between these two events, it has been suggested that an excess of folic acid could have promoted the growth of previously undetected preneoplasia (Song et al., 2000a; Song et al., 2000b). Although the success in prevention of NTD's has been dramatic, and folic acid is generally considered to be safe, there are concerns

that the levels of folic acid intake may be far higher than initially expected. This is causing some concern, as the long term effects of folic acid exposure and the long-term consequences of increased folate levels have yet to be extensively studied in any long term clinical trials.

In human studies conducted in metabolic units involving folate depletion of volunteers, global DNA hypomethylation was observed in circulating peripheral blood lymphocytes. After repletion of folate levels, DNA methylation levels returned to normal, indicating that the effect on DNA methylation was transitory and persisted only during the folate deplete period (Jacob et al., 1998). Studies involving folate depletion in rodents indicate similar effects. Folate deficient rats had significantly lower global DNA methylation levels in liver tissue compared to folate replete control rodents (Balaghi et al., 1993). It has also been demonstrated that rats fed a diet supplemented with folic acid showed increased levels of global DNA methylation in liver tissue (Choi et al., 1998). These experimental and observational studies of folate status argue strongly for the need to perform a long term clinical trial to assess the effects of folic acid supplementation.

Maternal Nutrition and Fetal Health

Poor maternal nutrition in the form of protein or protein-calorie restriction can also have a deleterious effect on offspring. In humans, intrauterine growth retardation (IUGR) effects approximately 3% to 5% of pregnancies in the United Sates, and this number is increased dramatically with poor maternal nutrition, general health, and environmental factors such as nicotine, alcohol, and drug abuse. Although complex in origin, poor maternal nutrition is the most likely candidate for IUGR, and worldwide, millions of pregnancies are affected by maternal low protein or calorie restriction, making the causal understanding of IUGR of great medical importance. In studies involving an animal model of IUGR utilizing a maternal low protein during pregnancy, the offspring exposed to a low protein diet had smaller birth weights as

compared to control diet offspring. In addition to low birth weight, these animals developed increased risk for hypertension and type II diabetes in adulthood, and had a larger adult BMI than control offspring in adulthood. Interestingly, these deleterious traits were passed on at least two generations even in the absence of poor nutrition during their pregnancies (Novak et al, personal communication), suggesting a heritable, epigenetic change is occurring.

There are several examples of experiments testing this hypothesis using various animal models; they are briefly summarized below. These studies have proved that nutritional insults during pregnancy and/or lactation have a major impact on tissue development and function, which leads to an increased risk of adult disease.

In a study performed by Rees et al., female rats were fed a normal protein diet containing 18% casein or low protein diet containing 8% casein for two weeks prior to mating. Female rats continued on their respective diet until 21 dpc, when they were euthanized along with the fetuses, and fetal tissues, liver, heart and kidney, were removed (Rees et al., 2000). The amount of food consumed during pregnancy did not differ significantly between groups of animals. At 21 dpc, the fetuses from the dams on the 8% casein diet were 13.7% smaller than the control group, and fetal livers from the 8% casein group were ~24% smaller. In addition to fetal and tissue weights, the authors examined the global methylation levels in the fetal tissues using the methyl acceptance assay. The results indicate a greater than 25% increase in methylation in the livers of the fetuses from the 8% casein diet. No significant changes were noted for heart or kidney tissues. The authors summarize by stating that protein restriction *in utero* causes genome wide changes in DNA methylation, at least in the liver, and these changes have the potential to alter the regulation of important genes in the offspring (Rees et al., 2000).

In another set of experiments, Lillycrop et al. use a similar animal model to show locus specific decreases in DNA methylation in liver tissue (Lillycrop et al., 2005). For this study, timed-pregnant rats were put into one of three diet groups at time of conception. One group was fed an 18% casein diet (Control), another a 9% casein diet (Restricted), and a third group consumed a 9% casein diet that was fortified with 5mg/kg folic acid (Restricted Fortified) (5 times the folic acid of the other diets). Each group was fed this diet until spontaneous birth at day 21 dpc, at which point the dams were fed a lactating diet that the pups were weaned onto for 28 days. At day 34 the pups were euthanized and the livers were removed. The methylation status and expression levels were examined for two hepatic genes, glucocorticoid receptor (GR) and peroxisomal proliferator-activated receptor (PPARα) through the use of methylation-specific PCR. The results showed that offspring exposed to a restricted diet *in utero* had 26% less methylation at the PPARα promoter, and 23% less methylation at the GR promoter, as compared to offspring in the control diet group. Methylation levels of GR and PPARα promoters were essentially the same for offspring of both the control group and the restricted fortified diet group. The authors also examined whether the changes in DNA methylation had a corresponding change in expression levels. mRNA levels of the PPARα gene increased by 945% and the expression of GR increased by 300% in the restricted diet group as compared to the control diet group. There was no statistical difference in the expression levels between the control and restricted fortified diet groups (Lillycrop et al., 2005). In a follow up study, it was shown through high resolution sodium bisulfite genomic sequencing that the hypomethylation observed at the promoter of the PPARα gene was occurring at specific CpG nucleotides (Lillycrop et al., 2008). The CpG sites that were affected were within the putative binding site of various transcription

factors, and the authors suggest that changes in DNA methylation at these sites may alter transcription factor binding and lead to changes in gene expression (Lillycrop et al., 2008).

The focus of this dissertation will be on analyzing the effects of a low protein diet *in utero* on DNA methylation levels at both the global level, through analysis of repetitive elements, and the locus-specific level. The DNA methylation statuses of imprinted domains, especially *Igf2*/*H19*, are analyzed in great detail due to their importance in proper fetal development. Additionally, the effects of folic acid supplementation and withdrawal in a folic acid naïve human population on DNA methylation are studied.

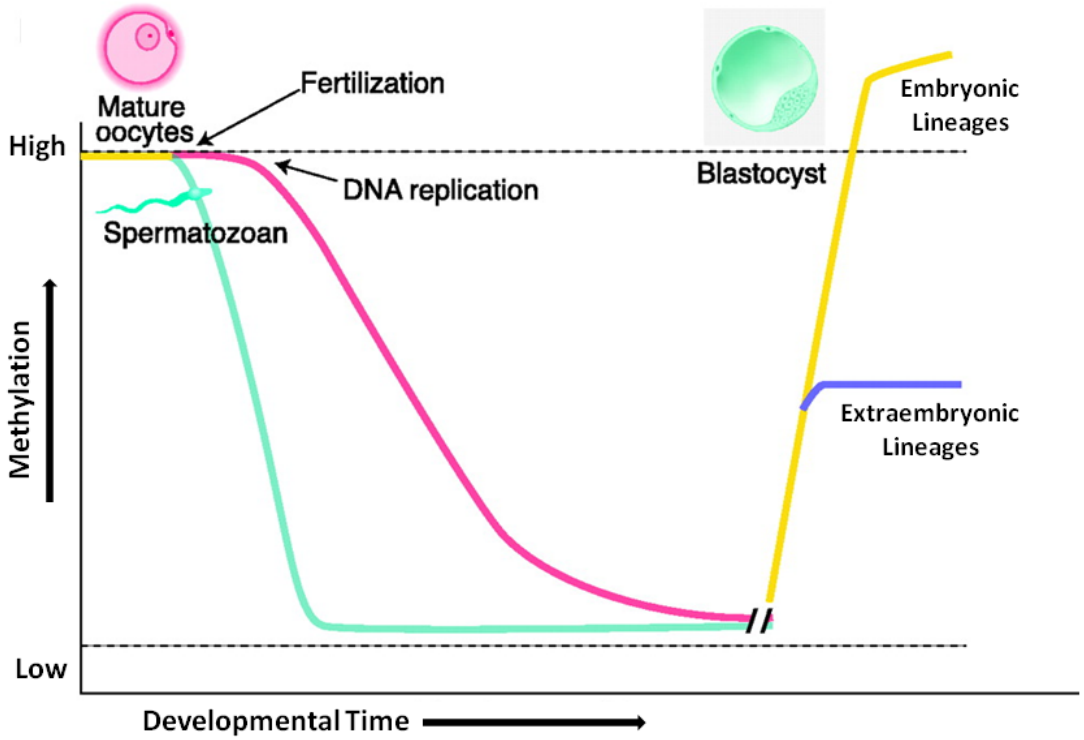


Figure 1-1. Parent of origin specific changes in DNA methylation during embryogenesis. The paternal genome (blue) is demethylated first by a putative active mechanism, followed by passive demethylation of the maternal genome (red). Both genomes become remethylated to different extents in the extraembryonic and embryonic lineages around the time of implantation. Figure adapted from Reik et al. 2001.(Reik et al., 2001)

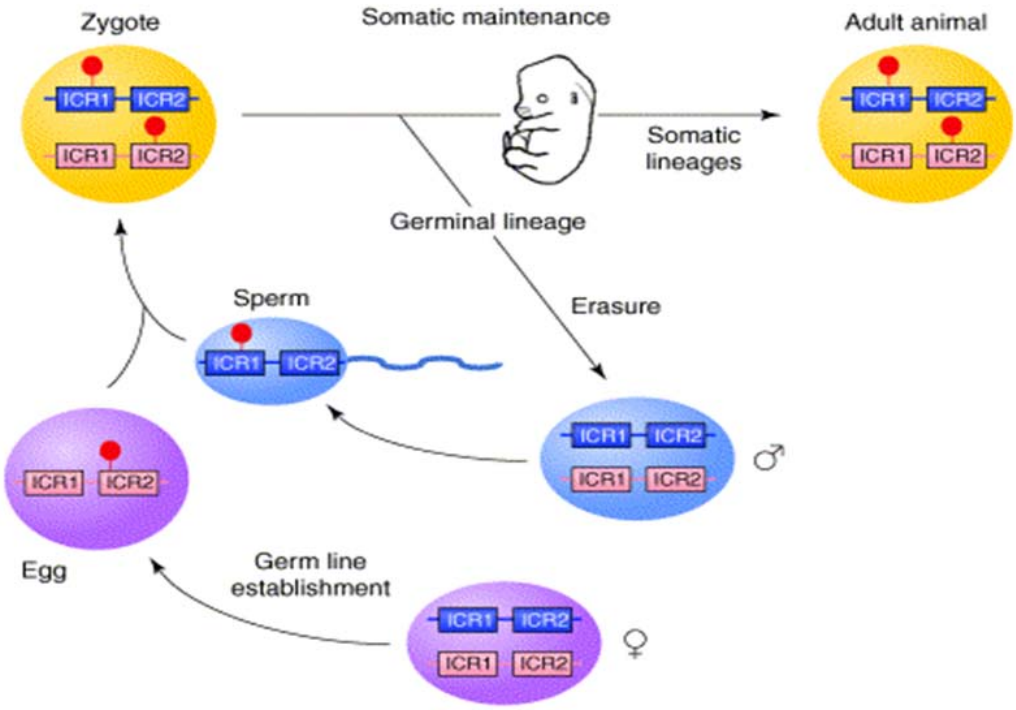


Figure 1-2. Erasure of DNA methylation during gametogenesis During spermatogenesis and oogenesis, parental imprints are established at the ICRs of imprinted domains. DNA methylation imprints are depicted by lollypops in two different imprinted domains. One paternally derived (ICR1) and the other maternally derived (ICR2). Figure adapted from Delaval et al. (Delaval and Feil, 2004)

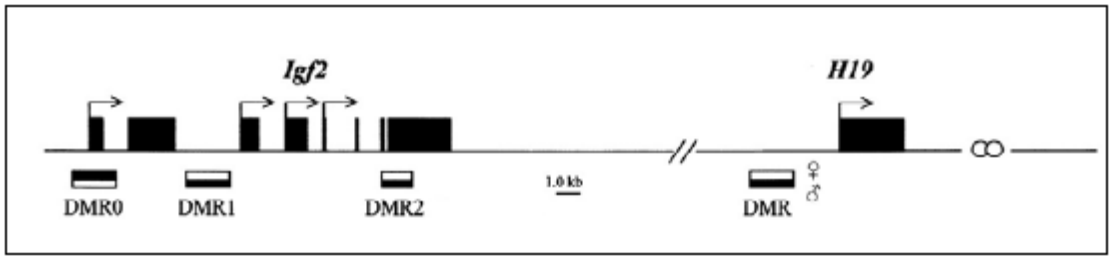


Figure 1-3. Schematic representation of the *H19*/*Igf2* imprinted domain showing the 3 paternally methylated DMRs and the placenta-specific maternally methylated DMR0. Adapted from Lopez 2003 (Lopes et al., 2003).

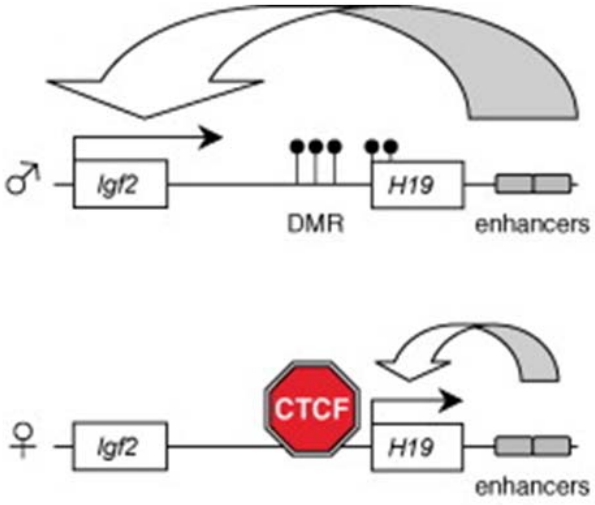


Figure 1-4. Schematic representation of the role of CTCF in long range transcriptional regulation in imprinted domains. Methylation of the *H19* DMR prevents CTCF from binding, allowing downstream enhancers to promote transcription of the paternal allele of *Igf2*. CTCF binds to the maternally unmethylated *H19* DMR, blocking access of enhancers to *Igf2* and allowing enhancers to drive expression of maternal *H19*.Figure adapted from Chao et al. (Chao and D'Amore, 2008)

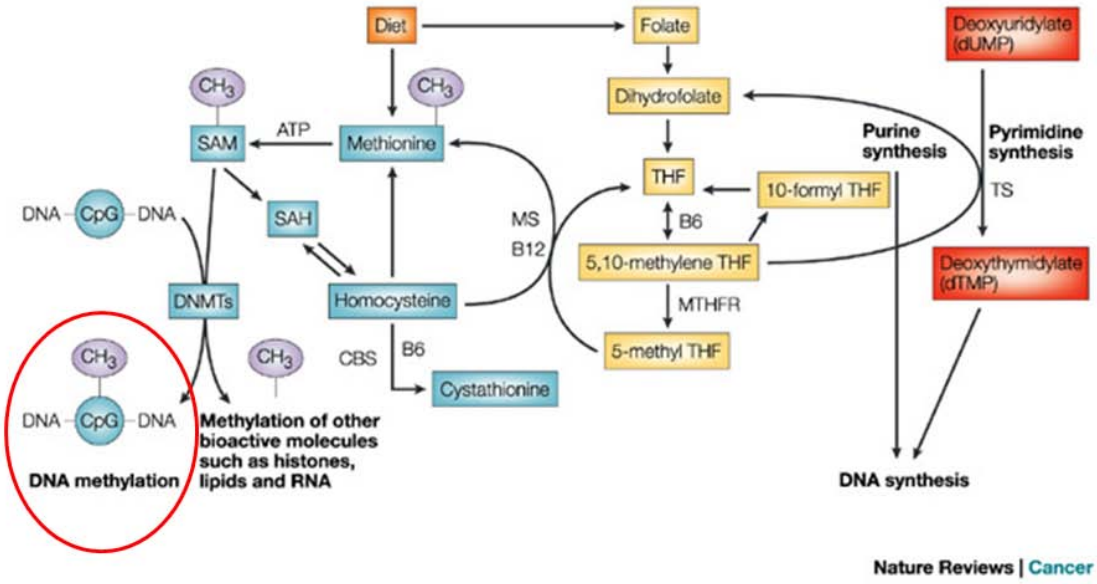


Figure 1-5. The role of folate in one-carbon metabolism. Schematic showing biochemical pathways and the role of folate in one-carbon metabolism, particularly the role of folate in DNA methylation, circled in red. Figure adapted from Lamprecht et al.(Lamprecht and Lipkin, 2003)

CHAPTER 2
EPIGENTETIC EFFECTS OF A LOW PROTEIN DIET IN UTERO

Introduction

There is an increasing awareness of the potential for deleterious effects of maternal malnutrition during pregnancy on the offspring, such as an increased susceptibility to metabolic syndrome and cardiovascular disease (CVD) in humans (Godfrey and Barker, 2001). In animal studies involving rodents, maternal malnutrition reinforces the observations that early environmental conditions can have adverse effects on the offspring throughout their life (Bertram and Hanson, 2001). There is a growing body of evidence, both epidemiological and experimental, in which nutrient deprivation *in utero* has been demonstrated to have adverse long-term effects on the metabolic and physiological states of offspring (Malandro et al., 1996; Osmond et al., 1993; Roseboom et al., 2001b). However, the mechanisms by which altered metabolism and disease susceptibility may arise as a consequence of dietary restriction during gestation remains unclear. One possibility is that epigenetic changes in the placenta and/or fetus in response to maternal malnutrition could lead to stable aberrant regulation of genes important for normal placental and/ or fetal development. Recent experimental data from the literature has demonstrated that exposure to a low protein diet (LPD) *in utero* can induce changes in DNA methylation at both the global and locus-specific scale (Lillycrop et al., 2005; Lillycrop et al., 2007; Rees et al., 2000). These findings are important in that they indicate that exposure to nutritional insults *in utero* can induce epigenetic changes in offspring. However, until recently, the focus has been limited to either a global view of genomic DNA methylation or a locus-specific analysis of DNA methylation of candidate genes only. In this study, we set out to expand the results of these initial findings to a much broader, genome wide view of potential changes in DNA methylation. We have used a modified experimental approach first developed by Weber et

al. (Weber et al., 2005), in which the authors utilized an immunocapture approach using antibodies against methylated DNA followed by microarray analysis. Using this method, the authors compared the DNA methylation levels of normal fibroblasts to a transformed colon cancer cell line on an 80-kb resolution bacterial artificial chromosome (BAC) array for all human chromosomes, and a CpG island array with an average resolution of 760 bases. The results of their study showed that they were able to detect large regions of hypomethylation in gene-poor genomic areas in the transformed cells compared to normal fibroblasts using the BAC array, and were able to detect 30 unique CpG island sites that showed hypermethylation in the transformed cells that were hypomethylated in normal fibroblasts on the CpG island array (Weber et al., 2005). We wished to expand this approach to the use of a high resolution Affymetrix Mouse Promoter array. This has been accomplished through the use of antibodies that specifically recognize 5-methylcytosine to obtain DNA fractions that are highly enriched for methylated DNA sequences from genomic DNA of experimental and control animals. The highly enriched DNA was then hybridized to Affymetrix Promoter Array Gene Chips to analyze changes in methylation on an array containing most of the known mouse promoters. The Affymetrix Mouse Promoter Array contains 4.6 million probes allowing the interrogation of 28,000 mouse promoters on a single gene-chip platform. These chips cover approximately 6 kb upstream and 2.5 kb downstream of the transcription start site at each promoter, which should include many regulatory elements and DMR's, as well as ~70% of all CpG islands in the mouse genome (Affymetrix). The probes are 25-mers that are tiled at an average 35 base resolution, allowing interrogation of genomic regions at high resolutions. Results from the gene chip experiments were then validated using high resolution sodium bisulfite genomic sequencing.

Results

Pair-fed Animal Feedings

Timed-pregnant C57BL/6J mice were received on day 4 dpc, weight matched, and put into either a control (NPD) or restricted protein (LPD) feeding group (Figure 2-1). The mice were weighed every 24 hours and their weight recorded starting from day 5 until day 18 (Figure 2-2). The amount of food consumed by the LPD animals was weighed every 24 hours and the corresponding amount of food consumed was given to the pair-fed control animal the following day to ensure equal consumption of calories (Figure 2-1). Although there was no significant statistical difference in the amount of weight gained during pregnancy between the NPD and LPD animals, there was a trend for the NPD animals to gain more weight compared to the LPD animals (Figure 2-3 A). However, there was no significant statistical difference in the amount of daily food consumption between the NPD and LPD animals (Figure 2-3 B). The difference in weight gains appears to be related to the average litter size, as the LPD group tended to have smaller litters compared to the NPD group (Figure 2-3 C). As the number of pups would be expected to have the greatest impact on weight gain, it is not surprising that the NPD group gained more weight. When weight gain per pup was analyzed there was no statistical difference between the two groups (Figure 2-3 D). These results indicate that the average weight gained, food consumed and numbers of pups did not differ significantly between the experimental and control animal groups.

Methylated DNA Immunoprecipitation (MeDIP)

In order to analyze DNA methylation using promoter tiling arrays, the DNA was first enriched for methylated DNA sequences. This was performed using a modified methylated DNA immunoprecipitation (MeDIP) procedure (Figure 2-4). Genomic DNA was first sheared in length to about 250-700 bases through sonication. These DNA fragments were then denatured and

single-stranded methylated fragments are immunocaptured using antibodies specific to 5-methylcytosine, along with a no antibody control IP reaction. The 5-Methylcytosine and no antibody control IP DNA was then captured using secondary antibodies attached to magnetic beads to purify the IP from unbound DNA. This complex was then washed and eluted. These highly enriched fractions were then labeled and hybridized to Affymetrix promoter arrays (Figure 2-4).

To validate the specificity of enrichment of methylated DNA fragments versus no antibody and non-specific antibody controls, quantitative RT-PCR was performed on methylated and unmethylated genomic sequence fractions. To test the efficiency of the MeDIP assay, several controls were performed. During the immunocapture, a non-specific control antibody (normal mouse IgG) reaction and a no antibody control reaction were always run in parallel to the α5-MeC IP reaction. Three genomic sequences known to be methylated were chosen as enrichment controls: *Xist*, *Hprt* and the *H19* DMR. X-inactive specific transcript (*Xist*) is an X-linked gene that is only expressed from the inactive X chromosome in female mammals, and is hypermethylated on the active X chromosome in females and males. Hypoxanthine phosphoribosyl transferase 1 (*Hprt*) is an X-linked housekeeping gene that is expressed from the active X chromosome and is hypermethylated on the inactive X chromosome in females. The *H19* DMR is a paternally imprinted differentially methylated region with hypermethylation on the paternal allele and hypomethylation on the maternal allele. As an additional test of efficiency, we performed the initial control experiments using genomic DNA from both male and female mouse livers. This was performed in order to observe the expected methylation differences between *Xist* and *Hprt* in male and female cells. The promoter region of *Xist* is hypermethylated on the active X chromosome and hypomethylated on the inactive X. Therefore we would expect

males to have approximately twice the enrichment of methylated DNA at this region as females. This is due to the fact that the only genomic contribution of *Xist* comes from the single, active X chromosome in males. Additionally, *Hprt* is a housekeeping X-linked gene that is hypermethylated only on the inactive X chromosome in female cells, so we would expect to see enrichment for methylation only in females. The *H19* DMR is differentially methylated and we would expect to see equal enrichment from both male and female cells. Two genomic sequences known to be unmethylated were chosen as negative controls. Adenine phosphoribosyl transferase (*Aprt*) and *β-Actin* (ActB) are housekeeping genes and contain CpG islands in their promoter regions that are known to be hypomethylated. A control genomic sequence (CSa) was chosen that contains no CpG dinucleotides as an additional negative control (Figure 2-5).

The results from our experiments to determine the efficiency of MeDIP enrichment of methylated DNA confirm that we were, in fact, obtaining a highly enriched fraction of methylated fragments as compared to non-methylated controls. The methylation of *Xist* in male DNA was ~30% of input compared to ~10% of input in females (Figure 2-5). This is in agreement with expected results. In males, which contain no inactive X chromosome, methylation of *Hprt* was undetectable, while females showed an enrichment of methylation of approximately 5% compared to input. Enrichment at the *H19* DMR was essentially equal between males and females. The unmethylated control sequences, *Aprt, β-Actin*, and CSa, had undetectable enrichment of methylated sequences in both males and females (Figure 2-5). These results indicate that the MeDIP protocol used was efficiently enriching methylated DNA fragments.

MeDIP on Experimental Animals

As MeDIP has demonstrated, we now can obtain highly enriched fractions of methylated DNA fragments from genomic DNA. We next wished to combine the detection of DNA methylation with the large scale capabilities of a microarray analysis. Fetal livers were harvested and pooled (four each) from litters obtained from four independent pair-fed animal studies, genomic DNA extracted, and MeDIP performed. The specificity and efficiency of enrichment for methylated DNA was again determined through the use of quantitative RT-PCR (qRT-PCR) using the same controls as before, plus the 5' LTR of IAP, which is known to be hypermethylated.

The qRT-PCR results for IAP show similar levels of enrichment between all four NPD and LPD samples (Figure 2-6). Although there are significant differences in enrichment of methylated DNA at *Xist* between NPD and LPD samples, the samples are pooled fetal livers from pups that were not sexed, therefore the ratio of male to females is unknown, and no meaningful conclusion can be drawn. However, there appears to be a slight decrease in the enrichment of methylated DNA at the *H19* DMR in the LPD samples compared to the NPD samples. The unmethylated control sequences showed negligible enrichment for all samples (Figure 2-6). These results again confirm that the MeDIP protocol produced highly enriched fractions of methylated DNA.

Amplification and Labeling of DNA

The amount of DNA recovered from a typical MeDIP is extremely low (~40ng/IP). In order to generate quantities of DNA necessary for use on a microarray, the MeDIP DNA must first be amplified in an unbiased manner. To achieve this, we performed a whole genome amplification (WGA) using a kit from Sigma. The Whole Genome Amplification kit (WGA2) is designed to generate an amplifiable fragment library from the MeDIP DNA, and amplify the

sample ~600 fold without introducing bias. For each sample, 25 ng of MeDIP DNA was amplified using WGA2, with an average yield after amplification of between 4-6 μg. Gel electrophoresis of amplified products indicate no noticeable change in the size distribution of the original MeDIP input DNA. *Xist* and *Aprt* remained highly enriched in randomly chosen WGA amplified samples according to a qRT-PCR analysis (data not shown).

To prepare the WGA-amplified DNA samples for hybridization on the Affymetrix promoter arrays, the samples were biotin labeled using a standard random-primed labeling reaction. Briefly, uracil was incorporated into the amplified DNA using a random primed labeling reaction containing a mixture of dNTPs (10 μM dATP, 10 μM dCTP, 10 μM dGTP, 8 μM dTTP, 2 μM dUTP). After uracil incorporation, DNA was fragmented through the actions of uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic (AP) endonuclease (APE 1), and end labeled with a biotinylated ATP analog using Affymetrix's Double-Stranded DNA Labeling Mix. To test for the efficiency of uracil incorporation, fragmentation and ultimately, biotin incorporation, we performed an Electro-Mobility Shift Assay (EMSA). The high affinity binding properties of avidin to biotin were exploited in this assay. Samples of fragmented and labeled DNA were either pre-incubated with avidin, or not, and run on a 4% to 20% gradient acrylamide TBE gel. The binding of biotin to avidin retards gel migration, and efficient labeling can be visualized by a shift in molecular weight of labeled fragments as compared to fragments without avidin. The results of the EMSA indicate that all samples were efficiently labeled (Figure 2-7). The labeled DNA was then hybridized to Affymetrix Mouse Promoter Arrays in Dr. Henry Baker's laboratory in collaboration with Dr. Baker and Cecilia Lopez. Briefly: Fragmented and labeled DNA was hybridized to the promoter arrays at 45^{o}C for 16 hours. Arrays were washed and then stained with a fluorescently labeled antibody conjugated to avidin which binds to

biotinylated nucleotides in the labeled samples. Fluorescent signal intensity was then recorded using the GeneChip Scanner 3000. A more thorough description of scanning the arrays can be found chapter 4.

Analysis of Promoter Array Data

The results of the gene-chip scan were analyzed using Partek's Genomic Suite Software package. This software allows sophisticated statistical analysis of multiple gene chips from multiple experimental groups. For our experiment, we had a total of 16 promoter arrays, four NPD and four LPD chips with α5-MeC, along with paired control chips using input DNA from each corresponding sample. The signal intensity for each chip was normalized using Robust Multi-chip Averaging (RMA), and average signal intensities were scaled across all chips. After normalization, the signals from input chips were subtracted from the corresponding antibody gene-chips to reduce noise due to background. The remaining eight data sets, representing four NPD and four LPD arrays with input subtracted were then compared. Signal intensities were compared between each experimental (LPD) array and the corresponding control (NPD) array. Differences in signal intensity were analyzed using a 300 base window (approximately 8 consecutive probe sets), using a threshold p-value of 0.001. Probe sets that met this threshold value in three out of four arrays were scored as a positive differential signal for subsequent analysis. Partek's software identified 176 positive genomic regions that showed changes in signal intensity meeting the above mentioned criteria. The genomic location of these probe sets were then used to annotate genes associated with them.

After analyzing positively identified probe sets within the UCSC Genome Browser, it was observed that the overwhelmingly vast majority of these positive regions were directly adjacent to a repetitive element in an intronic region of a gene. The sequence encompassing the positively

identified probe sets were analyzed for 30 regions with the lowest p-value for an increase and 30 regions with the lowest p-value for a decrease in signal intensity. The genomic sequence of the regions analyzed had very little to no CpG dinucleotides within them, suggesting that the resulting signal was due to the IP of a DNA fragment containing a repetitive element along with enough adjacent sequence to bind to the probes on the array. Two probe sets were identified that were in intronic regions of single copy genes that did contain a moderate density of CpGs, *Cdca8* and *Man1a*. These genes will be discussed in further detail later.

The Partek software generates a heat map to visualize the difference in signal intensities between the control and experimental samples for individual probe sets, and also shows the location of individual probes within a genome browser. For Mannosidase 1, alpha, (*Man1a*) a protein involved in the degradation of terminally misfolded proteins in the endoplasmic reticulum, the heat map shows a strong increase in signal intensity for the LPD samples, indicating an enrichment of methylated DNA fragments hybridizing to these probe sets (Figure 2-8). Cell division cycle associated 8 (*Cdca8*) is a protein that is critical for the proper segregation of chromosomes during mitosis (Yamanaka et al., 2008). In this case, the heat map indicates that there is increased signal intensity for the NPD samples compared to the LPD samples (Figure 2-9) A third gene that was also identified in the Partek screen, albeit with less stringent parameters, was RIO Kinase 1 (RIOkI), which is a ubiquitously expressed serine kinase (LaRonde-LeBlanc and Wlodawer, 2005) (heat map not shown).

After reviewing all the data from the promoter arrays, we proposed that there are two possible explanations for not finding significant changes in DNA methylation between the NPD and LPD groups. The first explanation is that the degree of any changes in DNA methylation between the NPD and LPD groups was below the limit of the array to detect. The design of the

tiling array, with 25-mer probes tiled at a 35 base resolution inherently has an elevated background and thus a reduced signal to noise ratio which could have masked small changes in DNA methylation between the NPD and LPD groups. Additionally, the α5-MeC antibody precipitates a large fraction of the genome as compared to an IP performed with an antibody against a specific DNA-binding protein. This is due to the fact that a large portion of the genome consists of methylated repetitive DNA (Lander et al., 2001; Rollins et al., 2006), which would IP along with single copy genes, and would also have contributed to an elevated background signal and a lower signal to noise ratio. These two factors would make it more difficult to detect small changes in DNA methylation. This seems to be a reasonable explanation as similar experiments in the literature have shown locus-specific changes in DNA methylation (Lillycrop et al., 2005; Lillycrop et al., 2007; Waterland et al., 2006), although the changes in methylation observed were modest at best, around 20% change. After the promoter array analysis had been performed, Lillycrop et al. (Lillycrop et al., 2008) demonstrated that the observed changes in methylation at the hepatic PPARα gene promoter in response to a low protein diet *in utero* were at individual CpG sites, thus confirming our notion that these types of changes would be difficult to detect using a tiling array approach. This led us to propose that any changes in DNA methylation between the two feeding groups would be minor, which is consistent with the observation that there is very little phenotypic difference between NPD and LPD offspring at birth.

The second explanation is that there was a technical issue in the implementation of the experiment. As the MeDIP and qRT-PCR showed highly enriched fractions of methylated DNA, and the EMSA results indicated that the labeling reaction was successful, it may be that the either the hybridization to the arrays was unsuccessful, or the chips themselves were defective in some manner. This explanation seems unlikely as statistical evidence and pair-wise matching of

the arrays during the quality control steps indicate that the arrays hybridized well and the collection of signal intensity was successful.

Locus-Specific DNA Methylation Analysis

In order to test our hypothesis that there may be modest or CpG site-specific differences in DNA methylation between the NPD and LPD groups, we analyzed the methylation status of two well studied and characterized imprinted domains, whose regulation are known to be controlled by DNA methylation. The first domain was the *H19*/*Igf2* imprinted domain. This region was chosen because *Igf2* is a critical growth factor involved in fetal and placental development, and both genes are highly expressed in fetal tissues (Constancia et al., 2002). The imprinted domain is under the regulation of an ICR located 2-4 kb upstream of *H19* whose methylation status is critical for interaction with two DMRs in *Igf2* which establish proper imprinted expression of these genes. The second imprinted domain was the Angelman/Prader-Willi syndrome (AS/PWS) imprinted domain. This is another well characterized imprinted domain whose imprinting control center (ICR) at the promoter of the *Snrpn* gene controls proper imprinted gene expression of genes as far away as 2 Mb, including the *Mkrn3* locus. Both *Snrpn* and *Mkrn3* expression are regulated by DNA methylation. Additionally, we analyzed the DNA methylation status of *Man1a*, *Cdca8* and *ROIkI*, the three genes identified using Partek' Genomic Suite Software from the promoter array analysis.

In order to determine if any changes in DNA methylation were occurring at these genes, high resolution sodium bisulfite genomic sequencing was performed in a region overlapping the location of the positive probe sets for *Man1a*, *Cdca8*, and *ROIkI*. For *Snrpn* and *Mkrn3*, GpG islands within the promoters of these genes were analyzed. For the *H19*/*Igf2* imprinted domain, differentially methylated regions were analyzed for the *H19* DMR, and *Igf2* DMRs 1 and 2. For each locus, two NPD samples and all four LPD samples were analyzed.

DNA Methylation Analysis by Sodium Bisulfite Genomic Sequencing

The results for *H19* DMR (Figure 2-10 A) indicate a moderate, but statistically significant reduction in DNA methylation in the LPD group as compared to the NPD group (Figure 2-10 B). Methylation dropped from 37% in the NPD group to 25% in the LPD group (Figure 2-10 C). The relative risk (*RR*) of demethylation is 1.44 times greater in the LPD group compared to the control group (Figure 2-10 D). However, it should be noted that these data are from one bisulfite treatment only, and attempts to repeat these experiments yielded highly variable results. In order to determine changes in the methylation status with any degree of certainty, a second technique, such as pyrosequencing, should be employed to verify the initial findings.

At the *Igf2* locus, both DMR1 (Figure 2-11 A) and DMR2 (Figure 2-12 A) were analyzed for changes in DNA methylation. There was no detectable change in DNA methylation at DMR1 between NPD (Figure 2-11 B) and LPD (Figure 2-11 C) and the *RR* had a value of 1.0 (Figure 2-11 D), indicating no increased risk to changes in DNA methylation based on exposure to the low protein diet. There was, however, a statistically significant increase in DNA methylation at DMR2 in the LPD group as compared to the NPD group. Methylation increased from 20% in the NPD samples (Figure 2-12 B) to 29% in the LPD samples (Figure 2-12 C). The relative risk of increased methylation was 1.32 times greater in the LPD group compared to the NPD group (Figure 2-12 D). This is the combined data from two independent bisulfite treatments which showed a large degree of variability between individual experimental results, though the combined general trend is towards an increase in methylation in the experimental group. To further verify these data by an independent means, pyrosequencing should be performed at DMR2.

The results for the AS/PWS imprinted domain showed no statistically significant change in DNA methylation levels at either the *Snrpn* or *Mkrn3* promoters. At the promoter of *Snrpn*,

which also functions as an ICR for the imprinted domain (Figure 2-13 A) DNA methylation increased from 17% in the NDP samples (Figure 2-13 B) to 22% in the LPD samples (Figure 2-13 C). This change in DNA methylation is not significant though, with a p-value of 0.06 and a *RR* of 0.91 (Figure 2-13 D). The results of the DNA methylation analysis at the promoter of *Mkrn3* (Figure 2-14 A) indicate a slight decrease in methylation, from 17% in the NPD samples (Figure 2-14 B) to 13% in the LPD samples Figure 2-14 C). This change was also not statistically significant with a p-value of 0.03 and a *RR* of 1.05 (Figure 2-14 D).

The DNA methylation analysis of the regions overlapping the probes identified by the Partek software analysis for *Man1a*, *CdCa8* and *ROIkI* all indicate that there is no statistically significant changes in methylation. At *Man1a* DNA methylation decreased from 85% in the NPD samples (Figure 2-15 A), to 83% in the LPD samples (Figure 2-15 B), with a p-value of 0.5 and a *RR* of 0.91 (Figure 2-15 C). *Cdca8* DNA methylation was essentially identical between the NPD samples, 79% (Figure 2-16 A), and the LPD samples, 80% (Figure 2-16 B), with no statistical significance to the change (Figure 2-16 C). Both the NPD samples (Figure 2-17 A) and the LPD samples (Figure 2-17 B) were hypomethylated at *ROIkI*, with DNA methylation levels less than 1% Figure 2-17 C).

Man1a, *Cdca8*, and *RIOkI*, were all identified by Partek's software analysis as regions indicating change in DNA methylation, yet at all three regions, no changes in DNA methylation were observed. The most likely explanation for their detection on the promoter array is their close genomic proximity to repetitive elements that may be undergoing differential methylation between the NPD and LPD samples. Due to the fragment size of the sonicated DNA, the labeled DNA will hybridize to the promoter array if the probe sequence is located within a few hundred bases of the repetitive element being immunoprecipitated. For *Man1a*, the locations of the

positives probes are within the first intron and located ~500 bases from a SINE and the probes for *Cdca8* are within the 5' UTR and within 200 bases of a micro-satellite simple repeat. This proximity to a repetitive element is well within the size range of the sonicated DNA fragments used in the MeDIP.

mRNA Expression

As the *H19* and the *Igf2* DMR's physically interact (Murrell et al., 2004; Wallace and Felsenfeld, 2007) during proper imprinted expression, we next wanted to analyze whether the observed changes in DNA methylation at the *H19* DMR and the *Igf2* DMR2 produced any effect on expression of these genes. Since CTCF binds the unmethylated maternal allele at *H19* DMR, blocking enhancer access to the upstream *Igf2* promoter and driving *H19* expression, could a decrease in DNA methylation of the *H19* DMR allow for more CTCF binding, thus decreasing *Igf2* and increasing *H19* expression? Another unknown is whether an increase of DNA methylation at the *Igf2* DMR2 has any effect on the transcription of *Igf2*. *Igf2* DMR2 acts as a methylation sensitive activator, and an increase in methylation leads to increased *Igf2* expression *in vitro* (Murrell et al., 2001), although this has not been tested *in vivo*. To investigate these questions, expression of *Igf2* and *H19* were analyzed using reverse transcriptase qRT-PCR. RNA from two NPD and 4 LPD samples were analyzed for *Igf2* and *H19* expression using *Gapdh* as a reference gene to calculate fold change. The results of the expression analysis show that there is a strong induction of *H19* expression in the LPD samples as compared to the NPD samples. Furthermore, the expression of *Igf2* was reduced by ten-fold in the LPD samples as compared to the NPD samples (Figure 2-18).

Discussion:

Research involving the effects of maternal malnutrition and the associated increased risk of adult onset diseases has until relatively recently relied on epidemiological data. Animal models

of maternal malnutrition, including protein restricted diets, have revealed intriguing evidence implicating epigenetic changes as a possible explanation of increased disease risk in adulthood (Lillycrop et al., 2005; Waterland and Garza, 1999; Waterland et al., 2006). However, these studies have been somewhat limited in their analysis due to the technologies available to perform these types of analyses. The analysis of DNA methylation has been either limited to methods that observe changes in global DNA methylation, or to the site-specific analysis of a limited number of candidate genes. While site-specific analysis of DNA methylation provides much more informative data, it is a time consuming and laborious process, which severely limits the number of loci that can be analyzed. In the proceeding work, we have adopted and adapted new technologies that allow us to expand our abilities to analyze DNA methylation in a site-specific manner on a genome-wide level. We have optimized techniques that enable us to obtain highly enriched fractions of methylated DNA fragments from genomic DNA, which can then be applied to subsequent analysis applications, such as various gene-chip platforms that are available, or by utilizing massively parallel sequencing technologies to generate data concerning changes in DNA methylation based on various experimental conditions. In this particular study, we have utilized the Affymetrix Mouse Promoter Array Gene Chip platform to analyze changes in DNA methylation in fetal tissue from offspring exposed to a low protein diet *in utero*.

In the preceding work, we have demonstrated that there appears to be no wide-spread large scale changes in DNA methylation within promoter regions in fetal livers of offspring exposed *in utero* to a maternal low protein diet. However, we have demonstrated that exposure to a low protein diet *in utero* can produce modest changes in DNA methylation at differentially methylated regions within certain imprinted domains, specifically the *Igf2*/*H19* imprinted domain. At the *H19* DMR, there was an observable, and statistically significant decrease in DNA

methylation, and at *Igf2* DMR2, there was an observable and statistically significant increase in DNA methylation in fetal livers. Analysis of the AS/PWS imprinted domain revealed no statistically significant changes in DNA methylation at either the imprinted *Snrpn* or *Mkrn3* loci. Genomic regions identified as being differentially methylated between NPD and LPD samples in the promoter array analysis revealed no observable changes in DNA methylation. The most likely explanation for this is due to the close genomic proximity of these probe sets to repetitive elements. The repetitive sequences are not included on the promoter array, but these elements may contain adjacent sequences that when immunocaptured by MeDIP, can hybridize to the array, giving a false positive.

We were also able to demonstrate altered expression of both *H19* and *Igf2* between the NPD and LPD groups. There was a strong induction in the expression of *H19* in the LPD samples, and a 10-fold decrease in expression of *Igf2* in the LPD samples compared to the NPD samples. The concomitant increase in DNA methylation at *igf2* DMR2 and decrease in expression of *Igf2* is inconsistent with the role of DMR2 as a methylation-sensitive activator. A more detailed examination and possible explanation for this will be discussed in greater detail in Chapter 5.

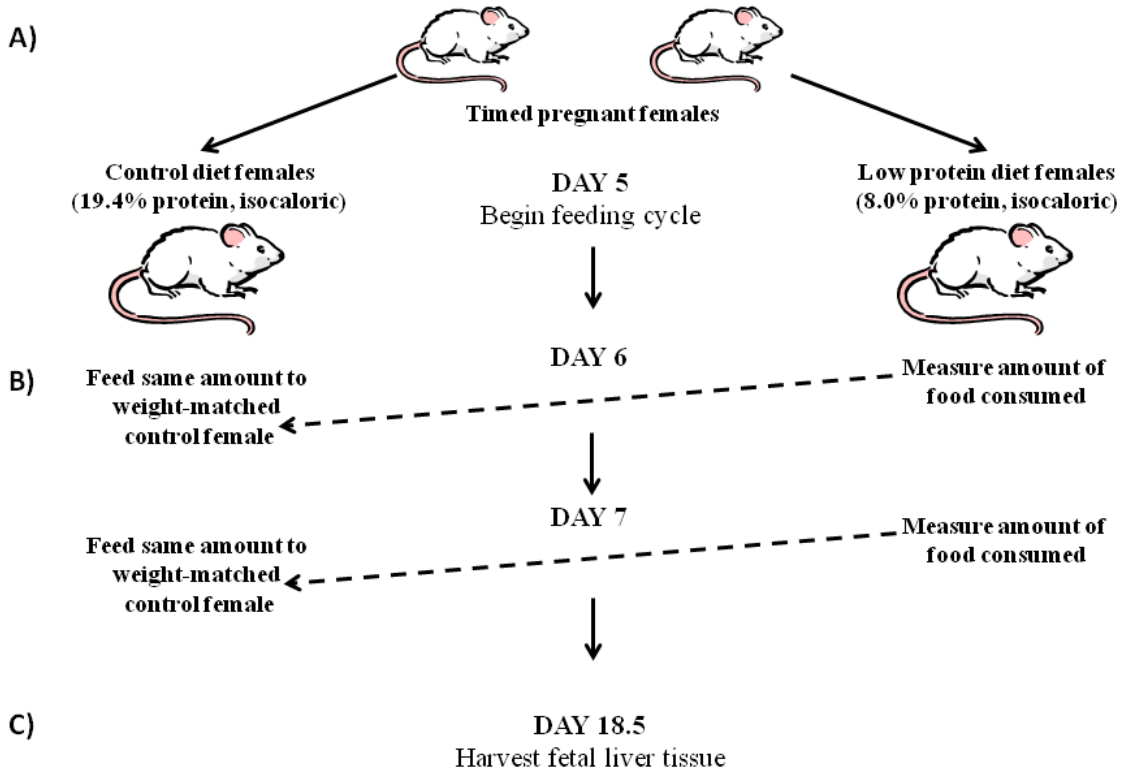


Figure 2-1. Schematic representation of pair-feeding study. A) Timed pregnant females are weight matched and separated into pair fed groups. B) Amount of food consumed by experiment group animals is recorded and the corresponding amount is fed to control group animals the following day. C) This feeding procedure continues until day 19 of pregnancy, when dams and pups are euthanized and fetal liver tissue is harvested.

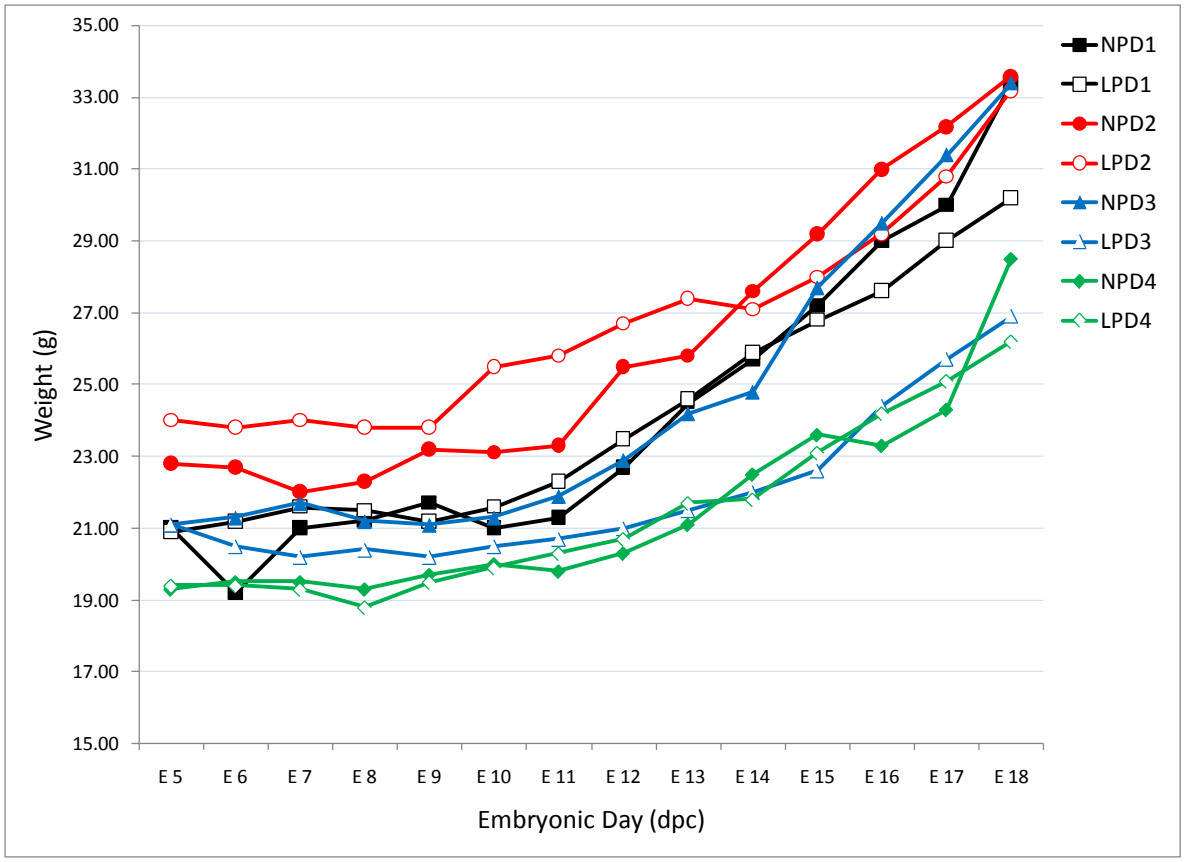


Figure 2-2. Weight gain of pregnant dams throughout pregnancy. Dams were measured every 24 hours from day 5 until day 18.5. Weight was recorded in grams. All four weight-matched pair-fed animals are represented by matching colored lines, with solid points indicating NPD animals, and open points representing LPD animals.

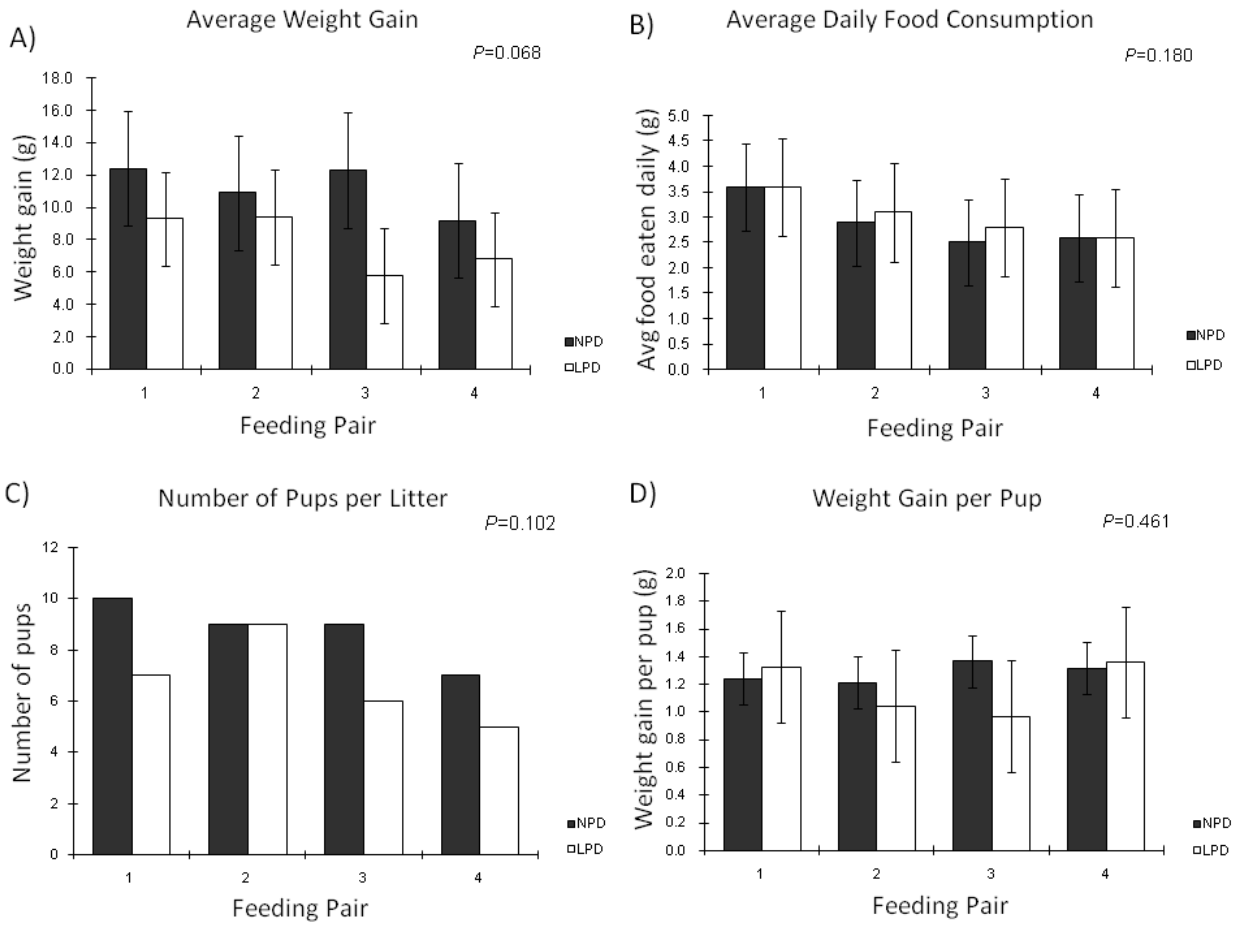


Figure 2-3. Pair-Feeding Study. A) Average weight gain per dam for each feeding pair. B) Average daily food consumption per dam for each feeding pair. C) Number of pups per litter for each feeding pair. D) Average weight gain per pup for each dam for each feeding pair.

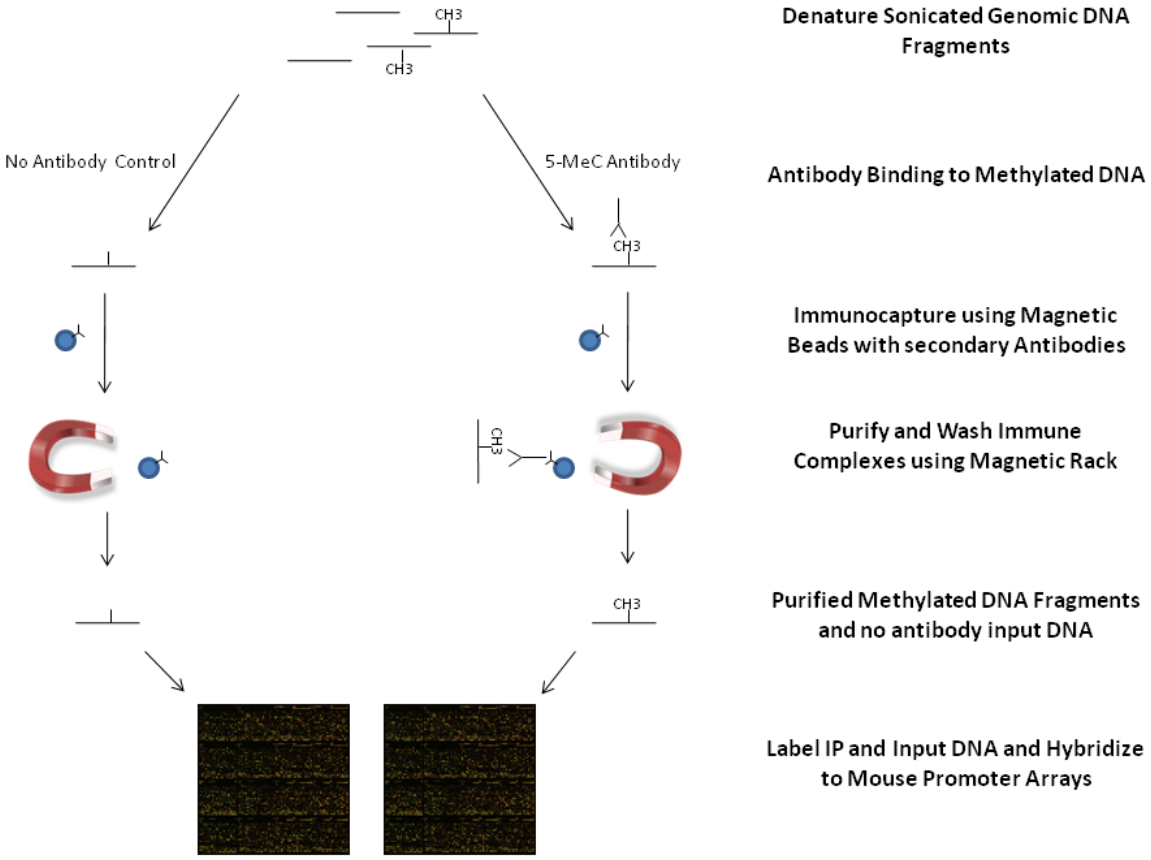


Figure 2-4. Outline of methylated DNA immunoprecipitation (MeDIP) and schematic of work flow.

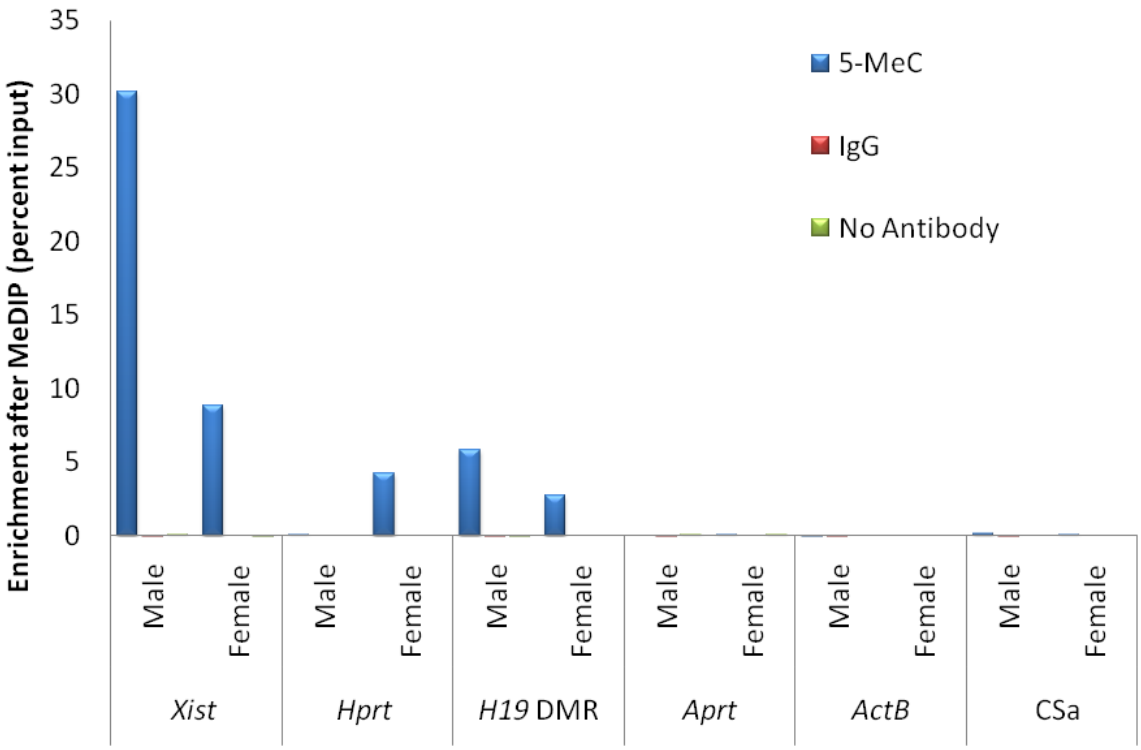


Figure 2-5. Verification of enrichment of methylated DNA by qRT-PCR. *Xist* is an X-linked gene that is hypermethylated only on the active X chromosome. *Hprt* is an X-linked gene that is hypomethylated on the active X chromosome. *H19* DMR is a paternally imprinted gene. *Aprt* and *ActB* are housekeeping genes known to be unmethylated at their CpG islands within their promoters. CSa is a control sequence containing no CpG dinucleotides.

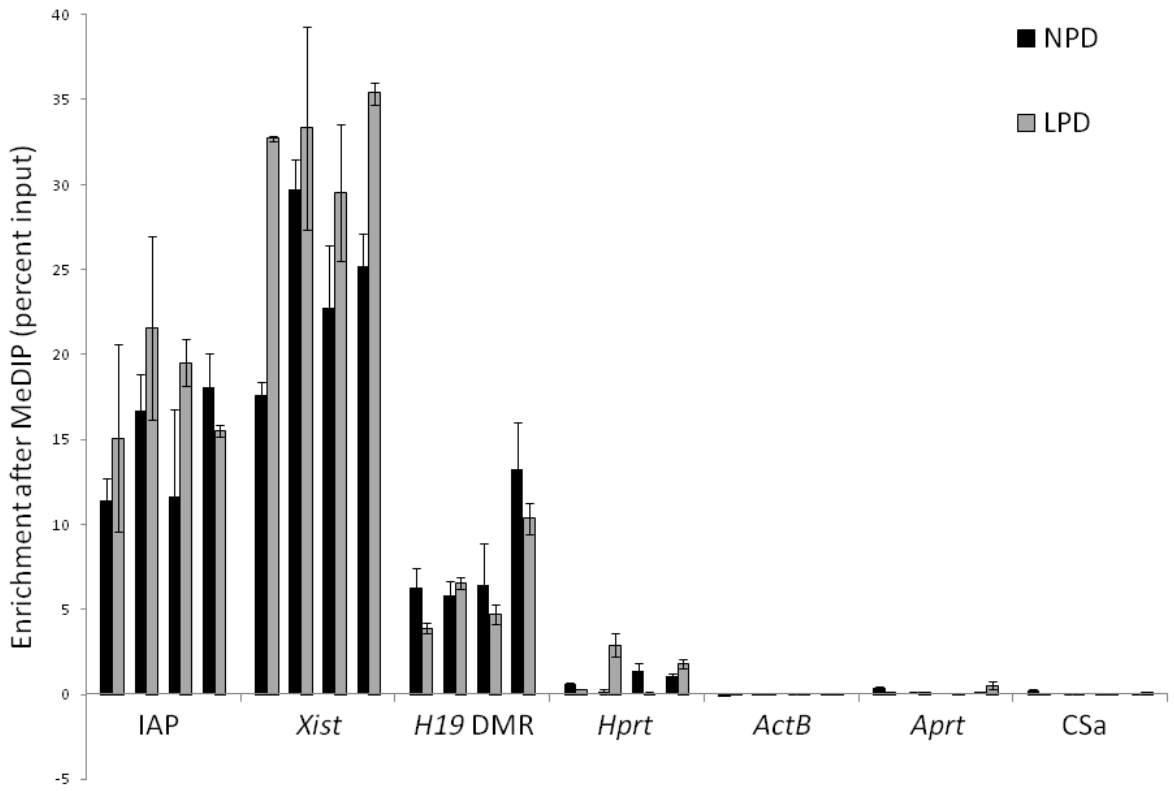


Figure 2-6. qRT-PCR results of MeDIP enrichment of NPD and LPD samples. Regions analyzed are the same as described in figure 2-4, except for the addition of IAP, which is hypermethylated. Each of the four feeding pairs is represented at each region as four pairs of bars. Black bars represent NPD samples and grey bars represent LPD samples. No antibody control and IgG were removed from this graph for the sake of clarity in presentation

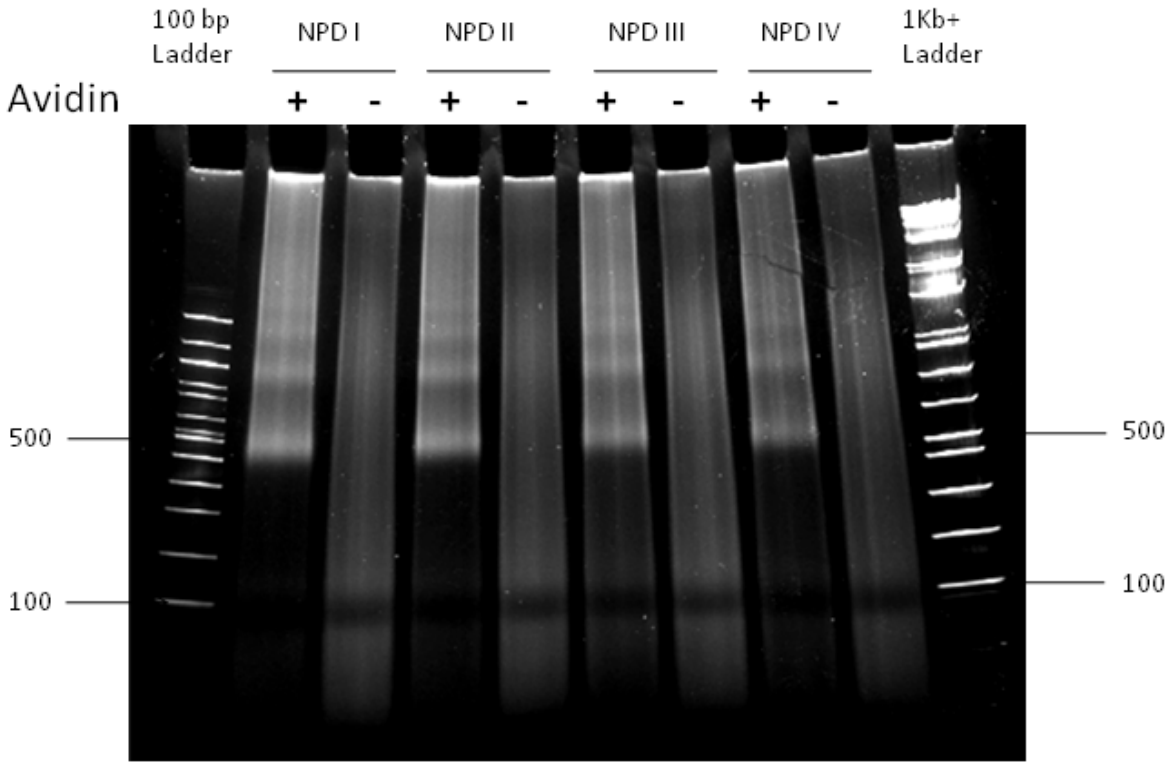


Figure 2-7. Electro-mobility shift assay. Representative EMSA gel verifying efficient biotin labeling of DNA. Fragmented and labeled DNA from 4 normal protein diet samples were incubated with or without Avidin prior to gel electrophoresis. Efficient labeling can be visualized as a shift in molecular weight in the Avidin treated lanes.

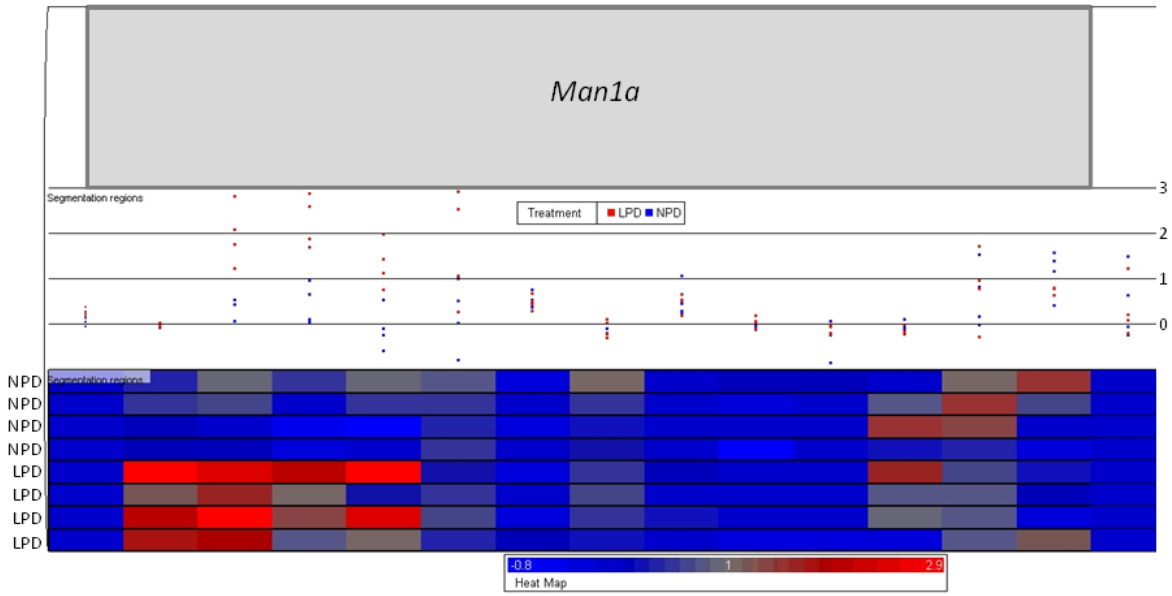


Figure 2-8. Heat map of *Man1a* Partek analysis. The gray rectangle at top represents the region view in Partek's genome browser of *Man1a*. The points in the dot plot represent individual probes (NPD in blue, LPD in red), whose height represents signal intensity. The rows at bottom indicate probes in individual samples, labeled at left. Red indicates increased signal intensity and blue decreased signal intensity.

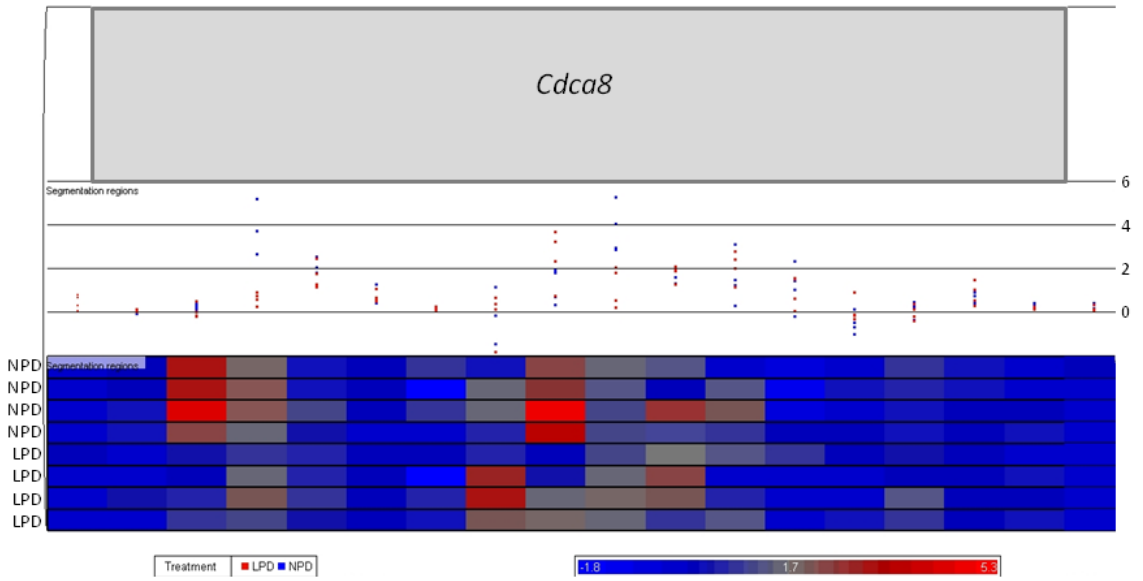


Figure 2-9. Heat map of *Cdca8* Partek analysis. Figure is labeled as in figure 2-8

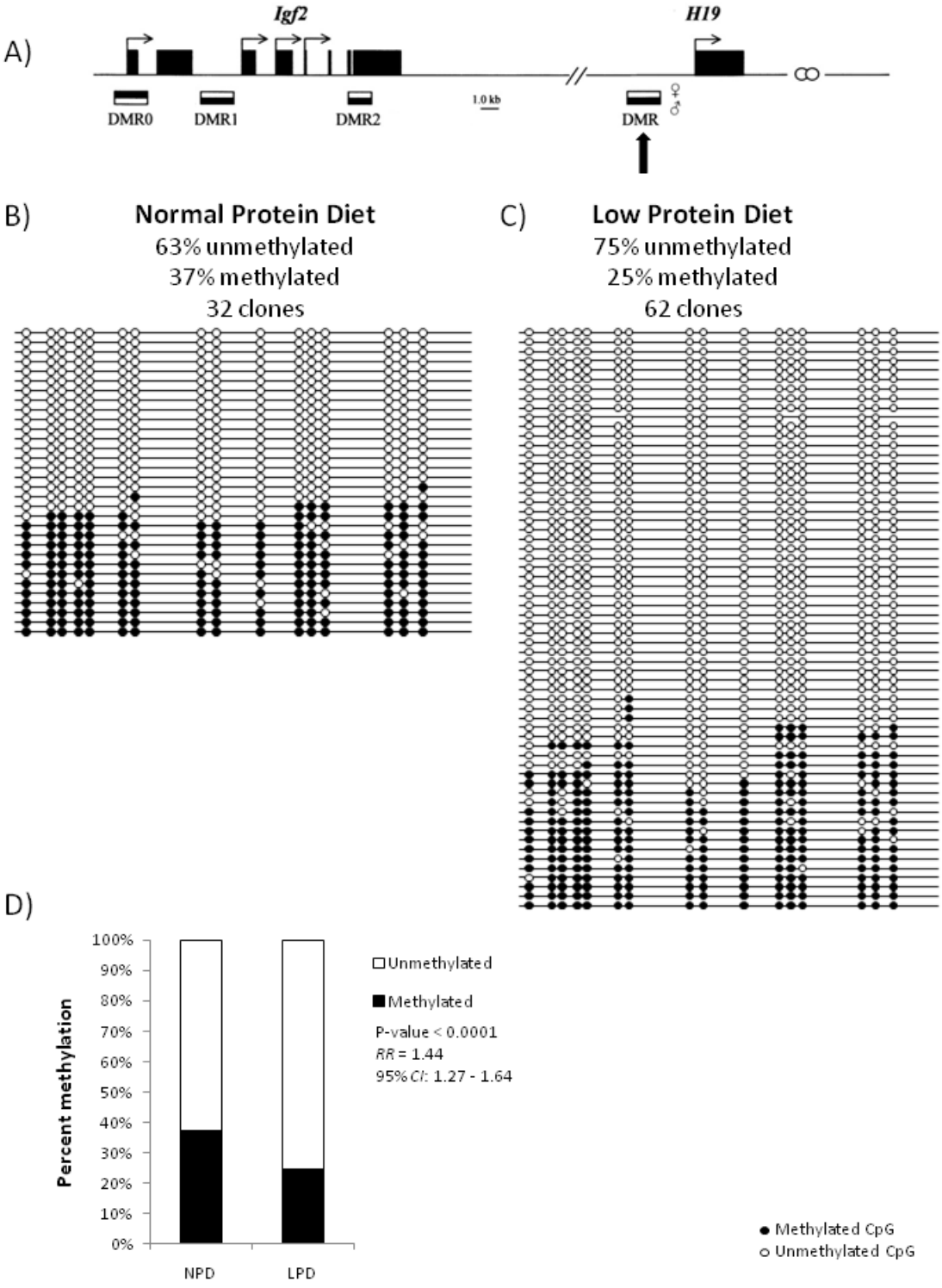


Figure 2-10. Bisulfite Genomic Sequencing Data of *H19* DMR. A) Schematic representation of the *H19*/*Igf2* imprinted domain (Lopes et al., 2003).Location of *H19* DMR indicated by black arrow. B) Percent CpG methylation for NPD samples. C) Percent CpG methylation of LPD samples. D) Statistical analysis of changes in CpG methylation between NPD and LPD samples.

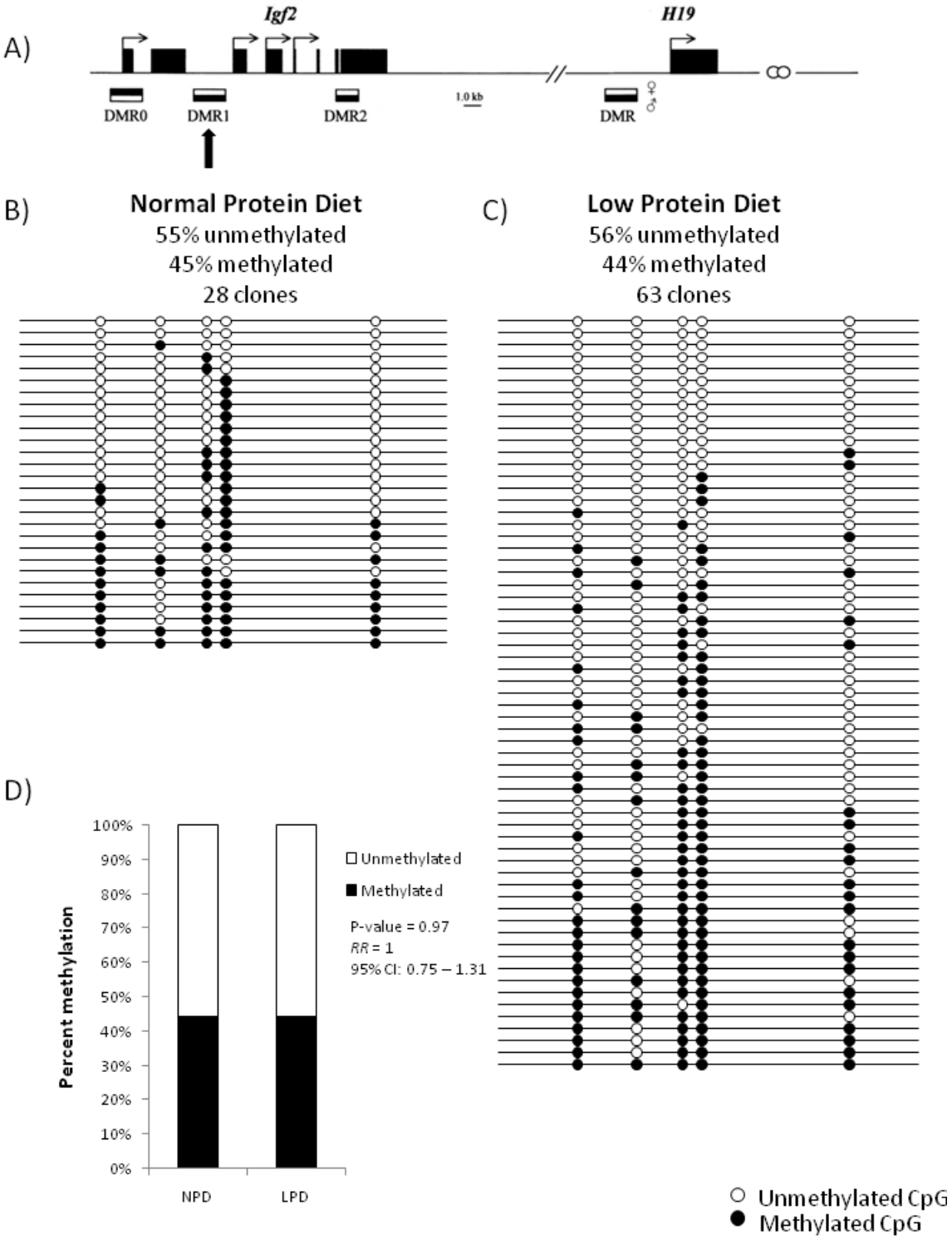


Figure 2-11. Bisulfite Genomic Sequencing Data of *Igf2* DMR1. A) Schematic representation of the *H19*/*Igf2* imprinted domain (Lopes et al., 2003).Location of *Igf2* DMR1 indicated by black arrow. B) Percent CpG methylation for NPD samples. C) Percent CpG methylation of LPD samples. D) Statistical analysis of changes in CpG methylation between NPD and LPD samples.

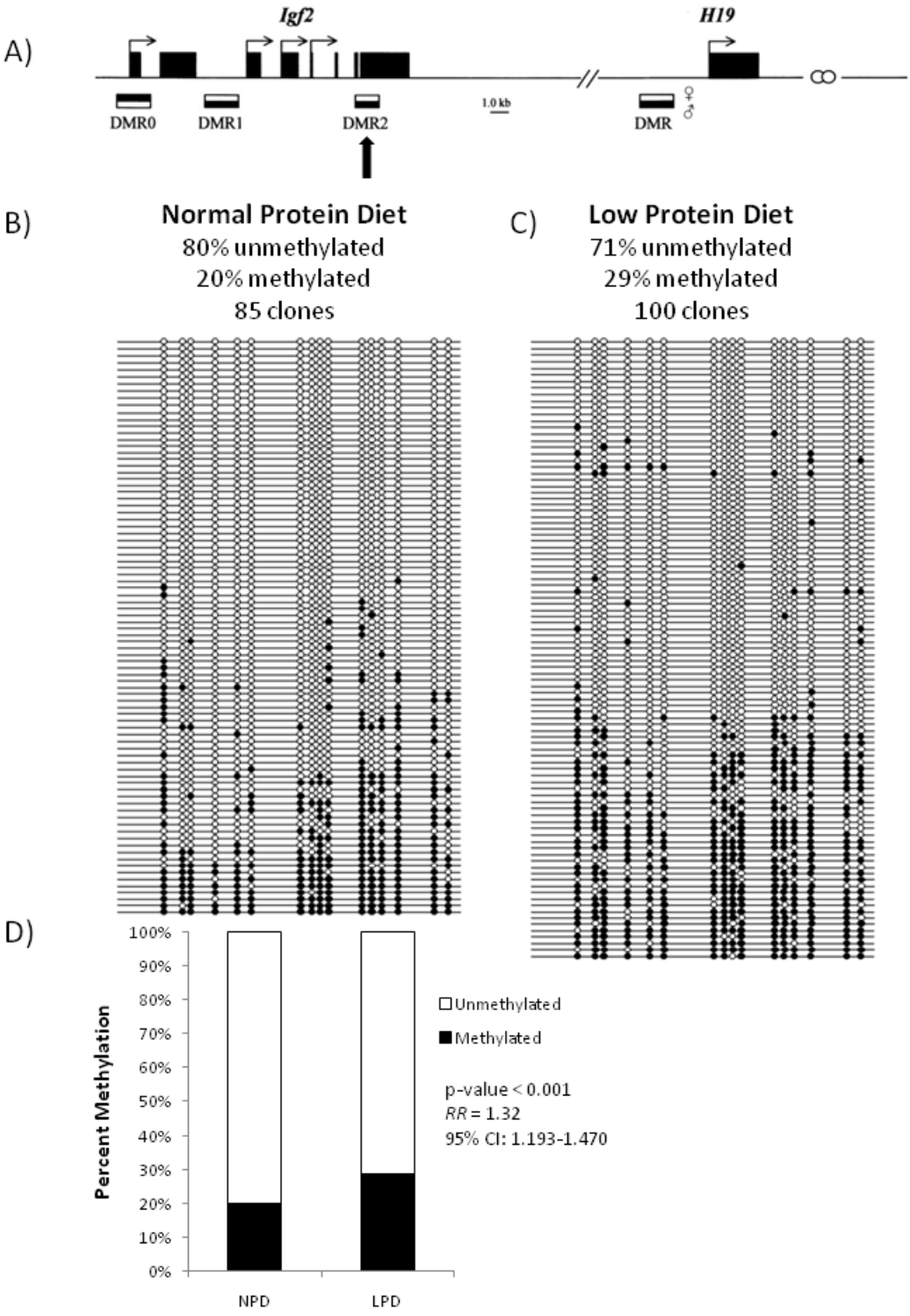


Figure 2-12. Bisulfite Genomic Sequencing Data of *Igf2* DMR2. A) Schematic representation of the *H19*/*Igf2* imprinted domain (Lopes et al., 2003).Location of *Igf2* DMR2 indicated by black arrow. B) Percent CpG methylation for NPD samples. C) Percent CpG methylation of LPD samples. D) Statistical analysis of changes in CpG methylation between NPD and LPD samples

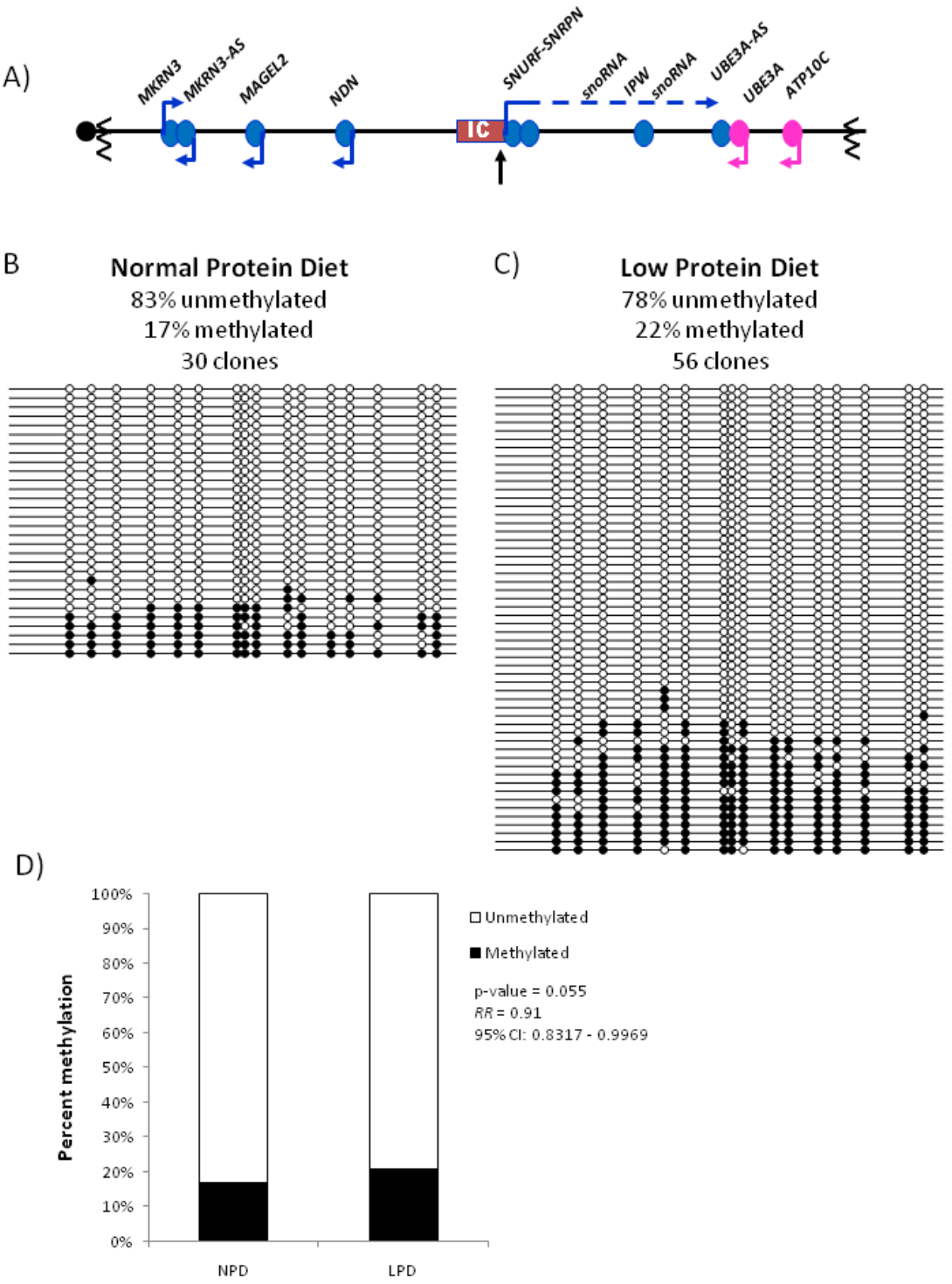


Figure 2-13. Bisulfite Genomic Sequencing Data of *Snrpn*. A) Schematic representation of the AS/PWS imprinted domain. Blue ovals represent paternally expressed genes and Pink oval represent maternally expressed genes. Location of *Snrpn* indicated by black arrow. B) Percent CpG methylation for NPD samples. C) Percent CpG methylation of LPD samples. D) Statistical analysis of changes in CpG methylation between NPD and LPD samples

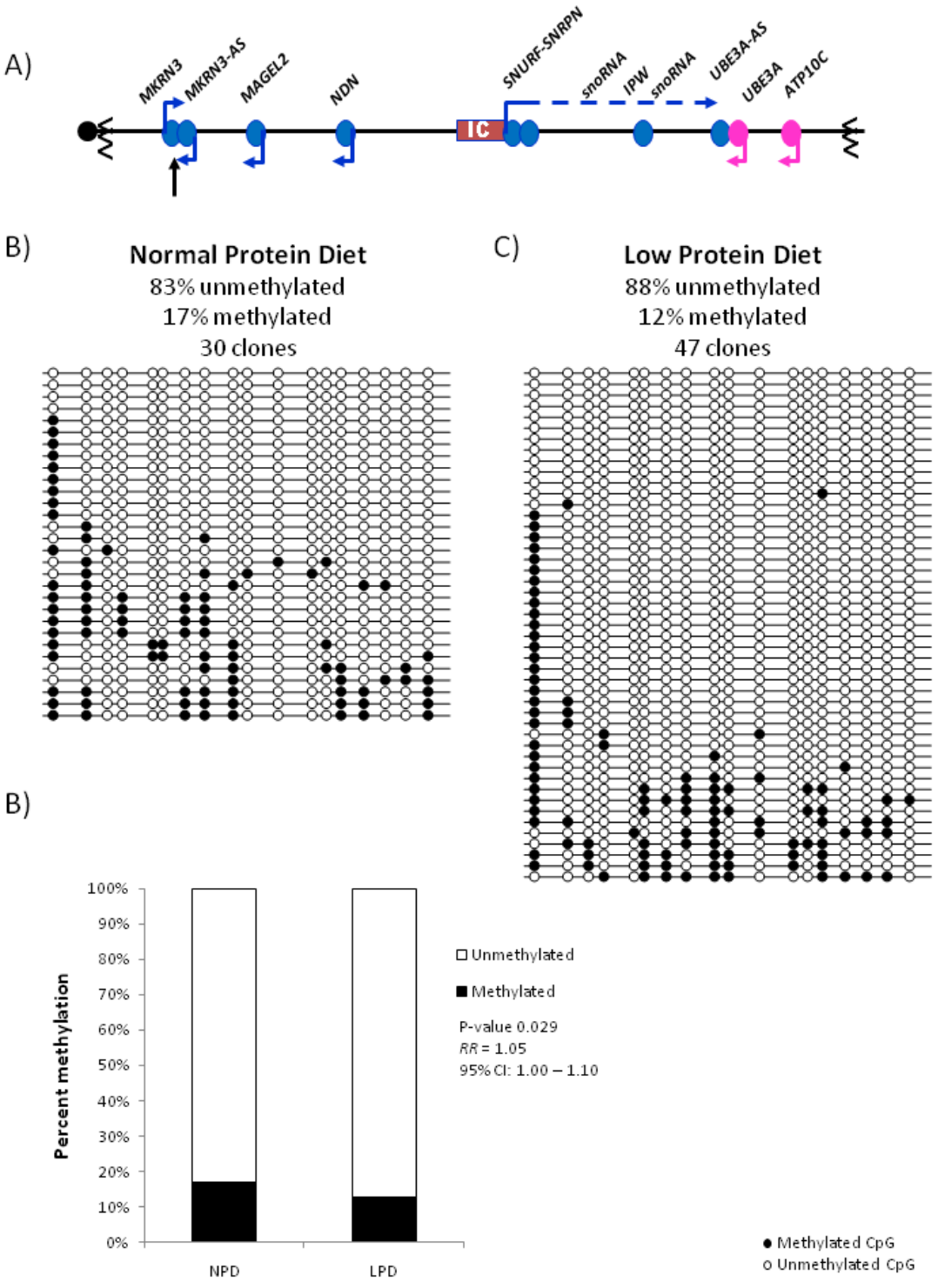


Figure 2-14. Bisulfite Genomic Sequencing Data of *Mkrn3*. A) Schematic representation of the AS/PWS imprinted domain. Blue ovals represent paternally expressed genes and Pink oval represent maternally expressed genes. Location of *Mkrn3* indicated by black arrow. B) Percent CpG methylation for NPD samples. C) Percent CpG methylation of LPD samples. D) Statistical analysis of changes in CpG methylation between NPD and LPD samples

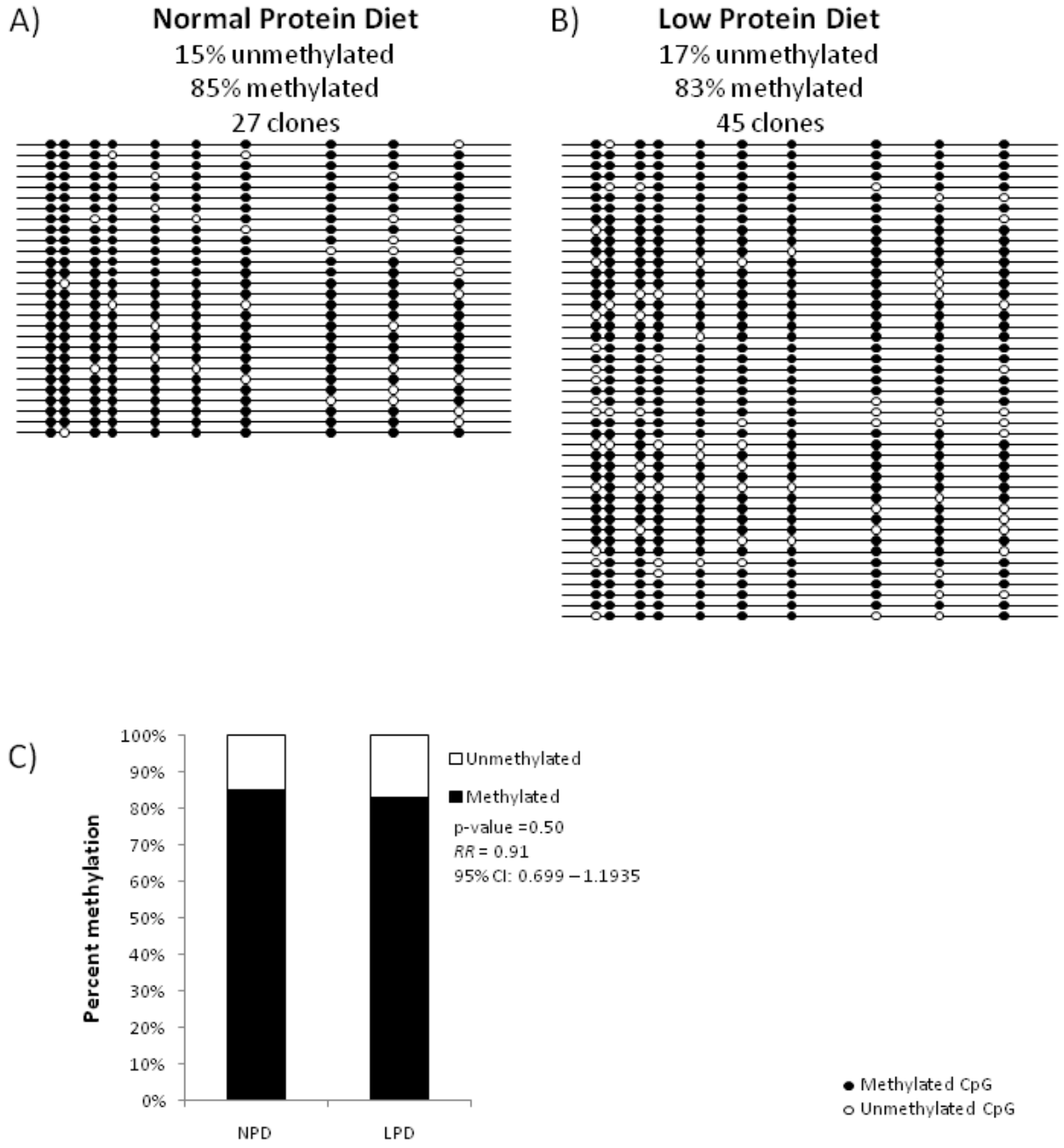


Figure 2-15. Bisulfite Genomic Sequencing Data of *Man1a*. A) Percent CpG methylation for NPD samples. B) Percent CpG methylation of LPD samples. C) Statistical analysis of changes in CpG methylation between NPD and LPD samples

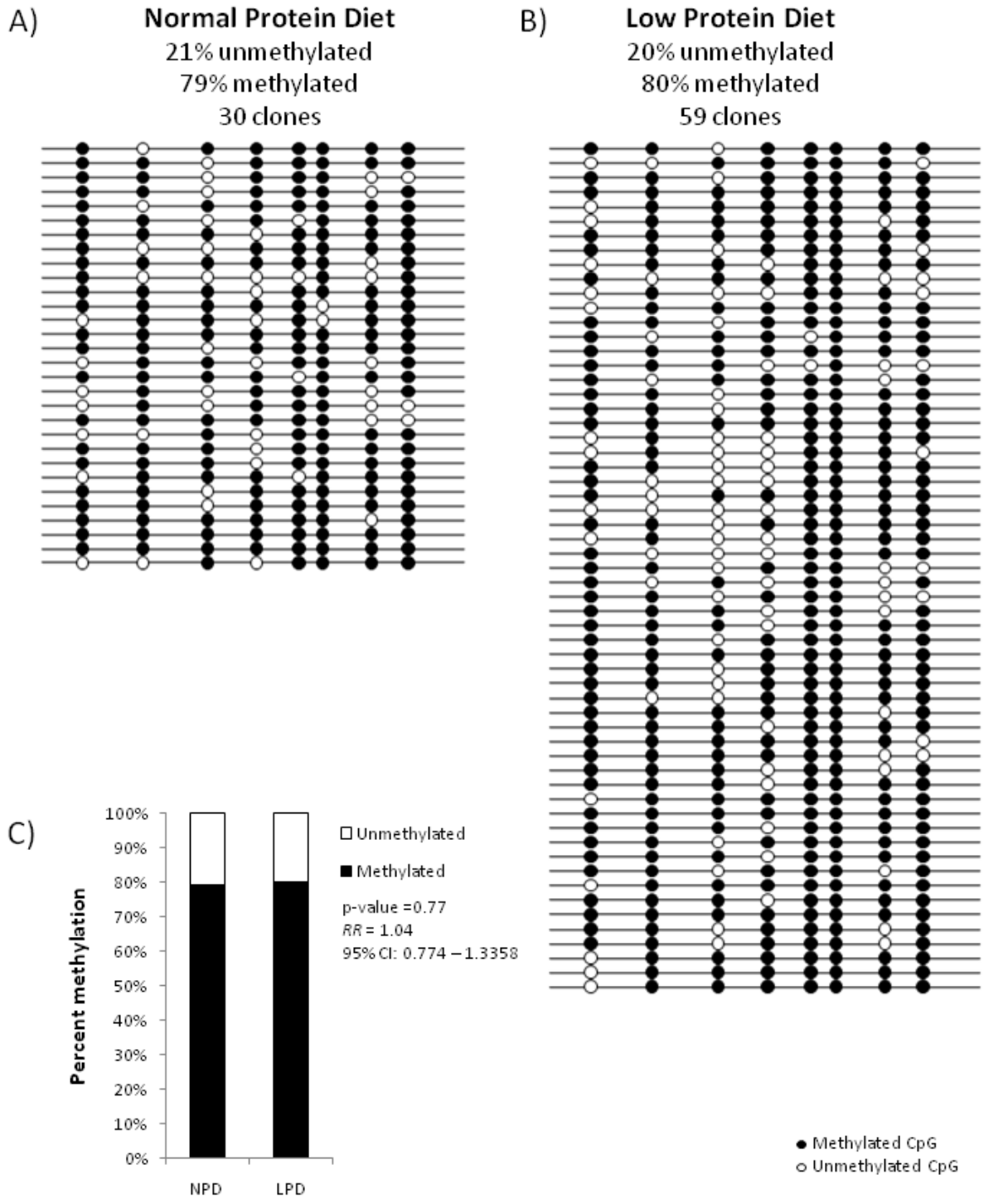


Figure 2-16. Bisulfite Genomic Sequencing Data of *Cdca8*. A) Percent CpG methylation for NPD samples. B) Percent CpG methylation of LPD samples. C) Statistical analysis of changes in DNA methylation between NPD and LPD samples.

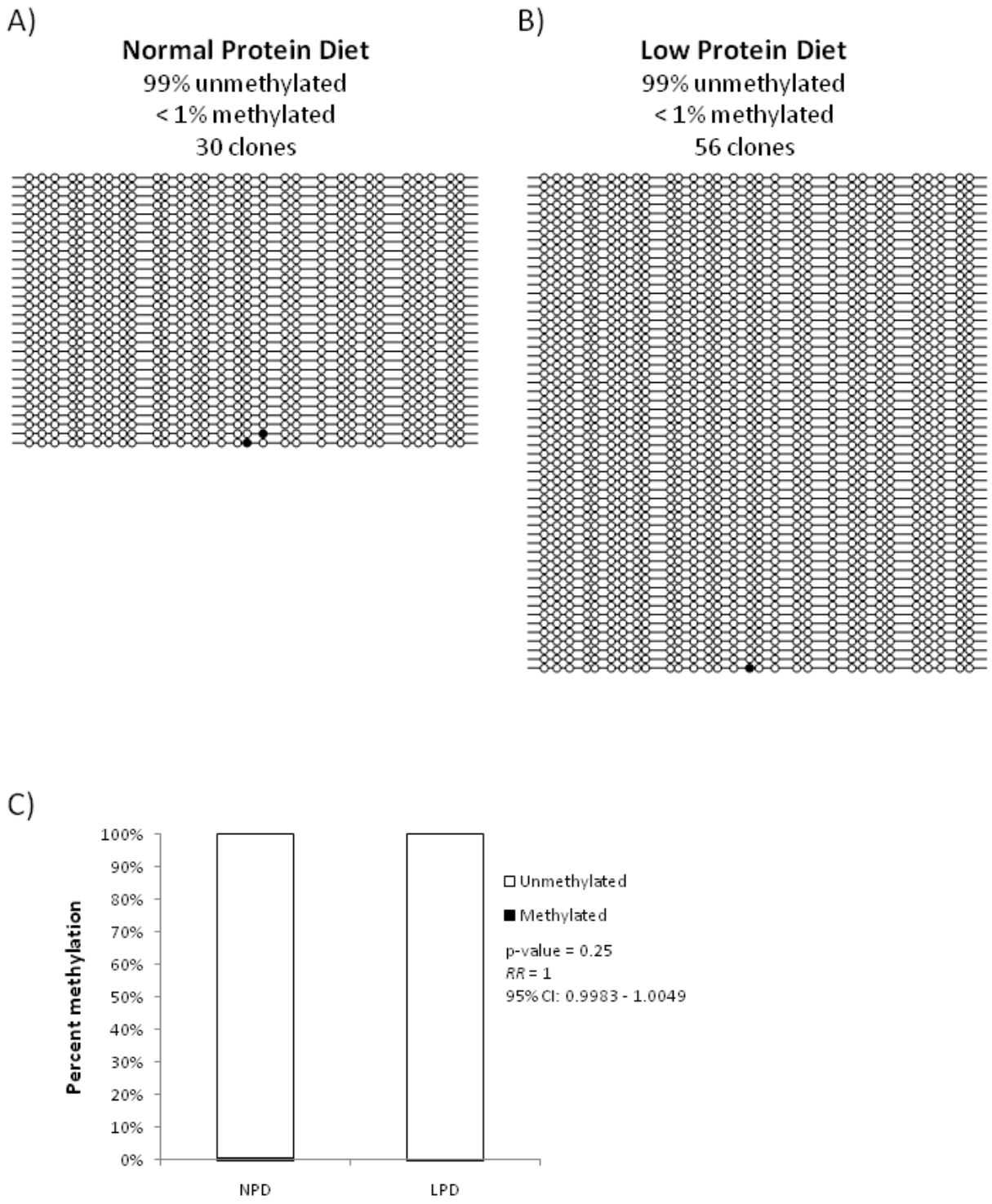


Figure 2-17. Bisulfite Genomic Sequencing Data of *ROIkI*. A) Percent CpG methylation for NPD samples. B) Percent CpG methylation of LPD samples. C) Statistical analysis of changes in CpG methylation between NPD and LPD samples.

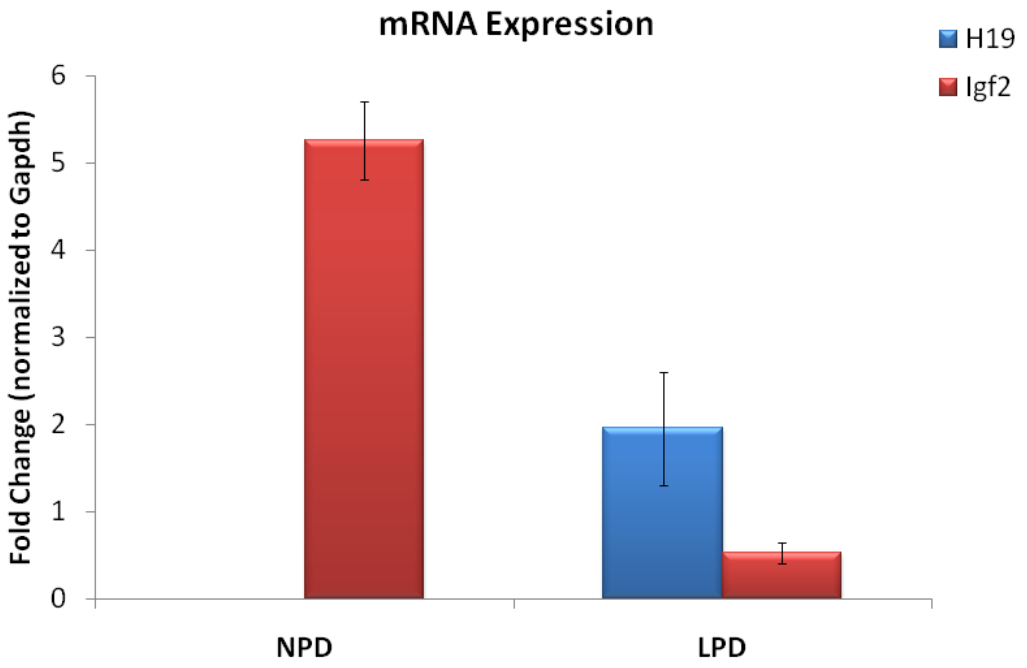


Figure 2-18. mRNA Expression. Expression levels are fold change normalized to reference gene, *Gapdh*. *H19* expression are displayed as blue bars, *Igf2* expression is displayed as red bars. Error bars are the calculated standard error of the mean of two independent duplicate reactions.

CHAPTER 3
ANALYSIS OF DNA METHYLATION IN RESPONSE TO CHRONIC FOLIC ACID SUPPLEMENTATION AND WITHDRAWAL IN CHINESE WOMEN

Introduction

The benefits of periconceptional supplementation with folic acid, a synthetic, oxidized form of folate, in the reduction in neural tube defects (NTD's) have been well studied, and it is recommended that women of child-bearing age consume 400 μg of folate daily (Berry et al., 1999). Although the success in prevention of NTD's has been dramatic, and folic acid is generally considered to be safe, there are concerns that the levels of folic acid intake may be far higher than recommended (Pfeiffer et al., 2005). As the long-term consequences of increased folate levels have yet to be extensively studied in long term clinical trials, this is becoming an area of increased concern and research.

Folate plays a major role in one carbon metabolism (Mackenzie, 1984), with its most prominent roles being involved in nucleotide synthesis and the remethylation of homocysteine to methionine, a precursor of S-Adenosylmethionine (SAM), which serves as the universal one-carbon donor involved in methylation of DNA, RNA, lipids and proteins (Lamprecht and Lipkin, 2003; Lucock, 2000). Because of this essential role, perturbations in the levels of folate can influence methylation of DNA, which is critical in maintaining the integrity of DNA, the stability of the genome, and the proper regulation of gene expression (Dolinoy and Jirtle, 2008; Robertson, 2005).

In order to examine the effects of increased folate levels on DNA methylation, we analyzed blood samples from a population-based, randomized trial of folic acid supplementation and withdrawal in Chinese women of child bearing age (Hao et al., 2008). This large population-based study set out to determine the effects of folic acid supplementation and withdrawal on homocysteine levels in a folic acid deplete population, based on dosages of folic acid ranging

from 100 μg to 4000 μg daily. Blood samples were collected at baseline (0 months), 1, 3, and 6 months, at which time folic acid supplementation ceased. Blood samples were again collected 3 months after cessation of supplementation (Hao et al., 2008). In addition to examining homocysteine levels, another study utilizing these blood samples was performed to determine changes in global DNA methylation levels based on dosage of folic acid and the presence of a common single nucleotide polymorphism (C677→T) within the methylenetetrahydrofolate reductase (MTHFR) gene. The C to T polymorphism at position 677 codes for an enzyme with reduced activity, and individuals homozygous for the TT genotype have elevated levels of blood serum homocysteine and decreased levels of genomic DNA methylation (Friso et al., 2002). Preliminary data obtained from the laboratory of Dr. Lynn Bailey at the University of Florida's Food Science and Human Nutrition Department, including work by Dr. Eoin Quinlivan and David Maneval, demonstrate a 13% reduction in global DNA methylation after 6 months of supplementation, with an additional 23% decrease after 3 months of withdrawal. The global reduction in DNA methylation appeared to be independent of dosage and genotype, but with a TT genotype dependent recovery in DNA methylation status after withdrawal. To further characterize this decrease in global DNA methylation, a subset of subjects, those receiving 400 μg of folic acid once daily, were chosen randomly and analyzed for changes in DNA methylation of tumor suppressor genes, imprinted genes and repetitive elements in response to folic acid supplementation and withdrawal. For this particular study, a total of ten subjects were analyzed, five with the CC genotype, and five with the TT genotype.

Results

Methylation Specific PCR

As global DNA hypomethylation and site-specific hypermethylation are a hallmark of the cancer genome, the initial findings that there was a substantial decrease in global DNA methylation was a concern. In order to investigate the possibility of site-specific DNA hypermethylation occurring simultaneously with global DNA hypomethylation, the promoter regions of five tumor suppressor genes (TSG) were analyzed by methylation-specific PCR (MSP). These genes were chosen based on a variety of factors, including the fact all these genes are known to be silenced by promoter hypermethylation, and these genes are all known to be silenced in a high proportion of colon cancer tumor cells (Ohm et al., 2007). This was an important determinant as the link between folate and colon cancer has been well studied (Kim, 2003). These genes were also found to be silenced in the broadest range of tumor types (Ohm et al., 2007), thus making them ideal candidate genes to be screened for possibly increased promoter methylation within our subjects. The goal of our initial screen was to observe if folic acid supplementation and subsequent withdrawal had any impact on the DNA methylation status of tumor suppressor genes, as methylation-mediated silencing of these genes could lead to increased risk of cancer development. Methylation-specific PCR allows us to perform a rapid screen of several different tumor suppressor genes in order to find candidate genes for a more detailed and in depth analysis.

The results of the MSP analysis indicate that there were two subjects with the CC genotype, 3150 and 3309, that did show methylation at the death-associated protein kinase 1, (DAPK1) and tissue inhibitor of metalloproteinase 3 (TIMP3) promoters, respectively, at 6 months (Figure 3-1A and 3-1B.). This increase in DNA methylation compared to 0 month samples appears to be transient, as it is no longer detectable at 9 months. This raises the

interesting possibility that there may be an increase in site-specific DNA methylation occurring during the folic acid supplementation phase, which is not maintained during the washout period. Although DNA methylation-mediated silencing of DAPK and TIMP3 may led to an increase in the development of cancer (Ohm et al., 2007), the biological significance of hypermethylation of these TSG promoters in circulating lymphocytes is unknown. There was also another subject, 2588 (CC) that showed hypermethylation at the estrogen receptor 1 (ESR1) gene promoter at all three time points analyzed, including baseline (Figure 3-1C), suggesting that this subject may have had hypermethylation at the ESR1 promoter before enrollment in the clinical study. Cyclin-dependent kinase inhibitor 2A (P16) (figure 3-1 D) and hypermethylated in cancer 1 (HIC1) (Figure 3-1 E) showed no detectable changes in methylation at 6 or 9 months as compared to 0 month samples.

Bisulfite Genomic Sequencing Analysis of Repetitive DNA Methylation

In order to determine where in the genome the reduction in global DNA methylation seen by Bailey et al. is occurring, we chose to focus our analyses on repetitive DNA elements as it is estimated that 35% to 40% of all DNA methylation is within repetitive elements (Bestor, 1998; Kochanek et al., 1993; Schmid, 1998). The L1 element is the most abundant non-LTR retrotransposable element, which account for approximately 21% of the human genome (Wilson et al., 2007). This made the L1 element a good candidate for analyzing changes in global DNA methylation levels. The L1 repetitive element was analyzed by high resolution sodium bisulfite genomic sequencing (BGS) to determine if any changes were occurring in DNA methylation. The initial BGS analysis focused on the 5 CC and 5 TT subjects that were analyzed by MSP. Approximately ten clones from each subject at 0, 6 and 9 months were sequenced, for a total of approximately 100 clones analyzed at each time point. For each subject, two bisulfite conversions and two PCR reactions were performed in order to reduce any potential bias from a

single reaction. It is critical when performing bisulfite sequencing to examine a sufficient number of clones in order to obtain an accurate representation of the population of L1 elements being interrogated.

Since L1 elements are evolutionarily quite old (Ostertag and Kazazian, 2001; Skowronski et al., 1988; Yang et al., 2004), there are many CpG sites which have become mutated over time by the spontaneous deamination of methylcytosine to thymine. This type of mutation is indistinguishable from an unmethylated CpG site, as analyzed by bisulfite genomic sequencing. If the deamination mutation occurs on the reverse strand, the mutated site will read as a TpA site during bisulfite sequencing (Yang et al., 2004), and will be scored as a reverse strand mutation. For this reason, when performing statistical analyses of changes in DNA methylation, CpG sites determined to be unmethylated or mutated sites during bisulfite genomic sequencing were grouped together as not methylated, and compared to methylated CpG sites for all samples analyzed.

The DNA methylation status of the L1 repetitive elements was analyzed both stratified by genotype and grouped together for 0, 6 and 9 months subjects. The bisulfite sequencing results for individual clones for 0, 6 and 9 months respectively, are represented in figures 3-2A, B and C. When stratified by genotype, there was no statistical difference in the proportion of methylated, unmethylated and mutated sites at all three time points analyzed (Figure 3-2 D). Additionally, when subjects are grouped together by time points, there was no statistical difference in the levels of DNA methylation between samples (Figure 3-2 E). This is a surprising result given the evidence of an overall global reduction in DNA methylation, and the fact that the L1 family of repetitive elements accounts for a large proportion of methylated DNA in the human genome (Ehrlich, 2002), The relatively large overall decrease in global DNA methylation

observed may be influenced by subjects with an extremely large decrease in global DNA methylation, however it needs to be noted that the global DNA methylation of these 10 randomly selected subjects is unknown at this time.

Bisulfite Genomic Sequencing Analysis of *SNRPN* Promoter

We next wanted to determine if any there were any changes in DNA methylation occurring in a locus specific manner. In order to determine whether folate supplementation and withdrawal induces any site specific changes in DNA methylation levels, we chose to analyze the *SNRPN* promoter. *SNRPN* is a maternally imprinted gene within the PWS/AS domain. Parent-of-origin methylation at the *SNRPN* promoter is critical to maintaining proper imprinted expression across the entire 2.5 Mb domain (Glenn et al., 1993; Zeschnigk et al., 1997). The expression of *SNRPN* itself is also under the regulation of promoter methylation (Zeschnigk et al., 1997).

Our initial analysis of this region was performed using high resolution sodium bisulfite genomic sequencing of the same 5 CC and 5 TT subjects analyzed for MSP and L1 DNA methylation. Approximately 10 clones for each subject were analyzed at each time point, and when possible replicate bisulfite treatments and PCR were performed. Due to difficulty in obtaining samples and problems with sample quality, not all subjects were able to be analyzed and or verified by a repeat bisulfite conversion and sequencing. A list of samples analyzed and whether verification for a particular sample was able to be performed are listed in Table 3-1.

As *SNRPN* is a maternally imprinted gene, the BGS results for the 0 month samples gave the expected ratio of approximately 50% hypermethylated clones and 50% hypomethylated clones. Without a known single nucleotide polymorphism (SNP) for these samples, it is impossible to determine the parent of origin for each clone, but we make the assumption that the hypermethylated clones are from the maternal allele.

When subjects are stratified by genotype, the percent methylation is fairly similar at 0 months, with CC subjects averaging 40% and TT subjects averaging 45% DNA methylation (Figure 3-3 A), and when samples are combined by genotype for analysis, the 0 months samples averaged 43% DNA methylation (Figure 3-3 B). The methylation data for 0 months samples are summarized in Figure 3-3 C. At 6 months there is a dramatic decrease in DNA methylation in the CC genotype, with methylation levels decreasing to 8% and a near complete loss of DNA methylation in the TT genotype, with methylation levels less than 1%. (Figure 3-4 A). When samples are combined by genotype for analysis, the 6 months samples averaged 4% DNA methylation (Figure 3-4 B, C). There is a further reduction in DNA methylation in the 9 month samples, with both CC and TT subjects averaging less than 1% methylation (Figure 3-5 A, B and C). The overall combined data for both stratified CC and TT analysis and grouped analysis for each time point for DNA methylation at SNRPN are summarized in Figure 3-6 A and B. These results are truly striking, in that we have demonstrated a possible connection between folic acid supplementation and withdrawal with a dramatic decrease in locus-specific DNA methylation at an imprinted control region. In order to more thoroughly analyze the global DNA methylation status of these samples, and to analyze a greater proportion of L1 sub-families, we analyzed these samples using pyrosequencing technology. This technique allows for the quantitative analysis of a small number of CpG sites by sequencing a large pool of individual DNA molecules simultaneously. The advantage of this technique is that a large number of DNA molecules are analyzed simultaneously, reducing the risk of bias compared to sequencing individual clones as in bisulfite genomic sequencing. Another advantage to this technique is that the DNA methylation levels are measured in a truly quantitative manner.

Pyrosequencing Analysis of L1 Elements

In order to analyze DNA methylation by pyrosequencing, we used a kit designed by Biotage to specifically analyze global methylation using the L1 element as a surrogate marker. The primers for this L1 kit were specifically designed to amplify the largest portion of L1 subfamilies as possible, thus allowing us to examine the broadest range of L1 elements and increasing our chance of observing a change in DNA methylation if it were to occur with the L1 family. Pyrosequencing was performed on 0 month samples and on 9 months samples, after 3 months of folic acid withdrawal. The results of the pyrosequencing show that in 0 month samples both CC and TT subjects averaged 72% and 71% DNA methylation respectively (Figure 3-7 A). The results are similar for the 9 month samples, with both CC and TT subjects averaging 72% DNA methylation (Figure 3-7 A). When all CC and TT subjects were combined, the pyrosequencing data indicated no change in DNA methylation between 0 and 9 month samples, with both groups averaging 72% methylation (Figure 3-7 B). Additionally, the percentage methylation was similar between the results obtained from the bisulfite genomic sequencing analysis of the L1 repetitive element and the pyrosequencing results using the L1 specific sequencing kit, with only a slight difference between bisulfite sequencing and pyrosequencing in the 9 months samples, which is most likely due to selection bias involved in sequencing individual clones using BGS (Figure 3-7 C).

Bisulfite Genomic Sequencing Analysis of Subjects with a Large Decrease in DNA Methylation after Washout

Additional data from Bailey et al. revealed that there are subsets of subjects that show either a dramatic decrease or a moderate increase in the levels of global DNA methylation in response to folic acid supplementation and especially to withdrawal of folic acid. Several of these subjects have global DNA methylation levels that are decreased by greater than 80% after

the folic acid washout period as compared to baseline DNA methylation levels. With this degree of loss of methylation, these subjects would likely have hypomethylation at repetitive elements, potentially leading to activation of transposable elements and genomic instability (Wilson et al., 2007). In order to determine if these subjects were indeed experiencing hypomethylation at repetitive elements, two subjects were chosen for further analysis, one CC (3126) and one TT (2624) genotype, which showed a greater than 85% reduction in DNA methylation levels. Sodium bisulfite genomic sequencing was performed for L1 elements in order to determine if any changes could be detected using this technique, given the large decrease in global DNA methylation of these samples. For each subject, 24 clones were sequenced for 0 and 9 months each.

The clones sequenced by BGS and analyzed for subject 2624 (TT genotype) at 0 and 9 months are shown in Figure 3-8 A and B. The results for 2624 show a statistically significant decrease in DNA methylation at L1 between 0 and 9 months (Figure 3-8 C). The 9 month sample is only 73% as methylated as the 0 month sample. The clones sequenced by BGS and analyzed for subject 3126 (CC genotype) at 0 and 9 months are shown in figure 3-9 A and B, and show no statistically significant change in DNA methylation between 0 and 9 month samples. This is a surprising result, given the greater than 85% decrease in global DNA methylation for this subject.

Although the degree of loss of DNA methylation observed at the L1 element for subject 2624 is a modest decrease, it does not account for the amount of decrease observed globally. It may be that other repetitive elements that represent a smaller fraction of the genome are in fact demethylated to a higher extent than the L1 element is. Another possible explanation is that our sample size is simply not large enough to accurately represent the methylation levels of the L1

family of repeats, or that our L1 primers may preferentially amplify DNA from a subset of cells that have not undergone demethylation. This may be due to degradation of DNA in cells with a dramatic loss of global DNA methylation.

Discussion

In the preceding work, we have demonstrated that folic acid supplementation and withdrawal can produce changes in DNA methylation in a locus-specific manner. Our analysis of the DNA methylation of the promoters of five tumor suppressor genes by methylation-specific PCR indicated that in at least two subjects, in two different genes, there was an observable increase in DNA methylation after 6 months of folic acid supplementation. Both of these subjects were homozygous for the MTHFR 677C genotype, indicating a potential genotype-dependent association with the observed changes in DNA methylation. It was also demonstrated that the changes in methylation appeared to be transient, as the increase in DNA methylation was no longer detectable in either subject after 3 months of folic acid withdrawal.

We were also able to demonstrate that folic acid supplementation and withdrawal can produce dramatic changes in DNA methylation in an imprinted gene. The DNA methylation of the maternally imprinted *SNRPN* promoter was shown to experience a near complete loss of DNA methylation after 6 months of folic acid supplementation, and to have become completely demethylated after 3 months of folic acid withdrawal. These results were obtained through the use of high resolution sodium bisulfite genomic sequencing at the promoter region of *SNRPN*. In addition to the overall decrease in DNA methylation observed at this locus, it was also demonstrated that there appears to be a genotype dependent manner to the loss of methylation, with subjects homozygous for the MTHFR 677C→T genotype showing a greater degree of loss of methylation as compared to those subjects with the homozygous CC genotype.

We have also determined that there is no detectable decrease in global DNA methylation levels caused by folic acid supplementation and withdrawal. This was demonstrated by the use of high resolution sodium bisulfite genomic sequencing of L1 repetitive DNA elements and verified though the use of pyrosequencing technology. In both experimental endeavors, there was neither a genotype dependent loss of DNA methylation nor an overall loss of DNA methylation in the subjects analyzed, although the global DNA methylation levels of these subjects is unknown. However, we did analyze two subjects whose DNA methylation levels were known to be decreased by more than 85% after folic acid withdrawal as compared to their baseline levels of DNA methylation. Through the use of bisulfite genomic sequencing, we were able to demonstrate a statistically significant decrease in DNA methylation in one subject homozygous for the TT genotype, with no change observed for the CC subject. Although the decrease in DNA methylation observed was moderate, it does not account for the greater than 85% reduction in global DNA methylation. A more detailed discussion of potential explanations for this will be included in Chapter 5.

Table 3-1. List of subjects analyzed by sodium bisulfite sequencing (BGS) at the *SNRPN* promoter. Subjects are listed by genotype and month of sample. Check mark in BGS Sequenced column indicates that data was obtained from a single bisulfite treatment, PCR and sequencing reaction. Check mark in Verified column indicates that data has been verified by a second, independent bisulfite treatment, PCR and sequencing reaction.

Genotype	Subject ID	Sample month	BGS Sequenced	Verified
CC	3150	0 months	✓	
CC	3150	6 months	✓	
CC	3150	9 months		
CC	2588	0 months	✓	
CC	2588	6 months	✓	✓
CC	2588	9 months	✓	✓
CC	3655	0 months	✓	
CC	3655	6 months	✓	
CC	3655	9 months	✓	
CC	3309	0 months	✓	
CC	3309	6 months		
CC	3309	9 months		
CC	2562	0 months	✓	
CC	2562	6 months	✓	✓
CC	2562	9 months	✓	✓
TT	2561	0 months	✓	
TT	2561	6 months	✓	✓
TT	2561	9 months	✓	
TT	2642	0 months	✓	
TT	2642	6 months	✓	✓
TT	2642	9 months	✓	✓
TT	2618	0 months	✓	
TT	2618	6 months	✓	✓
TT	2618	9 months	✓	
TT	3317	0 months	✓	
TT	3317	6 months	✓	✓
TT	3317	9 months	✓	✓
TT	3248	0 months	✓	
TT	3248	6 months	✓	✓
TT	3248	9 months	✓	

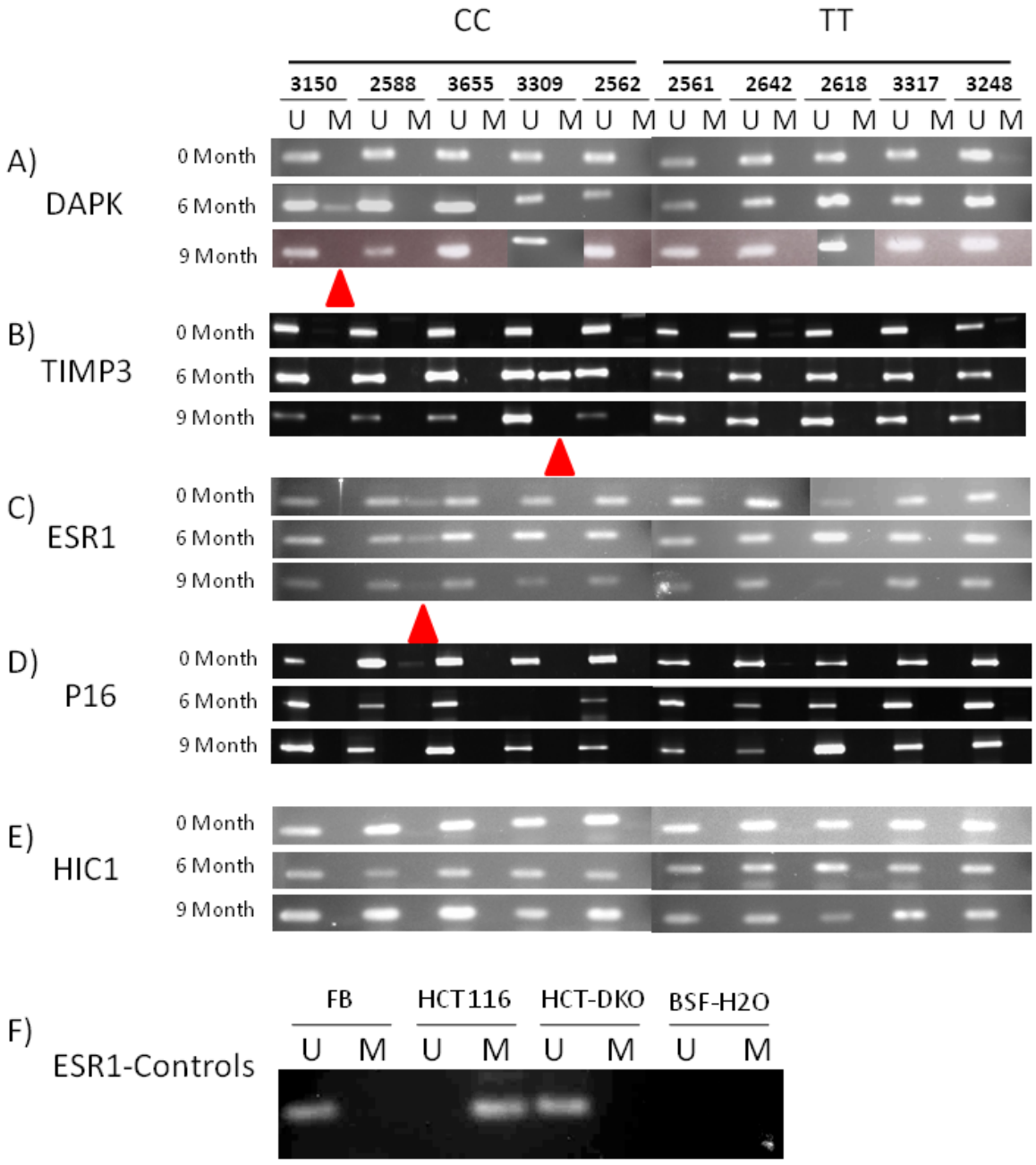


Figure 3-1. Methylation Specific PCR results for Tumor Suppressor Genes at 0, 6 and 9 months. Genotype is listed above each group of number coded subjects. U represents MSP performed with primers specific for unmethylated sequences. M represents MSP performed with primers specific for methylated sequences. For each tumor suppressor gene, methylation was analyzed for 0, 6 and 9 months as labeled at left of gel. Red triangles indicate subjects that had observed changes in methylation. F) Representative gel of MSP controls. FB: Normal human fibroblasts. HCT 116: Human male colorectal cancer cell line. HCT-DHO: HCT 116 cell line with DNMT3a and DNMT3b knockouts. BSF-H_2O: Bisulfite treated water negative PCR control.

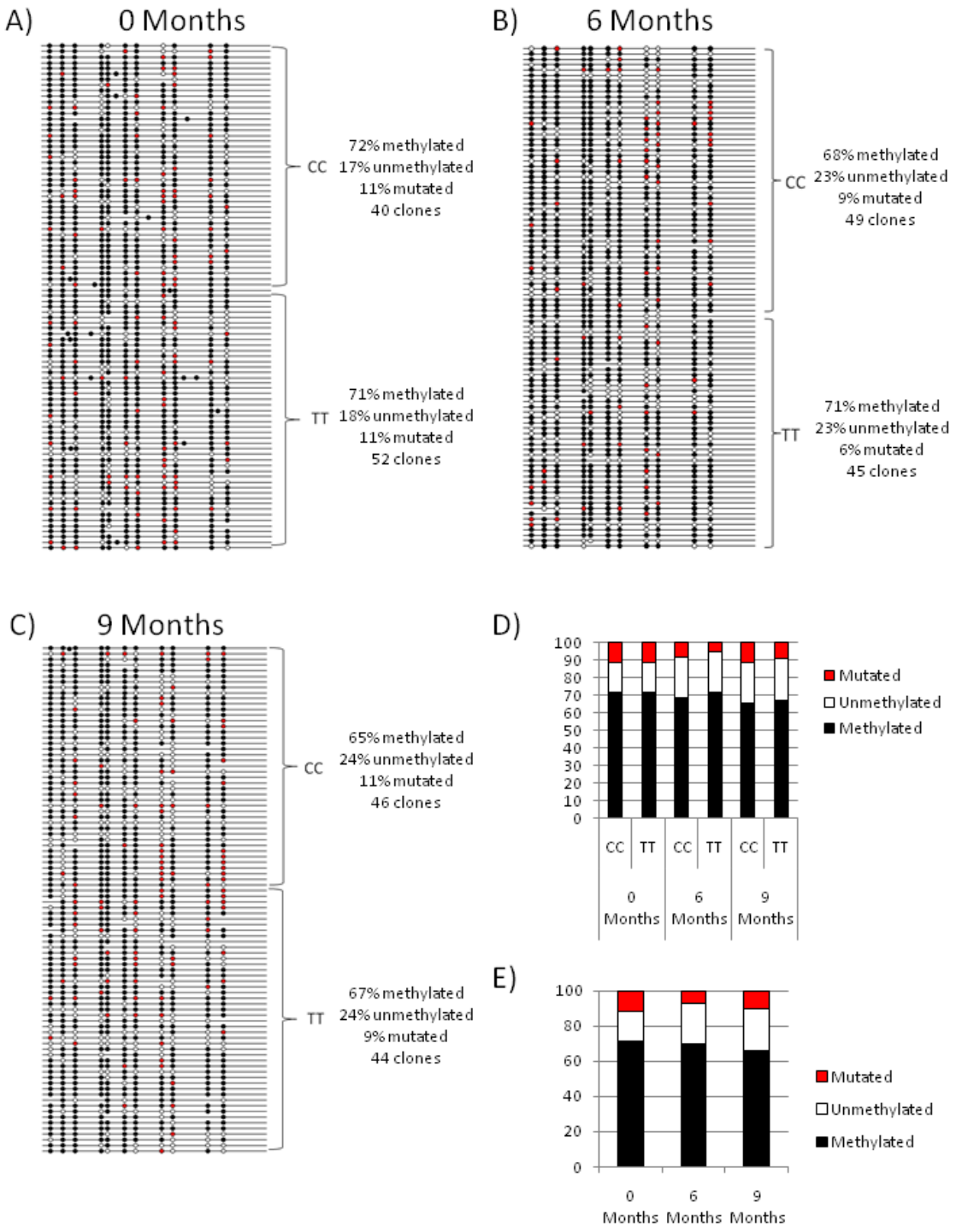


Figure 3-2. Bisulfite Genomic Sequencing of L1. A) 0 month samples grouped by genotype. Percentage values calculated as number of methylated, unmethylated or mutated sites divided by total CpG sites. B) 6 month samples, labeled as in A. C) 9 month samples, labeled as in A. D) Stacked-bar chart showing ratio of methylated, unmethylated and mutated sites stratified by genotype at each month. E) Stacked-bar chart showing ratio of methylated, unmethylated and mutated sites in grouped samples for each month.

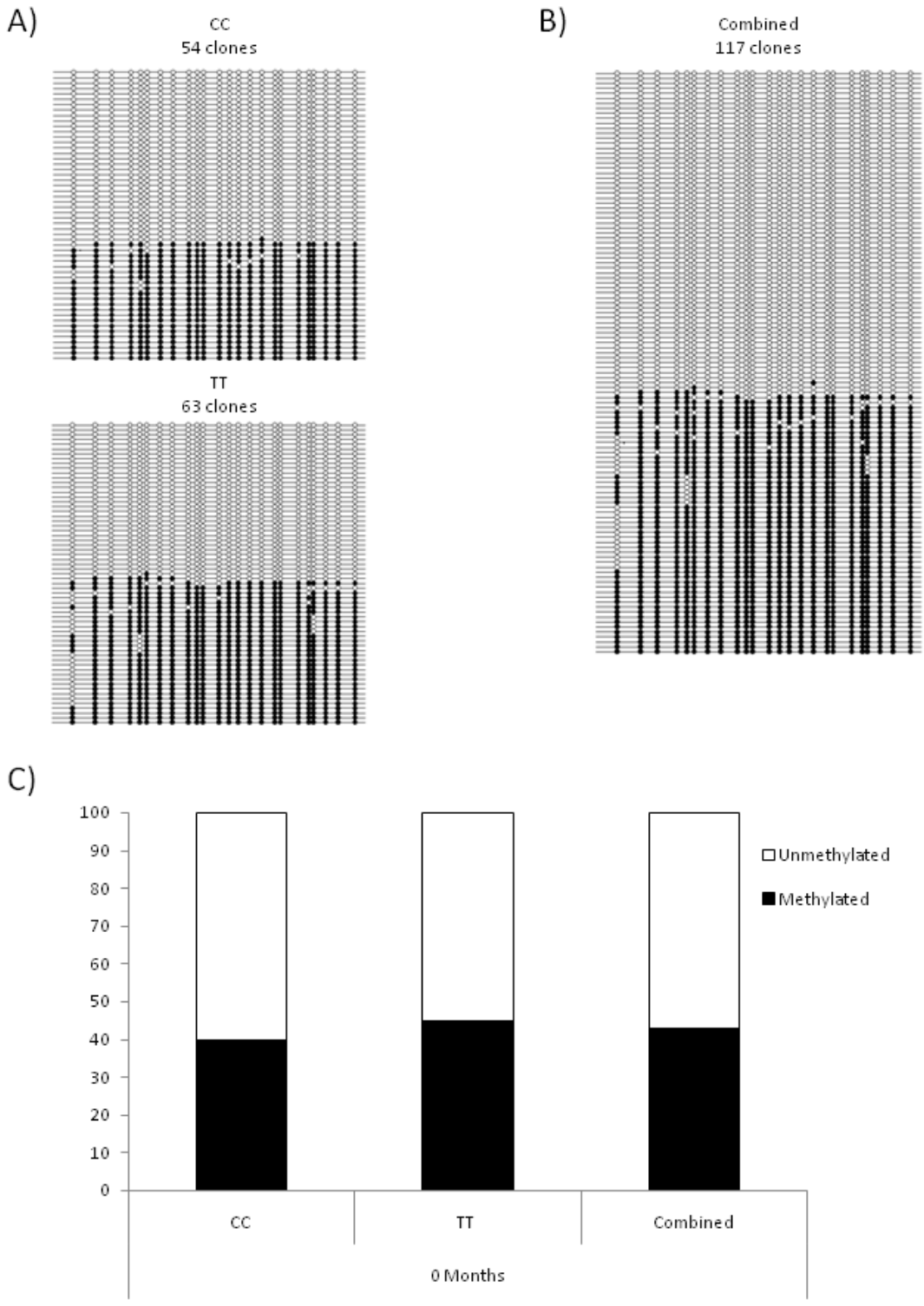


Figure 3-3. Bisulfite Genomic Sequencing of *SNRPN* at 0 months. A) 0 month samples stratified by genotype. B) Combined 0 month samples. C) Stacked-bar chart of ratio between methylated and unmethylated CpG sites by genotype and combined for 0 month samples. See Table 3-1 for samples analyzed.

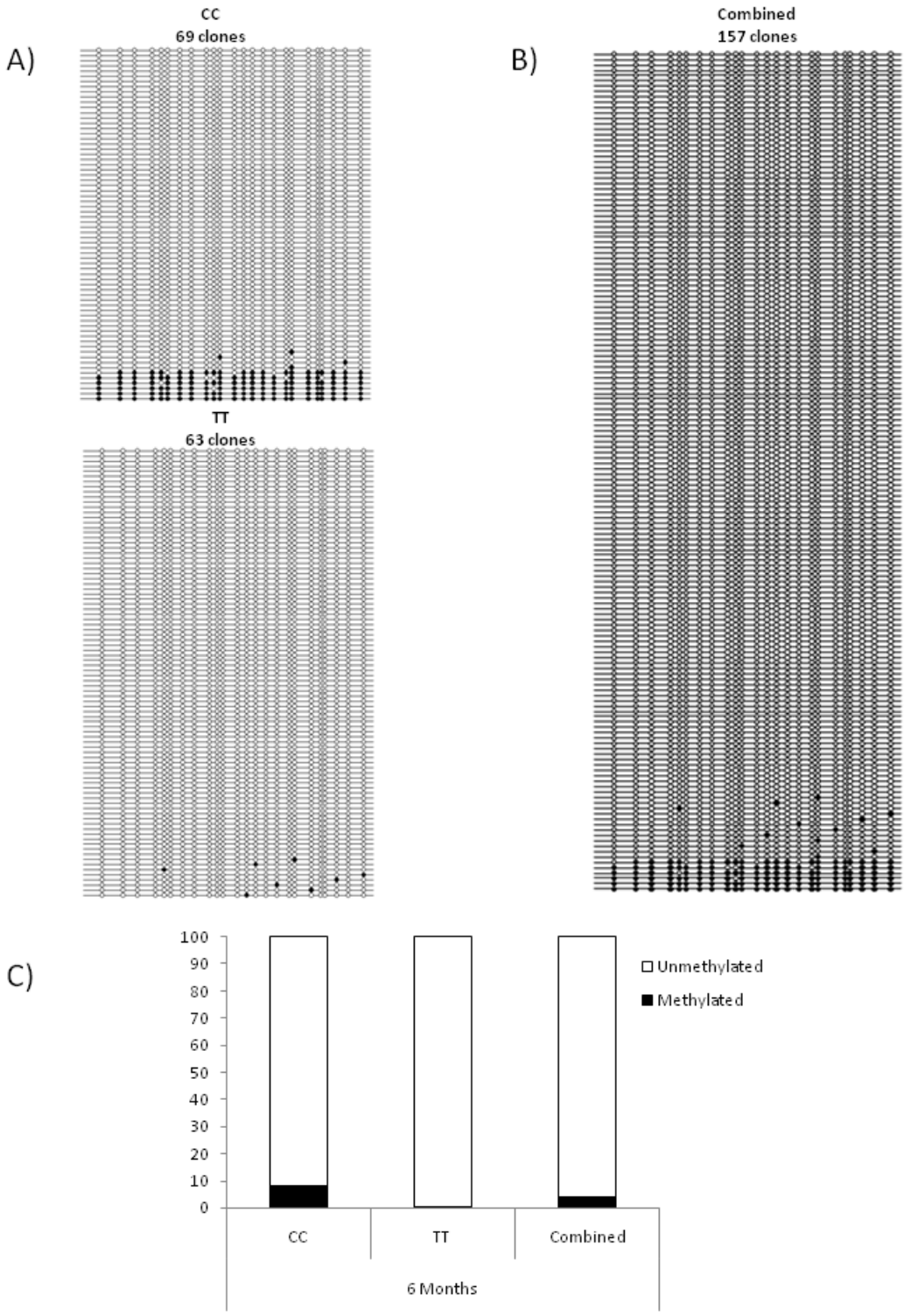


Figure 3-4. Bisulfite Genomic Sequencing of *SNRPN* at 0 months. A) 6 month samples stratified by genotype. B) Combined 6 month samples. C) Stacked-bar chart of ratio between methylated and unmethylated CpG sites by genotype and combined for 6 month samples. See Table 3-1 for samples analyzed.

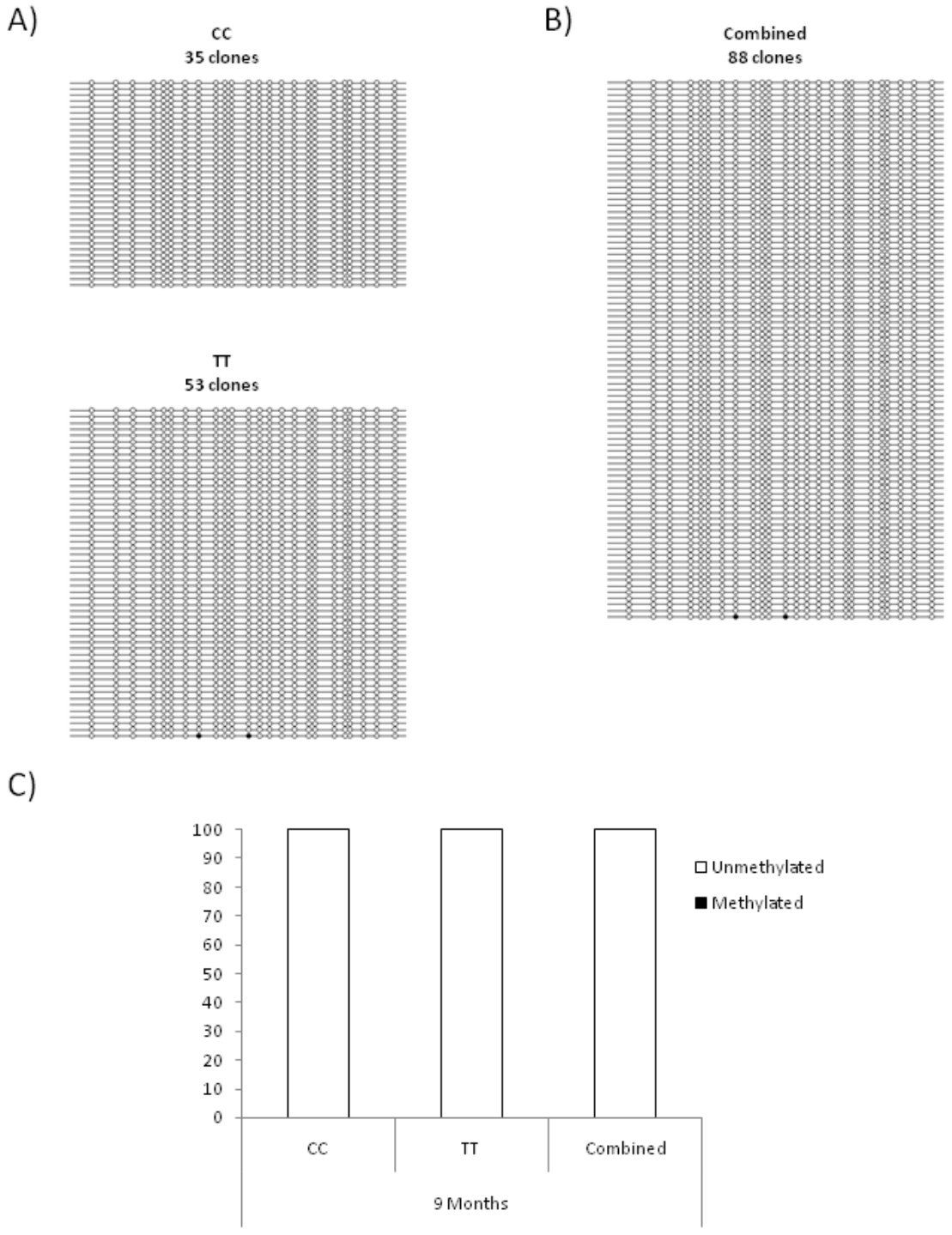
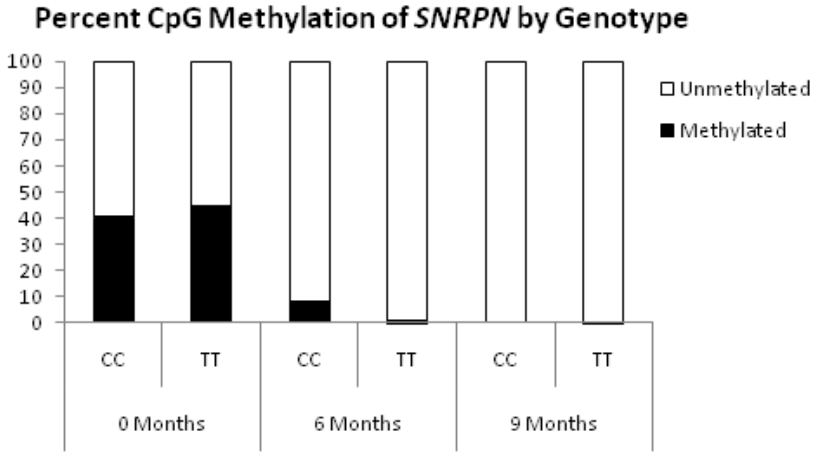


Figure 3-5. Bisulfite Genomic Sequencing of *SNRPN* at 9 months. A) 9 month samples stratified by genotype. B) Combined 9 month samples. C) Stacked-bar chart of ratio between methylated and unmethylated CpG sites by genotype and combined for 9 month samples. See Table 3-1 for samples analyzed.

A)



B)

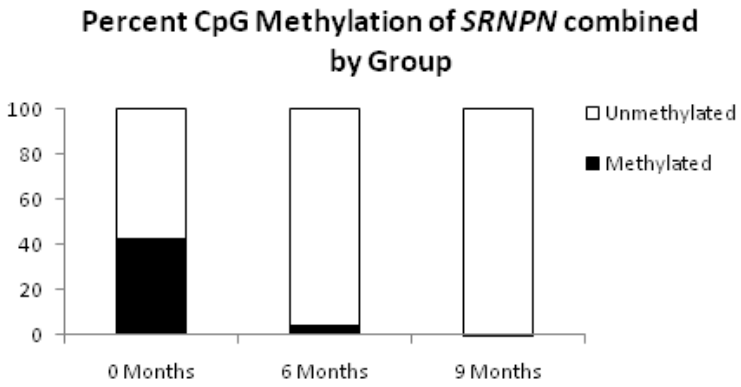


Figure 3-6. Bisulfite Genomic Sequencing of *SNRPN*. A) Stacked-bar chart representing percent CpG methylation stratified by genotype and by month for all subjects combined. B) Stacked-bar chart representing percent CpG methylation by month. See Table 3-1 for samples analyzed.

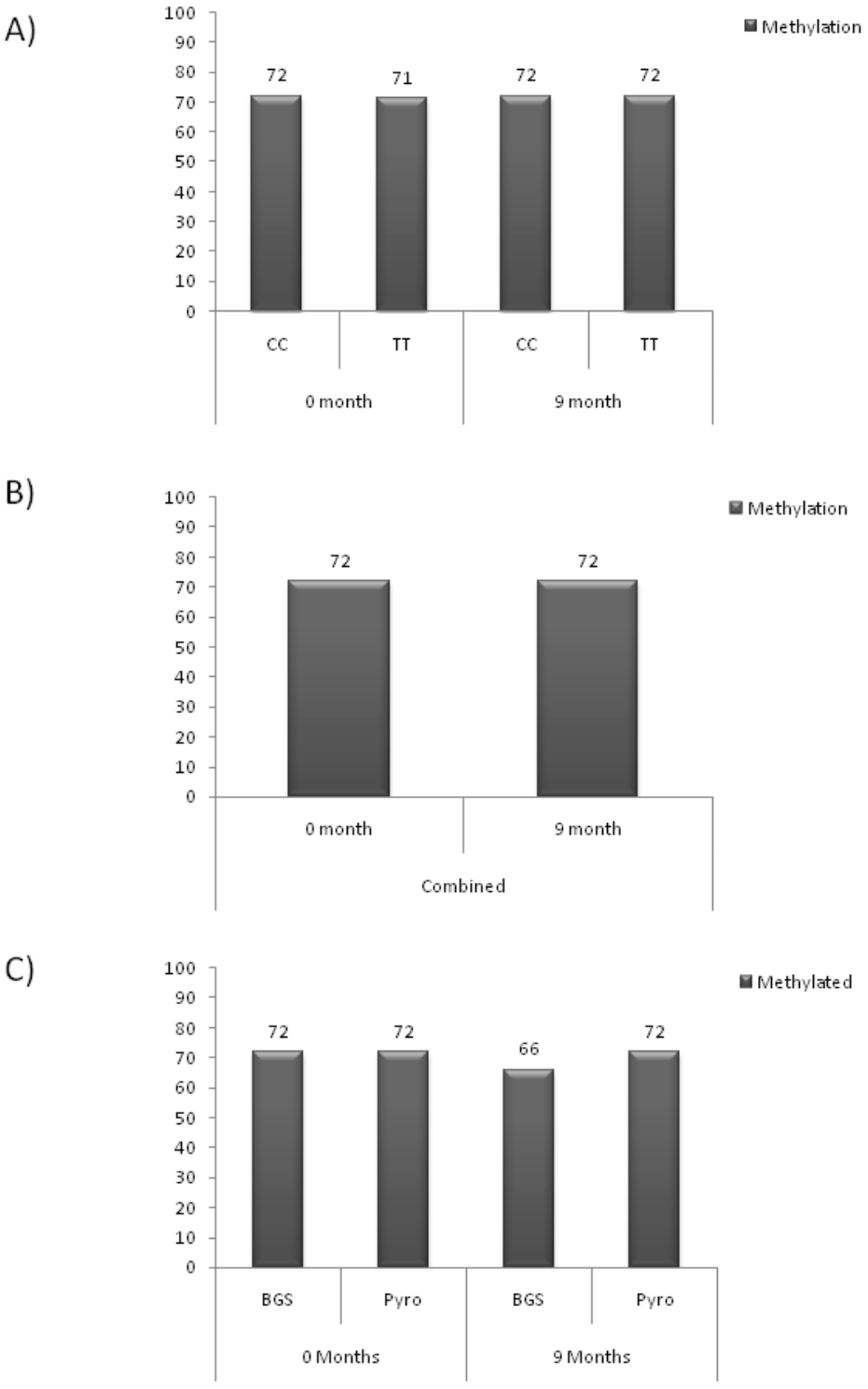


Figure 3-7. Pyrosequencing Analysis of L1 Repetitive Element. A) Percent methylation for 0 and 9 month subjects stratified by genotype. B) Percent methylation for combined subjects at 0 and 9 months. C) Comparison of bisulfite genomic sequencing (BGS) with pyrosequencing results.

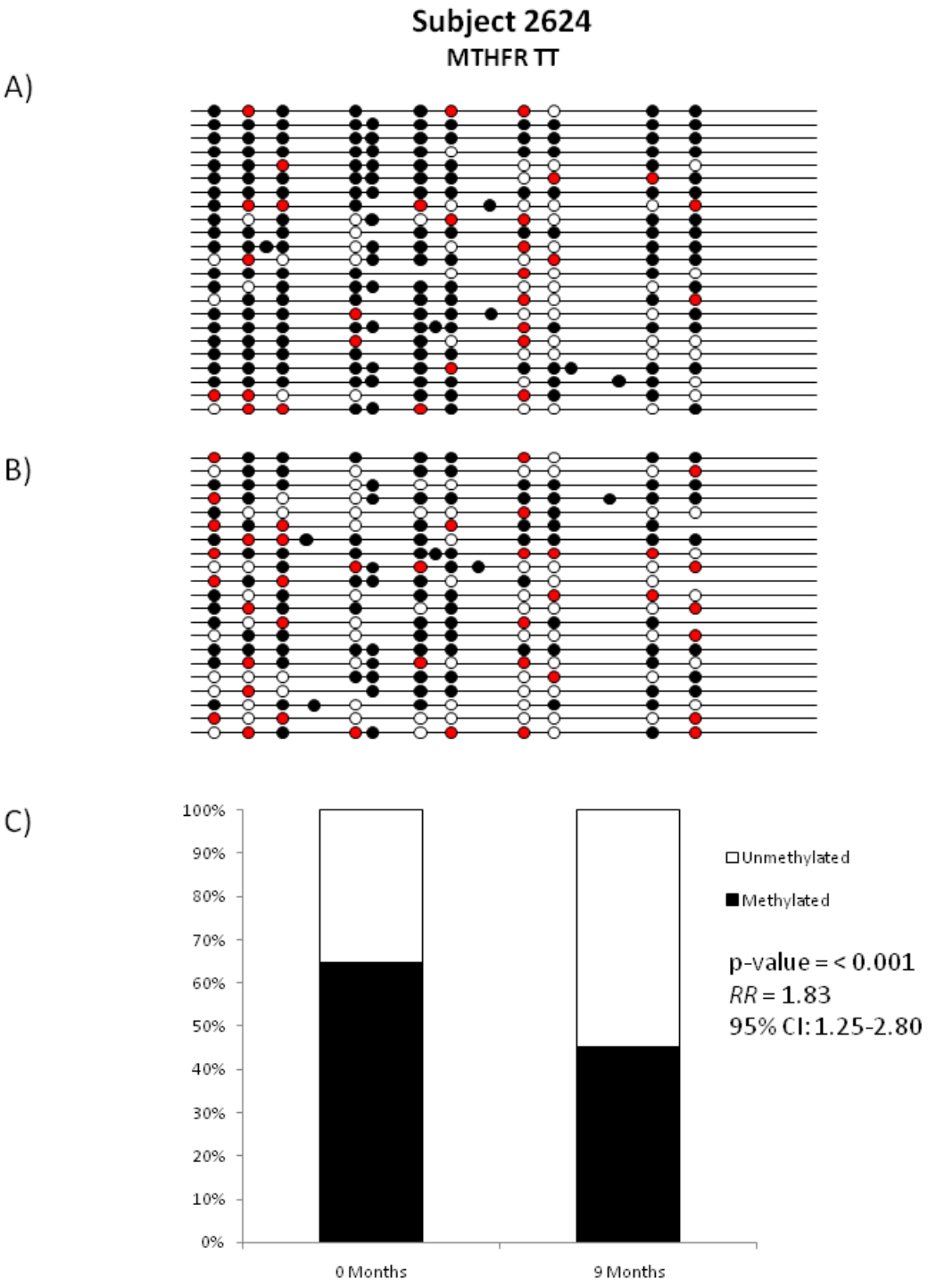


Figure 3-8. Bisulfite Genomic Sequencing of subject 2624. A) Bisulfite genomic sequencing of 0 months samples. B) Bisulfite genomic sequencing of 9 months samples. C) Stacked-bar chart representing ratio of methylated CpG sites to unmethylated (combination of unmethylated and mutated sites).

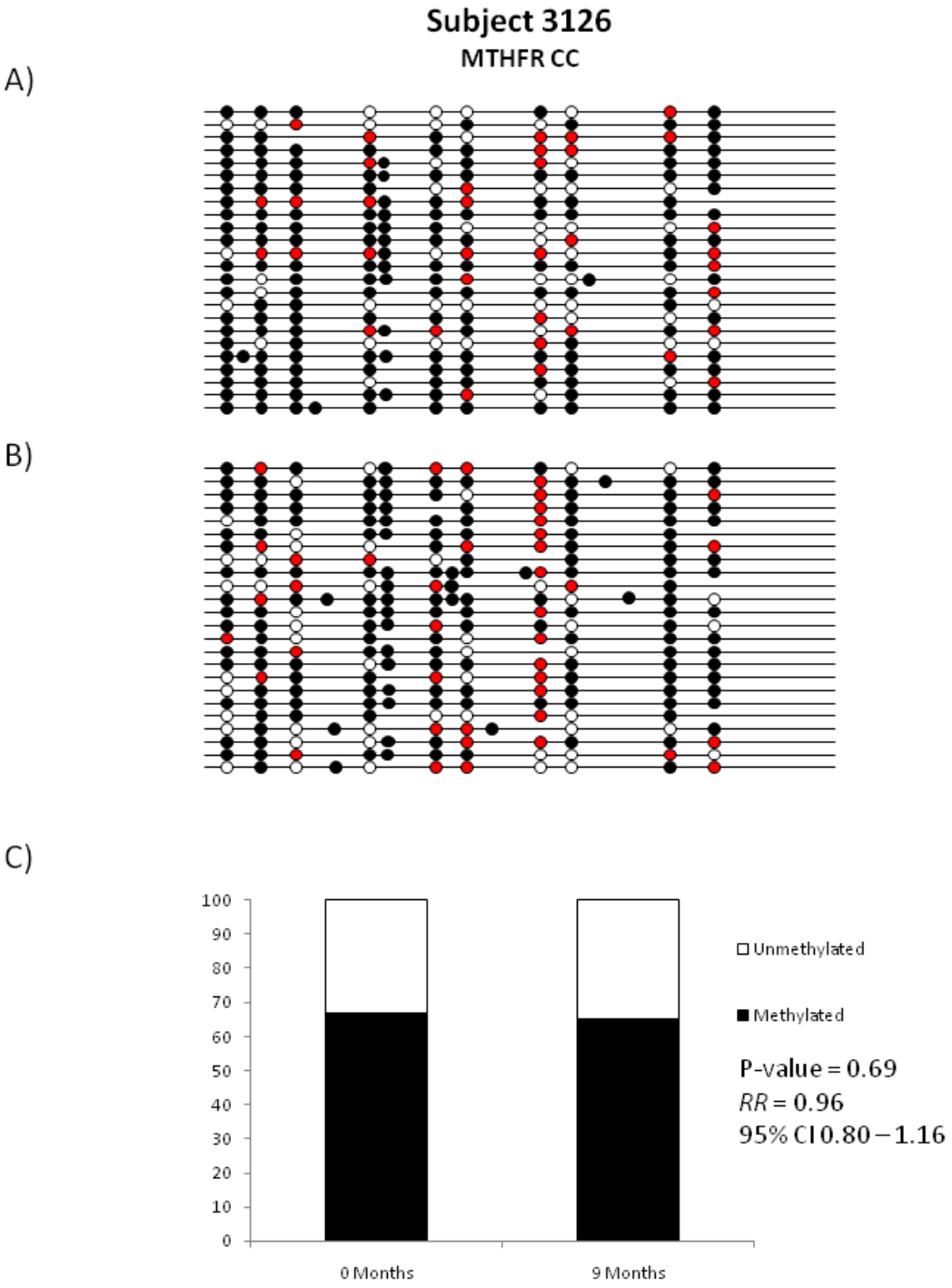


Figure 3-9. Bisulfite Genomic Sequencing of subject 3126. A) Bisulfite genomic sequencing of 0 months samples. B) Bisulfite genomic sequencing of 9 months samples. C) Stacked-bar chart representing ratio of methylated CpG sites to unmethylated (combination of unmethylated and mutated sites).

CHAPTER 4
MATERIALS AND METHODS

Animals

Timed pregnant C57BL/6J mice were ordered from The Jackson Laboratory and delivered on or before day 5 of pregnancy. Upon arrival, mice were weight-matched and placed into either control or experimental groups. At day 5 of pregnancy, weight-matched control and experimental mice were pair fed to ensure equal consumption between weight-matched pairs. The control group was fed a normal protein diet (19.39% protein) and the experimental group was fed a low protein diet (8% protein); both diets were prepared from TestDiet® (Purina Mills, LLC/PMI). Both diets are isocaloric by weight, with extra calories being supplied by sucrose in the low protein diet. The experimental group animals were given the low protein diet *ad libitum* and food consumption was weighed daily. The control group animals were then given normal protein diet equal in weight to the amount of food consumed for the pair-fed experimental animal during the previous 24 hours, such that food available to control mice is matched accordingly on a daily basis. Water was supplied *ad libitum* and both groups of mice were weighed daily to ensure that pair-feeding has no adverse affect on weight, as per IACUC mandates. All animals were housed in barrier cages (Specific Pathogen Free) in a climate controlled room (22C/20% humidity) with 12:12-hours light-dark cycles. Pair feeding was continued until sacrifice at day 18.5 of gestation, at which time individual maternal and fetal liver tissues were collected and immediately frozen in liquid nitrogen and stored at -80^{o}C.

Genomic DNA Extraction

To prepare fetal liver tissues for DNA extraction, previously frozen livers from each litter were ground together using a mortar and pestle. Tissues were kept frozen throughout this process by using liquid nitrogen in the mortar and keeping samples on dry ice. One-third of ground liver

samples were removed and stored at -80^{o}C for later RNA extractions. DNA was purified from the remaining tissues using standard phenol extractions and ethanol precipitation (Strauss, 2001). Briefly, frozen, ground tissues were transferred to a 50 ml conical tube to which 10 mls of DNA Extraction Buffer with Proteinase K (50 mM Tris-HCl pH 8.5; 25 mM EDTA pH 8.0; 150 mM NaCl; 300 μg/mL proteinase K) were added. Samples were rotated overnight at room temperature. Samples were then extracted sequentially using equal volumes of phenol:chloroform (1:1), followed by chloroform. Extracts were then treated with RNase (20 U/ml) (Ambion AM2286), followed by an additional round of sequential organic extractions. DNA was precipitated by addition of NaCl to a final concentration of 300 mM and 2.5 volumes of 100% ethanol (EtOH). Samples were allowed to precipitate overnight at -20^{o}C, followed by centrifugation. DNA was resuspended in 4 ml of TE (100 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0).

Methylated DNA Immunoprecipitation (MeDIP)

Sonication

Sonication was performed using a Sonic Dismembrator Model 100 sonicator to shear genomic DNA to a size of 300bp to 700bp using the following parameters: 80 μg of genomic DNA in 1 ml total volume IP Buffer (0.05% Triton X-100 in PBS). DNA was sonicated 3 times for 20 seconds at power setting 3 (8 watts power output), in a 4 ml plastic culture tube in an ethanol and ice bath with 1 minute in between pulses to cool samples. The samples were then pulsed an additional 2 times for 20 seconds each at power setting 4 (10 watts) with 1 minute of cooling in between pulses. 20 μl of sonicated sample were removed and run on an agarose electrophoresis gel apparatus to verify sonication efficiency and DNA fragment size.

Immunocapture

Immunocapture of methylated DNA was performed using an anti-5-methylcytosine (α5-C^{Me}) antibody (Epigentek Catalog # A-1014). For each IP, 4 μg of sonicated DNA was denatured at 95°C for 10 minutes and snap cooled on ice for 2 minutes, at which point cold IP Buffer was added to 500 μL total volume and 10 μl α5-C^{Me} was added to each methylated DNA IP. Normal mouse IgG (Millipore 12-371) and a no antibody negative control were performed for each sample. After addition of appropriate antibody, samples were rotated slowly at 4°C for 16 hours. For each IP sample, 40 μl magnetic beads (Dynal – Dynabeads M-280 Sheep anti-Mouse IgG) were prepared by washing twice in 500 μl Bead Washing Buffer (0.1% BSA, 0.02% NaN_3 in PBS) with 2 minutes on magnetic rack between each wash and resuspended in 30 μl Bead Washing Buffer. After antibody binding, each sample mixture was transferred to freshly prepared beads and incubated at 4°C for 6 hours while slowly rotating. Upon binding of DNA/antibody complex to Dynabeads, samples were allowed to settle in a magnetic rack for 5 minutes. The supernatant from the no antibody control sample was removed to a 1.5 ml tube and stored in ice for future use as input DNA sample. The supernatants were removed from the remaining samples and discarded.

Washing of the antibody/DNA/bead complexes was performed sequentially with 1 mL wash buffer at 4°C with slow rotation for 5 minutes each wash using the following wash buffers: Low Salt Wash (0.1% SDS, 1% Triton X-100, 2mM EDTA, 200mM Tris-HCl, pH 8.0, 150mM NaCl), High Salt Wash (0.1% SDS, 1% Triton X-100, 2mM EDTA, 200mM Tris-HCl, pH 8.0, 500mM NaCl), LiCl Wash (0.25M LiCl, 1% NP-40, 1% sodium desoxycholate, 1mM EDTA, 100mM Tris-HCl, pH 8.0) followed by two washes each of T.E. (10mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0).

Beads were then resuspended in Elution Buffer (100mM NaCl, 10mM Tris-HCl, 25mM EDTA pH 8.0, 0.5% SDS, 0.1mg/mL proteinase K) and incubated for 30 minutes at 50^{o}C with 550 RPM shaking in a thermomixer. After proteinase K treatment, SDS was added to a final concentration of 1.5% for all samples (including Input sample) and heated to 100^{o}C for 5 minutes and cooled briefly on ice, followed by bead separation on a magnetic rack. Supernatant, containing eluted DNA, was removed and purified using the QIAquick PCR Clean-up Kit (Qiagen Catalog # 28106), following the manufacturers provided protocol. Briefly: Five volumes of Binding Buffer PB was added to eluted DNA and applied to the clean-up column. After drawing sample through the column using a vacuum manifold, columns were washed with 750 µl Wash Buffer PE and then dried by centrifugation. DNA was eluted off the column with 150 µl 0.5X TE pH 8.0 and stored at -20^{o}C.

Quantitative RT-PCR of MeDIP

Enrichment of methylated DNA in the meDIP samples was determined by RT-qPCR using primers for the highly methylated LAP LTR sequences (Weber et al., 2005), the differentially methylated *H19* DMR, *Hprt* and *Xist* sequences, and for the unmethylated sequences of *ActB*, *Aprt*, and the control sequence (no CpG sites) CSa with both the input and immunoprecipitated samples. The protocol was adapted from Weber et al. (Weber et al., 2005).

Real-Time quantitative PCR was performed using the SYBR GREEN PCR Master Mix from Applied Biosystems (Part No.: 4309155) and a MJ Research DNA Engine Opticon 2 Continuous Fluorescence Detector. For each reaction, 1/60th of the input and immunoprecipitated DNA, and 5 pmol of each primer were used in a 20 µl reaction volume. A 5-point standard curve was generated from a serial dilution of DNA (100%-0.01%) for each primer pair, and percent input was calculated for each locus using Opticon 2 software. To determine relative enrichment

of methylated versus unmethylated sequences after immunoprecipitation, the calculated percent input values for each locus were normalized to the unmethylated sequences *ActB* or *Aprt*. Primers used are listed in Table 4-1.

Amplification of Immunoprecipitated DNA

Immunoprecipitated DNA was amplified using Sigma's Whole Genome Amplification Kit (Sigma Cat. WGA2). The manufacturer's protocol was followed with minor adjustments. The initial fragmentation steps of the protocol were not performed, as the IP DNA was previously sheared by sonication. For each sample, 25 ng of DNA in 10 μl volume were used for amplification, to which 2 μl Library Preparation Buffer and 1 μl Library Stabilization Buffer were added. Samples were vortexed briefly, heated to 95^{o}C for 2 minutes, and snap cooled in ice. 1 μl of Library Preparation Enzyme was added to each sample and placed in a thermocycler pre-chilled to 16^{o}C and run using the following protocol: 16^{o}C for 20 minutes; 24^{o}C for 20 minutes; 37^{o}C for 20 minutes; 75^{o}C for 5 minutes; 4^{o}C for at least 2 minutes. To each library sample, 7.5 μl of 10X Amplification Master Mix, 47.5 μl Nuclease-free H_2O and 5 μl of WGA DNA Polymerase were added. The samples were then incubated in a thermal cycler according to the following protocol: 95^{o}C for 3 minutes; 14 cycles of 94^{o}C for 15 seconds, 65^{o}C for 5 minutes; 4^{o}C hold. Amplified samples were then purified using the QIAquick PCR Purification Kit (QIAGEN catalog number 28106).

Uracil Incorporation, DNA Fragmentation and Labeling

To prepare DNA for subsequent fragmentation and labeling procedures, dUTP was incorporated through a random primed labeling reaction using octadeoxyribonucleotides in 10X Labeling Buffer from New England Bioloabs (NEB catalog number N1501L). 6 μg of WGA amplified DNA in 193.5 μL total volume was heated at 95^{o}C for 5 minutes and snap cooled on

ice. To this, 22.5 μL 10x Labeling Buffer, 6 μl of dNTPs (10 μM dATP, 10 μM dCTP, 10 μM dGTP, 8 μM dTTP, 2 μM dUTP) and 3 μl of Klenow fragment (NEB Catalog number M0212S) was added to each sample and incubated at 37°C for 2 hours. DNA from each reaction was purified using the QIAquick PCR Purification Kit.

To fragment the UTP containing DNA for labeling, the GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix #900812) was used according to the manufacturer's protocol. Briefly, 13 μg of DNA were fragmented using 15 Units UDG (Uracil DNA Glycosylase), 225 Units APE 1 (apurinic/apyrimidinic (AP) endonuclease) and the supplied buffer. The reaction was incubated at 37°C for 1 hour; 93°C for 2 minutes; 4°C for at least 2 minutes.

DNA was labeled with a nucleotide analog conjugated to biotin using the Double-Stranded DNA Labeling Mix from the Affymetrix GeneChip WT Double-Stranded DNA Terminal Labeling Kit. 45 μl of fragmented DNA were incubated with 2 μl Terminal Deoxynucleotidyl Transferase (TdT), 1 μl DNA Label (Biotin-labeled nucleotide analog), and 12 μl 5X TdT Buffer at 37°C for 1 hour; 70°C for 10 minutes; 4°C for 2 minutes.

Electrophoresis Mobility Shift Assay (EMSA)

In order to determine the efficiency of DNA fragmentation and labeling, a gel-shift assay was performed to qualitatively determine uracil incorporation. 1μL aliquots of labeled DNA (with incorporated biotin residues), were heated to 72°C for 2 minutes and then incubated with or without 5 μl of 2 mg/ml NuetrAvidin (Pierce Catalog # PI-31000) at room temperature for 5 minutes. Samples were then prepared for gel loading by addition of 5X Sucrose Loading Buffer (Amresco Catalog # E-274) to a final concentration of 1X. Samples were then loaded on a 4% - 20% gradient TBE acrylamide gel (Bio-Rad Catalog # 161-1235) in a Criterion Electrophoresis

Cell and run at 40 volts for 20 minutes; 100 volts for 30 minutes; 150 volts for 30 minutes with both a 100 bp and a 1 kb Plus DNA Ladder (Invitrogen Catalog # 15628-019 and 10787-018). After electrophoresis, the gel was stained in 1X SYBR Gold (Invitrogen Catalog # S-11494) in PBS for 30 minutes and visualized on a standard UV trans-illuminator. Efficient incorporation of labeled DNA was visualized as a slower migrating smear of DNA fragments compared to labeled DNA with no NuetrAvidin added.

Hybridization and Scanning Mouse Promoter Arrays

Hybridization of samples to Affymetrix Mouse Promoter Array 1.0R and scanning of arrays was performed in the laboratory of Dr. Henry Baker by Cecilia Lopez. Hybridization and scanning was performed following the manufacture's protocol for the GeneChip Scanner 7G. Briefly, samples were prepared using the GeneChip Hybridization, Wash and Stain Kit from Affymetrix (Affymetrix Cat. # 900720). For each array, 7.5 μg of fragmented and labeled DNA target was mixed with 50 pM Control Oligonucleotide B2 (Affymetrix Cat. # 900301), 1X Hybridization Mix and 7% dimethyl sulfoxide (DMSO) in a total volume of 200 μl Nuclease-free Water. Hybridization Cocktail was then heated to 95^{o}C for 5 minutes, cooled to 45^{o}C for 5 minutes and briefly centrifuged to collect sample. The sample was then injected into the array through the septa on the array case and incubated at 45^{o}C in hybridization oven at 60 RPM for 16 hours. Washing and staining of arrays is performed in the Fluidics Station using reagents from the GeneChip Hybridization, Wash and Stain Kit. The Fluidics Station is operated using protocols in the GeneChip Operating Software. After washing and staining, the arrays were scanned using the GeneChip Scanner 3000 7G, also controlled by the GeneChip Operating Software.

Data Analysis using Partek's Genomic Suite Software Package

Raw data from the Affymetrix GeneChip Scanner was analyzed using Partek's Genomic Suite Software Package (Partek Incorporated, 12747 Olive Blvd., Suite 205, St. Louis, Missouri 63141, U.S.A). using the Tiling Array Workflow option. Raw data (.CEL files) files were imported and arrays normalized with Robust Multi-chip Averaging (RMA) and quantile normalization with output set to log base 2. After normalization, the no antibody input chip data was subtracted from the corresponding antibody chip. After subtraction, the genomic data was segmented using the Genomic Segmentation option with the following parameters: minimum genetic markers set to 10, p-value threshold set to 0.001, signal-to-noise set to 0.3, and the Region Report set to report values above or below 0. The resulting segmented, overlapped regions were exported automatically into a new spreadsheet within the software. An observation of Variance (ANOVA) was performed between NPD and LPD data sets, and the resulting data was used to generate a list of positive genomic regions using the Create Region List option with the p-value threshold set to < = 0.05. A gene was list was then created from the Region List using the Find Overlapping Genes option and the most recent genome build of the mouse genome (mm9 – July 2007).

High Resolution Sodium Bisulfite Genomic Sequencing

High resolution sodium bisulfite genomic sequencing was performed essentially as described by (Clark et al., 1994; Kang et al., 2003). Briefly; 2.5 μg of genomic DNA was sheared by vortexing for 2 minutes at room temperature. DNA was then denatured in the presence of 300 mM NaOH for 30 minutes at 37^{o}C. A solution of sodium bisulfite and hydroquinone pH 5.0 was added to the denatured DNA to a final concentration of 1.55 M sodium bisulfite and 0.5 mM hydroquinone. The samples were then incubated in a thermocycler under the following conditions: 95^{o}C for 30 seconds; 55^{o}C for 90 minutes. These conditions were

cycled for 16-18 hours. After conversion, DNA was de-salted using the Promega's Wizard® DNA Clean-Up System following the manufacturer's protocol, desulphonated in 300 mM NaOH at 37°C for 15 minutes and precipitated by addition of 0.5 volumes 7.5 M ammonium acetate (NH_4AOC) and 2.5 volumes 100% EtOH. DNA precipitates were then washed in 70% EtOH and resuspended in 50 µl dsH_2O. PCR reactions were performed under the following conditions: $1/25^{th}$ to $1/10^{th}$ volume purified converted DNA; 1x PCR Buffer; 1 µM each primer; 200 µM dNTPs and 0.125 units of HotStarTaq™ Polymerase (Qiagen Cat. # 203205) in a 25 µl reaction volume. Thermocycler conditions were as follows: 95°C for 15 minutes; 45 cycles of 94°C for 45 seconds, primer specific annealing temperature for 30 seconds, 72°C for 90 seconds; 72°C for 10 minutes and hold at 4°C after completion. PCR primers were designed to amplify the upper stand of bisulfite converted target sequences using MethylPrimer Express Software. Primers are listed in Table 4-2.

RNA Purification

RNA was extracted using the RNeasy Mini Kit (Qiagen Cat. # 74104) following the manufacturer's recommended protocol. Briefly; 30 mg of previously frozen, ground fetal liver tissue was lysed in 600 µl Buffer RLT Plus, and lysate was centrifuged at 13,000 x g for 3 minutes. Genomic DNA was removed by transferring supernatant to gDNA Eliminator column and centrifuging at 8,000 x g for 30 seconds. 600 µl of 70% EtOH was added to flow-through, mixed, and sample was transferred to RNeasy mini column and centrifuged for 15 seconds at 8,000 x g. Samples were then washed by adding 700 µl of Buffer RW1 to RNeasy mini column and centrifuging for 15 seconds at 8,000 x g, followed by two sequential washes of 500 µl Buffer RPE centrifuged at 8,000 x g for 15 seconds each. RNeasy mini column was then transferred to

an RNase-free 1.5 ml tube and RNA eluted with two sequential elutions using 50 µl RNase-free water and centrifuging at 8,000 x g for 1 minute each. RNA was stored at -80^{o}C.

Reverse-Transcriptase Real Time PCR for Expression

First-strand cDNA was generated using SuperScript^{TM} III Reverse Transcriptase and Random Primers from Invitrogen (Invitrogen Cat. # 18080-093, #48190-011) following the manufacturer's recommended protocol. Briefly, 30 ng of RNA was mixed with 4.5 ng random primers (hexamers), 1.5 µl 10 mM dNTP's in a total volume of 21 µl with diethyl pyrocarbonate (DEPC) treated H_2O. Sample was heated to 65^{o}C for 5 minutes, and then snap cooled on ice for 1 minute. To the sample, 6 µl of 5X Buffer, 3 units of RNasin® Plus RNase Inhibitor (Promega Cat. # 9PIN261) and 300 units of SuperScript III were added for a total volume of 30 µl. Sample was mixed gently and incubated at 25^{o}C for 5 minutes, 50^{o}C for 1 hour, followed by heat inactivation of the reverse transcriptase by incubation at 70^{o}C for 15 minutes. RNA was then digested by addition of 20 units of RNase Cocktail (Ambion AM2286) incubated at 37^{o}C for 20 minutes. For quantitative Real-Time PCR, 1 µl of cDNA was used for each reaction using the RT-qPCR protocol described earlier. A no reverse transcriptase and a no template control were also added. Expression data was expressed as fold increase or decrease normalized to the reference gene *Gapdh*.

Table 4-1. Methylated DNA immunoprecipitation (MeDIP) primers. All primers listed 5' to 3'.

Primer name	Primer sequence	Annealing temperature
mLAP mDIP Upper	CTCCATGTGCTCTGCCTTCC	59^{o}C
mLAP mDIP Lower	CCCCGTCCCTTTTTTAGGAGA	59^{o}C
mXist mDIP Upper	CGCGGATCAGTTAAAGGCGT	59^{o}C
mXist mDIP Lower	AACCACGGAAGAACCGCAC	59^{o}C
MeDIP Hprt Upper	GCAGCGTTTCTGAGCCATTG	59^{o}C
MeDIP Hprt Lower	AAAAGCGGTCTGAGGAGGAA	59^{o}C
mH19 DMR mDIP Upper	GCATGGTCCTCAAATTCTGCA	59^{o}C
mH19 DMR mDIP Lower	GCATCTGAACGCCCCAATTA	59^{o}C
mAPRT mDip Upper	TGCTGTTCAGGTGCGGTCAC	59^{o}C
mAPRT mDip Lower	AGATCCCCGAGGCTGCCTAC	59^{o}C
mActB mDIP Upper	AGCCAACTTTACGCCTAGCGT	59^{o}C
mActB mDIP Lower	TCTCAAGATGGACCTAATACGGC	59^{o}C
mCSa mDIP Upper	TGGTTGGCATTTTATCCCTAGAAC	59^{o}C
mCSa mDIP Lower	GCAACATGGCAACTGGAAACA	59^{o}C

Table 4-2. Sodium bisulfite genomic sequencing primers. All primers listed 5' to 3'.

Primer name	Primer sequence	Primer sequence
mH19 DMR-BSF Upper	GAGTATTTAGGAGGTATAAGAATTTTGTAA	51^{o}C
mH19 DMR-BSF Lower	AAAACTAACATAAACCCCTAACCTC	51^{o}C
mIgf2-DMR1-BSF Upper	AGGTGAAGGTTTTGTGGGTAG	51^{o}C
mIgf2-DMR1-BSF Lower	CTCTACCTTTCCCCAAAAAAAA	51^{o}C
mIgf2-DMR2-BSF Upper	TGATGGAATTGTTTTTGTTTAA	51^{o}C
mIgf2-DMR2-BSF Lower	TAACACCTCCTCTCCAAAAC	51^{o}C
mSnrpn-BSF-Upper	TATTTGGGTTGTTAAAAATTTTAA	51^{o}C
msnrpn-BSF-Lower	TCCATTATTCCAAATTAACAAT	51^{o}C
mMkrn3-BSF Upper	AAGTAGTAGAYGGTAAAGGTAATGTGTGTA	51^{o}C
mMkrn3-BSF Lower	ACCTCAATAAAAACTATAAACTCTTCCAT	51^{o}C
Hu-SNRPN-BSF-Upper	GGAATTGGTTTTTTAGAATAAAGGATTTTAGGG	57^{o}C
Hu-SNRPN-BSF-Lower	CCCCCTCTCATTACAACAATACTATAAAACCC	57^{o}C
HuLINE1 BSF-Upper	ATTTTATATTTGGTTTAGAGGG	55^{o}C
HuLINE1 BSF-Lower	ATCAAAAATCAAAAACCCACTT	55^{o}C

Table 4-3. Reverse Transcriptase qRT-PCR primers. All primers listed 5' to 3'.

Primer name	Primer sequence	Annealing temperature
mGAPDH-RT-Forward	GCCTTCCGTGTTCCTACCC	60^{o}C
mGAPDH-RT-Reverse	CCTCAGTGTAGCCCAAGATGC	60^{o}C
mIgf2-RT-Forward	GTGCTGCATCGCTGCTTAC	60^{o}C
mIgf2-RT-Reverse	ACGTCCCTCTCGGACTTGG	60^{o}C
mH19-RT-Forward	GCCTCAATAACTGGAGAATGGAA	60^{o}C
mH19-RT-Reverse	CTCATGGGAATGGTGTGTCTG	60^{o}C

CHAPTER 5
DISCUSION AND FURTURE DIRECTIONS

This dissertation has focused on the epigenetic effects of exposure to a low protein diet *in utero* in a rodent model, and the epigenetic effects of folic acid supplementation and withdrawal in Chinese women of child bearing age. The overall focus of our laboratory is to gain insight into the mechanism by which environmental influences, particularly diet and nutrition, can affect the epigenome in both developing and adult organisms. DNA methylation has generally been considered a fairly stable epigenetic mark, associated with long-term repression of transcription. Changes in DNA methylation patterns were thought to normally only occur during embryonic and germ cell development. During embryogenesis, a single celled zygote progresses to a multicellular organism with over 200 functionally distinct and diverse cell types (Mann and Bartolomei, 2002). Each of these distinct cell types has undergone epigenetic changes to form a unique transcriptional memory that is then stably maintained throughout mitosis. (Nafee et al., 2008). During germ cell development, the DNA methylation marks at imprinted genes must be erased and reestablished in a parent-of-origin manner (Reik et al., 2001). It has been demonstrated that nutritional insults during these critical developmental periods can affect DNA methylation and influence gene expression levels (Lillycrop et al., 2005; Waterland et al., 2006). In order to investigate this further, we set out to explore changes in DNA methylation in fetal tissues of mice exposed to a maternal low protein diet *in utero*. Our experimental approach was to utilize newly developed techniques to enrich for methylated DNA fractions from genomic DNA (Weber et al., 2005) and combine them with the genome-wide scale analysis capabilities of microarrays. The results of our study demonstrated that there may be subtle changes in DNA methylation occurring at the *H19*/*Igf2* imprinted domain, however, there were no large wide spread changes in DNA methylation occurring in fetal livers due to maternal malnutrition. Given

the fact that there were no major phenotypic differences at birth between pups from a normal protein diet compared to a low protein diet, it is not surprising that there would be no dramatic changes in DNA methylation occurring. However, it may be that there are more subtle changes occurring in DNA methylation, which could have a biological effect on the offspring. The idea of subtle changes in DNA methylation due to maternal malnutrition has been demonstrated, at least for the PPARα gene in fetal rats. Lillycrop et al. demonstrated an approximately 20% decrease in DNA methylation at the PPARα promoter, with an increase in expression 10.5 fold higher in rats exposed to a low protein diet *in utero* as compared to normal protein diet control animals (Lillycrop et al., 2005; Lillycrop et al., 2008). Modest changes in DNA methylation may be difficult to detect using a high resolution promoter array combined with an antibody that immunoprecipitates such a large fraction of the genome. As discussed earlier, the Affymetrix Mouse Promoter Array inherently has an elevated background and the 5-MeC antibody will immunoprecipitate a large proportion of the genome due to the high content of methylated repetitive DNA in mammalian genomes (Lander et al., 2001; Rollins et al., 2006). These two factors might make it more difficult to detect small changes in DNA methylation. A second method to generate genomic representations for DNA methylation is the HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) assay. This technique takes advantage of differential digestion using methylation-sensitive restriction enzymes, followed by ligation-mediated PCR. The resulting library represents both hypomethylated loci and hypermethylated loci, which can then be used as probes to interrogate microarray platforms (Oda and Greally, 2009). For future studies, perhaps a better suited microarray platform would be the NimbleGen arrays. These arrays are tiled with 50 to 75-mer probes with 100 base spacing, resulting in a lower background and an increased signal to noise ratio. A second advantage to this platform is

that the arrays support two color scanning, allowing analysis of both input and IP samples on the same chip, reducing variations among sample sets. A second approach would be to utilize massively parallel sequencing to sequence an entire pool of highly enriched methylated DNA fractions. This technique has the advantage that the entire population of fragments that are immunoprecipitated or generated through other means, can be interrogated, without the limitation of array design. In this manner, repetitive DNA elements can be analyzed as well, an advantage over microarrays in which repetitive sequences have been removed from the arrays. The information obtained from massively parallel sequencing has the ability to reveal data concerning locus specific changes as well as global changes in DNA methylation.

In the preceding work, we were also able to demonstrate significant changes in expression of *H19* and *Igf2*. The LPD group showed a strong induction of expression of *H19*, and a 10-fold decrease in expression of Igf2. These expression data are seemingly inconsistent with the known roles of DNA methylation at the *Igf2* DMR2. Methylation on the maternal allele of the *H19* DMR facilitates the binding of CTCF which blocks enhancer access to *Igf2* (Bell and Felsenfeld, 2000; Hark et al., 2000). A decrease in methylation could potentially increase CTCF binding, leading to an increase in enhancer availability to *H19* and a decrease of enhancer availability at *Igf2*, consistent with our results demonstrating that an increase in DNA methylation at the *H19* DMR induces *H19* expression and reduces *Igf2* expression. However, *Igf2* DMR2 is a methylation sensitive activator, and increased methylation has been shown to increase *Igf2* expression in vivo (Murrell et al., 2001). Our results indicate an increase of DNA methylation of *Igf2* DMR2, which would be expected to increase *Igf2* expression. However, it has been reported that *Igf2* DMR2 functions at the level of transcription initiation, and it may be that with enhancer

competition occurring due to increased CTCF binding at the *H19* DMR, there is not enough enhancer activity at *Igf2* DMR2 to promote any increase in transcription of *Igf2*.

In order to gain a better understanding of the mechanisms leading to increased *H19* and decreased *Igf2* expression, we would like to repeat this experiment by crossing C57BL/6J and Cast/Ei mice. This would allow us to examine both changes in methylation and expression in a parent-of-origin allele-specific manner by utilizing single nucleotide polymorphisms between these two related but divergent mouse strains. This approach would allow us to determine if the increase in *H19* expression is occurring due to activation of the paternal allele, and whether the decrease in expression of *Igf2* in due solely to down regulation of the paternal allele, or if loss of imprinting is occurring at this locus leading to overall dysregulation of expression, with transcription occurring from both alleles, but at greatly reduced levels due to lack of enhancer access. There remains the possibility that the observed changes in expression may be unrelated to changes in DNA methylation, and any changes in expression levels may be a response to upstream signals due to maternal and/or fetal sensing of amino acid deprivation during the critical period of rapid growth associated with the developing embryo.

The DNA methylation status in somatic cells has been generally regarded as being stably maintained throughout the life of the organism. However, DNA methylation can in fact be labile and decreases in methylation have been observed to occur in somatic tissues as part of the aging process (Bjornsson et al., 2008; Richardson, 2003). In the preceding work, we have demonstrated that folic acid supplementation and withdrawal can produce changes in DNA methylation in somatic cells in a locus-specific manner at the promoters of *DAPK* and *TIMP3* tumor suppressor genes. Methylation-specific PCR indicated that there was an observable increase in DNA methylation after 6 months of folic acid supplementation. It was also demonstrated that the

changes in methylation appeared to be transient, as the increase in DNA methylation was no longer detectable in either subject after 3 months of folic acid withdrawal. We were also able to demonstrate that folic acid supplementation and withdrawal can produce dramatic changes in DNA methylation in an imprinted gene. The DNA methylation of the maternally imprinted *SNRPN* promoter was shown to experience a near complete loss of DNA methylation after 6 months of folic acid supplementation, and to have become completely demethylated after 3 months of folic acid withdrawal. These results demonstrate that the DNA methylation status in somatic cells can undergo changes in response to nutritional supplementation and withdrawal in adult organisms. The biological significance of loss of methylation at either tumor suppressor genes or at the *SNRPN* locus in circulating leukocytes is unknown, and future studies should include different cell types if possible. A rodent model recapitulating this study would allow the examination of various tissue types that would be otherwise unavailable in a human study.

We have also demonstrated that there is no detectable decrease in DNA methylation levels at the L1 repetitive elements caused by folic acid supplementation and withdrawal in ten subjects randomly chosen from the group receiving 400 μg of folic acid daily. It should be noted that the global methylation status of these subjects is unknown, so it is possible that these subjects did not experience significant changes in DNA methylation. However, we did analyze two subjects whose DNA methylation levels were known to be decreased by more than 85% after folic acid withdrawal as compared to their baseline levels of DNA methylation. Through the use of bisulfite genomic sequencing, we were able to demonstrate a statistically significant decrease in DNA methylation in one subject homozygous for the TT genotype, with no change observed for the CC subject. Although there was a modest 27% decrease in DNA methylation observed, it does not account for the greater than 85% reduction in global DNA methylation observed in

these two subjects. Repetitive elements make up about 45% of the human genome (Rollins et al., 2006), with the LINE elements accounting for 21% of the human genome (Wilson et al., 2007), and it is estimated that 35% to 40% of all DNA methylation occurs at repetitive elements (Bestor, 1998; Kochanek et al., 1993). A subject that has experienced an 85% reduction in global methylation would conceivably have had to come from loss of methylation at the L1 elements. One possibility is that the L1 primers we are utilizing for bisulfite genomic sequencing analysis are preferentially amplifying a subfamily of L1 elements that are protected from demethylation, or that DNA from cells that have not undergone demethylation are being preferentially amplified. Another possibility is that degradation of the DNA sample, due to handling and shipping of samples from China, or to incomplete purification during the extraction steps, is limiting the available pool of amplifiable fragments, and that we are not able to amplify a truly representative population of DNA molecules from these samples. The levels of global methylation were determined using LC/MS/MS (Laboratory of Dr. Bailey; University of Florida's Food Science and Human Nutrition Department; work by Dr. Eoin Quinlivan and David Maneval) on DNA that has been digested to nucleosides, so degradation of the DNA sample would not interfere with this type of assay The quantity of DNA that is available to us for this study are exceedingly small, and therefore precious, precluding the possibility of gel electrophoresis to determine the extent of sample degradation. A second approach would be to utilize pyrosequencing, which conceivably amplifies a greater proportion of L1 subfamilies than our BGS primers do. Additionally, in future studies, the analysis of DNA methylation at other repetitive elements, such as satellite repeats, will be performed. Overall future studies will involve the continuing optimization of new technologies to gain a better understanding of the

mechanisms involved in epigenetic alterations occurring due to nutritional or environmental insults.

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BIOGRAPHICAL SKETCH

Jason Orr Brant was born in Jupiter, Florida, to Garry and Christine Brant. He attended Florida Culinary Institute and had a short-lived but successful career as a chef in California and New York. Jason then decided to expand his love of learning, and after graduating magna cum laude with a degree in environmental science and forest biology from SUNY-ESF, he entered graduate school at the University of Florida. He studied epigenetic gene regulation in the Laboratory of Dr. Thomas P. Yang, where he will continue his education as a postdoctoral fellow. Jason ultimately wants to continue his research career as an officer in the United States Army.