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The Interplay Between Aging, Growth Hormone, The Methionine Pathway, And Epigenetic Methylation Marks And Mechanisms

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THE INTERPLAY BETWEEN AGING, GROWTH HORMONE, THE METHIONINE PATHWAY, AND EPIGENETIC METHYLATION MARKS AND MECHANISMS

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Chairperson

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Wayne Swisher, Dean of the School of Graduate Studies

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PERMISSION

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ABSTRACT

Methylation reactions are important for the establishment and maintenance of epigenetic methylation tags on DNA and histone molecules that are critical for the development and life-long function of an organism. While epigenetics has been studied extensively during embryological development and age-related disease such as cancer, little is known about whether epigenetic methylation enzyme expression change in normally aging or long-living mammals. Because growth hormone (GH) plays an important role in determining the lifespan of many organisms, we have focused our studies on the long-living growth hormone deficient model the Ames dwarf mouse. We focused our studies on epigenetic methylation in the liver, because of its role in GH signaling, IGF-1 signaling, and it's responsiveness to changes in the methionine pathway which govern DNA and histone methylation.

Our studies showed that DNA methyltransferase 1 (DNMT1) protein is basally expressed less in the Ames dwarf compared to their wild-type counterparts, despite higher or equivalent transcription, suggesting that some form of post-transcriptional regulation is taking place. Global DNA methylation levels in dwarf mice resisted age-related changes compared to wild type mice. Histone methylation of key regulatory transcriptional markers, and total histone H3 expression were also altered in dwarf mice.

We demonstrated that the protein abundance of a potential regulatory protein of DNMT1 (DEAD-box helicase 20) significantly higher in Ames dwarf mice at 3, 12, and 24 months of age. Metallothionein1 (MT1) mRNA expression was much higher in dwarf mice especially at 3 months of age. Also there were differences in the methylation of putative CpG islands of the MT1. Growth hormone administration influenced DNA methyltransferase and DDX20 protein expression in dwarf mice and areas of methylation in the predicted MT1 CpG island. MicroRNA 140-3p which pairs with DDX20 for post-transcriptional regulation of DNMT1 was also studied.

Studies using growth hormone receptor knockout mice and over-expressing growth hormone transgenic mice DNMT transcription and interspersed repeat methylation were also studied to discover continuities with previous experiments in Ames dwarf mice. Taken together, this study offers valuable insight about the changes in the epigenetic methylation mechanisms and markers due to aging.

CHAPTER I

INTRODUCTION

Epigenetics

Even though every cell type in the mammalian organism contains the same genome, each cell type expresses its own sets of genes and proteins which are necessary for function in complex multicellular organisms such as humans. Epigenetic refers to heritable forms of gene expression that cannot be attributed to a change in the DNA sequence (Robin Holliday, 2006). Epigenetic modifications on DNA and proteins contribute to cell differentiation, function, and gene expression and influence the observable phenotype of mammalian organisms. Epigenetic studies started with observations of heritable phenotypic traits that were passed on from mother to daughter cells during cell division. During embryological development, these traits determined cell type, organ formation, and eventually a multi-organ organism (Tost, 2010). It was understood as early as the 1950s that there were mechanisms responsible for the timing and regulation of gene expression and DNA packaging which were key to development (Robin Holliday, 2006). Epigenetics in this context led to the study of the epigenome, a heritable regulatory mechanism consisting of markers on DNA or proteins associated with DNA, that affects DNA packaging and expression of genes, non-coding RNA, and retroviral elements.

Epigenetics came into the social and scientific forefront following the publication of a few landmark studies. Nutritional insufficiencies were found to affect the phenotype of human offspring even to the second generation through the "Dutch famine studies" and Dr. Bygren's Swedish studies (Bygren et al., 2001; Heijmans et al., 2008). These reports changed the debate from nature (genetic control) versus nurture (environmental control) in phenotype expression to nature and nurture having equal importance on the phenotypic plasticity and fitness of an organism even during development (Jang & Serra, 2014). Maternal administration of demethylating agents such as bisphenol A or methyl-rich diets demonstrated that environmental maternal factors had an obvious and persistent impact on offspring phenotype (Jang & Serra, 2014; Morgan et al., 1999). Agouti mice are also prone to diabetes and cancer (Calvanese et al., 2009; Schellenberg et al., 2011). This challenged the rejection of Lamarck's principles of inheritable adaption and environmental influence that are continually being rethought, revised and reintroduced to the scientific community through the study of epigenetics (Gravina & Vijg, 2010; Robin Holliday, 2006; Jablonka & Lamb, 1989).

As early as the 1960's, it was thought that epigenetics may influence aging, but epigenetic regulation of human aging really came to the forefront with studying the phenotypic discordance among aging human monozygotic twins (Berdyshev et al., 1967; Fraga et al., 2005; Poulsen et al. 2007). Many of the most well-known epigenetic changes controlling gene expression have been studied in age-related diseases such as cancer, rheumatoid arthritis, and

Alzheimer's disease. Yet much remains to be done to understand how these changes are beneficial or detrimental to an aging organism and how this could be translated into medical treatments to enhance longevity and a higher quality of function during aging. Before these changes and their effects on gene expression and genome integrity can be discussed in detail, the mechanisms and markers related to these epigenetic changes must first be introduced and defined.

Epigenetic markers and mechanisms

There are several epigenetic markers including methylation, hydroxymethylation, which are covalent DNA modifications, and histone modifications that include but are not limited to methylation, phosphorylation, acetylation, ubiquitination, and sumoylation in mammals (Chia & Rotwein, 2010; Guibert & Weber, 2013; Strahl & Allis, 2000). Epigenetic markers are responsible for regulating the expression of the genome in each cell or cell-type. Without epigenetic regulation, the differentiation of cells and development of multicellular organisms wouldn't be possible. One well-studied epigenetic modification in somatic cells is DNA methylation. This marker consists of a covalent insertion of a methyl group onto the five prime carbon of the nucleotide cytosine typically preceding an adjacent guanine on the same strand known as a CpG dinucleotide. These dinucleotides make up around 1% of the total genome and are most densely packed in gene regions known as CpG islands (Bestor, 2000; Ehrlich et al., 1982; Tost, 2010). Gene regions make up approximately 2-3% of the entire genome and CpG islands, which are regions of CpG dinucleotides of between 100 and 5000 bps, are mostly located (50-60%) in gene promoter

regions. CpG islands are thought to regulate transcription positively or negatively depending on the gene but are generally hypomethylated in differentiated cells (Singal & Ginder, 1999). High levels of DNA methylation at the CpG island or promoter regions are mostly associated with the repression of gene expression, X chromosome inactivation, genomic imprinting, and the repression of repetitive elements that contain ancestral retroviral sequences able to transpose themselves into other areas of the genome if expressed (Shin et al., 2011; Tost, 2010). High levels of methylation are also typically associated with proteins that bind DNA into heterochromatin, a tightly packed form of DNA that is sequestered from transcriptional proteins such as transcription factors and RNA polymerases. DNA methylation is catalyzed by DNA methyltransferases, or DNMTs, which consist of three catalytically active families, DNMT1, DNMT3a, and DNMT3b. DNMT3L which is included in this family, contains homogeneity in sequence but lacks catalytic sequences and activity. However, DNMT3L affects the affinity of other DNMTs for their substrates and thus remains an important cofactor for DNMT activity (Bestor, 2000; Cheng & Blumenthal, 2008). DNA methyltransferases catalyze the removal of a methyl group from the substrate *S*-adenosylmethionine (SAM), which is the major methyl donor for every methyltransferase reaction in a cell, and transfers it to specific cytosines on the DNA molecule. This reaction relies on the availability of SAM in the nuclear compartment (Wallace & Fan, 2010). DNA methyltransferases have specific activity for methylating either hemi-methylated DNA, or can form *de novo* methylation reactions, such as DNMT3a and 3b. Since DNMT1 has the highest

affinity for hemi-methylated DNA that is used as a template and is more abundant during cell division and DNA synthesis, it has been termed a maintenance methyltransferase (Bestor & Verdine, 1994; Robertson et al., 1999). DNMT1 is considered the most active and abundant DNA methyltransferase in mammalian cells. DNMT3a and 3b are *de novo* methyltransferases not requiring a template strand for methyl marker replication. They preferentially methylate heterochromatin and are required for the methylation of repetitive DNA (Bachman et al., 2001; Liang et al., 2002a). However, DNMTs associate with different genomic areas upon differentiation and may play different roles (Challen et al., 2012; Jones & Liang, 2009). For instance, DNMT3a is enriched in intergenic heterochromatin, whereas DNMT1 and DNMT3b are more associated with intragenic regions and areas of transcription after induced differentiation of pluripotent cells with retinoic acid (Jin et al., 2012). In addition, different isoforms of each family of DNMT exist and are specific to certain periods of embryonic development or certain cell types that result in possible functional differences adding to the complexity of DNMT regulation (Bestor, 2000; Chen et al., 2002; Keith D Robertson, 2002). All three DNMTs have different isoforms and have gene expression regulatory effects independent of DNA methylation, adding yet another possible layer of function that has yet to be fully investigated (Bachman et al., 2001; Jin et al., 2012; Lan et al., 2010; Milutinovic et al., 2004). There are fewer CpG dinucleotides in the mammalian genome than statistically expected. This may be caused by nucleotide mutation through two deamination mechanisms, one is a spontaneous deamination and

the other is an enzymatically catalyzed deamination of methylated cytosines. Both processes render a new base, uracil, which is converted to thymine if no DNA repair enzymes intercede (Lindahl, 1974; Singal & Ginder, 1999). As a result, methyl binding proteins, which protect methylated cytosines from deamination, are thought to play a major role in the maintenance of methylation patterns and may be one mechanism of transcriptional repression (Hendrich & Bird, 1998; Lan et al., 2010).

If DNA in the human body were laid out in a string from each of the 46 chromosomes, it would measure about two meters long. This large amount of DNA must be packaged to fit into a nucleus which is only about six microns in diameter (Alberts et al., 2008). Most of our DNA is wrapped around and bound to proteins that produce a complex layered hierarchy of packaging that is known in its most condensed form as chromatin. DNA, and its associated proteins, plus the proteins that anchor chromatin to the inside of the nuclear membrane, make up the nuclear architecture, which provides compartmentalization for transcription and the sequestration of repetitive DNA in the nucleus. DNA methylation also impacts how DNA is packaged. DNA can be gathered into tightly wound prohibitive structures and around proteins that inhibit transcription factor and polymerase binding to suppress gene expression (heterochromatin) or less prohibitive structures that allow or even facilitate gene expression (euchromatin). Both utilize histones, and non-histone proteins. There are two types of heterochromatin, facultative and constitutive. Both types of heterochromatin markers are heritable from cell-to-cell, however they perform different functions.

Constitutive heterochromatin occurs at repetitive DNA that localize principally near telomeres and centromeres of chromosomes. This structure keeps chromosomes separated, and stabilizes the genome by preventing transposition events or mutations in the DNA sequence by keeping it silenced. Compartmentally it localizes to the nuclear periphery and historically, has been considered fixed and irreversible, but recently has been discovered to change with aging (Kreiling et al., 2011; Oberdoerffer & Sinclair, 2007; Sedivy et al., 2008).

Facultative heterochromatin (or inducible chromatin) is largely responsible for, the phenotype of a cell during differentiation, X- chromosome inactivation, and the phenotypic response of a cell to a changing environment. This type of heterochromatin is localized to promoter regions and is dependent on transcriptional repressors and silencing protein complexes, which may include non-coding RNA. Interestingly, senescent cells associated with aging phenotypes in culture, show alterations in facultative heterochromatin that disallow cell cycle re-entry. (Sedivy et al., 2008; Sinclair & Oberdoerffer, 2009)

Histones are proteins that, in binding DNA, form the most basic repeating unit of structure in chromatin known as a nucleosome. Nucleosomes are octameric structures made up of dimers of four families of core histone proteins labeled H2A, H2B, H3, and H4. Histones are highly basic and able to bind DNA due to its negatively charged backbone making it a very stable complex (Li et al., 2007). At its smallest repeating unit, approximately 147 base pairs of DNA are wrapped around each nucleosome. Another histone known as histone H1 is

associated with linker DNA, the DNA between each nucleosome and the nucleosome itself facilitating the next level of more compact nuclear condensation known as the 30-nm fiber. This structure is then looped and coiled into a higher order of condensation which is typical of transcriptionally repressive heterochromatin by non-histone proteins such as condensins, DNA topoisomerase, and heterochromatin protein 1 (HP1). Other non-histone proteins are chromatin remodeling proteins associated with the disruption of histone binding to DNA in order to promote the transcription of gene encoding sections of DNA (Alberts et al., 2008).

Histones contain large *N*-terminal tails of amino acids which are subject to covalent modifications that further regulate how tightly DNA is wrapped and sequestered from transcriptional proteins or other elements in the nuclear milieu. Histone modifications are reversible and include acetylation, methylation, ubiquitination, sumoylation, along with an abundance of other post-translational modifications to which large databases such as HIstome have been dedicated in cataloguing (Khare et al., 2012; Shin et al., 2011). These modifications appear to carry out specific patterns of transcriptional regulation that are synergistic or antagonistic depending upon the combination of markers in each histone at any given time. The acetylation marks and some facultative histone methylation marks are thought to be more dynamic and responsive to intrinsic or extrinsic regulation than DNA methylation and more "stable" constitutive heterochromatin marks which are heritable across cell generations (Barth & Imhof, 2010; Dunn, 2003; Jin et al., 2011). Histone methylation is one of the most well-studied

modifications and forms a large part of the regulatory “histone code” that has been consistently observed as either activating or repressive to transcription (Strahl & Allis, 2000).

Table 1: A list of histone methylation modifications.
Adapted from (Li et al., 2007).

Modifications	Histone and Position	Transcriptional Function
Lysine Methylation	H3 K4	Activation
	H3 K9	Repression, activation
	H3 K27	Repression
	H3 K79	Activation
	H4 K20	Silencing
Arginine Methylation	H3 R2	Activation
	H3 R17	Activation
	H3 R26	Activation
	H4 R3	Activation

Histone methylation also uses the universal methyl group donor, SAM, similar to DNA methylation. A list of well-known methylation markers and functions is shown in Table 1. Repressive and silencing markers such as the tri-methylation of Histone H3 lysine 9, Histone H4 lysine 20, and histone H3 lysine 27 contribute to constitutive and facultative heterochromatin. These markers are concentrated in areas of DNA that normally are heavily methylated throughout adulthood and are resistant to changes in methylation. These include areas of repetitive DNA, e.g. retroviral ancestral elements, that are thought to contribute transcriptional “noise”, genomic mutation through retrotransposon insertions, and cellular dysfunction when expressed (Christensen et al., 2009). Other tri-

methylation marks such as on Histone H3, lysines 4, 36 and 79, are associated with transcriptional activation, relaxing the tightly bound DNA allowing access of regulatory elements by transcriptional proteins. Just as there are variants of each protein in the DNMT family, there are histone variants of H3, H2, and H1 that are conducive to transcription or repression. These variants can also participate in other processes such as DNA repair and cell cycle regulation (Jin et al., 2011). There are many known histone H1 linker variants in somatic mammalian cells that stabilize the genome into heterochromatin states and higher compaction. Six variants of H1 (H1.0- H1.5) are found in somatic mammalian cells while two variants are specific to testes and oocytes. Histone H1.0 is the most abundant subtype in terminally differentiated cells while the other subtypes are associated with cell division and vary depending on cell type (Sancho, Diani, Beato, & Jordan, 2008). Histone H2 variants reside in heterochromatic areas and promoter regions poised for transcription (Histone H2A.Z) or areas of active transcription (H2ABbd) (Li et al., 2007). Histone H3 variants vary in abundance according to tissue type and may play a role in determining and maintaining cellular differentiation (Garcia et al., 2008). However, histone variant H3.3 differs in four amino acids from canonical histone H3, and deposition is associated with increased transcription. During transcription histones must be temporarily evicted for transcriptional machinery access to the DNA elements. Subsequent to histone methylation marks on histone H3K36 and H3K4, DNA is opened up and histones are displaced by RNA Polymerase II and chromatin remodeling proteins. Special histone chaperones act to redeposit histones onto DNA into their appropriate

placements (Li et al., 2007). There are many enzymes for adding and subtracting histone post-translational methylation and the enzymes are methylation marker specific (Rice & Allis, 2001). Histone post-translational modification is intrinsically linked to DNA methylation by DNMT association with histone deacetylases (HDACs), histone methyltransferases (HMTs), chromatin remodeling complexes, and other transcriptional corepressors altering chromosome structure in tandem with DNA methylation (Cheung & Lau, 2005; Cockerill, 2011; Shin et al., 2011). Similar to the complex function of DNMTs, many histone enzyme, histone variant, and associating protein functions are complex adding more facets to an already intricate mechanism of regulation.

Recently, a third form of epigenetic regulation has been discovered and involves the small non-coding RNAs (snRNA). Two types of snRNAs have been studied, small interfering RNAs (siRNAs) and microRNAs (also known as miRs or miRNAs). Both siRNAs and miRNAs are involved in mRNA or transcriptional control. Small interfering RNA is produced by RNA polymerase III, is transcribed from repetitive DNA, and is involved in heterochromatin formation. MicroRNAs are primarily transcribed in gene regions by RNA polymerase II and are involved in mainly posttranscriptional regulation (Nakahara & Carthew, 2004; Yu et al., 2014). MicroRNAs are approximately 22 nucleotides long and regulate post-transcriptional expression via targeted degradation or translational suppression. MicroRNA transcripts are highly conserved among different species of mammals demonstrating the evolutionary importance of this form of regulation (Chuang, 2007). MicroRNA transcription areas can be found in exonic and intronic regions

and are processed with a guanine cap and polyadenylated tail before editing. The discovery of new miRNAs transcripts has increased in surprising numbers, from 450 transcripts in 2007 to over 1100 transcripts in 2010, with more being continually discovered and predicted in databases such as *rna22* and miRBase (Chuang, 2007; Kozomara & Griffiths-Jones, 2014; Miranda et al., 2006; Sato et al., 2011).

MicroRNAs are processed after transcription by RNA polymerase II and are composed of long stem loop RNA structures known as pri-miRNA. These molecules then associate with RNase III endonuclease, Drosha, and DGCR8 that make up the microprocessor complex where it is cleaved into an approximately 70 base pair fragment which is now known as pre-miRNA. From here a complex of other proteins stabilize and export the pre-miRNA from the nucleus to the cytosol for further processing by associating with Dicer and Transactivation Responsive RNA-binding Protein 2 (TARBP2). Dicer has RNase III activity which facilitates double strand RNA cleavage. The final miRNA double stranded RNA strand, of approximately 22 base pairs is attached to Argonaut (Ago) which in participates in miRNA processing to a single stranded RNA. This complex is then loaded onto RISC (RNA induced silencing complex) for posttranscriptional regulation of mRNAs. This regulation can occur either through the complementary base pairing of mRNA transcripts to the miRNA where RISC degrades the mRNA transcript or through an incomplete complementation between the miRNA and the 3' UTR region of an mRNA molecule where other proteins decap and deadenylate the mRNA reducing its stability and promoting

degradation (Bian et al., 2013; Chuang Jody C., 2007; F. Sato et al., 2011). However, miRNA biogenesis, localization, turnover, and activity are all regulated by RNA binding proteins (RBPs) which can intercede at different processing stages. DEAD-box helicases, (DDXs) also known as Gem-associated proteins (Gemins), are a family of putative RNA helicases with a specific amino acid consensus sequence, which complexes with Ago to guide the miRNA onto RISC. These are potentially important regulators of miRNA to mRNA targeting and post-transcriptional control. The expression of DEAD-box helicases can be altered in certain disease states (Van Kouwenhove, Kedde, & Agami, 2011; Wilker et al., 2010). MicroRNA has the capability of controlling the expression of other epigenetic factors, such as the DNMTs, histone modifying enzymes, and methyl binding proteins (Bian et al., 2013; Denis, Ndlovu, & Fuks, 2011; F. Sato et al., 2011). MicroRNA can be controlled by epigenetic mechanisms through DNA methylation of miRNA coding regions and in the cytosol by RNA binding proteins.

Genetic and epigenetic stability, aging, and aging pathology

Aging is considered a temporally based physiological decline that occurs through the accumulation of cellular damage causing cellular dysfunction accompanied by inappropriate responses of cells to external or internal stimuli (Gravina & Vijg, 2010; López-otín et al., 2013). However, in genetic and epigenetic terms, the focus is the damage to molecules in the nucleus and the disruption of the genome and the epigenome whose regulation is interconnected. This dysregulation can affect RNA expression, repetitive element expression,

and miRNA expression, with effects that ripple outward to create excesses or deficiencies in cellular protein expression and function.

Proper gene expression of a somatic cell relies on the fidelity of gene sequences during replication, along with all of their regulatory elements, so that they may be properly transcribed and translated in concert, providing functional proteins affecting the overall function of a cell at any given time and condition. DNA sequence damage and mutations, which involve insertions or deletions, can cause genetic instability, changing the ability of the cell to function and respond to internal or external stimuli (Gravina & Vijg, 2010; López-Otín et al., 2013; Wolters & Schumacher, 2013). Extrinsic DNA sequence damage can be caused by mechanical, biological, and chemical stressors. Examples of extrinsic forces include ultraviolet radiation and mutagenic substances. Intrinsically caused DNA damage can occur through oxidative stress via the production of reactive oxygen species, spontaneous hydrolytic, deamination, or alkylating reactions, and DNA replication or repair errors of the cell (Hoeijmakers, 2009; Lindahl, 1974; Shin et al., 2011; Singal & Ginder, 1999; Wolters & Schumacher, 2013). Genetic instability accumulates with aging in humans and mice (Vijg & Suh, 2013). It also occurs in human accelerated aging diseases such as Werner syndrome, Bloom syndrome, and Hutchinson-Gilford Progeria syndrome (HGPS) and many of these mutations cause cancer susceptibility associated with aging (Moskalev et al., 2013; Wolters & Schumacher, 2013). Werner syndrome and HGPS stem from a mutation in the nuclear lamin A/C gene, from which translated nuclear membrane proteins define the arrangement and have a regulatory role in gene

expression. These mutations display phenotypes that correlate with both genetic and epigenetic instability and many markers of aging. Disruption of DNA repair mechanisms through mutation causes many aging phenotypes including retinal degeneration, progressive kyphosis, ataxia, photosensitivity, and neurodegeneration (Barzilai et al., 2008; Burtner & Kennedy, 2010; Laugel et al., 2010) Cellular mitotic or DNA replication errors producing chromosomal aneuploidies and variable copy-numbers also increase with aging (Faggioli et al.,2012;Forsberg et al., 2012) . Repetitive DNA, unlike genes which make up a small percentage of DNA, makes up 40 to 50% of the genome in mice and humans respectively (Shapiro & Von Sternberg, 2005; Waterston et al., 2002). Repetitive elements are also known as interspersed repeats, retroviral elements, and DNA transposons. Transposable elements (TEs) are a class of repetitive elements found in mammals including mice and humans which are able to copy and subsequently insert themselves in different areas of the genome when expressed (Tomilin, 2008). Transposition of these elements increases with aging in mouse tissue (De Cecco et al., 2013).

There are four classes of transposable elements in mammals, three of which appear to be important in cellular differentiation and can be altered in DNA methylation and expression due to nutritional stressors and age (De Cecco et al., 2013; Martens et al., 2005; Pogribny et al., 2009). The first class, known as long interspersed nucleotide elements (LINEs), is independently capable of transposition. They are considered non-retroviral elements and contain coding regions for reverse transcriptase (RT) and an endonuclease. They move through

an RNA intermediate, create DNA copies via RT, and insinuate themselves into the host genome via an endonuclease. Short interspersed nuclear elements (SINEs) do not contain RT or endonuclease coding regions, therefore these elements require LINE transcription to provide these enzymes. Interestingly, LINEs are located in A-T rich regions in gene poor regions and SINEs are located in C-G and gene rich areas (Alberts et al., 2008; Waterston et al., 2002). A third class of transposable element is the retroviral-like retrotransposon. These repeats are more like the retroviral ancestors from which they probably came. These include the intracisternal-A particles long terminal repeats (IAP-LTRs) whose transcription causes small particles to accumulate in the endoplasmic reticulum during embryogenesis and in tumors (Hojman-Montes de Oca et al., 1983). LTRs contain RT and integrase coding regions. Their transcription produces a DNA copy, similar to LINEs, and an integrase implants the DNA into a new area of the genome (Alberts et al., 2008).

Telomeres are repetitive sequences at the ends of chromosomes that are rich in guanine and protect DNA from degradation and recombination. Telomere loss is strongly associated with aging, progeria, and increases vulnerability to genomic instability (Londoño-Vallejo, 2010; López-Otín et al., 2013; Shin et al., 2011; Sinclair & Oberdoerffer, 2009; Wolters & Schumacher, 2013). Telomere sequences attract special proteins that loop the DNA and protect it from damage. Due to these proteins, DNA replication and repair enzymes have difficulty accessing these regions which consequently decrease in size over time until they become truncated enough that the cell enters a state called senescence

(Londoño-Vallejo, 2010). Cellular senescence is the cellular state where a cell has permanently left the cell cycle and cannot proliferate even upon exposure to stimulatory growth factors. Senescent cells also display an aberrant hyperactive expression and secretory state and aging tissue is thought to accumulate these types of cells (Blagosklonny, 2011; Campisi & d'Adda di Fagagna, 2007).

During adulthood, epigenetics plays an important role in maintaining cell differentiation, gene expression, genetic stability, and contributes to the ability of a somatic cell to change its functions or its state in response to a variable environment. This is termed phenotypic plasticity. Epigenetic changes are likely to occur in a group of cells responding to the same stimulus unlike genetic changes defined by the maintenance of a single cell's genetic integrity during DNA synthesis and cellular division, or exposure to chemical, environmental, or biological stressors (Gravina & Vijg, 2010; López-Otín et al., 2013; Shin et al., 2011). Epigenetic (unlike genetic changes) are dynamic, which allow phenotypic plasticity for cellular responses to a stimulus, or reversal of cellular responses upon the stimulus cessation. Epigenetic stability involves maintaining the fidelity of DNA and histone markers and replications over time but also the plasticity of epigenome marker addition, subtraction, or replacement, to changes in intrinsic and extrinsic cellular signaling. If parts of the epigenome are no longer able to appropriately change according to the condition in which these cells exist, cell genome and protein expression can become unresponsive or hyper-responsive, meaning that necessary genes either are not expressed or are over-expressed in a manner that is ultimately deleterious to cell function (Burzynski, 2005; Gravina

& Vijg, 2010). This concept is what is currently proposed to happen during aging through several mechanisms contributing to epigenetic and genetic instability. Because genome and epigenome instability are both present in aging cells, it is currently debated which event precedes the other as a potential early biomarker of cell dysfunction (Pegoraro & Misteli, 2009). When do epigenetic changes start to coincide with aging pathology? As yet, there are no firm answers to this question however, there is surprising evidence that DNA methylation changes occurring early in the life of mammals (i.e. zero to one month in mice) are amplified during adulthood and the aging process (Takasugi, 2011). This begs the question as to which of these DNA methylation changes could be predictive for later detrimental age-related changes or are part of normal aging development. Epigenetic stability can be threatened in several ways. The environment has a well-established effect on epigenetic markers, especially DNA methylation. Chronically present intrinsic or extrinsic stressors, such as altered nutritional intake can make lasting differences in epigenetic markers which can permanently influence signaling in cells (Pogribny et al., 2006). In addition to environmental inputs or insults, epigenetic markers can be affected by random or stochastic changes over time which may be defined as epimutations, epigenetic drift, or even epigenetic recombination. These can result in observable phenotypic traits that cannot be explained by genetics or the environment that cause divergent transcriptomes over time (Fraga & Esteller, 2007; Gravina & Vijg, 2010; R Holliday, 1987; Poulsen et al., 2007). In addition, just as in genetic replication, epigenetic replication is subject to error as well. In regularly

proliferating cells, errors can make a significant contribution to epigenetic aging (Laird et al., 2004). This can promote epigenetic heterogeneity between monozygotic twins and in in-bred animal strains that are exposed to similar environments and nutritional access (Wong, Gottesman, & Petronis, 2005).

There are several mechanisms by which the epigenome protects or enhances genome function in a cell that can go awry during aging causing genetic instability and ultimately cell dysfunction. Many parts of the DNA sequence are protectively sequestered from mutations and undesired transcription by DNA methylation and epigenetic proteins, such as methyl-binding, histone, and non-histone proteins. Areas of normally heavily methylated DNA regions in adult somatic cells are interspersed repeats and oncogenes. Two major events appear to be important factors in aging and aging pathologies via DNA methylation. The first is global hypomethylation in CpG dinucleotides and concurrent hypomethylation in repetitive elements (Asada et al., 2006; Berdasco & Esteller, 2012; V Bollati et al., 2011; De Cecco et al., 2013; Jintaridth & Mutirangura, 2010; Mustafina, 2013; Singhal, Mays-Hoopes, & Eichhorn, 1987; Thompson, Atzmon, & Gheorghe, 2010). The second event is the hypermethylation of tumor suppressor genes and other normally expressed genes such as housekeeping genes (Burzynski, 2005; Christensen et al., 2009; Chuang Jody, 2007; Fraga & Esteller, 2007; Gentilini et al., 2012; Gravina & Vijg, 2010; López-otín et al., 2013; Sedivy et al., 2008; Teschendorff et al., 2010). While interspersed repeat expression is postulated to threaten genomic stability and create transcriptional noise, the expression of oncogenes spurs the cell into

unwarranted cellular proliferation, and appears to be concurrent with silencing of tumor suppressor genes by increased methylation, laying the foundation for cellular transformation and tumorigenesis during aging. Global loss of DNA methylation is thought to contribute to chronic inflammation and autoimmune diseases by making DNA more immunogenic to surrounding cells. Therefore more "self" reactivity can occur resulting in less apoptosis of aberrant cells and increasing necrosis during aging (Agrawal et al., 2010). DNA methylation changes during aging have been demonstrated to polarize T cell differentiation patterns and alter receptor expression contributing to autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Goronzy et al., 2010). Age-related DNA hypomethylation, transcription, translocation, or even partial translation of endogenous retroviral elements may contribute to the development of autoimmune diseases (Balada et al., 2010; Perl, 2003). Brains of Alzheimer's patients show global changes in DNA methylation, changes in DNA methylation at repetitive elements, gene specific methylation changes, and changes in metabolic pathways that affect DNA methylation (Bollati et al., 2011; Coppieters et al., 2014; Groen, 2010). Gene specific changes in DNA methylation are also associated with osteoarthritis (Barter et al., 2012). Global DNA hypomethylation has been associated with aging and is a widespread phenomenon; however there are exceptions to the accepted paradigm. *In vivo* tissue does not always show global hypomethylation with age or in progeroid tissues (Osorio et al., 2010). As a result, the aging epigenome paradigm might be different for different tissues and different species of mammals.

Chromatin structure, histones, and histone modifications can also play large roles in protecting the cell from genetic and epigenetic instability. As stated earlier, chromatin structure, histone alterations, and DNA methylation work cumulatively or synergistically to regulate gene function and DNA accessibility. There is a strong reorganization of chromatin structure during aging that impacts global RNA expression. In aging drosophila there is a loss of heterochromatin and a gain of ribosomal RNA expression associated with aging. In murine tissue, there is a gain of heterochromatin and loss of total RNA correlated with aging (De Cecco et al., 2013). Senescent cell types also exhibit changes in heterochromatin formation at normally euchromatin associated areas (Sedivy et al., 2008).

Histone proteins play a role in genomic stability. For instance, DNA damage repair triggers H2AX phosphorylation, which is a histone H2 variant that senses DNA damage and helps negatively regulate the cell cycle for DNA repair. This mechanism helps maintain genomic stability and proper cell function and is associated with cellular senescence in aging tissues and progerias (Campisi & d'Adda di Fagagna, 2007; Gravina & Vijg, 2010; Hanasoge & Ljungman, 2007; Wang et al., 2009). It is also a convenient marker for global DNA damage because of this role (Pogribny et al., 2010). Histone methyltransferases and DNMTs form complexes and both attain methyl groups from SAM, the universal methyl donor. Therefore, it's not especially surprising to discover that there are many histone methylation modifications that change with age, just as there are DNA methylation changes with age across mammalian species and tissue types.

The constitutive heterochromatic markers, histone H3K9 methylation, H3K27 methylation, and protein HP1 decrease in elderly human cells, senescent cells, and progeroid cells (Pegoraro & Misteli, 2009; Sedivy et al., 2008; Shin et al., 2011). Histone H3K27 tri-methylation levels decrease while messenger RNA increases with age in nematodes but not flies (Han & Brunet, 2012). Histone H4K20 tri-methylation is also a heterochromatic transcriptionally repressive histone marker that increases in senescent cells, rat liver, and progeroid cells but is decreased in cancer (Fraga & Esteller, 2007; Shin et al., 2011). Loss of histone H3K4 methyltransferase, which produces the transcriptionally active tri-methylation marker, extends lifespan in nematodes. In addition, loss of the demethylase for the same marker decreases lifespan for both nematodes and drosophila. However, in murine and human brain tissue, histone H3K4 tri-methylation is decreased with aging (Cheung et al., 2010; Kuzumaki et al., 2010a). Thus some of the histone markers involved in aging appear to be specific to mammalian aging and disease. Histone acetylation, which is a more transient marker, shows some differences in age such as increases in H4K16 acetylation in yeast. In theory, stochastic changes in histone modifications can occur just as in DNA methylation, which would correlate with increased stochastic gene expression with age (Gravina & Vijg, 2010). Global acetylation of histones H4 and H2A also occur in progeroid mice (Osorio et al., 2010). Cognitive decline in mice is associated with the loss of chromatin plasticity via deregulated histone acetylation (Peleg et al., 2010).

Non-coding RNAs have recently been discovered to alter their expression with age in nematodes, with a decline in total microRNA expression and an increase in specific miRNA transcripts (Boehm & Slack, 2005; Ibáñez-Ventoso et al., 2006). However, no specific miRNA over- or under-expression effects have been discovered to modulate aging directly in mammals, though many have been identified for the regulation of aging pathways and in cross-sectional studies of aging mammals that may be potential targets (Bates et al., 2010; Grillari & Grillari-Voglauer, 2010; Maes, An, Sarojini, & Wang, 2008). Some of these targets are shown to have effects on DNA methylation regulation, cancer and autoimmune regulation as well (Bian et al., 2013). Other miRNA transcripts are regulated by other epigenetic mechanisms and are involved in many different types of cancer, apoptosis, neurogenesis, neurodegenerative disease, senescence, and genomic imprinting all of which are affected by, or are correlated with, aging. (Grillari & Grillari-Voglauer, 2010; Sato et al., 2011). Histone H2AX expression is involved in the DNA damage response and cellular senescence. This histone variant is also regulated by miRNA expression and is implicated in DNMT expression as well (Denis et al., 2011; Grillari & Grillari-Voglauer, 2010; Ugalde et al., 2011). However, though some bioinformatical and predictive queries have been started, miRNA expression and functional crossovers between aging, aging diseases, and epigenetics are still lacking (Inukai & Slack, 2013). Finally, the transcription of repetitive elements, possibly into small interfering RNA, has been suggested to potentially serve some function in the mammalian cell and aging in particular, since transcription of some of these families increases with aging in mice (De Cecco et al., 2013; Grillari & Grillari-Voglauer, 2010).

The methionine pathway, methylation, and epigenetics

The methionine pathway is central to many important biochemical reactions and regulation of cellular function. All of the myriad methylation reactions of a cell and the nucleus are connected to the methionine pathway, also known as one carbon metabolism or thiol metabolism, through the production of the methyl donor molecules *S*-adenosylmethionine (SAM or AdoMet). As discussed previously, DNA methylation is very important to transcriptional regulation. Histone methylation and it's "code" for transcriptional activation or repression is well studied. Histone methylation marks are more stable posttranslational modifications than either acetylation or phosphorylation and have specific measurable markers that contribute to all forms of chromatin (euchromatin, facultative heterochromatin, and constitutive heterochromatin) (Barth & Imhof, 2010). The first step of the methionine pathway involves the conversion of methionine to SAM by methionine adenosyltransferase (MAT). This step requires ATP and is the rate limiting step of this pathway due to a triphosphate intermediate (Wallace & Fan, 2010). SAM is then converted to SAH by many different methyltransferase enzymes unless it is shunted to a salvage pathway and polyamine synthesis where it is decarboxylated (Cavuoto & Fenech, 2012). *S*-adenosylmethionine on its own can act as a riboswitch involved in translation inhibition and transcriptional termination by binding RNA affecting cellular function. However for methylation reactions, in which it is mostly involved, SAM is converted to *S*-adenosylhomocysteine (SAH) by methyltransferase enzymes which perform different vital functions in the cell. In fact, the use of

methyl groups is second only to the use of ATP in the cell and is used for the methylation of DNA, RNA, histones and other proteins, polyamines, and lipid methylation as well as the methylation of some small molecules, demonstrating the diverse number of uses for methionine that contribute heavily to cellular metabolism and homeostasis (Loenen, 2006). The availability and competition for SAM as a substrate affects methyltransferase activity and expression and consequently DNA methylation. For instance, induced or inhibitory alteration in glycine-*N*-methyltransferase activity, which competes for methyl groups with DNMT, causes alterations in DNA methylation and DNMT activity (Huang et al., 2007; Martínez-Chantar et al., 2008; Rowling et al., 2002; Ulrey, et al., 2005; Wang et al., 2011). The methionine pathway is also subject to nutritional supplementation or deficiency of methionine, or other nutrients, cofactors, and hormones as well. These molecules include choline, vitamins B6 and B12, folate, insulin, glucagon, growth hormone and glucocorticoids (Aida et al., 1997; Brown-Borg, Rakoczy, & Uthus, 2005; Ulrey et al., 2005). Diets that are methyl-deficient alter the availability of SAM and usually lack one or a combination of choline, methionine, or folate. These diets are thought to alter the ratio of SAM: SAH abundance which is widely accepted as a reference for methylation capacity (Brown-Borg et al., 2005; Cantoni & Chiang, 1980). Nutritional deficiencies or supplementation of folate, choline and vitamin B12 have been shown to alter the expression of several methionine metabolic enzymes, which in turn induced changes in SAM:SAH, global DNA methylation, and histone modifications in humans, rabbits, and mice in different tissue types (Choi & Friso, 2010; Ulrey et

al., 2005). Altered SAM and SAH levels are known to contribute to human disease (Loenen, 2006). *S*-adenosylhomocysteine is also capable of inhibiting methyltransferase activity and possibly DNA methylation (Davis & Uthus, 2004; De Cabo, Santos, & Fernández-Piqueras, 1995).

How is the methionine pathway affected by aging? One of the most studied hormones involved in aging is growth hormone (GH). Growth hormone is known to regulate components of the methionine pathway. Several murine studies, in diverse background strains, show mutant animals deficient in GH signaling live significantly longer than their non-mutant siblings (Brown-Borg, 2009). Furthermore, there are reports that GH administration to GH deficient mutants shortens lifespan to wild type levels and greatly affects the enzyme activities in the methionine pathway, including many methyltransferases (Brown-Borg et al., 2005; Panici et al., 2010).

Growth hormone, methionine pathway, and aging

Growth hormone is a protein hormone that is released by somatotrophs in the anterior pituitary in a pulsatile fashion (Hartman et al., 1993; Vijayakumar et al.,2011). Several factors diminish or stimulate its release in humans and mice including exercise, sleep, starvation, steroid hormones, and stress (Cornford et al., 2011; Hartman et al., 1993; Weltman et al., 2006). GH secretion is affected by estrogens and androgens and therefore exhibits sexual dimorphism (Ohlsson et al., 2009). Growth hormone also affects DNA methylation of many genes based on gender (Takasugi et al., 2013). One molecule of growth hormone from the serum stimulates the dimerization of two tyrosine kinase receptors which

facilitates the phosphorylation of the Janus family of tyrosine kinases (JAK2) and the serine threonine phosphorylation of the GH receptors. A phosphorylation cascade is initiated which can stimulate the Signal Transducers and Activators of Transcription (STATs) and many other pathways such as ERK1/2 and MAPK (Bartke et al.,2013; Vijayakumar et al., 2011). STAT localizes to the nucleus where it can act as a transcription factor through STAT responsive elements on many different genes involved in proliferation. GH signaling affects other pathways including the phosphotidylinositide 3-kinase/Akt pathway (PI3K/Akt) which is primarily responsible for regulating cellular proliferation, cell survival, and differentiation (Herrington et al., 2000; Rawlings et al., 2004; Schumacher et al., 2008) and GH administration to GH deficient dwarf mice affects many components of this pathway in liver tissue (Masternak et al., 2010; Miquet et al., 2010). During aging, GH levels decline starting after the age of twenty years in humans, and since GH stimulates IGF-1 production and secretion from the liver, this hormone declines as well. IGF-1 is important to somatic and central nervous system development and brain function (Aberg et al., 1998; Muniyappa et al., 2007; Ye & D'Ercole, 2006). Though most IGF-1 is produced in the liver, some is produced by local tissues with autocrine/paracrine effects (Ohlsson et al., 2009). IGF-1 can also affect GH secretion (Bartke et al., 2013; Vijayakumar et al., 2011). Insulin shares similar pathway mechanisms with IGF-1 signaling and has also been implicated in aging across species, including worms, flies, and mice (López-Otín et al., 2013). Overexpression of growth hormone in mice leads to reduced lifespans and early onset of aging markers (Bartke et al., 1999; Steger

et al., 1993). In humans there are a number of indicators that the GH, IGF-1 and insulin signaling pathways are involved in aging from Laron dwarfs, deficient in GH signaling, to centenarians. The lack of many age-related diseases such as cancer and diabetes in Laron dwarfs, and delayed cancer among centenarians, indicates potential promising cross-over between mice and humans (Bartke et al., 2013; Guevara-Aguirre et al., 2011; Heemst, 2010; Longo & Finch, 2003; Salvioli et al., 2009; Suh et al., 2008). However, Laron dwarfism (GH receptor defect) does not offer any decided prolongation of lifespan in humans as it does in mice (Aguiar-Oliveira et al., 2010). Although there is a difference between humans and mice in aging phenotypes, some bioinformatics inquiries are tackling the species specific similarities and differences in the murine and human genomes to help guide the scientific community to more translatable paradigms and hopefully a better understanding of somatotropic signaling and human aging (Yuan et al., 2011).

The liver as an organ of study for aging and methylation

In some mammalian tissues, cells continue to readily divide in a fully formed adult organism, such as intestinal wall or skin cells, whereas others are terminally differentiated and have lost the capacity to divide, such as neurons. One exception is liver cells, which upon insult or partial removal can reenter the cell cycle from a quiescent differentiated state to proliferate and replace lost tissue (Pennisi et al., 2004; Schmucker & Sanchez, 2011). The liver loses volume, blood flow, and the ability to regenerate with age. Also more Kupffer cells are activated in aged liver, which may be indicative of some basal inflammation

associated with aging (Hilmer et al., 2007; Sheedfar et al., 2013). The liver is responsible for the first pass metabolism of many pharmaceuticals that can increase drug half-life and drug toxicity in aged humans (Klotz, 2009; Pond & Tozer, 1984). Therefore, the liver is a very important organ in aging and there is an excellent reservoir of scientific evidence demonstrating that epigenetic changes occuring in this organ are related to the methionine pathway and methyl availability.

Many diseases such as hepatitis steatosis (fatty liver), cirrhosis from alcoholism, and vascular diseases and inflammation can be correlated with an altered production of S-adenosylmethionine (SAM) and its corresponding byproduct after methyl group subtraction, S-adenosylhomocysteine (SAH). Liver disease and risk of hepatocellular carcinoma increases in humans and animals with age as well (Sheedfar et al., 2013). The most abundant methyltransferase in the liver, glycine-*N*-methyltransferase (GNMT), displays diminished expression in human hepatocellular carcinoma and GNMT knockout mice show a propensity for hepatocellular carcinoma accompanied by differences in DNA and histone methylation (Martínez-Chantar et al., 2008). Experimentally increased or decreased expression of GNMT in hepatocytes alters DNMT methylation and activity, which can further be affected by adding another variable, methionine supplementation (Wang et al., 2011). The activation of GNMT using retinoic acid also causes global hypomethylation in rats (Rowling et al., 2002). GNMT is one of the methyltransferases in the methionine pathway mentioned earlier, and because it is so abundant, it regulates SAM availability in the liver for other

methyltransferases (Yeo & Wagner, 1994). By altering separate nutritional components of the methionine pathway, such as choline, and methionine itself, many differences can observed in the expression of epigenetic markers and mechanisms in murine hepatocytes (*in vitro*) and murine liver (*in vivo*). Methyl-deficient diets induce miRNA expression changes in mice that are associated with diseases and aging (Choi & Friso, 2010). However, common groups of miRNAs which simultaneously regulate aging pathways, the methionine pathway, and the regulation of the epigenome, still remain to be discovered. Methyl-deficient diets also alter repetitive element expression which is associated with genetic instability, hepatic steatosis, and hepatocellular carcinoma (Asada et al., 2006; Hsieh et al., 1989; Pogribny et al., 2009). Methyl-deficient diets significantly alter DNA and histone methylation in long-lived inbred strains of mice compared to short-lived strains and show some interesting differences (Pogribny et al., 2009). However, none of these epigenetic modifications have been studied in mutant long-lived mouse models compared to their wild type siblings, exposing a large gap in the scientific knowledge between aging, the methionine pathway, and epigenetic mechanisms.

As stated earlier, the liver is very responsive to growth hormone and primarily responsible for IGF-1 secretion in the body (Bartke et al., 2013; Brown-Borg, 2009). Liver fibrosis, a condition that increases with aging, displays differences in DNA methylation and miRNA expression. DNA methyltransferase 1 negatively regulates the phosphatase and tensin homologue (PTEN), that negatively regulates PI3K/AKT and ERK pathways, and coincidently are

stimulated by GH through JAK/STAT pathways (Bian et al., 2013). The protein PTEN is also associated with increased lifespan in many organisms (López-otín et al., 2013; Salminen & Kaarniranta, 2010; Scrable et al., 2010). However the connection between GH pathways and DNMT expression has not yet been demonstrated. Aging mouse liver show higher amounts of γ-H2AX, a marker of DNA damage demonstrating decreased genomic stability (Wang et al., 2009). Liver tissue from old mice exhibits multinucleated hepatocytes that increase in size but without cytokinesis (known as polyploidy). This phenomenon is not well understood but is similar to senescence in other cell lines and occurs with aging (Lebel, De Souza-Pinto, & Bohr, 2011).

Ames dwarf mice as a model for epigenetic study and aging

Ames dwarf mice result from a single nucleotide polymorphism in the *Prophet of Pit-1* gene (*prop-1*) rendering a loss of function mutation that is unable to stimulate *Pit-1* gene expression (Andersen et al., 1995; Schaible & Gowen, 1961; Sornson et al., 1996). This results in an underdeveloped anterior pituitary gland devoid of secreting somatotropes, lactotropes, and thyrotropes causing the absences of plasma growth hormone, prolactin, and thyroxine stimulating hormone, respectively (Bartke, 1964; Sornson et al., 1996). Ames mice exceed wild type sibling lifespans by 49-68%, with females showing the largest gain (Brown-Borg et al., 1996). Ames dwarf mice have many physiological and developmental phenotypic differences from wild type mice including, smaller size, lower body temperature, female infertility, delayed incidence of neoplastic disease and less age-related tissue and cell injury in toto at death, and they

maintain a juvenile appearance in adulthood (Bartke et al., 2001; Bartke et al., 2013; Brown-Borg et al., 1996; Hunter et al., 1999; Ikeno, Bronson et al., 2003; Schaible & Gowen, 1961). Other important biochemical characteristics related to aging in Ames dwarf mice include heightened insulin sensitivity even in old age, lower glucose levels, very low IGF-1 plasma levels, enhanced resistance to oxidative stress, and an atypical methionine pathway with altered basal expression and activity of many enzymes (Brown-Borg & Rakoczy, 2003; Brown-Borg, 2009; Chandrashekar & Bartke, 1993; Louis et al., 2010; L. Y. Sun et al., 2011; Uthus & Brown-Borg, 2003, 2006). GH is known to oppose the actions of insulin and contributes to insulin resistance. Another interesting feature of dwarf mice is that sexual liver dimorphism gene expression seen in wild type mice disappears between male and female dwarf mice (Amador-Noguez et al., 2005; Takasugi et al., 2013). When Ames dwarf mice are treated with GH beginning at 2 weeks of age for 6 weeks, their lifespan becomes shorter and more similar to wild type mice. Thyroxine injections however have no effect on lifespan, only body weight, illustrating the effects of longevity in dwarf mice is mostly due to a lack of GH (Panici et al., 2010).

While many of the phenotype differences are interesting, the enzymatic differences within the methionine pathway have a large potential to alter epigenome methylation in Ames dwarf mice, and thereby change phenotypic gene expression. GNMT is an important regulator of methyl donor availability and dwarf mice show increased activity and expression of this enzyme. There are also increases in methionine adenosyltransferase, which affects the

production of methionine into SAM, the universal methyl donor (Uthus & Brown-Borg, 2003). Ames dwarf mice have a reduced SAM:SAH ratio, that is usually associated with reduced methylation capacity, but lack the increased disease pathology seen in other mice or in humans (Chen et al., 2010; Elmore & Matthews, 2007). However, Ames mice show greater activity in methylation enzymes responsible for SAM to SAH conversion, and higher rates of transmethylation and transulfuration, demonstrating that they most likely do not have a reduced methylation capacity (Uthus & Brown-Borg, 2006). Also, due to the fact that the administration of GH in dwarf mice can reverse methionine enzyme activity and expression to wild type levels suggests growth hormone may be a powerful factor in epigenomic stability and aging.

The presence of GH diminishes the ability to resist oxidative stress (Brown-Borg, 2009). Ames dwarf mice also exhibit a high resistance to oxidative stress from reactive oxygen species and an increased expression and activity of many antioxidant enzymes including superoxide dismutases (SODs), catalase, glutathione, peroxidase, and on enzymatic antioxidants such as glutathione, and metallothionein (Amador-Noguez et al., 2004; Brown-Borg & Rakoczy, 2000; Brown-Borg & Rakoczy, 2002; Brown-Borg et al., 2005; Meyer et al., 2003; Sharma et al., 2010; Sun et al., 2011; Swindell et al., 2010). While glutathione expression is regulated through enzymes connected to the methionine and transulfuration pathways, the heavy metal and free radical scavenger, metallothionein can be regulated by the DNMTs through DNA promoter methylation (Kumari et al., 1998; Majumder et al., 2006;

Majumder, Ghoshal et al., 1993). This is yet another reason why the Ames dwarf mouse may have the potential for important epigenetic alterations contributing to aging and longevity.

There are other models deficient in GH signaling, such as Laron dwarf mice, which lack a functional growth hormone receptor and binding protein resulting in a mouse resistant to GH (GHRKO mice) (Zhou et al., 1997). These mice show increased life spans (38-55% longer) along with many other phenotypic similarities with Ames dwarf mice including small body size, delays in sexual maturation, oxidative stress resistance, enhanced insulin sensitivity and an altered expression of methionine enzymes including GNMT and MAT (Brown-Borg et al., 2009; Brown-Borg, 2009). However, while these mice display low levels of plasma IGF-1, they also express high levels of plasma GH and normal levels of thyroid stimulating hormone and typically prolactin (Al-Regaiey et al., 2005; Coschigano et al., 2000; Coschigano et al., 2003; Hauck & Hunter, 2001; Zhou et al., 1997). As the Ames dwarf mice exhibit multiple endocrine deficiencies, growth hormone receptor knockout mice are thought to be a good model to validate GH effects of aging. However, GHRKO mice are created on inbred laboratory strains, whereas Ames dwarf mice come from a genetically heterogeneous background. Epigenetically speaking there is great variation between laboratory strains alone and in response to nutritional stressors such as methyl-deficient diets, so this caveat must always be considered (Hsieh et al., 1989; Pogribny et al., 2009; Stanley et al., 2001).

Another model used to study the effects of GH and aging are the GH overexpressing transgenic mice (GH Tg). These mice live approximately half as long as their wild type littermates (Steger et al., 1993). Phenotypically these mice are much larger than wild type counterparts, hyperglycemic, insulin resistant, sexually mature earlier, and have increased cellular oxidative stress and oxidative damage due to increased metabolic activity (Brown-Borg, 2009). Also GH transgenic mice show differences in methionine enzyme expression and metabolite differences of the methionine pathway (Brown-Borg, unpublished data).

There are human epigenetic differences attributable to longevity. It's been reported that centenarians and their offspring tend to maintain higher DNA methylation levels than people without a family history of longevity demonstrating additional scientific reasons studying the epigenetic differences of normal and long living organisms (Gentilini et al., 2012). One of the most highly conserved (top ten) gene sequences among centenarians is DNMT3a, demonstrating a potential conservation of important methylation mechanisms. However there are more heterozygous IGF-1R mutations in centenarians that may alter the IGF-1 pathway in these individuals (Suh et al., 2008). Centenarians also have genetic telomerase variants that allow retained telomere length contributing to genetic stability (Atzmon et al., 2010). Human centenarians are known to have delayed cancer and in liver, the very elderly (>75 years of age) exhibit a reduction in hepatosteatosis and incidence of hepatocellular carcinoma (Salvioli et al., 2009; Sheedfar et al., 2013). The GH deficient and transgenic models mentioned have

not been studied in the context of epigenetic changes due to methyl-donor capacity and aging. Most aging research in epigenetics is in a developmental or diseased context, such as in embryonic stem cells and immortal cell lines derived from cancers. However, given that there are differences in “normal aging” and “longevity” such as between average life spanned and long-lived humans and mice, the context of epigenetic information and discovery with aging is enormously incomplete. The methionine pathway is strongly correlated with epigenetic differences in mice due to mutational or nutritional stressors, thus we were interested in potential differences in well-known methylation mechanisms and markers between GH deficient signaling mice (Ames dwarf and GHRKO mice) and GH transgenic mice and begin to catalogue important differences due to GH and aging. We expect to see differences in epigenetic markers that show increased epigenetic stability indicative of the already greater genetic stability noted in long-lived models such as Ames dwarf mice (Brown-Borg et al., 2001; Salmon et al. 2008; Sanz et al., 2002). We also expect to see increased maintenance of the heterochromatin and DNA methylation and a reduction or reversal of changes in markers that are considered to promote or correlate with aging. This research is extremely novel and should have profound implications and impacts on the study of epigenetics of aging and potential therapeutic targets that enhance epigenetic stability.

CHAPTER II

MATERIALS AND METHODS

We designed our cross-sectional age studies to examine DNA methylation enzyme activity, expression, and DNA methylation in growth hormone signaling deficient mice and GH overexpressing mice. We investigated interspersed repeat mRNA expression and methylation in these mice as a tool to investigate genetic stability and DNA methylation in gene-poor versus gene-rich regions. We also treated Ames dwarf mice with GH and looked for differences in DNMT and repeat expression. Primary hepatocytes from Ames mice were isolated and treated with growth hormone to validate our *in vivo* DNMT and DNA methylation studies as a possible future model of pathway investigations. We treated GHRKO (Laron dwarf) mice with IGF-1 to examine the effects down-stream of GH signaling through mRNA expression of DNMTs. We also studied a putative post-transcriptional pathway involved in DNMT1 regulation via miRNA expression, protein expression, and used methylation specific PCR analysis to probe the methylation difference of gene regions reported to be regulated by DNMT1 in Ames dwarf and age-matched wild type mice, and mice treated with growth hormone or saline. We began the characterization of histone methylation modifications cross-sectionally between Ames dwarf mice and their wild type

Siblings by immunoblotting of histone extract and included an mRNA expression pilot study to locate histone modification enzyme targets.

Experimental animals

All mutant and age-matched wild type mice were taken from colonies at the University of North Dakota animal facility. Ames dwarf mice (df/df) result from crossing heterozygous (df/+) or homozygous (df/df) male mice with carrier females (df/+) that are on a heterogenous background. The growth hormone receptor knockout mice (Laron dwarf or GHRKO) that were originally generated on an OLA/Balb/cJ background and used in our studies were kindly provided by Andrzej Bartke (SIU) (Zhou et al., 1997). They were developed by crossing 129 OLA/Balb/cJ wild type (GHR+/-) animals with mice derived from C57BL/6J and C3HJS strains. These mice were maintained in a closed colony (Panici et al., 2009). Wild type siblings of both the Ames dwarf and GHRKO mice have median lifespans of approximately 2 years (24 months) of age and this age was chosen to represent the oldest group for both strains in cross-sectional studies. Three months of age was chosen to represent young adult development and twelve months was chosen for middle-aged adult mice. The GH transgenic (GH Tg) mice used in these studies were derived from a single male founder (strain B6SJL) produced by microinjection of the phosphoenolpyruvate carboxykinase (PEPCK) promoter region (300 bp)/bGH hybrid gene into the male pronucleus of single-cell embryos. The production and characterization of transgenic animals (transgenic males crossed to C57Bl/6J x 3C3H/J F1 females) has been previously described (McGrane et al., 1988; Steger et al., 1994). The GH Tg line

has a median lifespan of approximately 12 months and at this age we and others have observed large numbers of liver tumors (Steger et al., 1993; Brown-Borg, unpublished data). Therefore, to avoid using pathological tissue in our studies, the oldest group of GH Tg mice used was 10 months of age. Young adult mice at three months of age and 6-month-old mice (GH Tg middle aged) with age-matched wild type controls were used for cross-sectional studies. All of the mice were kept under standard laboratory conditions with a twelve hour light/dark cycle, room temperature of 22℃, and fed *ad libitum* (8640 Teklad 22/5l: 22.6% crude protein, 5.2% fat, Harland Laboratories) with free access to water. Only males of GHRKO, GH Tg and their respective wild type siblings and only wild type males of the Ames dwarf line were used to avoid any variability caused by sex steroid effects. Groups of Ames dwarf mice groups contained both female and male mice. Female dwarf mice are infertile and exhibit biochemical characteristics similar to males in many studies. In addition, it has been reported that there is a loss of sexual liver dimorphism gene expression in these mice (Amador-Noguez et al., 2005). All procedures involving animals were reviewed and approved by the UND Institutional Animal Care and Use Committee in accordance with the NIH guidelines for the care and use of laboratory animals.

For cross-sectional basal expression studies, liver tissue was collected from mice at 3, 6, 12 and 24 months of age. Liver tissue was then divided into portions, rapidly frozen, and stored at -80℃. Liver tissues were utilized to evaluate mRNA and miRNA expression, DNA methylation and methylation enzymes, histone methylation, and protein abundance.

In vivo GH treatment of Ames dwarf mice and IGF-1 treatment of GHRKO mice

Porcine growth hormone (GH) (NIDDK, National Hormone and Peptide Program, Torrance, CA,USA) was administered to 4-6 month old Ames dwarf mice and IGF-1 was administered to 6 month GHRKO mice using methods published previously to determine whether GH and IGF-1 affects protein expression, miRNA and mRNA transcripts, and methylation of our enzymes of interest in the liver listed in later sections (Brown-Borg & Rakoczy, 2003; Rojanathammanee, Rakoczy, & Brown-Borg, 2013). Porcine GH in alkaline saline mixed with 50% polyvinylpyrrolidone (pH 9, 1:1, saline–PVP) was injected subcutaneously twice daily (25 µg in 50 µl /injection) into one group of dwarf mice (n= 8-12). The vehicle, saline-PVP was injected subcutaneously (50 µl/injection) into another group of dwarf mice (n=8-11) and age-matched wild type mice (n=8-11) in two separate experiments. Insulin-like growth factor 1 was dissolved in saline solution and one group of GHRKO mice (n=8-12) were administered 13µg of IGF-1 in 50 µl/injection (Catalogue # CYT-216, ProSpec, Ness-Ziona, Israel). The vehicle, saline, was injected subcutaneously (50 µl/injection) into another group of GHRKO mice (n=8-12) and age-matched wild type mice (n=8-12) similar to the GH administration for Ames dwarf mice. All mice were injected two times daily (8:00 a.m. and just before lights out at 8:00 p.m.) for 7 days (total of 13 injections). This is done to more closely mimic the pulsatile manner in which GH is secreted, which consequently is responsible for the secretion of IGF-1 from the liver (Panici et al., 2010; Waxman et al., 1995). On the morning of the seventh day, one hour following the last injection of saline-PVP or GH, liver tissue was

collected from each group of mice, weighed, frozen, and stored at -80℃ until analysis. This experimental protocol has been shown to raise IGF-1 plasma levels in the dwarf mouse and the somatic changes due to GH are apparent by a gain in body weight of 1-4 grams (Chandrashekar & Bartke, 1993). To confirm GH action in the dwarf, the body weight of each mouse was recorded prior to starting the experiment, during, and following the last GH injection to validate weight gain in GH-treated animals compared to untreated animals. Liver weights were also recorded and compared. Liver was collected similarly for GHRKO treated and untreated GHRKO and wild type mice.

Hepatocyte isolation and GH treatment *in vitro*

Primary hepatocyte cultures were prepared with liver tissue isolated from 5 – 6 month old Ames dwarf and wild type mice using previously published pre-perfusion, collection, and culturing methods (Brown-Borg & Rakoczy, 2003; Kreamer et al., 1986). Briefly, each animal was anesthetized with 2.5% tribromoethanol (i.p. 100 µl/10 g body wt), the inferior vena cava was cannulated, and the liver was perfused with 37℃ calcium-free HEPES pre-perfusion buffer (160.8 mM NaCl, 3.15mM KCl, 0.7 mM Na2HPO4-7H2O, 33 mM HEPES, and 0.5mM; pH 7.6) containing 1% penicillin-streptomycin for 15 min followed by perfusion with a 37℃ collagenase solution (pre-per fusion buffer and 1.0 mM CaCl) until livers displayed a swollen appearance and were altered in color indicating blood loss and liver digestion (0.033% collagenase [Worthington, Lake Park, NJ]). Collagenase separates the lobular parenchyma of the liver to release hepatocytes. Following perfusion, livers were removed, placed into warm transfer buffer and 1%

pen/strep (37℃), and the outer membrane of the liver was carefully excised into a petri dish with warm media under a sterile hood. The remaining connective tissue was agitated by hand until the media became cloudy indicating the presence of freed hepatocytes and the connective tissue (extracellular matrix and epithelial tissue) was discarded. The cells were transferred to a conical centrifuge tube and pelleted for 3 min at 600 rpm at 4℃ and washed three times with ice-cold hepatocyte wash buffer containing 1% penicillin-streptomycin until the supernatant was clear (Gibco® Life tecnologies, Grand Island, NY). The final cell pellet was resuspended in ice-cold serum-free HepatoStim Media containing 1% penicillin-streptomycin (Becton Dickinson, Mountain View, CA, USA). Trypan blue dye exclusion was used to assess cell viability and integrity. Cell collections with ≥ 95% viability were used. Cells for each genotype were pooled (n=2-5 mice/genotype), resuspended in attachment medium containing 1% pen/strep and 15% fetal bovine serum (to aid in cellular adhesion) and seeded (2×10^6) into MatriGel coated dishes (Life Technologies, Grand Island, NY, USA). Following cell attachment (2 hours at 37℃ and 5% CO_2), the medium was exchanged for serum-free HepatoStim media (Life Technologies) containing 1% pen/strep and 2 mM glutamine. Serum-free media favors differentiation and suppresses growth and proliferation. Following overnight recovery, fresh serum-free media was added and the cells were allowed to equilibrate (2 hrs.) before GH treatment (0.1, 1.0, 10 and 20 µg porcine GH per ml media). Twenty-four hours following treatment (37℃ and 5% CO_2), cells were collected by washing with cold (4℃) HEPES and applying MatriSperse (BD Biosciences, Franklin Lakes, NJ, USA) for 1 hour on ice.

Dishes were aspirated to remove dead cells and media, scraped, and cells were pelleted and washed with ice-cold phosphate buffered saline (PBS) via centrifugation. Cell pellets were stored at -80℃ or homogenized immediately for downstream assays (see immunoblotting).

Immunoblotting

Liver tissue and hepatocyte samples were analyzed using standard immunoblotting procedures previously published (Brown-Borg & Rakoczy, 2003). Liver tissue was homogenized in lysis buffer (10 mM Tris, 0.15 M NaCl, 5 mM EDTA, 1% Triton X) containing serine, cysteine, threonine and trypsin protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 25 mg/ml leupeptin, and 25 mg/ml aprotinin, or Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail, Catalogue # 78442, Thermo Scientific, Rockford, IL, at 10µl per milliliter of lysate) at 4° C using the 24 sample Bullet Blender (Protocol for Hepatic (Liver) Tissue Homogenization, Next Advance Inc. Averill Park, NY). Protein abundance was determined using the Bradford assay (Bradford, 1976). Briefly, a protein standard, bovine serum albumin, with a starting concentration 80 µg/ml was serially diluted in sterile water produce a standard curve of six concentrations (80, 60, 40, 20, 10, 5 µg/ml). Sample liver lysates were diluted in sterile water at a 1:700 dilution to gain absorbance measurements close to our central concentration, 40 µg/ml, for best detection. Standards (in triplicate) and samples (in duplicate) were loaded at 50 µl per well of a 96-well plate. Bradford Reagent (Catalogue # 500-0006, BioRad, Hercules, CA) was diluted with sterile water according to manufacturer directions and 150 µl per well was loaded to the standards and samples. The plate was read

on a spectrophotometer 96-well plate reader (BioTek Instruments, Winooski, VT) at a wavelength of 595 nm. Standard curves of $R^2 \geq 0.95$ were used. Protein dilution was calculated by comparison to the standard curve. Equivalent protein samples (50 µg per 10 µl volume) were assembled using Laemmli Buffer solution and β-mercaptoethanol was added according to manufacturer's directions (Catalogue # 161-0747, BioRad, Hercules, CA). Before loading samples were briefly centrifuged and heated in a dry-bath at 95-100℃ for 5 minutes and 10 minutes for target proteins >100kDa. Loading samples were centrifuged again and loaded at 50-75 µg per lane, unless otherwise specified. Loading samples were resolved by SDS-PAGE (Criterion™ TGX™ Precast Gels Any kD™, BioRad, Hercules, CA) running at 100 volts for 3.5-4 hours for proteins >100 kDa (40 minutes to 1 hour for smaller proteins). PVDF (polyvinylidine diflouride) membranes for transfer were activated in 100% methanol for at least 1 hour before transfer. Proteins were transferred to PDVF membrane in cold transfer buffer at 100 volts for 30 minutes in cartridges backed by filter paper and sponges soaked in cold transfer buffer in a transfer case (Criterion™, Hercules, CA). Transfer buffer contained 10% methanol for larger proteins (>100 kDa) and 20% methanol for smaller proteins. After transfer, the blots were rinsed briefly in tris-buffered saline containing Tween 20 (25mM Tris-Cl, pH8.0; 125mM NaCl; 0.1% Tween 20) and blocked in 5% nonfat dry milk in 0.1% T-TBS for 1 hour at room temperature. Blots were incubated for DNA methyltransferases DNMT3b and DNMT1, staining in primary antibody in 1% milk/0.1% T-TBS and in primary with antibody in 5% milk/0.1% T-TBS for all other antibodies. All blots were rotated overnight at 4° C. Primary antibody (Abcam ®, Cambridge, MA, USA)

concentrations were as follows: DNMT3a 1:1,000 (ab14291), DNMT1 1:400 (ab13537), DNMT3b 1:250 (ab13604), and DEAD-box helicase 20 1:1000 (ab166913) and glycine-*N*-methyltransferase (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA, sc-166834). After incubation with the primary antibody blots were washed 4-6 times for 10 minutes to reduce background signaling. Blots were then incubated and probed for 1.5 hours rotating at room temperature in the appropriate horse radish peroxidase or alkaline phosphatase conjugate secondary between 1:2000 to 1:5000 dilutions as necessary for each optimized antibody (BioRad, Hercules, CA). Solutions for secondary antibody probes were 1% milk/0.1%T-TBS for Dnmt1 and Dnmt3b blots and 5% milk/0.1% T-TBS for all other blots. Blots were washed 5-6 times in 0.1% T-TBS for 10 minutes to reduce background. Blots were developed with chemiluminescent substrate for 5 minutes rotating at room temperature and mean optical density was measured and used for comparative analysis (UVP Bioimaging system (Upland) with Labworks 4.5 analysis software and also Omega Lum™ G imaging system (Aplegen™) with UltraQuant Analysis Software version 13.04.05). Ponceau S staining was used to assess equivalent loading.

Nuclear extraction and DNMT activity assay

Liver nuclear extracts were prepared from frozen liver tissue according to manufacturer's directions using an Epiquick Nuclear Extraction Kit containing premixed proprietary buffers, dithiothreitol (DTT), a positive DNMT enzyme control, and a protease inhibitor cocktail mix (OP-0002, Epigentek, Farmingdale, NY, USA). Briefly, liver was homogenized at 4° C (Bullet Blender, Next Advance Inc. Averill Park, NY) in a nuclear pre-extraction buffer

containing DTT to lyse the cellular membrane and release intact nuclei from the cells. Lysate was then incubated on ice for 15 minutes and centrifuged for 10 minutes at 12,000 rpm at 4℃. The supernatant (c ytosolic fraction) was either kept for negative control in fresh tubes or discarded. To the remaining nuclear pellet, nuclear extraction buffer, DTT, and a protease inhibitor cocktail was added. The nuclear extraction was incubated on ice and vortexed for 5 seconds every 3 minutes during incubation for a total 15 minutes. The suspension was then centrifuged for 12 minutes at 13,200 rpm at 4℃ and the supernatant containing nuclear proteins was transferred to a fresh microcentrifuge tube. Protein abundance was measured using the Bradford assay as previously mentioned (Bradford, 1976). Protein was immediately aliquoted for DNMT activity assessment and the remaining nuclear lysate was stored at -80℃ until further use. Activity of DNMT was measured using Epigentek DNA methyltransferase Activity/Inhibition Assay Ultra Kit following manufacturer's directions. This assay contains proprietary buffers, colorimetric solutions, SAM substrates, antibodies, and 8-well microplate strips containing cytosine-rich DNA substrate that can be used in a 96 well format frame for spectrophotometry. (P-3009, Epigentek, Farmingdale, NY, USA). These assays are colorimetric similar to ELISA assays that use color changing reactions, such as 3,3',5,5'-tetramethylbenzidine, that react to horse radish peroxidase to yield a blue color when detecting HRP. Then an acidic solution is applied turning the reaction yellow with a maximum absorbance of 450 nm. Optimization was determined to be 20 µg of lysate per well by serial dilution of

sample to detect an appropriate magnitude of absorbance for maximum detection. Previously aliquoted protein was diluted to a final concentration of 6.67 µg/µl. Assay buffer was aliquoted into each well (24 µl per well) and 3 µl of protein solution was added. Blank wells were assayed in buffer alone and positive wells contained 50 ng of DNMT control and assay buffer. S-adenosylmethionine substrate was diluted to a 1:5 solution and 3 µl added to each well (final volume of 30 µl per well). Wells were incubated at 37℃ for 1.5 hours and the plate was mixed during incubation so the cell sample DNMT could methylate the coated plate substrate cytosine residues. Wells were aspirated and washed 3 times with wash buffer and 50 µl of the 5'-methylcytosine capture primary antibody (1:1000 wash buffer dilution) was added. The plate was incubated at room temperature (22℃) for 60 minutes on an orbital shaker. Wells were aspirated and washed 4 times with wash buffer and 50 µl of the detection secondary antibody (1:1000 wash buffer dilution) was added and incubated at room temperature for 30 minutes. Wells were then aspirated and washed 5 times with wash buffer. Developer solution was added to each well (100 µl) and incubated at room temperature for 2-10 minutes (with the plate protected from light) until a medium blue hue was seen indicating a reaction between the solution and the secondary antibody. An acidic stop solution was added turning the reactions yellow. Samples were run in triplicate, included one blank well, and one positive control well and analyzed using dual-wave spectrophotometric analysis (630nm and 450nm). The amount of methylated DNA is proportional to the enzyme activity and is

calorimetrically quantified by an ELISA-like reaction. Dnmt activity which is measured in optical density/hour/milligram was calculated by subtracting the negative control optical density from the optical density of the reaction. This value was then divided by the amount of sample protein added in micrograms that was multiplied by the initial incubation time in hours. This dividend was then multiplied by 1000 to convert micrograms to milligrams for the final value.

Messenger RNA expression via one-step RT-PCR

Total RNA was isolated using Ultraspec® RNA (Catalogue # BL-10050, Biotecx, Houston, Texas), based on a previously described method and measured with a spectrophotometer for quality and purity using the optical density ratio of 260nm/280nm from 1.8-2.0 (Brown-Borg et al., 2009; Chomczynski & Sacchi, 1987). The RNA was then diluted and used to measure expression of genes of interest in 3, 12, and 24 month old Ames dwarf and GHRKO mice and also 3, 6, and 10 month old GH Tg mice and compared to age-matched wild type controls. Ames dwarf and wild type 4-6 month old mice used in the GH administration experiments were also studied for mRNA expression. Genes of interest are listed along with the primers used for these experiments (Table 2). Primers were obtained from previous literature (Chen et al., 2005; Ray et al., 2006; Sharma et al., 2010; Uthus & Brown-Borg, 2006), screened against the whole mouse genome for unintended targets using NCBI primer blast, and optimized. Reaction substrates were contained in the QuantiTect SYBR® Green RT-PCR Kit (Catalogue # 204243, Qiagen, Valencia, CA).

TABLE 2: Primer pairs utilized for gene specific one-step RT-PCR

Gene	GeneBank accession no.	Primer 5'- 3'	Annealing Temperature
β2M	NM_00975	For - AAGTATACTCACGCCACCCA	60℃
		Rev - AAGACCAGTCCTTG	
Histone H4		For- ACAACATCCAGGGCATCACG	60℃
		Rev- GAAGACCTTCAGCACACCGC	
GNMT	NM_010321	For - GCTGGACGTAGCCTGTGG	60℃
		Rev - CACGCTCATCACGCTGAA	
DNMT1	NM_010066	For - AAAGTGTGATCCCGAAGATCAAC	60℃
		Rev - TGGTACTTCAGGTTAGGGTCGTCTA	
DNMT3a	NM_007872	For – TGCTACATGTGCGGGCATAA	58℃
		Rev- GGAGTCGAGAAGGCCAGTCTT	
DNMT3b	NM_001003961	For – CCCAAGTTGTACCCAGCAATTC	59℃
		Rev – TGCAATTCCATCAAACAGAGACA	
DDX20	NM_ 017397	For - ACATGCATCGGATTGGCAGA	60℃
		Rev - CCCAGGCGTTCTTTGAGACT	
MT1	NM_013602	For - CCTTCTCCTCACTTACTCC	60℃
		Rev -TCCATTCCGAGATCTGGTGAA	
MT2	NM_008630	For - TCCTAGAACTCTTCAAACCGATCT	65℃
		Rev- AAAAGGCTAGGCTTCTACATGGTC	

The reaction mixtures contained SYBR green, forward and reverse primers, and QuantiTect RT mix, a Taq DNA polymerase, and assayed using a SmartCycler instrument (Cepheid, Sunnyvale, CA) or a CFX 96^{TM} Real-Time PCR Detection System (BioRad, Hercules, CA). Polymerase chain reactions included an initial reverse transcription step of 50° for 30 minutes, a *taq* polymerase activation and denaturation step at 95℃ for 15 minutes, PCR cycling of 95℃ for 15 seconds and an annealing temperature of 50-60℃ (Table 2) for 30 seconds. These reactions were run for 40-45 cycles. Melt curve analysis and standard curve optimization was used for quality control of the PCR reaction and detection. No template controls and no reverse transcriptase treated template controls were used to check for cross-contamination and genomic DNA contamination respectively. Data was analyzed using the $\Delta\Delta C_t$, also known as the comparative C_t method, which calculates in reference to the control (the genomic DNA control specific to mouse, the reverse transcription control from the cDNA reaction in triplicate, and the positive PCR control in triplicate). The amount of tested gene cDNA relative to a control gene (β2-microglobulin) was determined using the $\Delta\Delta C_t$ (comparative C_t method) as previously published (HM Brown-Borg et al., 2009; Lupberger et al., 2002). The C_t value is the number of cycles it takes for a sample to reach the level where the rate of amplification is the greatest during the exponential phase and the value is inversely proportionate to the amount of RNA or cDNA in the sample. ΔC_t for each mouse type is calculated as $\Delta Ct = Ct_{\text{(gene of interest)}} - Ct_{\text{(average of β2m RNA)}}$. $\Delta\Delta C_t$ is calculated as

$\Delta\Delta Ct = \Delta Ct_{(Ames\ experimental)} - \Delta Ct_{(wildtype\ control)}$ of the same age group and the fold change in expression will be calculated as $2^{(-\Delta\Delta Ct)}$. A one-fold change indicates no change in transcription, a greater than one-fold change suggests increased transcription, and a less than one-fold change indicates decreased transcription (Heid et al., 1996).

miRNA expression via two-step RT-PCR

The miRNA 1st-Strand cDNA Synthesis Kit (600036, Agilent Technologies, Wilmington, DE) was used on isolated total liver RNA (1 µg per reaction**)** to polyadenylate and elongate miRNA at the 3' end of the transcript in the sample with a bacterial polyadenylate polymerase (PAP). A no PAP control was included to screen for contamination. All steps were done with samples on ice except during incubation periods. Then the miRNA is reverse transcribed using a genetically engineered Moloney murine leukemia virus reverse transcriptase into cDNA according to the manufacturer's directions. RNase inhibitors are also included to prevent miRNA degradation during the process. The RT portion of the kit also provides a proprietary "RT adaptor primer" which anneals to the 3' end of the poly A tail and creates a universal sequence tag during the amplification that is incorporated into the 5' region of the transcripts. This tag allows the use of a proprietary universal reverse primer to be used during real-time qPCR amplification (Agilent Technologies, 600037, Wilmington, DE). Forward primer sequences for miR-140-3p and the 5S rRNA control were verified for use in mouse using miRBase and NCBI GenBank (Table 3; Takata et al. 2012; Bates et al. 2010). For miRNA expression validation a commercially prepared validated

control for U6 splicoeosomal RNA (RNU6) (MiScript Primer Assay,Catalogue # MS00033740, Qiagen, Valencia, CA). RNU6 is an approximately 100 basepair non-coding RNA that complexes with RNA splicing proteins for mRNA processing. RT-PCR was performed using the PerfeCta® SYBER® Green SuperMix (Catalogue # 95054, Quanta Biosciences, Gaithersburg, MD), forward and reverse primers, nuclease free water and polyadenylated cDNA. Reactions were run using CFX 96^{TM} Real-Time PCR Detection System (BioRad, Hercules, CA), reactions beginning with denaturation step of 95 °C for 3 minutes, followed by cycling conditions of 95 °C for 15 seconds, 60° C for 30 seconds, for 45 cycles. Controls for cross-contamination and genomic DNA contamination were utilized. Melt curve analysis was used for quality control of the PCR reaction and detection. The amount of tested gene cDNA relative to a control gene, 5S rRNA, was determined using the $\Delta\Delta C_t$ (comparative C_t method).

Interspersed repeat transcription two-step RT-PCR

Total RNA was treated with DNase I according to the manufacturer's protocol to eliminate genomic DNA contamination through a non-specific endonuclease action, which degrades DNA in the RNA samples (Catalogue #M0303S/L, New England Biolabs, Ipswich, MA). All steps were performed with samples on ice except during incubation. Briefly, 10 µg of RNA was suspended in DNase I reaction buffer which includes enzyme activating cations such as Ca^{2+} to a final volume of 100 µl. Two units of DNase I were added to each sample mixture and were mixed thoroughly by inverting samples roughly 20-30 times. Reactions were

briefly centrifuged and put into a dry bath at 37℃ for 10 minutes. To terminate the reaction, 1 µl of 0.5M EDTA was added and the samples were then heat inactivated for 10 minutes. RNA was converted to cDNA by use of Protoscript M-Mulv Taq RT-PCR reagents from New England Biolabs, Ipswich, MA) along with control reactions to verify cDNA synthesis and the absence of gDNA contamination (Filkowski et al., 2010; Martens et al., 2005; Pogribny et al., 2009). DNase I treated RNA (1 µg) was put into a mix with a random primer mix (2 µl, 60 µM), a deoxynucleotide (dNTP) solution mix (1 µl, 10mM), and nuclease free water to a final concentration of 16 µl (Catalogue #S1330S, #N0447S/L, New England Biolabs). The random primers mix is designed to allow the reproduction of the complete complement of messenger RNA to complimentary DNA in a sample. This mix was briefly centrifuged and put in a dry bath at 70℃ for 5 minutes after which the samples were promptly chilled on ice. Then, to each reaction 2 µl of 10x RT buffer, 20 units of Murine RNase inhibitor, 200 units of Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV), and 0.5 µl of nuclease free water were added. Samples were again briefly centrifuged and incubated at 25℃ for 5 minutes then incubated at 4 2℃ for one hour. To terminate the reaction and inactivate the enzyme, the samples were then transferred to a dry bath at 80℃ for 5 minutes. Nu clease free water was added to bring the final volume to 50 µl and the reactions were placed on ice. Successful reaction were validated by PCR using a control primer set (β2-microglobulin) and running a negative control (reactions without the reverse transcriptase added) alongside reverse transcriptase treated samples on a 2%

agarose gel. Complementary DNA (cDNA) products were used immediately or stored at -20℃ for downstream PCR applications. Table 3 lists the primers used which were taken from previous publications or designed via NCBI primerblast (Lane et al., 2003; Martens et al., 2005). All primers were verified via NCBI primerblast and/or Repbase, which is a database established purely for repetitive sequences. Reactions were performed using the QuantiTect SYBR® Green PCR Kit. The reaction mixtures contained, forward and reverse primers, and QuantiTect SYBR Green PCR Buffer, a *taq* DNA polymerase, and assayed using a SmartCycler instrument (Cepheid, Sunnyvale, CA). Polymerase chain reactions included an initial *taq* polymerase activation and denaturation step at 95°C for 15 minutes, PCR cycling of 95℃ for 15 seconds, an annealing temperature of 60-63℃ (Table 3) for 30 seconds, and an extension step of 72℃ for 30 seconds. These reactions were run for 40 cycles. Standard curves were performed between the transcripts of interest and β2m, RNA and primer concentration and reaction conditions were optimized, and standard RT-PCR controls, similar to the aforementioned experiments, were used. The amount of tested gene cDNA relative to a control gene (β2m) was determined using the $\Delta\Delta C_t$ (comparative C_t method) as previously mentioned for one step RT-PCR experiments (Bates et al., 2010; Brown-Borg et al., 2009; Heid et al., 1996).

McrBC-qPCR DNA methylation assay

McrBC is an endonuclease which cleaves DNA containing 5-methylcytosine or 5-hydroxymethylcytosine or N4-methylcytosine on one or both strands of genomic DNA. McrBC will not act upon unmethylated DNA and

depends on the presence of guanosine tri-phosphate (GTP) for cleavage (Sutherland, Coe, & Raleigh, 1992). This allows for the enrichment of non-methylated genomic DNA which can then be compared to non-digested genomic DNA following qPCR using the absolute standard method (Filkowski et al., 2010; Martens et al., 2005; Pogribny et al., 2009).

TABLE 3: Primer pairs utilized for McrBC-PCR and miRNA two-step RT-PCR.

Interspersed Repeat	GeneBank accession no.	Primer 5'- 3'	Annealing Temperature
LINE1 3'UTR	D84391	For - TTGATCACAGGGCTCCCAATGGAG	60℃
		Rev - GTACCGGGGCACACAAAGTCTGCG	
IAP-LTR	M17551	For - TGCGCATATGCCGAGGGTGGTT	63℃
		Rev - TCTCACGCCCGGCCAGGAAGAAC	
SINE B1	J00630 1-203 bp	For - GTGGCGCACGCCTTTAATC	63℃
		Rev -GACAGGGTTTCTCTGTGTAG	
SINE B2	J00630 506-700 bp	For –GAGATGGCTCAGTGGTTAAG	60℃
		Rev- CTGTCTTCAGACACTCCAG	
miRNA 140-3p	NR_029553	For – TACCACAGGGTAGAACCACGG	59℃
5S rRNA	NR_046144	For-CAGGGTCGGGCCTGGTTAGTACTTG	60℃

DNA was extracted from the liver using a Gentra® Puregene® Tissue Kit (Catalogue # 154667, Qiagen, Germantown, MD, USA) and was homogenized using the Bullet Blender^{TM}**.** Briefly, 10-15 mg of liver tissue was homogenized in 100 uL of Cell Lysis solution from the Gentra Puregene kit on ice. After the initial homogenization another 200 µl of Cell Lysis solution from the Gentra Puregene kit was added to each sample (on ice) and run in the Bullet Blender at a lower speed for 1 minute to mix thoroughly. Genomic DNA was measured

spectrophotometrically using OD of 260nm/280nm for purity and samples measuring 1.8-2.0 were used for the assay (Epoch Micro-Volume Spectrophotometer System, BioTek Instruments,Inc., Winooski, VT). DNA was aliquoted into 25-50 µl and kept in -80º C until needed. Genomic DNA was diluted in TE buffer (10 mM Tris, 1 mM EDTA, at pH8) and sonicated using the UCD500-Bioruptor XL® (Life Research ©, Scoresby, Victoria, Australia) user manual version 2.1 to increase the efficiency of downstream restriction enzyme digestion. After genomic sonication was complete samples were run on a 1% agarose gel to validate DNA fragment sizes of 3-5 kilobase pairs. Restriction and mock restriction reactions were set up with nuclease free H_20, buffer provided by New England Biolabs, a GTP substrate, bovine serum albumin, and 1µg of genomic DNA. Lastly, 3 µl (30 U) of McrBC was in a 50% glycerol solution for restricted gDNA and for mock restrictions 3 µl 50% glycerol in nuclease free water. Plasmid DNA was also provided as a positive control for enzyme activity. (Catalogue # M0272S or M0272L, Ipswich, MA). Final reaction volumes were 100 µl. Sample reactions were gently mixed by tapping the tube lightly, or inverting the tube gently several times, and incubating overnight at 37 ℃ (Holemon et al., 2007; Martens et al., 2005). Reactions were inactivated by heating the samples to 65℃ for 20 minutes. The positive control plasmid DNA, mock restricted, and restricted samples were verified through electrophoresis on a 1% agarose gel. Table 3 shows all primers that were used which are the same primer sets used for two-step RT-PCR transcriptional analysis of the interspersed repeats as previously mentioned. Reactions for mock-restricted and restricted

samples were assembled and run together in duplicate. Reagent contents, primers, and thermocycling conditions were equivalent to the previously stated qPCR measurement of interspersed repeat transcription parameters. As in previous publications, the absolute quantification analysis of qPCR was used where the threshold cycle (C_t) is defined as the fractional cycle number that passes the fixed threshold. C_t values for each repetitive element were converted into the absolute amount of input DNA using the absolute standard curve method. An increased amount of input DNA after digestion with McrBC compared to mock-restricted DNA is indicative of hypomethylation, whereas a decreased amount of input DNA is indicative of hypermethylation (Filkowski et al., 2010; Pogribny et al., 2009)

Global methylation assay

Global methylation of isolated genomic DNA (see above in the McrBC-qPCR methylation assay) was determined using the Methylamp™ Global DNA Methylation Quantification Ultra Kit (Catalogue # P-1014B, Epigentek, Farmingdale, NY, USA) according to the manufacturer's instructions. This kit is a colorimetric assay similar to ELISA reactions that probes for 5'-methylcytosine using antibodies. The kit includes proprietary buffers including wash buffer, binding buffer, blocking solution and antibodies, developer and stop solutions and also unmethylated DNA (negative control, 100 ng per well) and fully methylated DNA (positive control, 10 ng per well). The DNA sample (200 ng per well), positive control and negative control DNA are aliquoted into wells coated with a DNA affinity substance and the DNA binding solution is added to a final volume of 30 µl per well. The plate was gently

shaken and incubated uncovered at 37℃ for 40 minut es in room air. Afterwards the samples were incubated at 60℃ for 35-40 minutes ev aporating all the solution and drying the wells. If the wells were not completely dry, wells were aspirated of the remaining solution with 10 or 20 µl pipette tips or incubated at 60℃ for 5-10 minutes more according to manufacturer directions. Blocking solution was then added at 150 µl per well and incubated at 37℃ for 30 minutes. Wells were aspirated and washed three times with 150 µl of wash buffer at room temperature. The capture antibody was added in a 1:1000 dilution (1 µg/ml, 50 µl/well) in wash buffer. The wells were covered and incubated at room temperature (22℃) for 1 hour. Wells were aspirated and washed four times with 150 µl of wash buffer at room temperature. The detection antibody was added in a 1:5000 dilution (0.08 µg/ml, 50 µl/well) in wash buffer. The wells were covered and incubated at room temperature (22℃) for 30 minutes. Wells were then aspirated and washed five times with 150 µl of wash buffer at room temperature. A signal enhancer was added in a 1:5000 dilution (50 µl/well) in wash buffer. Wells were again, aspirated and washed five times with 150 µl of wash buffer at room temperature. A developer solution was added (100 µl/well) and incubated at room temperature (22℃) for 1 to 5 minutes (protected from light). As in the DNMT activity assay previously mentioned, color development was monitored and stop solution (50 µl/well) was applied after the reactions had reached a medium blue color. Samples and controls were run in triplicate and analyzed using dual wave spectrophotometric analysis (630 nm and 450 nm). To calculate the percent methylation of the samples, the negative control absorbance was subtracted from the sample absorbance. This

number was then divided by 0.42 because there is a 42% DNA GC content in mouse DNA. This number was then divided by the difference between the positive control minus the negative control multiplied by 20 since there is a 20 fold difference in concentration between the sample genomic DNA and the fully methylated positive control. This number is then multiplied by 100 to obtain a percentage calculation of methylated DNA. Methylation was normalized to the 3 month wild type mice for GH signaling deficient mice, where in 2 assays, the positive and negative controls were similar in absorbance and had the same lot numbers, were averaged (within 2 standard deviations) between assays and thus were used for subsequent cumulative analysis. For GH Tg assays, differences between the control absorbance measurements between the two plates were too large (58% difference), therefore the first assay was normalized to 3 month old wild type mice, and the second assay was normalized to 6 month wild type mice.

Methylation-specific PCR analysis

Genomic DNA was isolated from dwarf and wild type liver tissue (Gentra Puregene Tissue Kit; Qiagen, Germantown, MD), and bisulfite treated using the BisulfFlash^{TM} DNA Modification Kit (Catalogue # P-1026, Epigentek, Farmingdale, NY). Bisulfite treatment of genomic DNA results in the deamination of cytosines not protected by methylation, to uracil. Upon PCR amplification of this product, uracil is converted to thymine thus 2 sets of primers are used to probe for methylated and unmethylated cytosines of CpG dinucleotides to assess the methylation status of the target region. This kit contains a proprietary conversion buffer, a conversion enhancer solution, and a denaturation enhancer which

protects DNA from degradation and keeps it denatured during the bisulfite conversion. These three agents are first mixed together and 200 ng of genomic sample DNA was added to each reaction and subsequently used for methylation-specific PCR (Herman, Graff, Myöhänen, Nelkin, & Baylin, 1996). Primers (Table 4) were designed for predicted methylated and unmethylated sequences of CpG dinucleotide islands using the MethPrimer program (Li & Dahiya, 2002). Thermocycling conditions and primer concentrations were optimized using RT-PCR and PerfeCta® SYBR® Green SuperMix™ (Catalogue # 95054, Quanta Biosciences, Gaithersburg, MD) to ensure end products for agarose gel analysis were produced during the exponential phase of the reaction for methylation comparison. This mix contains the same AccuStart™ taq DNA polymerase as the AccuStart™ II PCR SuperMix used for the MS-PCR experiment reactions (Catalogue #95127, Quanta Biosciences, Gaithersburg, MD). Reactions contained AccuStart™ II PCR SuperMix, the forward and reverse primers for each methylation state (methylated versus unmethylated; 1 µM), and 1µL of bisulfite treated DNA (10 ng) for a total volume of 15µl. Thermocycler (Eppendorf Mastercycler) conditions consisted of an initial denaturation step at 95℃ for 15 minutes, PCR cycling of 95℃ for 15 seconds, an annealing temperature of 55℃ for 30 seconds, and an extension step of 72℃ for 30 seconds. Reactions were held at 4℃. Samples were run on a 2% agarose gels, stained with ethidium bromide and band intensities of methylated PCR products were compared to unmethylated PCR products using Omega Lum™ G imaging system (Aplegen™, Pleasanton, CA) and UltraQuant version 13.04.05.

TABLE 4: Primer pairs utilized for MS-PCR.

Gene	Predicted CpG Island	Primer 5'- 3'	Annealing Temperature
MT1 methylated	177-328	For - TTTTAGGGAGTTTTGTATTTCGTTC	55℃
		Rev - AACAACCTACCCTCTTTATAATCGTT	
MT1 unmethylated	177-328	For -TTAGGGAGTTTTGTATTTTGTTTGA ACATGCATCGGATTGGCAGAACATG	55℃
		Rev - AACAACCTACCCTCTTTATAATCATT	
MT1 methylated	465-570	For - GAATATTAAGTTGGGATCGTAGAGC	55℃
		Rev - AAAACGAATTCTAAAAAAAACCGA	
MT1 unmethylated	465-570	For -ATATTAAGTTGGGATTGTAGAGTGG ACATGCATCGGATTGGCAGAACATG	55℃
		Rev - AAAACAAATTCTAAAAAAAACCAAA	
MT2 methylated	105-280	For - TTGTTTTACGAGTAAAAAGAGGTC	55℃
		Rev -TAACGAAAAATATTAAATCGAACGC	
MT2 unmethylated	105-280	For - TTGTTTTACGAGTAAAAAGAGGGTC	55℃
		Rev- ATAACGAAAAATATTAAATCGAACG	
LINE1 3' UTR methylated	150-324	For- GGATTAGAGGATAGGTGTTCGTTC	60℃
		Rev- TACCTCAATACCTCTATACTTCCGC	
LINE1 3'UTR Unmethylated	150-324	For- GGATTAGAGGATAGGTGTTTGTTTG	60℃
		Rev- ACCTCAATACCTCTATACTTCCACC	
LINE1 3'UTR Methylated	528-676	For- GTTGAGGTAGTATTTTGAGTGGGTC	60℃
		Rev- AAACTAATTTCCTAAATTCGACGAA	
LINE1 3'UTR Unmethylated	528-676	For- GTTGAGGTAGTATTTTGAGTGGGTT	60℃
		Rev- AAACTAATTTCCTAAATTCAACAAA	
IAP-LTR UTR Methylated	105-298	For- TTATGTGTTTTGTTTTTTTCGTGAC	60℃
		Rev- CACAAACCAAAATCTTCTACGAC	
IAP-LTR UTR Unmethylated	105-298	For- ATGTGTTTTGTTTTTTTTGTGATGT	60℃
		Rev- ACCACAAACCAAAATCTTCTACAAC	

Histone extraction and immunoblotting

Histones were extracted from 3, 12, and 24 month old Ames dwarf and wild type age matched livers using the EpiQuik™ Total Histone Extraction Kits (OP-0006, Epigentek, Brooklyn, NY, USA) according to the manufacturer's protocol. Briefly, weighed pieces of tissue sample were homogenized in a pre-lysis buffer then centrifuged at 3000 rpm for 5 minutes at 4℃. After discarding the supernatant, the cellular pellet was re-suspended in lysis buffer and incubated on ice for 30 minutes. Samples were then centrifuged at 12,000 rpm for 5 minutes at 4℃ and the supernat ant fraction containing the acid-soluble proteins was transferred into a new microtube. A balance buffer containing DTT solution was added to the supernatant immediately and chromatin was quantified using a spectrophotometer using the Bradford method. Histone extracts (50 µg/ lane) were be separated via electrophoresis, transferred to PVDF membrane, and specific proteins detected using standard immunoblotting techniques and chemiluminescence. Antibodies to pan-Histone H3 (07-690) 1:2000 dilution, trimethyl-Histone H3Lys4 (07-473), trimethyl-Histone H3Lys27 (07-449), trimethyl-Histone H3Lys9 (07-442), trimethyl-Histone H4Lys20 (04-079, Millipore, Temecula, CA) were used. Densitometric analysis was conducted using an Omega Lum™ G imaging system (Aplegen™) and UltraQuant Analysis Software version 13.04.05. GelCode® Blue Stain reagent (ThermoScientific, Rockford, IL, USA) was used to assess equivalent protein loading (Kingston, 2010)

Histone chromatin modification enzyme RT-PCR pilot study

Histone modification enzyme mRNA expression was probed in 3-month-old Ames dwarf and age-matched wild type mice to look for differences in expression of these enzymes. For this study the murine Epigenetic Chromatin Modification Enzymes RT^2 Profiler PCR Array was used (Catalogue # PAMM-085A, Qiagen, Valencia, CA) which contains a 384 well plate coated with mRNA amplification primers for 4 sets of 84 genes, allowing 4 samples to be assayed at one time (n=2 mice/genotype). The 384 (4 replicate x 96 well) option contains 4 replicate primer assays for: each of 84 genes in the pathway (DNA methyltransferases, histone acetylases, deacetylases, methylases, methyltransferases, phosphorylases, kinases, and ubiquitination enzymes), primer assays for 5 housekeeping genes, for genomic DNA controls, 12 wells for reverse-transcription controls, and 12 wells contain positive PCR controls. Purified RNA was used to perform a reverse transcription reaction with the RT^2 First Strand Kit. Complimentary DNA is used with the RT^2 SYBR Green mix. The reaction mixtures contained SYBR green, forward and reverse primers, a Taq DNA polymerase, and assayed using a Bio-Rad CFX384™ (BioRad, Hercules, CA). Briefly, reagents of the RT^2 First Strand Kit were thawed on ice and tubes from the kit were centrifuged (10–15 seconds) to bring the contents to the bottom of the tubes. A genomic DNA elimination mix for each RNA sample was prepared containing, a proprietary buffer and RNase-free to a final volume of 10 µl. The reaction was mixed gently and briefly centrifuged. The genomic DNA elimination mix was incubated for 5 min at 42℃ then placed immediately on ice. The reverse-transcription mix was prepared with proprietary

buffers, a reverse transcriptase, RNase-free water to a total volume of 40 µl for 4 reactions. For the RT reaction 10 µl of the reverse-transcription mix was added to each tube containing the 10 µl genomic DNA elimination mix (containing sample or a positive control RNA). This was mixed gently and incubated at 42℃ for exactly 15 min. All reactions were stopped simultaneously by incubating at 95℃ for 5 min. RNase-free water (91 µl) was added to each reaction which were gently mixed and placed on ice. The PCR components mix were prepared in a 5 ml tube, containing a SYBR Green master mix, the cDNA synthesis reaction, RNase-free water to total volume of 1300 µl for each sample. The remaining 9 µl cDNA synthesis reaction was stored at –20℃, for quality control analysis of the RT reaction. The mixed components were dispensed into the RT2 Profiler PCR Array 10 µl/ well for 96 wells per sample. Special covers were provided by the manufacturer for ease of pipetting and prevention of sample cross-contamination. Thermocycling conditions were provided by the manufacturer as a program which consisted of 10 min at 95℃ for *Taq* polymerase activation, followed by 40 cycles of 15 s 95℃, and 30 seconds at 55℃, 30 seconds at 72℃. Data was analyzed using PCR Array Data Analysis Software (Excel based) provided by Qiagen which automatically performs all $\Delta\Delta C_t$ based fold-change calculations from the uploaded raw threshold cycle data.

Statistical analysis

Real-time quantitative PCR, interspersed repeat hypomethylation, immunoblotting or MS-PCR data among three or more groups, and global methylation data were analyzed with one-way ANOVA or two-way ANOVA (factors: genotype and age; GraphPad Prism 5.0) with Bonferroni's or Dunnett's post hoc tests as appropriate

with a $p < 0.05$ adjusted significance level. Global methylation of *in vivo GH* administration did not pass Levene's test for equal variances and was analyzed using a Kruskal-Wallis test and Dunn's multiple comparison post-test. Immunoblotting, end-point MS-PCR, and DNMT activity data between genotypes were analyzed using Students *t-test (*GraphPad Prism 5.0). All data are shown as mean ± standard error of the mean (SEM). Sample values (*n*) are listed in the figure legends.

CHAPTER III

RESULTS

Ames dwarf liver exhibits alterations in components of the methionine pathway in the liver. In hepatocellular carcinoma cells and in animal models these alterations are known to change DNA methylation. Most epigenetic studies are either in abnormally proliferating cells, such as cancers, or in pre-disease or diseased states, such as methyl-deficient diets causing hepatosteatosis. To offer a fresh perspective on epigenetic mechanisms in aging, we examined the epigenetic methylation mechanisms and markers in Ames dwarf mice that are known to outlive their wild type counterparts, which is attributable to GH deficiency. We studied DNA methyltransferase expression basally in cross-sectional studies (3, 12, and 24 months), and with the addition of growth hormone, to study this hormone, and its effects on epigenetic mechanisms. We also examined global methylation, histone methylation, and studied as whether one pathway that regulates DNMT1 in hepatocellular carcinomas, also regulates DNMT1 in dwarf mice. We ended our studies by performing similar experiments in another GH signaling deficient long-living mouse, the growth hormone receptor knockout, and a growth hormone over-expressing short-lived model. We were evaluating these mice for differences and similarities between the strains that might show patterns of epigenetic methylation related to aging and growth hormone.

GNMT transcription and translation is higher in dwarf mice

Glycine-N-methyltransferase regulates SAM in the liver and the over- or under-expression of this enzyme has been linked to changes in DNA methylation. Our lab has previously shown that GNMT mRNA expression and activity is increased in Ames dwarf mice compared to wild type mice. However, changes in GNMT expression due to aging had not been determined, which could affect DNA methylation over the lifespan of an animal. Given that GNMT is linked to DNA methylation and possible DNA methyltransferase activity via the methionine pathway, we investigated GNMT expression in dwarf and wild type mice in our cross-sectional studies. Similar to previous findings, GNMT liver mRNA is more abundantly expressed across all three age groups in the Ames dwarf compared to wild type mice with significance at 3 and 12 months of age (Figure 1). The GNMT mRNA expression is influenced by genotype ($p=0.0001$) and age ($p=0.0176$). Protein levels of GNMT are also higher in dwarf mice at 12 months of age when compared to age-matched wild type controls ($p=0.0408$).

DNA methylation enzyme basal expression and activity is significantly altered in Ames dwarf mice compared to wild type mice

Next we examined mRNA expression of DNMT1, DNMT3a, and DNMT3b using RT-PCR analysis of isolated liver RNA. Surprisingly, we found that dwarf mice show a much higher mRNA expression of both DNMT1 (105%) and DNMT3a (238%) at 3 months of age ($p<0.0001$; Figure 2).

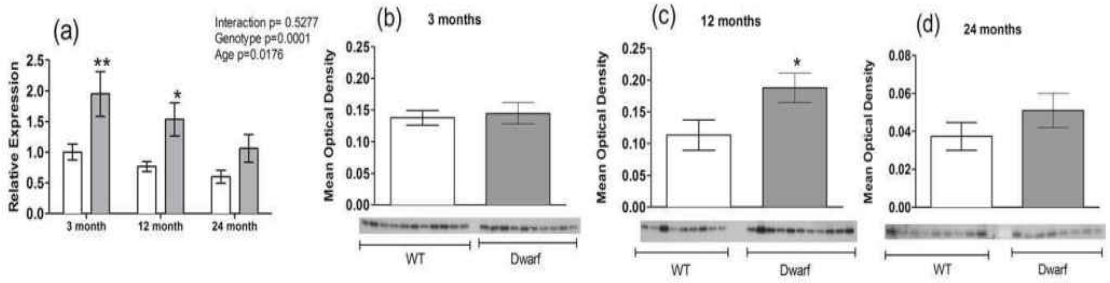

FIGURE 1. GNMT mRNA and protein expression are higher in Ames dwarf mice compared to their wild type siblings. a) Liver GNMT mRNA expression is shown for dwarf (gray bars) and wild type mice (open bars) at 3, 12, and 24 months of age (n=7-8). b) GNMT protein levels at 3 (n=10-11, p=0.7248) and c) 12 months (n=8-10, p=0.0408) and d) 24 months of age (n=6-8, p=0.6618). Mean optical densities are shown ± SEM. *p<0.05 and **p<0.01. Ponceau S was used for equal loading.

Gene expression of DNMT3b mRNA was similar between genotypes at all age groups studied. Age appeared to be an important factor in the expression of all three DNMTs. A significant effect of genotype was detected for DNMT1 and DNMT3a however, the genotype by age interaction was significant confounding the independent effects. The DNMT expression overall shows an interesting U-shaped pattern with the lowest levels at 12 months of age in both genotypes compared to the linear decrease in GNMT mRNA expression (Figure 1). Previous literature noted DNA methyltransferase RNA expression declined with age and when the expression was normalized to histone H4, which was used to rule out a more or less proliferative background (Ray et al., 2006). We compared histone H4 to β2m mRNA expression to detect any effects of background cellular proliferation that might influence DNMT transcription results and determined no discernable differences. Therefore β2m was considered an appropriate transcript for all mRNA normalization in the liver (Figure 3).

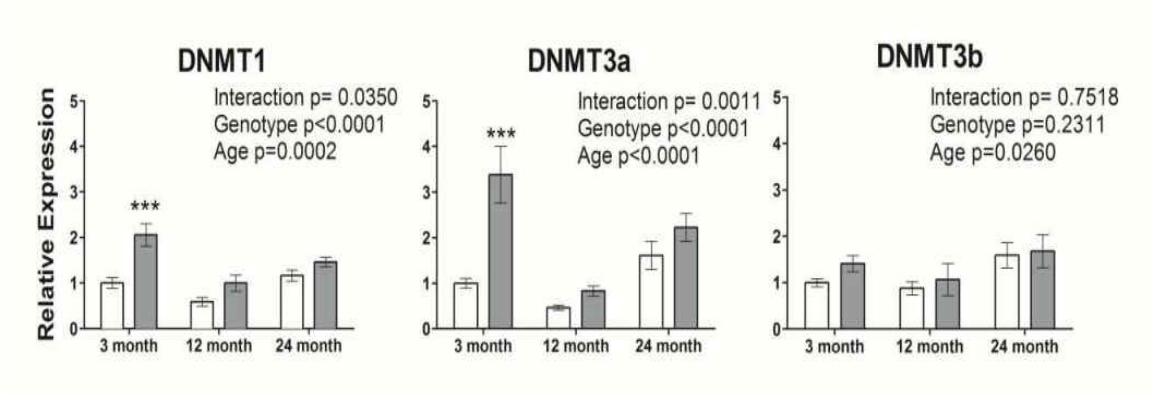

Figure 2. DNMT1 and DNMT3a mRNA expression are higher in Ames dwarf mice at 3 months of age. Liver DNMT mRNA expression is shown for wild type (open bars) and dwarf mice (gray bars) at 3, 12, and 24 months of age (n=7-8) for DNMT1, DNMT3a, and DNMT3b. Mean relative changes in expression are shown ± SEM. ****p<0.0001.

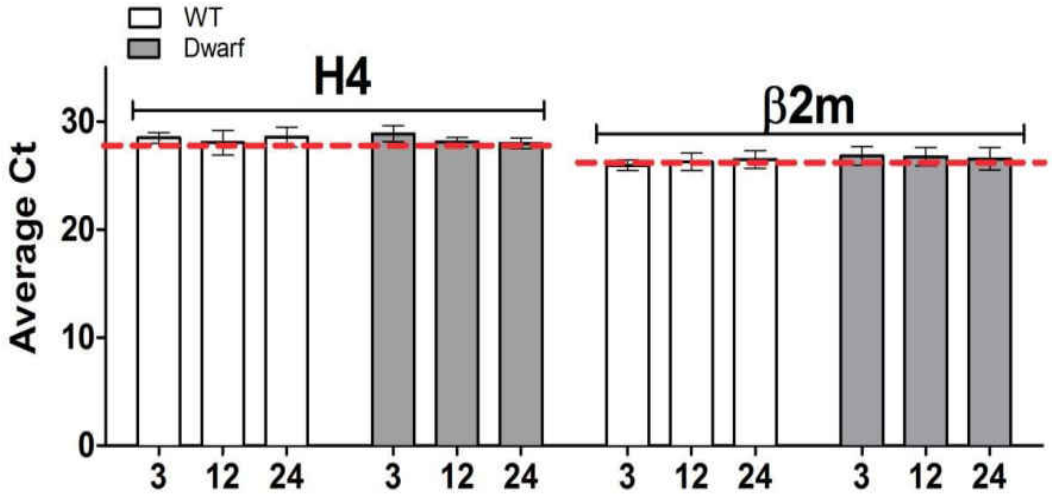

Figure 3. Average Ct counts for H4 or β2m transcripts showed similar biological expression between all age groups and genotypes. Age groups compared are 3, 12, and 24 months (wild type mice, open bars; dwarf mice, grey bars). β2m normalization was therefore used for all transcriptional studies. Bars represent mean± S.D.

Protein levels of DNMT1, DNMT3a, and DNMT3b were also compared between dwarf and wild type mice at 3, 12 and 24 months of age (Figure 4). To maximize the genotypic comparisons, independent immunoblots for each age were assayed. Very low levels of DNMT1 protein were observed in the liver tissue of dwarf mice compared to wild type mice at each age group examined.

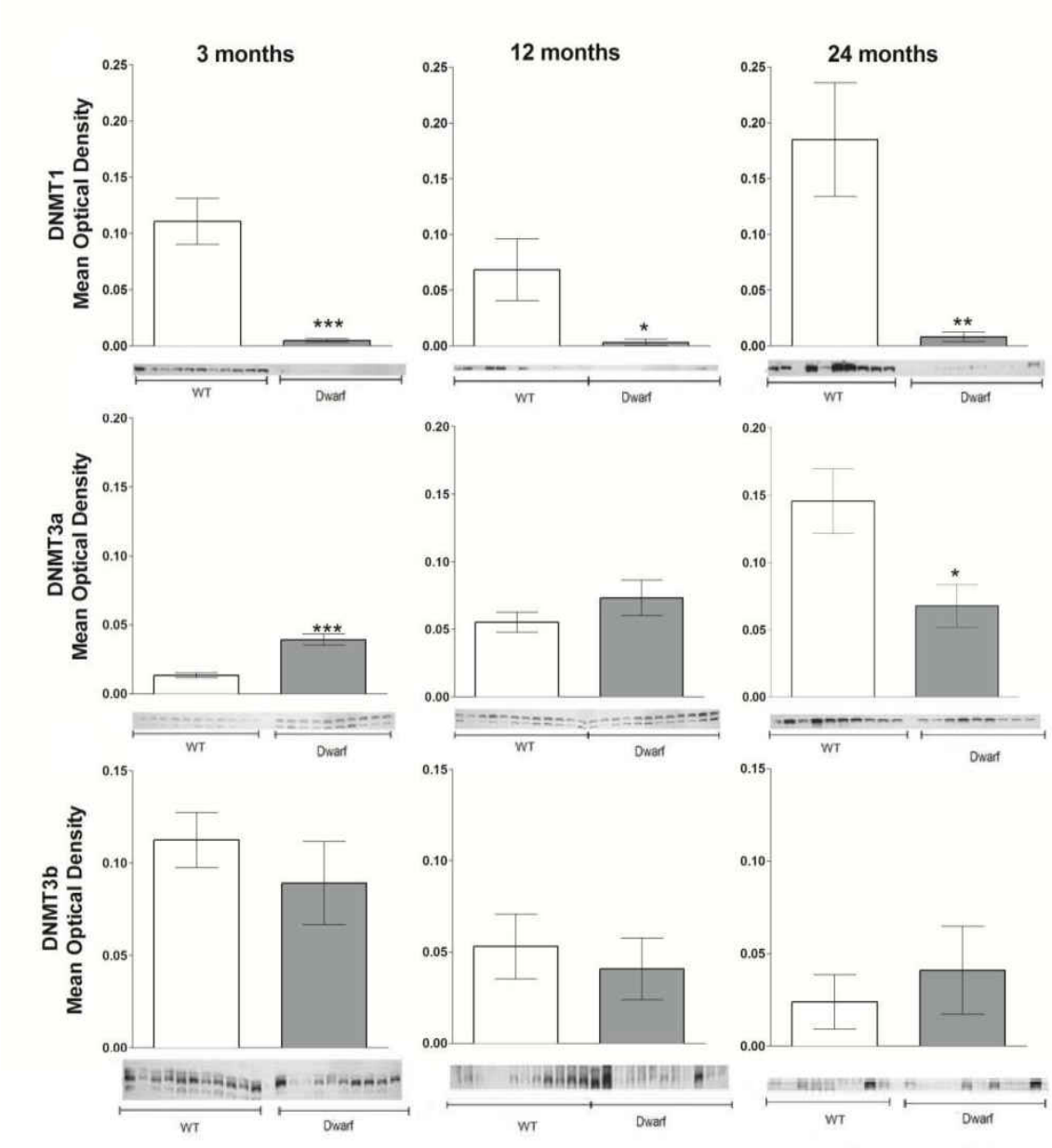

Figure 4. DNMT protein levels are expressed differently in Ames dwarf and wild type liver tissue at 3, 12, and 24 months of age. Wild type mice represented by open bars and dwarf mice by gray bars. DNMT1 protein expression was much lower that wild type mice at 3 months (n=10-11, $p<0.0001$), 12 months (n=9, $p=0.0427$) and 24 months (n=7-9, $p=0.0015$). DNMT3a protein was significantly higher in dwarf mice at 3 months (n=10-11, $p<0.0001$), statistically equivalent at 12 months (n=12, $p=0.2464$) and much lower in dwarf mice at 24 months (n=8, $p=0.0172$). DNMT3b protein was not different between the genotypes at 3 months (n=10, $p<0.4000$), 12 months (n=11-12, $p=0.6244$) or 24 months (n=9-10, $p=0.5621$). Mean optical densities are shown ± SEM. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

The variability of protein levels between animals was more pronounced in wild type DNMT1 and DNMT3a as compared to dwarf mice. DNMT3b expression was variable in both genotypes at every age. Due to a high variability of DNMT protein expression among *in vivo* groups, each immunoblotting assay was done independently for each group to increase murine sample sizes. DNA methyltransferase 3a protein levels were higher (193%) in the dwarf mice at 3 months of age ($p<0.001$), similar at 12 months of age ($p=0.2464$), and significantly lower (54%) at 24 months of age ($p=0.0172$). Compared to wild type controls, DNMT3b levels were not different between genotypes at any age, similar to mRNA findings at each age group. We also asked whether there were differences due to aging in DNMT1 and DNMT3a within genotypes. We evaluated expression at all three ages within each genotype where we were limited to a sample of 8 per age group (24 well gel capacity, Figure 7). We doubled the amount of our dwarf liver lysate and re-optimized for the detection of DNMT1 since the basal signal was so low. We observed an increase of DNMT1 expression in the dwarf mouse ($p=0.0012$) and in the wild type mouse ($p=0.0512$) with age. DNMT3a however, had opposite expression patterns over all three ages when comparing genotypes. Remarkably, dwarf mice displayed higher DNMT activity in young and similar DNMT activity in middle aged (3 months, $p=0.0214$; 12 months, $p=0.1704$) liver compared to wild type mice but lower activities in old Ames dwarf liver (24 months, $p=0.0491$) (Figure 6). The DNMT activity in the liver more closely resembles DNMT3a protein expression than DNMT1 or DNMT3b.

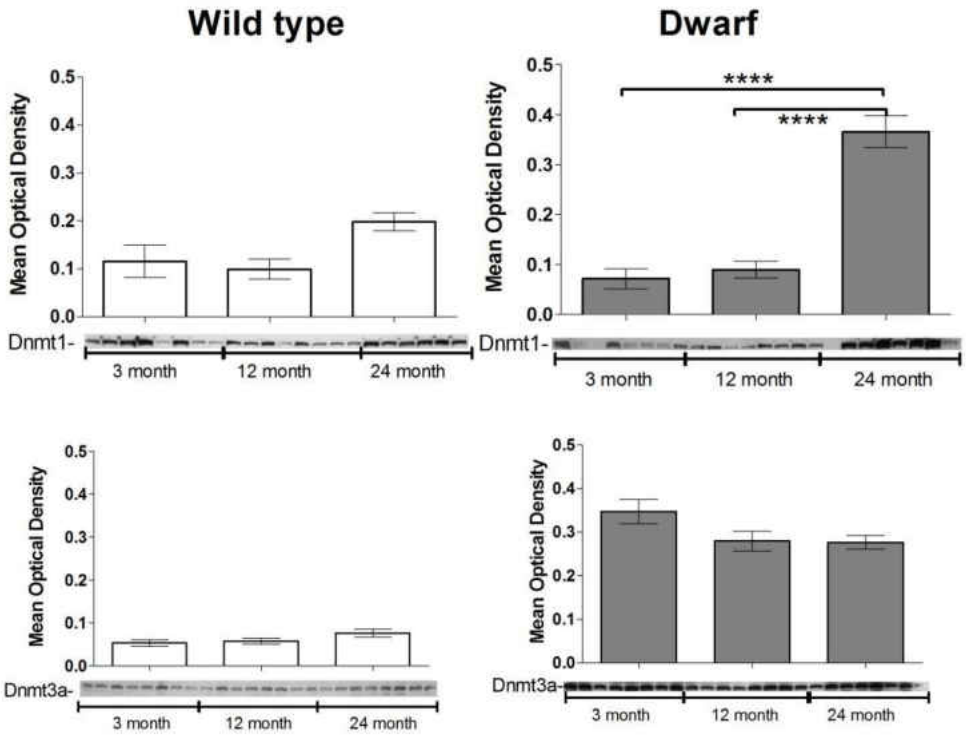

Figure 5. DNMT expression genotype differences due to age run in separate gels. The figures on the left wild type mice show an increase in DNMT1 and in DNMT3a (One way ANOVA p=0.0512 and p=0.1056 respectively, n=6-8). Dwarf mice also show a significant increase from 3 to 24 months of age in DNMT1 but a no significant decrease in DNMT3a (One way ANOVA p=0.0012 and p=0.0926 respectively, n=6-8). However, for DNMT1 in the dwarf the amount of liver protein used for dwarf tissue was doubled (and immunoblotting conditions re-optimized) in comparison to the wild type tissue to generate enough signal to detect by immunoblotting. Bars represent mean±SEM, ****p<0.0001.

Overall DNMT activity was measured with an ELISA-based colorimetric assay using nuclear lysates extracted from liver tissue, as DNMT isoform specific activity assays are not available. Independent assays comparing dwarf versus wild type for each age group were performed. All samples were performed in triplicate for accuracy. In conclusion, it appears that DNMT1, DNMT3a, and GNMT mRNA and protein expression are not directly correlated with each other though both show significant effects of age and genotype in transcription.

DNMT1 protein expression is greatly diminished in dwarf mice at every age and DNMT3a protein appears to follow DNMT activities, which are all novel findings. DNMT3b shows no genotype differences in transcription or protein; however an age difference was noted in transcription.

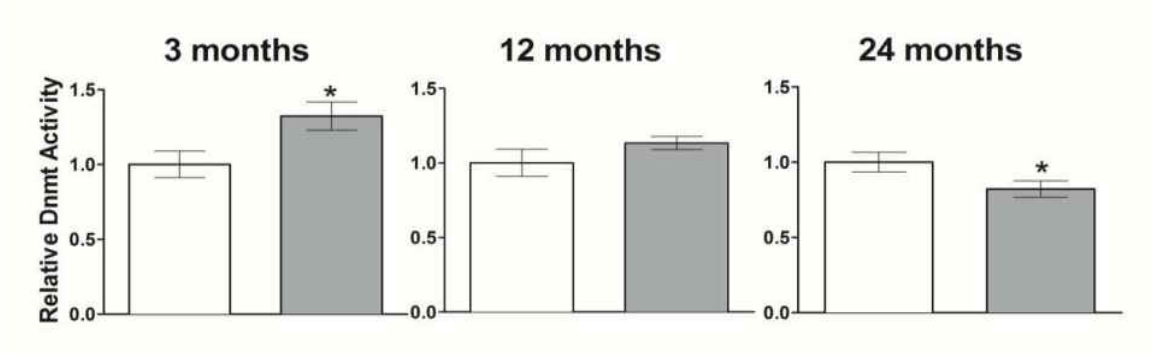

Figure 6. DNMT activity is higher in Ames dwarf mice at 3 months and lower at 24 months than age-matched wild type siblings matching the pattern of DNMT3a protein expression. ELISA-based colorimetric activity assay of nuclear liver lysate showing differences in total DNMT activity in 3 month (n=11, p=0.0214),12 month (n=8-11, p=0.1704) and 24 month old (n=9-12, p=0.0491) wild type (open bars) and Ames dwarf mice (gray bars). Mean activities expressed as optical density per mg per hour are shown ± SEM. *p<0.05.

Global DNA methylation is higher in dwarf mice at every age and resists changes due to age

Global DNA methylation in liver tissue is subject to DNMT activity alterations and abundance, SAM availability, and alterations of enzyme expression or activity in the methionine pathway. Because we observed such interesting differences in DNMT activity and expression, we measured global methylation using an ELISA-based method with antibodies for methylated cytosines across all three age groups (Figure 7). Comparison of dwarf versus wild type mice showed independent factors of genotype (p=0.0635) and age (p=0.0534) approaching significance. At 3 and 12 months of age, dwarf mice retained higher methylation than age-matched wild type mice, becoming equivalent at 24 months of age. Because much of the global loss of DNA

methylation with aging is thought to take place in interspersed repeats, also known as repetitive elements and transposable elements, transcription of these elements is thought to increase and contribute to genetic instability. We measured the hypomethylation status and transcription of these repeats between Ames dwarf and wild type mice in another cross-sectional study to see if this was indeed the case.

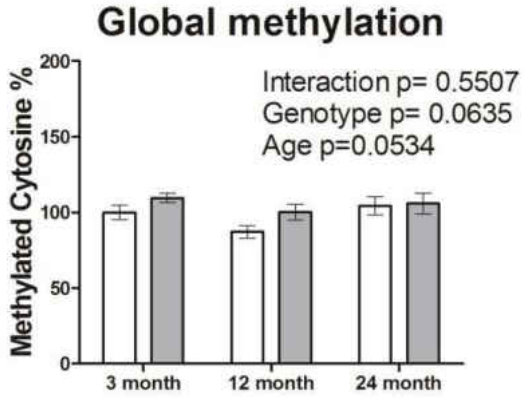

Figure 7. Global DNA methylation of Ames dwarf mice is higher at 3 and 12 months of age. ELISA-based colorimetric activity assay of nuclear liver genomic DNA showing global DNA methylation in wild type (open bars) and Ames dwarf mice (gray bars). Percentage methylation was derived using control and sample optical densities, which were then normalized to 3 month wild type mice± SEM.

We used a well-published technique called the McrBC-qPCR method. *McrBC* is an endonuclease that restricts methylated DNA and degrades it. The treated DNA sample is compared to “mock restricted” sample of the same genomic DNA which does not contain the restriction enzyme but has been processed in the same way at the same time. Genomic DNA that is restricted is enriched for unmethylated DNA, therefore DNA that is less methylated shows greater abundance levels during quantitative PCR (Figure 8, left panels). Both intracisternal A-particle long transposable repeat (IAP-

LTR) and short interspersed nuclear element B2 (SINE B2) are increasingly hypomethylated with age in the wild type mouse. Hypomethylation in SINE B2 also increases in the dwarf mice with age. Long interspersed nuclear element 1 (LINE1) hypomethylation approached significance for genotype differences ($p=0.0753$) displaying higher levels of hypomethylation in dwarf mice that decreases over time, whereas wild type mice appear to maintain or only slightly gain methylation levels. This lack of difference in methylation of wild type mice is consistent with the most recent literature. Differences in IAP-LTR hypomethylation between dwarf and normal mice also show genotypic differences moving toward significance ($p=0.0656$) where dwarf mice have more methylation at 3 months and 24 months compared to wild type mice. The repetitive element SINE B2, which is rodent specific, exhibited statistically different levels of hypomethylation affected by age and genotype independently ($p=0.0140$, $p=0.0415$, respectively). Short interspersed nuclear element B2 was clearly more methylated in dwarf mice at every age compared to wild type, yet both genotypes demonstrated a linear increase of hypomethylation with age. Short interspersed nuclear element B1, which is homologous to the human *Alu* repeat, did not display differences in methylation due to age or genotype, but remained relatively similar at all three ages studied.

While methylation does regulate transcription in many cases, for the four interspersed repeats studied, transcription and methylation appear unrelated or uncoupled. However, there are transcriptional differences

attributable to age or genotype (Figure 8, right panels). All four repetitive element transcripts were significantly affected by age, whereas only SINE B1 displayed a significant genotype difference, most likely due to the increase of transcription in dwarf mice at 24 months of age compared to wild type mice. Remarkably, transcriptional expression of repetitive elements resembles the u-shaped curve of expression similar to DNMT transcription. For IAP-LTR this pattern of mRNA expression due to aging has been noted in mouse liver before (Gaubatz, Arcement, & Cutler, 1991).It also appears the transcriptional expression of repetitive elements is greatest at 3 months of age in wild type mice but not dwarf mice, which can regain an equivalent or increased amount of transcription in LINE1, SINE B1, and SINE B2.

Since LINE1 makes up approximately 20% of the 40% of genomic DNA comprised of interspersed repeats in the murine genome, we decided to use LINE1 and MS-PCR to validate our hypomethylation results (Figure 9). Two primer sets were used, however, only the set with CpG island prediction between base pairs 150-324 showed any unmethylated CpGs. The other LINE1 primer set that probed between base pairs 525-676 only showed highly methylated products with no difference between dwarf and wild type mice at any age (data not shown). However, the loss of methylation at 3 months of age corresponds with our hypomethylation data using the McrBC-qPCR technique. At 24 months of age, dwarf mice appear to have increased methylation compared to wild type mice ($p=0.0586$).

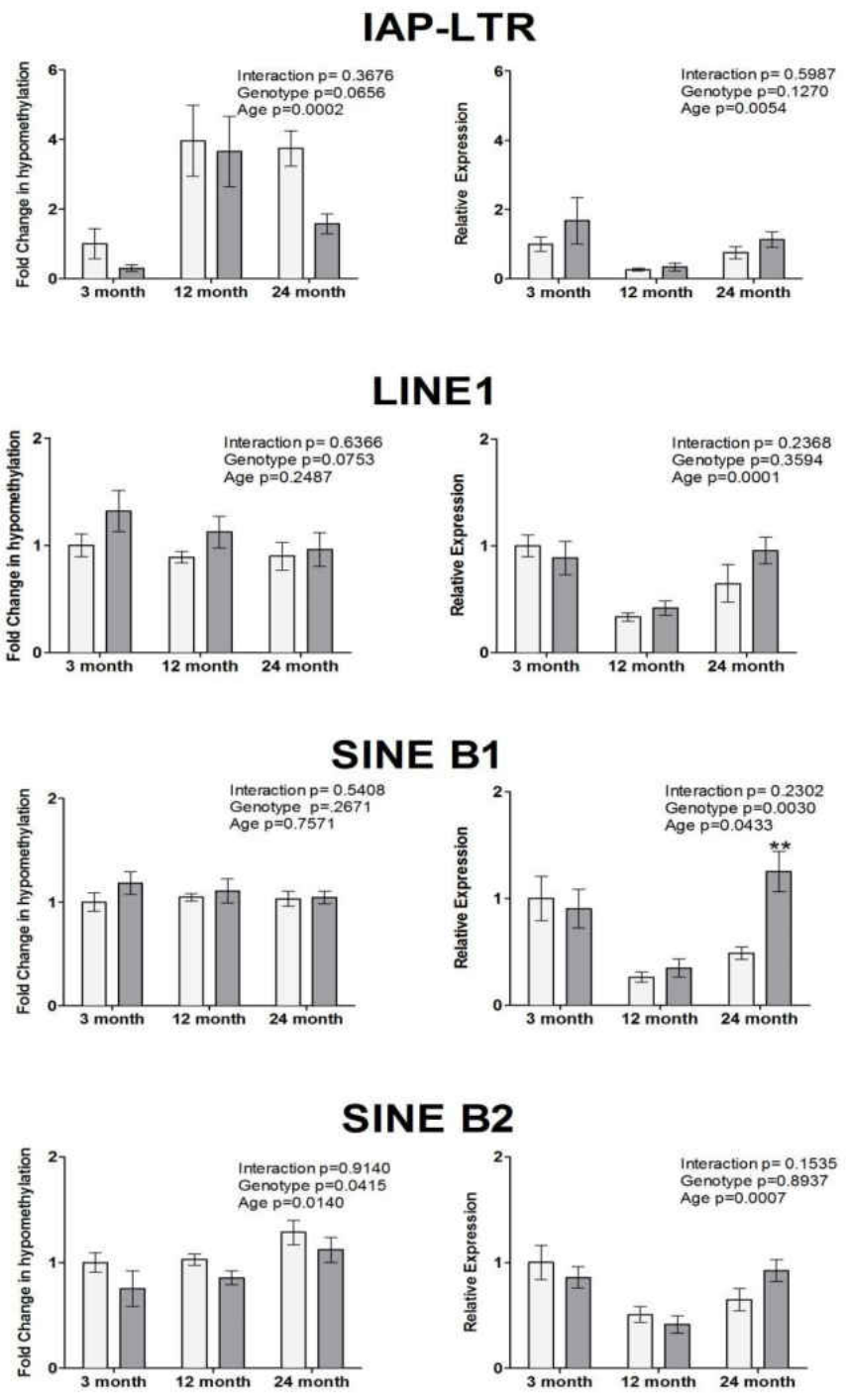

Figure 8. Ames dwarf mice show differences in hypomethylation and transcriptional expression of repetitive elements that may contribute to genetic stability. Using the McrBC-qPCR technique differences in liver genomic DNA hypomethylation of four interspersed repeats are shown for dwarf (gray bars) and wild type mice (open bars) (left panels, n=6-8). Liver IR mRNA expression is shown using two step RT-PCR at 3, 12, and 24 months of age (right panels, n=7-8). Mean relative changes in expression or hypomethylation are shown ± SEM. **p<0.01.

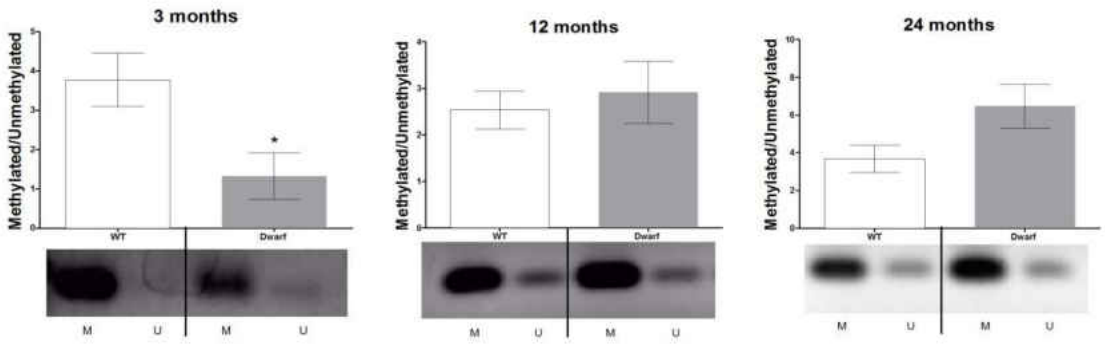

Figure 9. Methylation patterns of LINE1 are different in Ames dwarf mice. Ames dwarf mice display lower methylation at 3 months of age (gray bars, p=0.0351), equivalent methylation at 12 months of age, but higher at 24 months of age (p=0.0586). Wild type mice are represented by open bars. Mean relative changes in expression and optical densities are shown ± SEM. *p<0.05.

DNA methylation enzyme expression is significantly affected in growth hormone treated dwarf mice

Our next question was whether growth hormone, which also alters methionine pathway enzymes such as GNMT, could significantly affect DNMT expression and DNA methylation in the dwarf liver. To begin, porcine GH was administered to one group of 5-6 month old Ames dwarf mice while saline vehicle was administered to wild type mice and an additional group of dwarf mice as controls. Animals received GH or saline twice daily for seven days and was replicated with a second experiment. Growth hormone treated dwarf mice gained 3-4 grams of body weight over the course of one week compared to saline-treated dwarf mice which is typical of this protocol (Students *t-test*; Experiment1:+4.19 ± 0.06 g; p<0.0001, Experiment 2: +3.153±0.2184g, p<0.0001, Figure 10). Wild type mouse body weights were not different pre- and post-saline injection. Liver weights were recorded as an additional indicator of GH action and the mean liver weights of GH injected dwarf mice were approximately 60-76% higher than saline injected dwarf mice [Experiment 1: 0.70 ± 0.13 g and 0.44 ± 0.05 g respectively (p<0.0001); Experiment 2: 0.60 ±0.08 g and 0.34 ± 0.09 g respectively

(p<0.0001)]. The expression of DNMTs was compared between GH and saline-treated Ames dwarf and wild type controls. Liver DNMT1 and DNMT3b mRNA levels were higher in saline-treated dwarf mice than saline-treated wild type mice and increased further after GH administration (Figure 11). Transcription of DNMT3a mRNA abundance was higher in saline-treated dwarf mice compared to saline-treated wild type mice, and no change was observed after GH treatment.

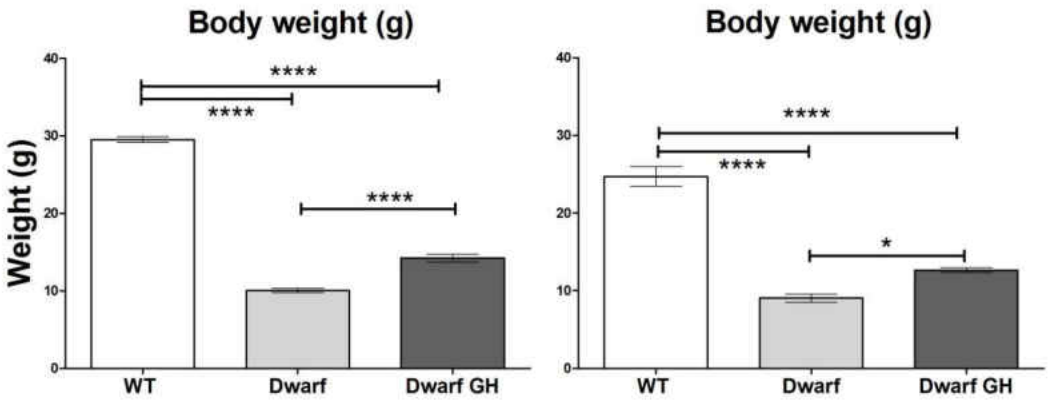

Figure 10. Body weight is increased by one week of growth hormone injections in Ames dwarf liver. Liver weight in grams is shown for wild type mice injected with saline (n=9-11, open bars), dwarf mice injected with saline (n=8-11, gray bars), and dwarf mice injected with growth hormone (n=8-12, black bars) at 5-6 months of age. One-way ANOVA for body weight p<0.0001 for both left (Experiment 1) and right graphs Experiment 2). *p<0.05, and ****p<0.0001 using Bonferroni's Multiple Comparison test. Bars represent means ± SEM.

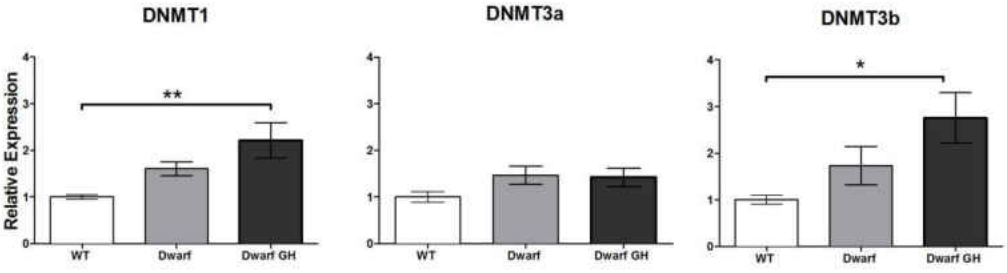

Figure 11. Liver DNMT mRNA expression For DNMT1 and DNMT3b is increased upon GH administration. Shown for wild type mice injected with saline (n=10-11, open bars), dwarf mice injected with saline (n=10-11, gray bars), and dwarf mice injected with growth hormone (n=10, black bars) at 5-6 months of age. mRNA expression is normalized to β2- macroglobulin. One-way ANOVA for DNMT1 p=0.0054, for DNMT3a p=0.1202, and for DNMT3b p=0.0129. Bars represent means ± SEM. *p<0.05 and **p<0.01.

We next examined DNMT protein. Protein levels of DNMT1 were significantly lower in saline-treated dwarf than in the saline-treated wild type mice similar to our aforementioned findings (Figure 12). However with GH treatment, protein expression of DNMT1 increased in dwarf mice ($p<0.05$) though not to wild type levels. DNMT3a protein levels were significantly decreased following GH treatment in Ames dwarf mice ($p<0.05$). Yet again, transcriptional differences did not correlate with protein expression, indicating a post-transcriptional pathway that may be influenced by growth hormone. DNMT3b protein in growth hormone treated mice was not assessed due to the absence of genotype differences from previous experiments between dwarf and wild type mice.

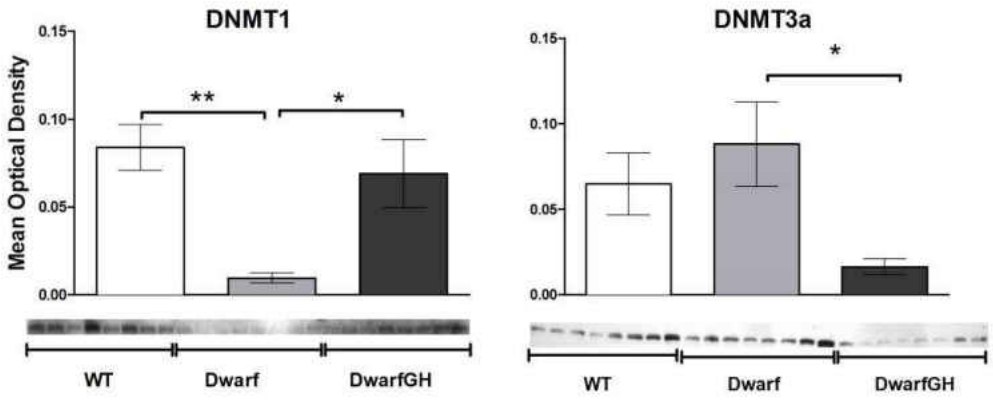

Figure 12. DNMT protein expression is susceptible to growth hormone treatment in Ames dwarf mouse liver. One-way ANOVA for DNMT1 protein $p=0.0021$ and for DNMT3a protein $p=0.0367$($n=5$-8). *$p<0.05$ and **$p<0.01$ using Bonferroni's Multiple Comparison test. Bars represent means ± SEM.

DNA methylation enzyme expression is significantly altered in hepatocytes following growth hormone treatment

For *in vitro* study of GH administration effects on DNMT protein expression, we isolated hepatocytes from 4-6 month old mice and treated them for 24 hours with different concentrations of porcine GH growth hormone (Figure 13). For this study

our goal was to demonstrate and verify that the *in vitro* hepatocytes reflected the *in vivo* results for future pathway studies of DNMT regulation that remain to be elucidated. Significant differences were detected for DNMT1 due to increasing GH concentrations in dwarf (p=0.0026) and wild type (p=0.0014) mouse hepatocytes. Ames dwarf mice showed a significant increase in DNMT1 protein expression following 1.0 and 10µg of GH per plate compared to a media only control. Though the higher concentration of GH (20 µg) also showed visibly greater protein expression, only 2 samples were present for quantification and thus statistically difficult to compare. Protein expression of DNMT1 in hepatocytes from wild type mice did not show a significant increase compared to a media only control until a dose 10 times higher than that used in dwarf mice (10 µg GH per plate). The levels of DNMT3a protein were decreased in primary dwarf hepatocytes following GH treatment (One-way ANOVA, p=0.0464) independent of GH concentration. Wild type hepatocytes showed little to no protein expression of DNMT3a and no difference following GH treatment that could be quantified by our methods.

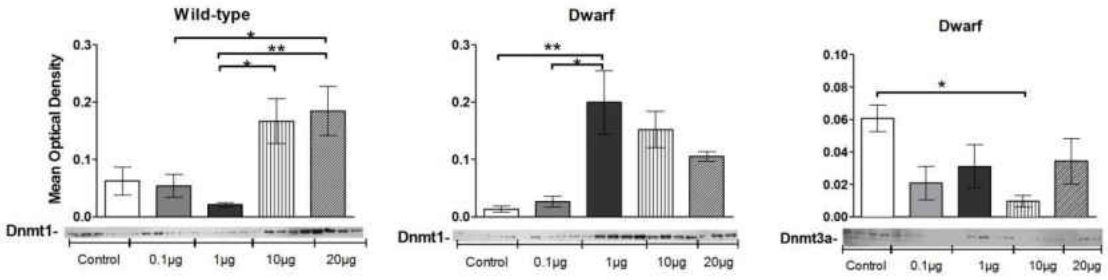

Figure 13. Ames dwarf hepatocytes are more sensitive to growth hormone treatment which alters DNMT expression. DNMT1 expression in wild type (One-way ANOVA,p=0.0014) and dwarf (One-way ANOVA,p=0.0026) hepatocytes with increasing concentrations of growth hormone (0, 0.1,1.0, 10, and 20 µg/ml). DNMT3a expression in dwarf (One-way ANOVA, p=0.0464) hepatocytes with increasing concentrations of growth hormone (0, 0.1, 1.0, 10, and 20 µg/ml). DNMT3a not present in wild type hepatocytes.*p<0.05 and **p<0.01 using Dunnett's Multiple Comparison Test to a media only control. Error bars represent means ± SEM.

Since growth hormone appears to affect DNMT expression, we were interested to see if the altered expression of methylation mechanisms could affect global methylation. Thus, we next examine global DNA methylation by the same ELISA colorimetric methods as described for the cross-sectional studies (Figure 14). Dwarf mice treated with growth hormone for one week lost approximately 15% of global DNA methylation. Because of the dramatic loss of methylation we next asked whether growth hormone administration could disrupt the methylation of interspersed repeats observed in many methyl-deficient diets which affect the methionine pathway.

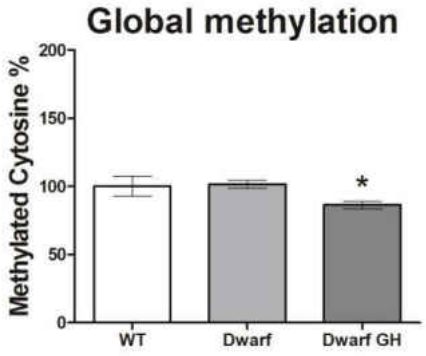

Figure 14. Growth hormone administration alters global DNA methylation in dwarf mice. Methylated cytosine by ELISA-based colorimetric assay shown for wild type mice injected with saline (n=10-11, open bars), dwarf mice injected with saline (n=10-11, gray bars), and dwarf mice injected with growth hormone (n=10, black bars) at 5-6 months of age (Kruskal-Wallis test, p=0.0392). *p<0.05 using Dunn's Multiple Comparison Test. Error bars represent means ± SEM.

We used MS-PCR to examine LINE1 in dwarf mice that had been treated with growth hormone as well as our saline injected wild type and dwarf controls (Figure 15). We found the GH administration did not seem to alter LINE1 methylation, although there was a large amount of deviation between the means for wild type and GH injected mice. Dwarf mice were consistent in mean methylation to each other.

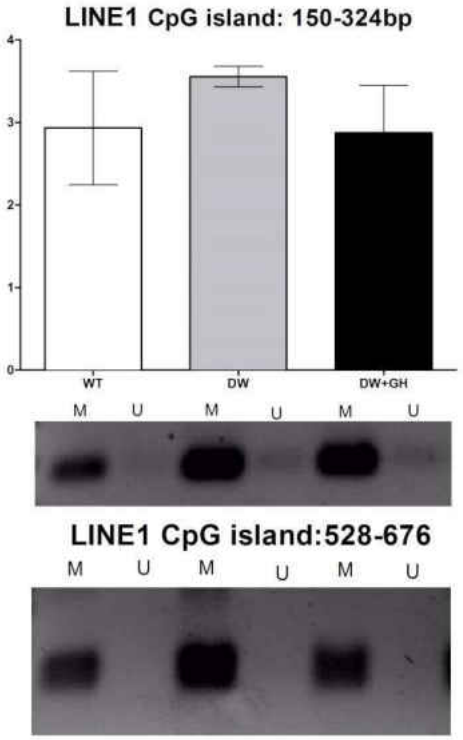

Figure 15. Growth hormone treatment did not change methylation patterns of LINE1 in Ames dwarf mice. LINE1 was probed with MS-PCR for wild type mice injected with saline (WT, open bars), dwarf mice injected with saline (DW, gray bars), and dwarf mice injected with growth hormone (DW+GH, black bars) at 4-6 months of age (One-way ANOVA p=0.6584, n=6). Mean relative changes in expression and optical densities are shown ± SEM.

DNMT1 post-transcriptional regulation in Ames dwarf mice

A recent line of investigation observed that DDX20 (a DEAD-box helicase) contributes to transcriptional up regulation of metallothionein 1 (MT1) and metallothionein 2 (MT2) via the repression of DNA methylation by DNMT1. Purportedly, DDX20 assists in pairing miRNA 140-3p with the DNMT1 mRNA transcript and in conjunction with RISC complex, allows for either degradation of the mRNA transcript or de-capping the mRNA transcript repressing translation (Takata et al., 2013; van Kouwenhove et al., 2011).

Interestingly, our lab and others showed that Ames dwarf mice express more metallothionein protein and mRNA in liver as well as other tissues (Amador-Noguez et al., 2004, 2005; Meyer, M.M., Swinscoe, J.C., Brown-Borg, H.M., Carlson, 2003; L. Y. Sun et al., 2011; Swindell et al., 2010). Coupling the high MT levels with altered DNMT1 expression in Ames mice, we investigated the expression DDX20 and miRNA 140-3p involvement in a potential novel regulatory pathway and whether it may be utilized by dwarf mice to repress DNMT1 translation in the liver.

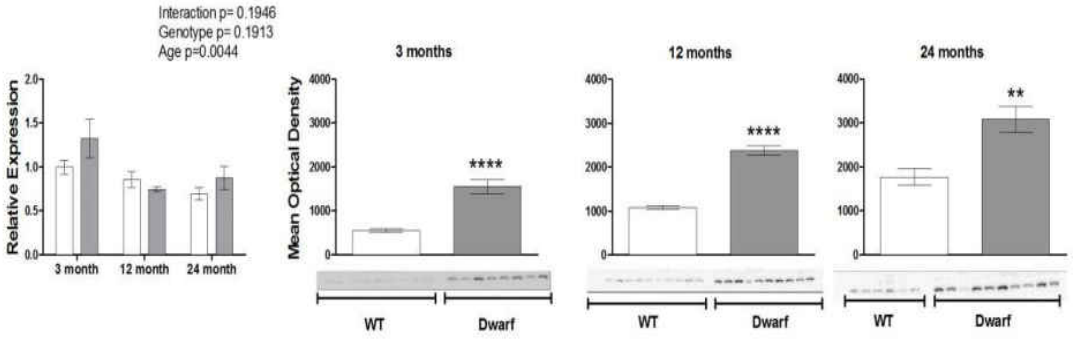

Figure 16. Liver DDX20 mRNA expression is affected by age but not genotype and DDX20 protein is more abundant in dwarf mice. Grey bars represent dwarf mice and open bars represent age-matched wild type mice at 3, 12, and 24 months of age. For mRNA n=7-8 per group and for protein studies n=6-10 per group. Mean optical densities are shown ± SEM. *$p<0.05$, **$p<0.01$, and ****$p<0.0001$.

We first looked at DDX20 transcriptional and protein expression in dwarf mouse liver to see if there were basal differences (Figure 16). While we found no differences to DDX20 transcriptional expression due to genotype, our study showed age was a significant factor for both genotypes of mice. Dwarf and wild type mice appear to lose transcription abundance of DDX20 with age somewhat reminiscent of GNMT mRNA levels, with a more linear loss observed in wild type mice. The basal expression of DEAD-box helicase 20 protein levels is more

abundant at all three age groups in dwarf mice. This correlates with the decrease in DNMT1 abundance suggesting that this may be a potential pathway for DNMT1 post-translational regulation. We next asked if basal expression of miR-140-3p was also changed in dwarf mice and could be contributing to DNMT1 regulation (Figure 17).

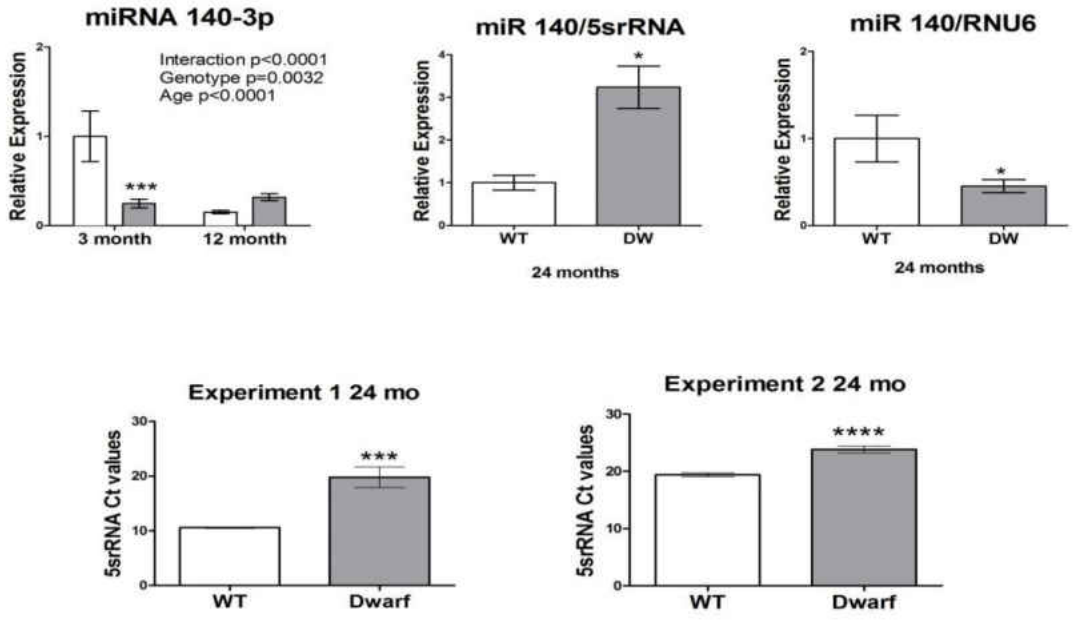

Figure 17. Dwarf mice display decreased expression of miRNA 140-3p at 3 and 24 months of age but also loss of 5S rRNA transcription at 24 months of age, compared to age-matched wild type mice. The top left panel displays age and genotype related differences in miRNA 140-3p expression between dwarf and wild type mice at 3 and 12 months of age, n=6-8. Using Student's t-test miRNA 140-3p expression is greater at 24 months in dwarf mice when normalized to 5S rRNA (Top middle panel, n=7-8, p=0.0207) but upon validation by normalization with RNU6, transcription of miRNA 140-3p is significantly decreased (Top right panel, n=6-8, p=0.0104). Differences in transcription can be seen in 5S rRNA Ct values for two separate experiments (Bottom panels, n=8, p=0.0002 and p<0.0001 respectively). . Error bars represent means ± SEM. *p<0.05, ***p<0.001and****p<0.0001.

We utilized 5S rRNA for normalization of miRNA 140-3p as suggested in previous literature (Bates et al., 2010). This ribosomal RNA is part of the translational machinery of ribosomes. We found that miRNA 140-3p was significantly decreased at 3 months and increased at 24 months of age.

However, upon validation with another miRNA normalization transcript, U6 splicoeosomal RNA (RNU6), we showed opposing results, where miRNA 140-3p was actually decreased, for the 24 month group dwarf only. RNU6 is a small non-coding RNA that is part of the RNA splicing machinery (spliceosome) that processes pre-messenger RNA in the nucleus. As a result of this finding, we investigated the normalization Ct values of 5S rRNA and found they were similar except at one age group in one group of mice.

Our dwarf mice show decreased abundance in 5S rRNA compared to wild type mice at 24 months of age, which is indicated by higher Ct values. We replicated this Ct difference with a different set of 24 month old liver samples from wild type and dwarf mice (Figure 17 Bottom panels, Experiments 1 and 2). This is a novel finding as increased rRNA expression has been linked to a loss of heterochromatin in drosophila (Larson et al., 2012).

DDX20 and miRNA-140 suppress DNMT1 translational repression and affect metallothionein (MT) expression via loss of methylation in CpG regions of the MT gene and promoter regions in hepatocytes. Ames dwarf mice exhibit increased basal expression of MT1 and MT2. However, no age-related differences in MT expression had been studied in the liver. Therefore, we examined metallothionein transcriptional expression and methylation in dwarf and wild type mice to determine age-related changes in metallothionein expression and identify whether methylation of the metallothionein gene and promoter region in dwarf mice correlated with increased DDX20 protein. We observed that liver MT1 expression exhibited age-related decreases in dwarf

mice after 3 months of age (Figure 18). However, there is an approximate 8-fold increase in wild type transcription at 24 months of age. At 3 months of age MT1 mRNA expression is nearly 150-fold higher in dwarf mice. MT2 transcription was not detected in the wild type liver, thus expression levels were normalized to dwarf 3-month-old mice (Figure 18).

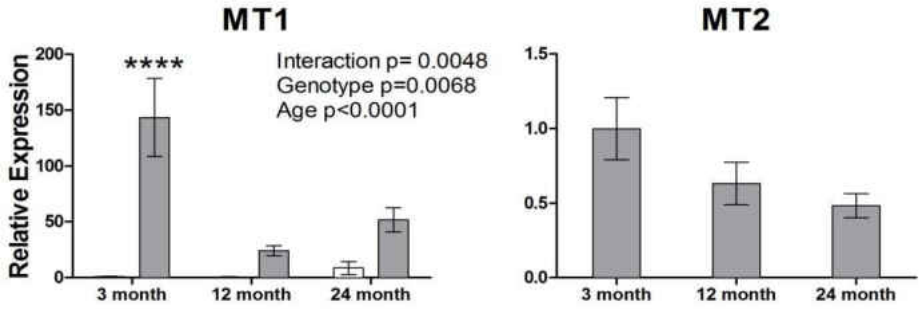

Figure18. Liver MT1 mRNA basal expression was higher in dwarf mice and affected by age and genotype. MT2 was only present in dwarf mice and the linear loss is not significant. MT1 mRNA expression for wild type (open bars) and dwarf mice (gray bars) at 3, 12, and 24 months of age (Left panel, n=6-8). Liver MT2 mRNA expression is shown for dwarf mice (gray bars) at 3, 12, and 24 months of age (Right panel, One-way ANOVA p= 0.1199, n=5-8).

Next we examined whether basal levels of DNA methylation in MT1 and MT2 gene and promoter regions were different in the Ames dwarf mice compared to wild type mice as might be expected from the higher transcriptional rates. Since the largest difference in transcription occurred at 3 months of age, we used tissues from this age group for determining methylation differences within predicted CpG islands of the MT1 and MT2 genes.

Using MethPrimer to predict CpG islands and primers sequences, MT1 showed a putative CpG island, including its promoter sequence, of approximately 600 base pairs in length and thus, two sets of primers were designed and used to maximize coverage (Table 5, Materials and Methods).

Bisulfite treated DNA is only protected from the cytosine to uracil conversion if the cytosine is methylated. Therefore two primer sets were designed, one set probed for converted cytosines (unmethylated cytosines) and the other set probed for unconverted cytosines (methylated cytosines). Coverage of the 600 base pair posited an MT1 CpG island between base pairs 177 to 328 that showed an equivalent methylation status between dwarf and wild type mice ($p=0.4422$, Figure 19, left panel). This sequence contains several transcription factor binding sites including metal response elements a,b,c, and d (MRE) and the metal response transcription factor 1 (MTF-1) that is responsible for an increase in MT1 due to heavy metal stress, such as the administration of zinc. The antioxidant response element (ARE) that is responsible for the oxidative stress response of MT1 is also located in this area (S. R. Davis & Cousins, 2000; Majumder et al., 2002; Park et al., 2013). From base pairs 464 to 570 of the MT1 predicted CpG island, dwarf mice exhibited decreased methylation compared to wild type mice (Figure 19, middle panel). This sequence covers an intergenic region between exons one and two of the MT1 gene. Metallothionein 2 has a putative CpG island between base pairs 86-395 in the MT2 gene. The MT2 gene is highly unmethylated in dwarf and wild type mice alike (Figure 19, right panel). Primers designed for this area showed no amplification of methylated primer sets in both normal and dwarf mice, therefore a ratio comparison could not be made. The area of regulation for MT2 may lie outside the gene and promoter regions or may not be subject to methylation regulation.

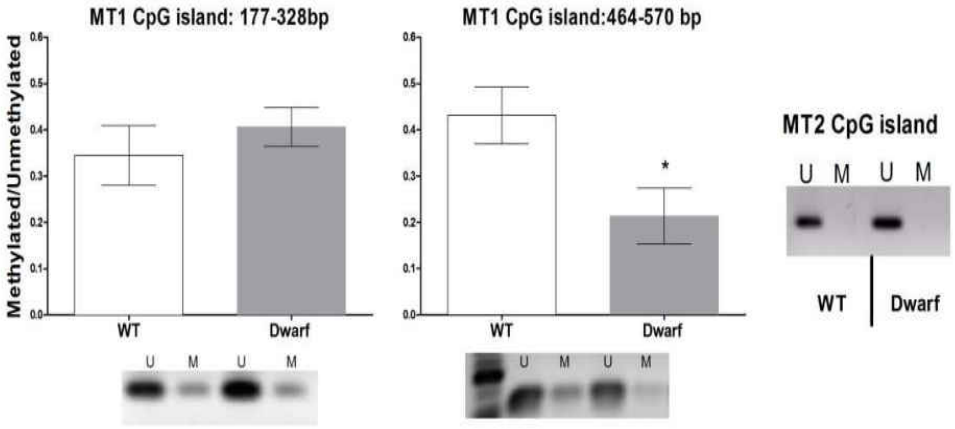

Figure 19. Three month old dwarf liver shows less methylation in MT1 at 464-570 bp of the putative CpG island. U is the product from primers designed to probe CpGs in the same regions that are unmethylated, M is the product from primers design to probe CpGs that are methylated. MT1 CpG Island methylation sites from base pairs 177-328 are shown for wild type (open bars) and dwarf mice (gray bars) at 3 months of age (Left panel, p=0.4422, n=7) and MT1 CpG Island methylation sites from basepairs 464-570 at 3 months of age (middle panel, n=6, *p<0.05). The right panel shows MT2 equally unmethylated (U) with no methylation (M) detected. Gels shown are representative to the mean optical densities of methylated primer products verses unmethylated primer products. Graphs represent mean ratios ± SEM.

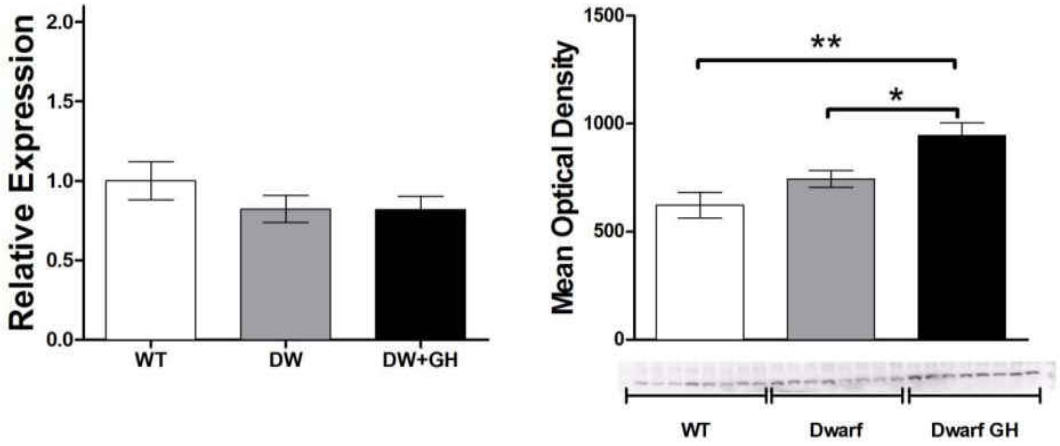

Figure 20. Protein expression of DDX20 is more abundant in dwarf mice treated with growth hormone. Liver DDX20 mRNA expression is shown for wild type mice injected with saline (WT, open bars), dwarf mice injected with saline (DW, gray bars), and dwarf mice injected with growth hormone (DW+GH, black bars) at 5-6 months of age (Left panel, One-way ANOVA p= 0.3490, n=9-10). DDX20 protein expression for wild type mice injected with saline (WT, open bars), dwarf mice injected with saline (DW, gray bars), and dwarf mice injected with growth hormone (DW+GH, black bars) at 5-6 months of age. Mean optical densities are shown ± SEM. *p<0.05, **p<0.01, and ****p<0.0001.

Growth hormone administration affects DDX20, metallothionein expression and methylation

Previously we identified differences in DNA methylation enzyme expression following one week of growth hormone administration; therefore we queried whether DDX20, MT1, and MT2 expression and possibly metallothionein methylation would be affected by GH as well. Growth hormone did not appear to have an effect on DDX20 transcription in 5-6 month old Ames dwarf mouse liver (Figure 20). Because DNMT1 protein levels are increased due to GH administration, we expected DDX20 to decrease with growth hormone expression. However, protein levels of DDX20 unexpectedly increased significantly with GH treatment.

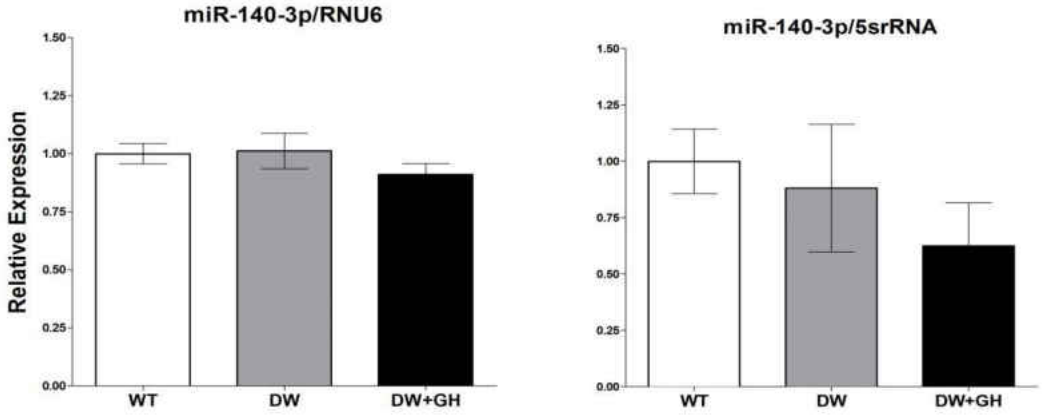

Figure 21. Liver miR-140-3p expression does not significantly change with GH administration. miRNA expression shown for wild type mice injected with saline (WT, open bars), dwarf mice injected with saline (DW, gray bars), and dwarf mice injected with growth hormone (DW+GH, black bars) at 5-6 months of age normalized to RNU6 (Left panel, One-way ANOVA p= 0.4139, n=8) or was normalized to 5S rRNA (Right panel, One-way ANOVA p=0.4631, n=7).

We examined transcription levels of the putative DNMT1 regulator miRNA 140-3p to ascertain whether GH administration would induce effects similar to that observed with DDX20 protein abundance (Figure 21). We did not see any significant effects when normalizing to U6 spliceosomal RNA (RNU6) or 5S ribosomal RNA (5S rRNA). Similar to our 3 and 12 month old miRNA studies, Ct

values or 5S rRNA were similar at 6 months of age in dwarf and wild type mice. The two studies produced very similar results indicating that miR-140-3p is not significantly changed following the administration of GH for one week.

Since DDX20 protein expression was enhanced in dwarf mice treated with growth hormone but DNMT1 levels were also increased, we measured the response of metallothionein expression and methylation following growth hormone treatment (Figure 23). Transcription of MT1 and 2 was increased in dwarf mice following one week of growth hormone injections, corresponding to the increase in DDX20 protein.

The methylation of several heavy metal response transcription factor binding sites including MRE-a,b,c,d, MTF-1, and ARE that are contained in the 177-328 base pair region of the MT1 CpG island, became more methylated with growth hormone treatment. There was no significant change found in the putative CpG island fragment between 464-570 base pairs of the MT1 gene region. There are two binding elements for the signal transducers and activators of transcription (STAT), one of which is shared with interleukin-6 (IL-6) a cytokine that can induce transcription. These binding sites are located within the first 150 base pairs of the MT1 gene, and were missed by the primer sets that probed from 166 to 570 base pairs of CpG dinucleotides we utilized in previous experiments. Therefore we designed an MT1 STAT primer pair set to probe this region as it may be sensitive to GH administration and thus activate the JAK/STAT pathway (Figure 23).

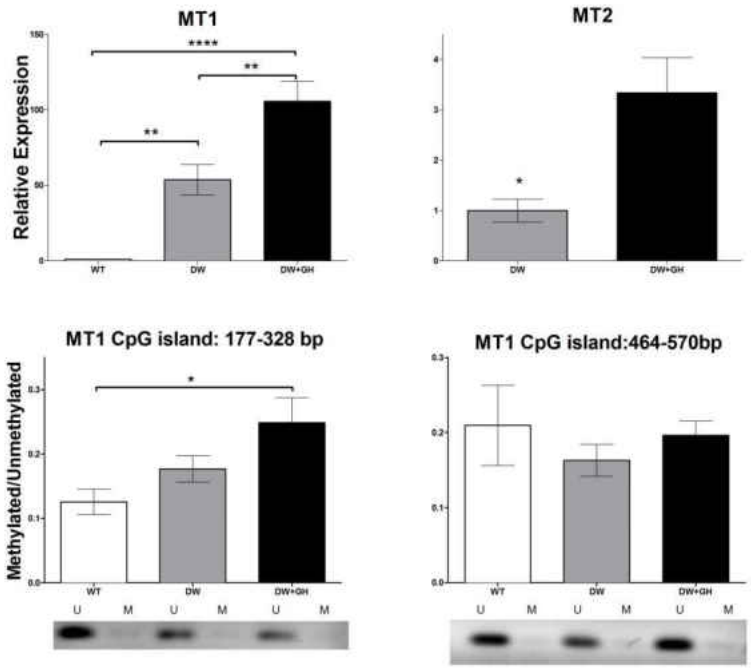

Figure 22. Growth hormone treatment increases MT1 and MT2 expression and changes methylation patterns in Ames dwarf mice. Upper-left panel shows MT1 mRNA expression for wild type mice injected with saline (WT, open bars), dwarf mice injected with saline (DW, gray bars), and dwarf mice injected with growth hormone (DW+GH, black bars) at 4-6 months of age (One-way ANOVA $p<0.0001$, n=9-10). Upper-right panel shows MT2 dwarf mice injected with saline, and dwarf mice injected with growth hormone at 5-6 months of age (n=8-10). Left-lower panel shows MT1 CpG Island: 177-328 bps methylation for wild type mice injected with saline, dwarf mice injected with saline and dwarf mice injected with growth hormone at 5-6 months of age (One-way ANOVA p= 0.0260,n=6-7). Lower-right panel shows MT1 CpG Island methylation for wild type mice injected with saline, dwarf mice injected with saline, and dwarf mice injected with growth hormone at 5-6 months of age (One-way ANOVA p= 0.6338,n=7). Mean relative changes in expression and optical densities are shown ± SEM. *$p<0.05$, **$p<0.01$, and ****$p<0.0001$.

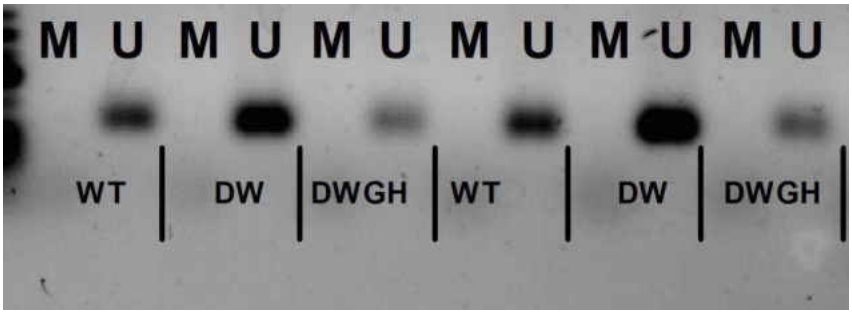

Figure 23. Panel shows two representative samples MT1 STAT binding CpG Island methylation. Samples are ordered as follows: wild type mice injected with saline (WT), dwarf mice injected with saline (DW), and dwarf mice injected with growth hormone at 5-6 months of age (n=6-7).

Based on the literature, a reasonable expectation would be that methylation should be diminished in this area at these two binding sites due to the actions of GH (Figure 23). However, using MS-PCR techniques and primer design, methylated and unmethylated regions were not quantifiable in relation to each other and thus no determinations could be made about methylation status of the STAT binding region of MT2.Even though a pattern is visually apparent when observing the unmethylated primers of the samples in relation to each other; this result must be validated by another technique such as pyrosequencing of the STAT binding site regions before this difference can be substantiated as non-artifact.

Histone markers associated with aging are differentially expressed and distributed differently in dwarf mice compared to age-matched wild type mice

Like DNMTs, histone modification enzymes also use methyl groups from SAM just as DNMT and therefore histone methylation marks are also subject to methionine pathway disruptions or alterations in enzyme activities or expression. Based on the literature we chose to examine histone methylation markers between Ames dwarf mice and age-matched wild type mice. Methylation markers from dwarf and wild type liver histone extracts were probed to determine the levels of histone H3 methylation.

Two repressive markers, histone H3 lysine 9 tri-methylation (H3K9me3) and histone H3 lysine 27 tri-methylation (H3K27me3), and one marker known for transcriptional activation histone H3 lysine 4 tri-methylation (H3K4me3), were measured using immunoblotting and coomassie staining (to confirm equal loading). Interestingly, histone H3 Pan, an antibody used to determine the total

amount of histone 3 was much higher in liver tissue dwarf mice at three months of age (~70%), lower at 12 months of age and was equal at 24 months of age when compared to age-matched wild type controls (Figure 24, top three panels).

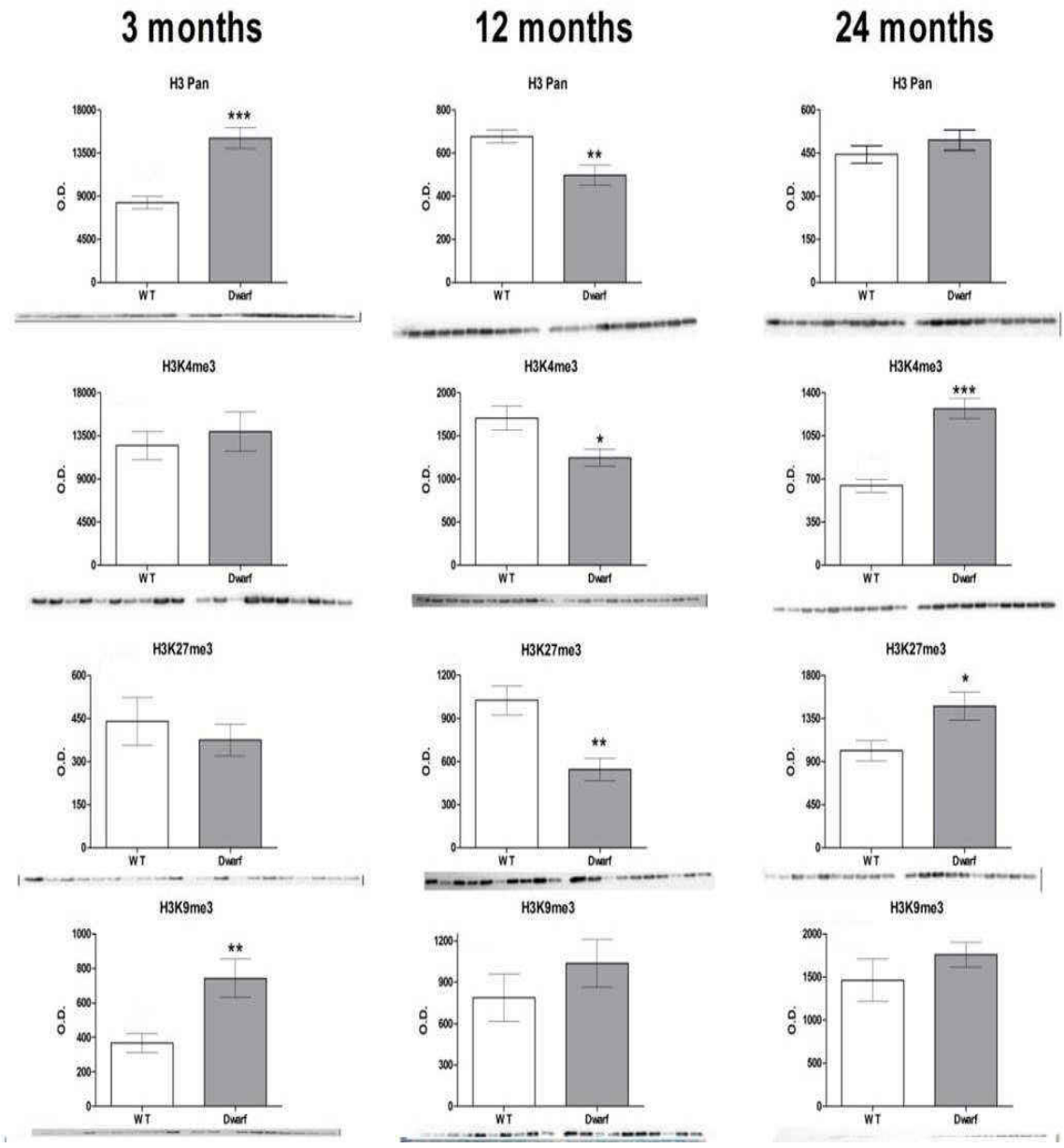

Figure 24. Histone H3 methylation and pan histone levels are expressed differently in Ames dwarf and wild type liver tissue at 3, 12, and 24 months of age. Wild type mice represented by open bars and dwarf mice by gray bars (n=7-10). Mean optical densities are shown ± SEM. *p<0.05, **p<0.01, and ***p<0.001.

Though used in many manuscripts as a loading control, total histone H3, has been shown to change with age, and is therefore dynamic. (Larson et al., 2012; Levine, Worrell, Zimnisky, & Schmauss, 2012). However, with each genotype, it appears that histone H3 fluctuates more with age in wild type mice than in dwarf mice (Figure 25).

For the transcriptionally active marker histone H3K4me3, total abundance in dwarf mice was equivalent at 3 months of age, significantly lower at 12 months, and higher at 24 months of age compared to age-matched wild type controls. Increases or decreases of this marker through mutations of methyltransferases or demethylases have marked effects on lower life forms such as *C. elegans* and *Drosophila* and have been noted to increase with aging (Han & Brunet, 2012). The repressive marker histone H3K27me3, is connected with levels of facultative heterochromatin, and this histone marker is purported to decrease with age (López-Otín et al., 2013). When dwarf mice were compared to wild type mice, this marker was observed to be decreased at 12 months of age, and significantly increased at 24 months of age. The constitutive H3K9me3 marker is significantly increased in dwarf mice at three months of age (~ 2 fold) only and only slightly higher at 12 and 24 months of age. This marker decreases with aging in worms (Berdasco & Esteller, 2012). When histone markers are normalized to H3 pan, this exposes the distribution of histone H3 for each genotype (Figure 26).

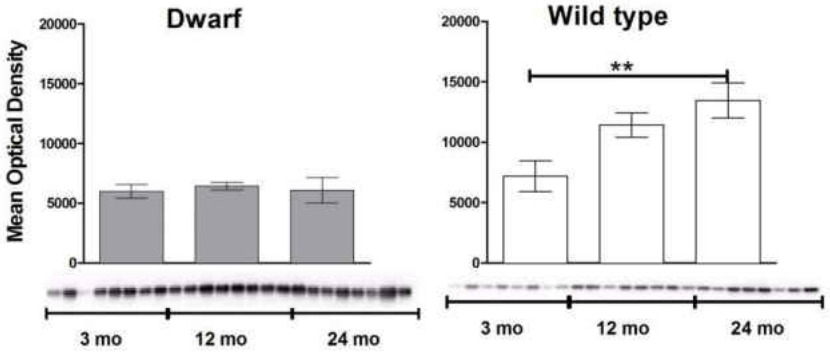

Figure 25. Age-related total histone H3 expression within Ames dwarf and wild type genotypes. In a separate experiment which compared total liver histone H3 abundance, levels appeared to have stayed equivalent in dwarf mice over all three age groups (left panel, grey bars One-way ANOVA p=0.7648, n=7-8) while levels increased linearly in wild type mice (right panel, open bars, One-way ANOVA p= 0.0065, n=8). Mean optical densities are shown ± SEM, **p<0.01.

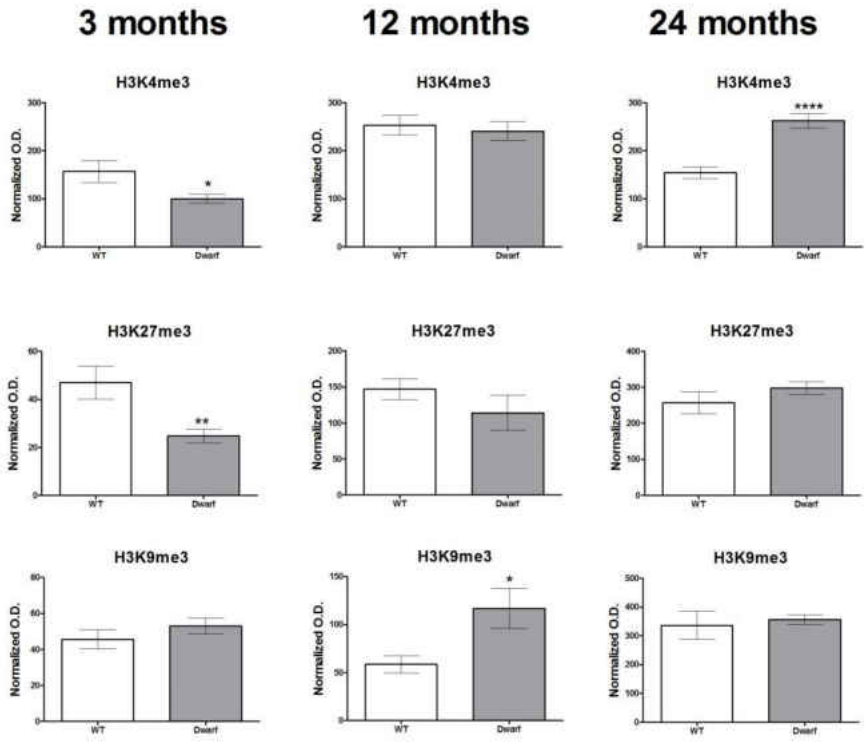

Figure 26. When normalized to H3 pan, Histone H3 methylation markers were distributed differently in dwarf mice at different ages. Histone tri-methylation expressed as normalized to total histone 3 for each age group, dwarf (gray bars) and wild type mice (open bars). Mean optical densities are shown ± SEM. *p<0.05, **p<0.01, and ****p<0.0001.

The abundance of H3 tri-methylation markers compared to total histone H3 shows that at three months of age, dwarf mice used less total histone H3 for the active transcription marker H3K4me3 and the facultative marker H3K27me3. However, amounts of H3K9me3 remained similar in dwarf and wild type mice. At twelve months of age the distribution of histone H3 to H3K9me3 was much higher in dwarf mice whereas the other markers distributions remained similar between genotypes. At 24 months of age, histone H3 is significantly increased in distribution to the marker H3K4me3 in dwarf liver compared to wild type mice.

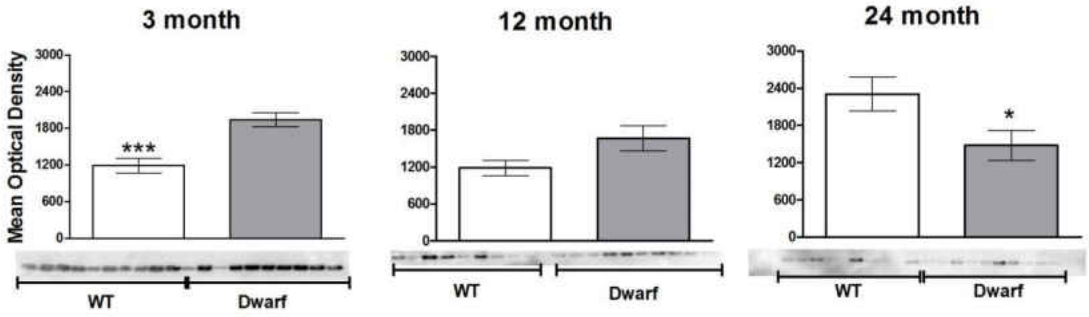

Figure 27. Histone H4 lysine 20 tri-methylation and are expressed differently in Ames dwarf and wild type liver tissue at 3, 12, and 24 months of age. Wild type mice represented by open bars and dwarf mice by gray bars. H4K20me3 protein expression was much higher that wild type mice at 3 months and lower at 24 months (n=7-10,). Mean optical densities are shown ± SEM. *p<0.05 and ***p<0.001.

We also probed liver histone extracts for histone H4K20me3 markers via immunoblotting (Figure 27). This transcriptionally repressive marker is known to increase with age in rat liver (Sarg, Koutzamani, Helliger, Rundquist, & Lindner, 2002). In our study dwarf mice at 3 months age had significantly more of the H4K20me3 marker compared to wild type mice (p=0.0005). However, at 24 months, the oldest age studied, dwarf mice had less H4K20me3 (p=0.0389).

Finally, in a pilot study, we examined 84 genes that included histone methyltransferases (HMTs) and lysine demethylases (KDMs) to ascertain possible targets for future mRNA and protein studies between dwarf and wild type mice (n=2/genotype; Table 5). Since we saw many significant differences at 3 months of age in epigenetic expression overall, we chose to examine 3 month old mice in this study. We observed a fold-increase of >2.5 in mRNA expression in many histone modifying enzymes in dwarf compared to wild type mice. Lysine demethylase 5b (Kdm5b) and histone methyltransferase Setdb2, highlighted in Table 5, are involved in demethylation of H3K4 tri-methylation and tri-methylation of H3K9, respectively. Both proteins have the potential to be repressors of transcription if more abundantly expressed at the translational level and are thus potential targets that help to explain our results in Figure 24. Whether GH modulates these enzymes, as suggested by the genotype differences, remains to be determined.

Table 5: Histone modification enzymes

Gene Symbol	Function	Fold-change (Dwarf/Wild type)
Ciita	Acetylation	2.65
Kat2a	Acetylation	2.59
Ncoa6	Acetylation	3.74
Hdac4	Deacetylation	2.98
Kdm5b	**Demethylation**	**2.91**
Prmt7	Methylation	2.70
Setd6	Methylation	2.83
Setdb2	**Methylation**	**3.20**
Nek6	Phosphorylation	2.74
Dzip3	Ubiquitination	2.89
Mysm1	Ubiquitination	2.86
Ube2a	Ubiquitination	2.86

DNA methylation enzymes are differentially expressed in other mutant mouse models with deficient or excessive GH signaling

To further explore the role of GH signaling, we examined another GH signaling mutant, the GHRKO mouse and the GH Tg mouse that exhibits an excess of GH signaling. These mice show respective increases and decreases in lifespan related to the absence or excessive GH signaling. This makes them very useful to our studies to elucidate any differences or similarities in epigenetic methylation or mechanism expression.

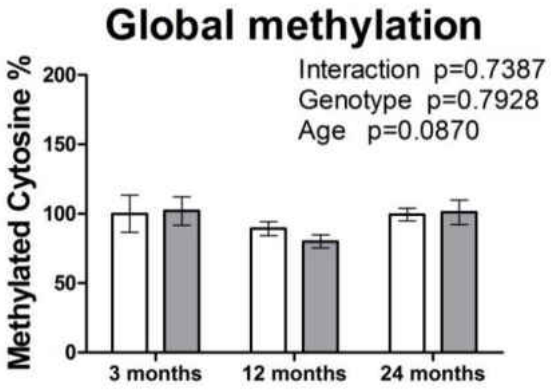

Figure 28. GHRKOmice show no genotypic difference in global DNA methylation compared to wild type mice. GHRKO represented by open bars and wild type mice by open bars (n=7-12). Percentage methylation was derived using control and sample optical densities, which were then normalized to 3 month wild type mice± SEM.

To see if overall DNA methylation was different between the two strains we performed the same colorimetric assay that was used for the dwarf line in a cross-sectional study. GHRKO mice at ages 3, 12, and 24 months were used along with their age-matched wild type counterparts. These mice exhibit high plasma levels of GH but are unable to respond to it due to a mutation in the GH receptor gene. We first studied global DNA methylation and interspersed repeat methylation to detect any similarities or differences with Ames dwarf mice (Figure

28). GHRKO mice showed no genotypic differences in global methylation, whereas dwarf mice tended to have high levels of methylation consistent across all three age groups (Figure 7). Our data also suggested age might be a factor ($p=0.0870$), but we were limited by sample size in the 24 month old group ($n=7$) at the time of this study. To probe for possible differences in interspersed repeat expression and methylation across age groups and genotype, we applied the McrBC-qPCR technique and measured hypomethylation of the repetitive elements IAP-LTR, LINE1, SINE B1, and SINE B2 (left panels, Figure 29). Similar to our observations in dwarf mice versus wild type mice, GHRKO mice also show some resistance to the hypomethylation of IAP-LTR. Age-related differences were also apparent (Figure 29, $p=0.0691$). In LINE1 there were genotype effects, especially at 24 months of age, and age effects of hypomethylation were observed however a significant interaction was detected that confounds the main effects. The wild type in this strain of mouse shows a large pattern of hypomethylation over age that was not observed in the Ames dwarf wild type mice and therefore could be influenced by genetic background. There was a significant interaction between genotypes and age in hypomethylation of SINE B1 in GHRKO and wild type mice. SINE B2 was only affected by age and not genotype, as these elements became hypomethylated in both genotypes. Similar to dwarf mice, there is higher transcription of IAP-LTR at three months of age that is significant between GHRKO mice compared to their wild type counterparts (Figure 29, right panels). The transcription of LINE1 is affected independently by both age and genotype.

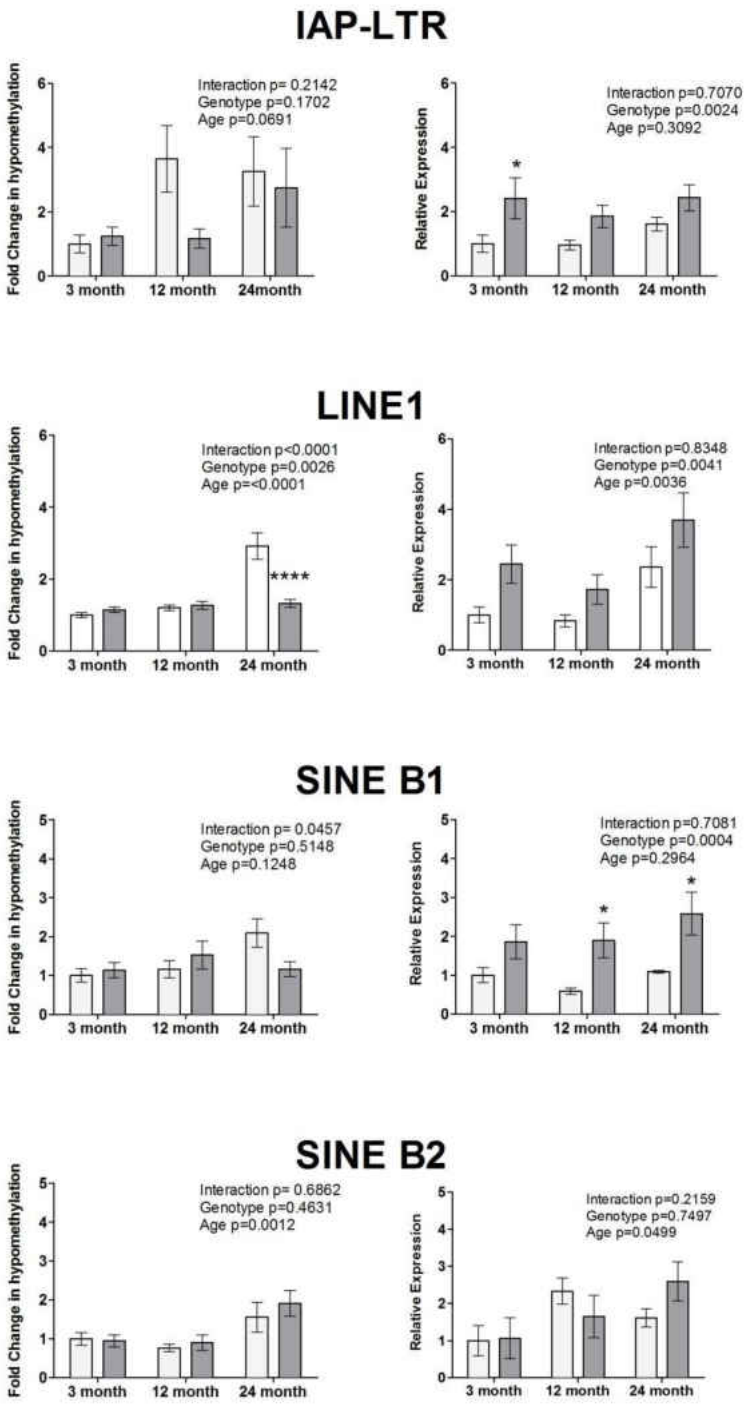

Figure 29. GHRKO mice show differences in hypomethylation and transcriptional expression of repetitive elements that may contribute to genetic stability. Using the McrBC-qPCR technique differences in liver genomic DNA hypomethylation of four IRs are shown for GHRKO(gray bars) and wild type mice (open bars, left panels, n=6-8). Liver IR mRNA expression is shown using two step RT-PCR at 3, 12, and 24 months of age (right panels, n=6-8). Mean relative changes in expression or hypomethylation are shown ± SEM. *p<0.05 and **p<0.01.

Overall, transcription in the GHRKO mouse is higher compared to wild type mice. For the dwarf mouse strain, primarily age effects were seen for the cross-sectional study. SINE B1 transcription is significantly higher for GHRKO mice at both 12 and 24 months of age, for Ames dwarf mice, this increased transcription was only significant at 24 months of age. No age-related differences in SINE B1 were observed in the GHRKO but genotype significantly affected transcription. SINE B2 also showed age-related changes in the GHRKO strain, similar to the Ames dwarf strain, although the transcriptional profile is different. Ames dwarf mice along with their wild type siblings present a consistent u-shaped transcriptional profile between the ages of 3, 12, and 24 months whereas GHRKO mice do not.

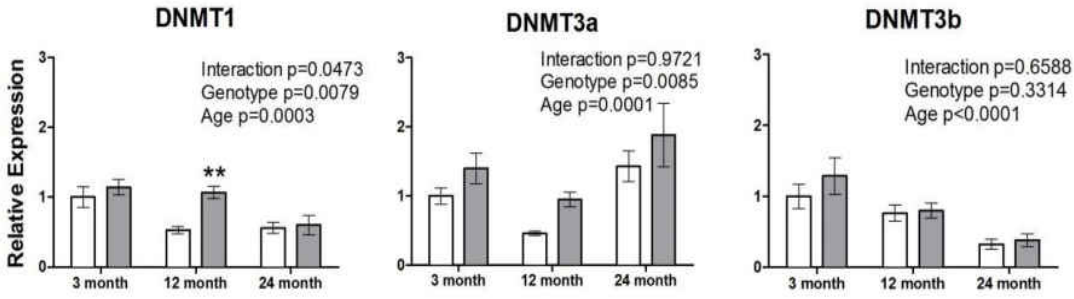

Figure 30. DNMT1 and DNMT3a mRNA expression are higher GHRKO mice at 12 months of age. Liver DNMT mRNA expression is shown for wild type (open bars) and GHRKO (gray bars) at 3, 12, and 24 months of age (n=5-8) for DNMT1, DNMT3a, and DNMT3b. Mean relative changes in expression are shown ± SEM. **p<0.01.

Twelve month old GHRKO mice exhibited alterations in transcriptional levels of GNMT and other methionine related enzymes, thus we measured the cross-sectional DNMT expression to determine if there were genotype differences in transcription between GHRKO and wild type mice (Brown-Borg et al., 2009). The results in Figure 30 show that DNMT1 and DNMT3a gene

transcription is higher at 12 months of age in the GHRKO mutant mice compared to wild type mice of the same age. Similar to Ames dwarf mice, DNMT3b expression was not different between genotypes, but was impacted by age in this strain of mouse. However, it is interesting to note that while DNMT3b transcription seems to increase with age in the Ames dwarf line, the expression in the GHRKO strain decreases with age, demonstrating the importance of the genetic background of our mice on epigenetic mechanisms.

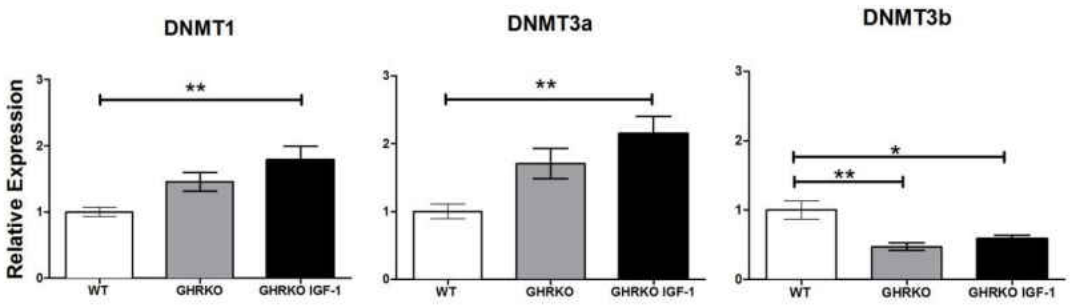

Figure 31. Liver DNMT mRNA expression For DNMT1 and DNMT3a is increased upon IGF-1 administration in GHRKO mice. Shown for wild type mice injected with saline (n=7-9, open bars), GHRKOmice injected with saline (n=7-9, gray bars), and GHRKOmice injected with IGF-1 (n=8-9, black bars) at 4-6 months of age. One-way ANOVA for DNMT1 p=0.0056, for DNMT3a p=0.0026, and for DNMT3b p=0.0009). Bars represent means ± SEM. $p<0.05$ and $^{**}p<0.01$.

GHRKO mice express growth hormone but lack a functional receptor, therefore secretion of IGF-1 from the liver is absent. This strain of mouse allows the unique opportunity of identifying and observing potential independent effects of IGF-1 apart from GH. We therefore decided to measure DNMT transcriptional expression in GHRKO mice that have been administered IGF-1 in the same manner we studied GH in Ames dwarf mice. These mice were also treated at 4-6 months of age. Interestingly, DNMT1 displayed a small increase in transcription in IGF-1 treated GHRKO mice which without treatment, already showed a greater

mRNA levels compared to age-matched wild type mice at 6 months of age. This finding is similar to that observed in GH injected dwarf mice. DNMT3a transcription, was increased in GHRKO mice, was further elevated by IGF-1 administration, whereas in dwarf mice it was not affected by GH. Surprisingly, at 6 months of age, GHRKO mice expressed less DNMT3b mRNA compared to wild type mice and these levels remained unaltered with IGF-1 treatment. In comparison to dwarf mice DNMT3b was more highly expressed basally, and increased even more with GH administration.

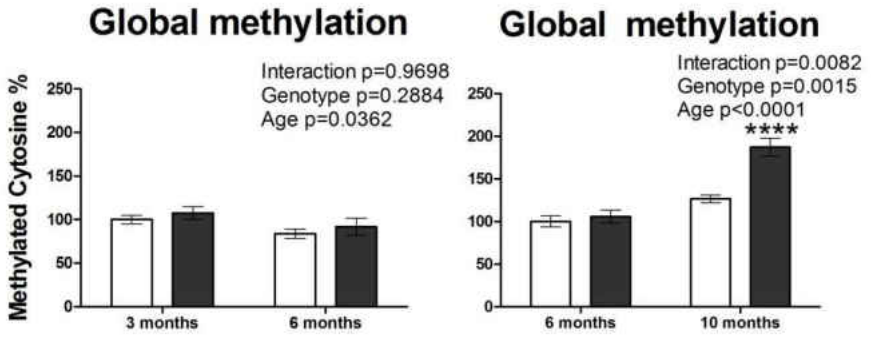

Figure 32. GH Tg mice show a genotypic difference in global DNA methylation compared to wild type mice at 10 months of age. GH Tg mice are represented by black bars and wild type mice by open bars (n=7-12). Percentage methylation was derived using control and sample optical densities, which were then normalized to 3 (left) or 6 (right) month wild type mice± SEM.

Growth hormone transgenic mice (GH Tg) are an excellent model to study the overexpression of GH *in vivo* on epigenetic mechanisms. Growth hormone transgenic mice express approximately 20-50 fold more growth hormone measured by radioimmunoassay compared to wild type control mice (Sotelo et al., 1998). GH Tg mice also express more premature aging phenotypes which are readily apparent as early as 6 months of age and live approximately half as long (12 months) as their wild type counterparts (Steger et al., 1993; Steger et

al., 1994). We were interested in evaluating epigenetic differences in GH Tg mice compared to their wild type counterparts. Because GH Tg mice exhibit excessive liver tumors at 12 months of age, which is near median lifespan, we chose to study these animals at earlier ages (3, 6, and 10 months). Unlike the Ames dwarf and GHRKO mouse assays, assays were not pooled due to excessive variability between 3 and 6 month and 6 and 10 month assays. The mean raw absorbance values for the 6 month wild type mice used in the first assay were 0.1882±0.01240, and the same wild type samples measured 0.1386±0.0092 for the second assay (p= 0.0123, Student's t test). We saw no real differences between genotype, although there was a potential age-related difference (Figure 32). However, at 10 months of age, GH Tg mice exhibited significantly increased amounts of global DNA methylation in liver tissue when compared to wild type mice, suggesting a large shift in methylation enzyme activity. The assay between 6 and 10 months also showed a potential age difference between the two groups, but effects of age and genotype were interacting confounding any independent effects.

We next studied the hypomethylation of GH Tg mice in interspersed repeats probing for possible genetic instability as we had in dwarf and GHRKO mice (left panels, Figure 33). Whereas there were lower levels of hypomethylation in dwarf mice and similar levels in GHRKO mice at 3 months of age, compared to their respective age-matched controls, GH Tg mice start out with more hypomethylation, which is essentially less methylation, of these elements in young adulthood.

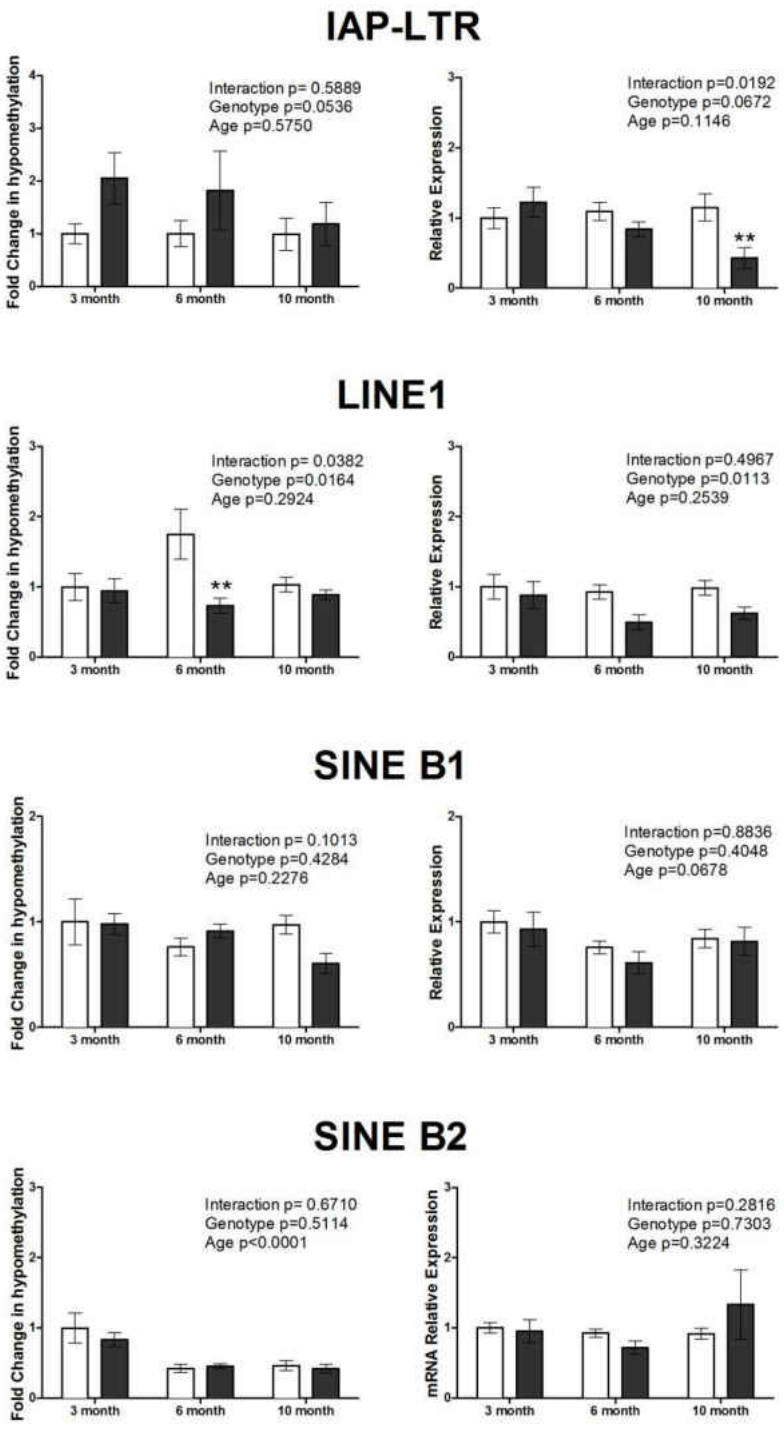

Figure 33. GH Tg mice show differences in hypomethylation and transcriptional expression of repetitive elements that may contribute to genetic stability. Using the McrBC-qPCR technique differences in liver genomic DNA hypomethylation of four IRs are shown for GH Tg (black bars) and wild type mice (open bars) (left panels, n=6-8). Liver IR mRNA expression is shown using two step RT-PCR at 3, 12, and 24 months of age (right panels, n=7-8). Mean relative changes in expression or hypomethylation are shown ± SEM. **p<0.01.

This genotype difference appears linear with age and approaches significance (p=0.0575) whereas age is not seen as a factor possibly due to the loss of differences seen in the wild type mice which age normally. In LINE1 elements GH Tg mice are surprisingly stable in methylation, while in wild type mice a significant increase in hypomethylation of this element is detected at 6 months of age. However, genotype differences are confounded by interactions with age effects even though age itself as a factor was not significant. SINE B1 did not show significant changes in methylation due to genotype or age. SINE B2 showed a marked difference of hypomethylation in this strain of mouse shared between the GH Tg mutants and wild type counterparts which was dependent on age, nevertheless, there were interactions between the factor approaching significance (p=0.0671).

We also examined transcriptional differences in the four repetitive elements (right panels, Figure 33). Interestingly, whereas the longer lived mutant mice tended to show more transcription of IAP-LTR at 3 months of age, GH Tg mice had similar transcription until 10 months of age. At this older age transcription dropped noticeably in the GH Tg mice. There were no differences in transcription attributable to age in these mice, but an interaction between age and genotype was apparent. For the interspersed repeat, LINE1, transcription was lower at 6 and 10 months in the GH Tg mice compared to their wild type controls. This contrasted with our other mouse lines where dwarf mice were similar in expression and GHRKO mice had increased transcription in LINE1. Also the GH deficient mutant strains showed age-related differences in

expression, whereas this line does not. Still, it must be noted that these studies are at relatively young ages for the normal mice of this strain, which may have to be studied more thoroughly at a later date for comparison in age to other strains. Transcription of SINE B1 did not show any differences attributable to genotype, as had been seen previously in both GH deficient signaling strains where transcription was increased and age-related differences in expression approached significance (p=0.0678). In the repetitive element, SINE B2, we did not observe age or genotype related differences in mRNA expression.

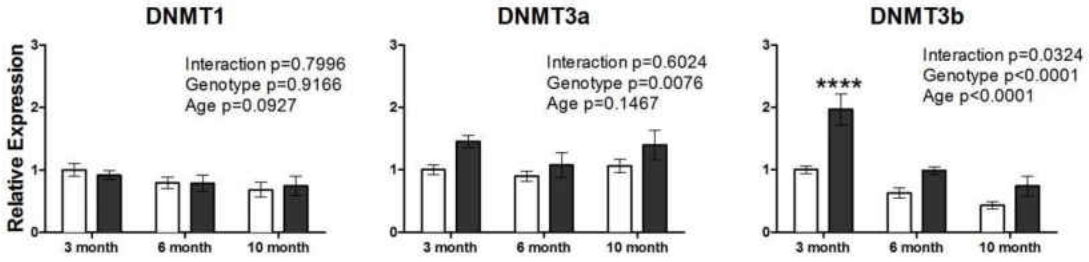

Figure 34. DNMT3b mRNA expression was higher in GH Tg mice at 3 months of age. Liver DNMT mRNA expression is shown for wild type (open bars) and GH Tg mice (black bars) at 3, 12, and 24 months of age for DNMT1, DNMT3a, and DNMT3b (n=6-8). Mean relative changes in expression are shown ± SEM. ****p<0.0001.

We evaluated DNMT transcription in GH Tg mice at 3, 6, and 10 months in a cross-sectional study, similar to our GH signaling deficient mouse strains with their age-matched wild types. For DNMT1 there were no genotype differences in transcription, in contrast to our previous experiments in GH signaling deficient mice. DNMT3a however showed significant genotype differences in transcription with higher levels overall in the GH Tg mice at any particular age. Age-related effects were not seen for this transcript. However, in stark contrast to both GH signaling deficient genotypes, DNMT3b transcription was approximately 2-fold

higher in GH Tg mice at 3 months of age. Age and genotype effects were significant along with a significant confounding interaction between the two factors. We completed our studies with some preliminary data on DNMT protein expression in GH Tg mice versus their wild type counterparts. Interestingly, DNMT1 expression is decreased in GH Tg mice at 10 months of age, DNMT3a expression lower in GH Tg mice at every age compared to age-matched wild type mice, and DNMT3b remains to be probed.

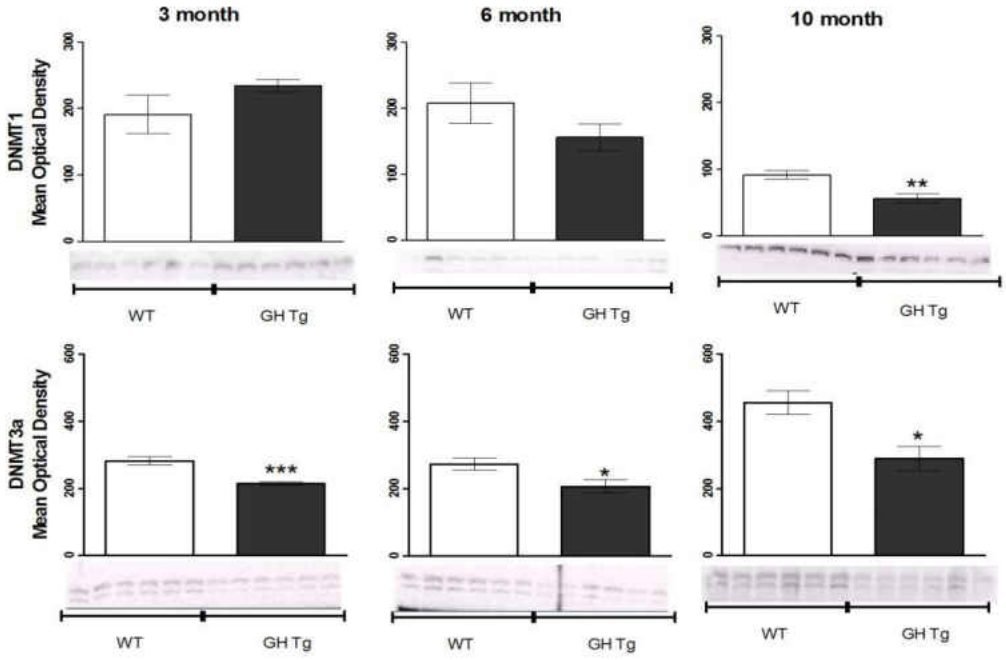

Figure 35. DNMT protein levels are expressed differently in GH Tg and wild type liver tissue at 3, 6, and 12 months of age. Wild type mice are represented by open bars and GH Tg by black bars. DNMT1 protein expression was much lower than wild type mice at 10 months (n=5-6, p=0.004). DNMT3a protein was significantly Lower in GH Tg mice at 3 months (n=5-6, p=0.0007), at 6 months (n=5-6, p=0.0397) and lower in GH Tg mice at 24 months (n=8, p=0.0172). DNMT3b protein was not different between the genotypes at 3 months (n=10, p<0.4000), 12 months (n=11-12, p=0.6244) or 24 months (n=9-10, p=0.5621). Mean optical densities are shown ± SEM. *p<0.05, **p<0.01, and ***p<0.001.

Thus for each parameter evaluated in the GH Tg mice epigenetic markers, mechanisms, and elements contrast with expression and methylation observed in the GH signaling deficient mice. These findings suggest that GH signaling pathways influence DNA methylation patterns and the expression of epigenetic methylation mechanisms.

CHAPTER IV

DISCUSSION

The main hypothesis of this study was that long-living Ames dwarf mice exhibit differences in epigenetic mechanisms and markers, specifically DNA and histone methylation. The rationale supporting this hypothesis was that Ames mice have an altered methionine pathway that is known to contribute to epigenetic processes. Ames mice are GH-deficient and GH has been shown to affect expression of components of methionine metabolism. The second part of this hypothesis was that these alterations in epigenetic markers lead to increased genetic and epigenetic stability in dwarf mice. Evidence in the literature consistent with our findings will be discussed. The epigenetic patterns established in the dwarf appear to remain highly stable through the dwarf life span, protect the DNA from damage, reduce transcriptional noise, and participate in an altered pattern of gene expression that resists damage and diseases associated with aging, favoring the longevity of Ames dwarf mice. This is a novel hypothesis since as to date, the preponderance of epigenetic studies and seminal literature has been studied in diseased or pre-disease states. This dissertation lays essential foundational groundwork to elucidate the epigenetic markers and mechanisms that promote longevity and disease resistance, and

how the master hormone, growth hormone, a chiefly metabolic regulator, can drastically affect methylation modifications in the epigenetic landscape.

The first aim of our study was to examine age-related and genotype differences in DNA methylation enzymes and global DNA methylation and differences of transposable element methylation and expression that could contribute to genetic stability in Ames dwarf mice compared to wild type mice. GNMT expression affects DNA methylation so we examined whether GNMT expression was positively correlated with DNMT epigenetic enzyme expression in a cross-sectional aging study (Martínez-Chantar et al., 2008; Rowling et al., 2002; Y. Wang et al., 2011). Activation or suppression of the GNMT enzyme has been implicated in many studies involved in global DNA methylation changes. We also studied whether this potential competitor for methyl groups changed expression with age. We found while GNMT mRNA expression was age-related in dwarf and wild-type mice, our data did not suggest any correlative relationship to DNMT mRNA expression. Then we examined the abundance and activity of DNMT protein between three age groups, and global abundance of DNA methylation. We found less DNMT1 protein expression in Ames dwarf mice compared to wild type controls and also that DNMT3a protein expressional differences in dwarf mice were reflected in overall DNMT activity levels. Next we evaluated transposable elements (IAP-LTR, LINE-1, SINE B1, and SINE B2) whose hypomethylation or expression is thought to affect transcriptional function and genetic stability as it relates to aging (Asada et al., 2006; Valentina Bollati et al., 2009; Gentilini et al., 2012; Jintaridth & Mutirangura, 2010; Mustafina, 2013).

In these repetitive areas we found interesting differences due to genotype and age when studying Ames dwarf mice versus their wild type counterparts. We also employed another technique to investigate the differences found in LINE-1 between the two genotypes at different ages.

Our second aim was to establish whether growth hormone administration to a GH deficient mouse model (Ames dwarf) affected DNA methylation and the enzymatic mechanisms responsible. We have looked on a global scale to define major differences in possible GH regulation of epigenetic methylation processes. A sub-aim was to validate the use of primary hepatocyte culture for specific pathway studies by testing whether dwarf mice showed any alterations in DNMT signaling at the hepatocyte level similar to the *in vivo* results. We found that hepatocyte primary cultures reflected the differences seen in expression in the *in vivo* experiments.. In addition, we explored one possible and novel pathway of DNMT post-transcriptional regulation that has been very recently introduced and never studied in the context of a longevity mouse model as an additional sub aim based on our findings and whether it was modulated by GH administration.

For our third aim, we characterized basal histone methylation marker differences in a cross-sectional study between Ames dwarf and age-matched wild type mice and identified possible histone modification enzyme targets for future study. Our final aim consisted of characterizing other mutant mouse strains with altered GH signaling. Within this aim, we utilized the Laron dwarf or growth hormone receptor binding protein knockout mouse and the GH transgenic mouse. This allowed us to identify possible similarities between longevity models

(Ames dwarf and GHRKO mice) and a short-lived premature aging model (GH Tg mice), and further establish the potential role of growth hormone in processes affecting the expression of epigenetic markers and in epigenetic stability.

Specific Aim 1- To examine GNMT, DNMT expression, DNMT activity, and global DNA methylation cross-sectionally in Ames dwarf and age-matched wild type mice

Several lines of long-living mice display deficiencies in growth hormone or growth hormone signaling (Brown-Borg, 2009). Growth hormone influences the methionine pathway by decreasing the expression and activity of several enzymes, including methionine adenosyltransferase and glycine-*N*-methyltransferase, which promote the production of S-adenosylmethionine and S-adenosylhomocysteine, respectively. Glycine-*N*-methyltransferase is the most abundant protein in the liver, about 1% of total protein, and facilitates the conversion of SAM to SAH. Thus, GNMT is a major regulator of SAM in the liver(Yeo & Wagner, 1994). S-adenosylmethionine is the major methyl donor for most methyltransferase reactions in cells. While many methyltransferases, including DNMTs, are negatively regulated by rising SAH levels, GNMT is thought to regulate DNMT by an unknown mechanism (Wang, Tang, Chen, Chen, & Chiang, 2011). Previously, we showed that GNMT activity decreased between 3 and 12 months of age in Ames dwarf mice (Holly M Brown-Borg et al., 2005). In our first aim, we investigated cross-sectional GNMT expression in both genotypes, looking for age-related differences, and expanded our findings to include protein levels. We found age-related decreases in GNMT mRNA expression in both genotypes and confirmed increased GNMT gene expression

in dwarf mice at all three age groups, most significant at 3 and 12 months of age, in comparison to wild type mice (Figure 1). Protein levels of GNMT were significantly elevated in dwarf mice (12 months) therefore, our GNMT expression relates well with current literature and known GNMT activity level differences between dwarf and wild type mice (Brown-Borg et al., 2009; Holly M Brown-Borg et al., 2005)

To determine if alterations in DNMTs were associated with GNMT expression, we examined DNMTs in liver tissue of GH-deficient dwarf and normal age-matched wild type mice (Figure 2 and 4). Interestingly, all three known catalytically active DNMTs (1, 3a, and 3b) showed a significant difference in age-related transcriptional expression but did not correlate with the linear decrease exhibited in GNMT mRNA expression. Though in dwarf mice, DNMT1 and 3a expression was significantly higher at 3 months of age and decreased thereafter. In both genotypes, the lowest levels of DNMT mRNA were observed at 12 months of age with a subsequent increase at 24 months, indicating a u-shaped pattern of age-related expression. In dwarf mice, the highest levels of DNMT1 and DNMT3a were expressed in the youngest mice. Consequently, the expression of methyltransferases may be related to aging in both long-living and normal mice.

The elevated DNMT transcription in 3-month-old dwarf mice is a novel finding. Compared to the present literature, very few studies have reported age-related differences in murine DNMT expression. Similar to our findings, DNMT1-deficient mouse T cells showed a decrease in DNMT1 transcriptional expression

with age (Ray et al., 2006). However this landmark study used histone H4 as the normalizing transcript to rule out increased cellular proliferation skewing the findings. We also tested H4 in comparison to our normal standardization transcript β2m to see if cellular proliferation could have influenced our results (Figure 3). We found that alteration of cellular proliferation was not a factor in our results and therefore β2m was used for normalization of all DNMT transcription between mice. Folate and methyl-deficient diets altered methionine metabolism in rats that, in turn, also decreased DNMT1 and 3a gene expression with age (Ghoshal et al., 2006). This evidence corroborates a link between DNMT transcription, the methionine pathway, and age-related expression of DNMTs.

To determine if DNMT transcription was predictive of DNMT protein abundance, immunoblotting for all three DNMT families was implemented. Liver protein levels of DNMT1 in dwarf mice were much lower than in their wild type counterparts at every age examined, even though transcriptional expression was significantly higher in the dwarf (Figure 4). These particular protein assays were designed to detect primarily genotype differences and maximize sample numbers thus; age-related differences were ascertained later in a different experiment (Figure 5). DNA methyltransferase 3a protein expression in our cross-sectional study also yielded novel results. At 3 months of age, dwarf mice had considerably more of this enzyme (almost 2-fold), but equivalent amounts as wild type mice at 12 months of age. Also at 24 months of age, dwarf mice had approximately half of the amount of protein expressed compared to age-matched wild type counterparts. In previous literature, a comparison between long-living

(C57Bl/6) and short-living (DBA2J) mouse strains indicated that liver DNMT1 decreased in both lines when fed a lipogenic methyl-deficient diet but the longer living C57Bl/6 mice showed higher liver DNMT3a protein levels. High levels of DNMT3a were thought to be protective against the more pronounced demethylation of repetitive elements observed in the DBA2J thereby protecting epigenetic stability (Pogribny et al., 2009). Additionally, ablation of DNMT3a in the nervous system of mice causes shortened lifespans even though at birth they appear healthy and similarly shortens the lifespan of conditional *K-ras* knockout mice (Gao et al., 2011; Nguyen et al., 2007). Our results in addition to previous literature suggest that DNMT3a may be important to longevity and aging.

Our studies also included DNMT protein expression of DNMT1 and DNMT3a within each genotype to determine age related-differences. Re-optimization of both protein amounts and antibody concentrations was conducted based on our previous finding that DNMT1 protein expression was extremely low in dwarf mice. We observed DNMT1 protein expression increases in both genotypes with age although the change across the three age groups was statistically more dramatic in dwarf mice (Figure 5). DNA methyltransferase 3a levels increased in wild type mice with age and decreased in dwarf mice. However the constraint in sample numbers per gel (n=8) may have undermined any statistical significance for this enzyme. Mutant mice deficient in DNMT1 expression showed no detriments or benefits to longevity (Ray et al., 2006). DNMT1 deficient mice exhibited delayed autoimmunity due to aging. They also exhibited protective effects from cancer

or exhibited a higher incidence of cancer compared to wild type mice depending on the organ studied (Ray et al., 2006). Therefore, DNMT1 does not appear to have any independent effects in regards to lifespan. In totum, our data indicates that DNMT3a levels in young mice may indeed be protective but expressed later in life may be detrimental, thus DNMT3a protein abundance during aging may correlate with longevity. Whether the lower expression of DNMT1 is required for this action still remains to be verified. Finally, whether Ames dwarf mice exhibit low DNMT1 in other tissues remains to be studied but because methylation enzymes and global DNA methylation changes with age are tissue specific, we suspect that DNMT1 and DNMT3a differences will be tissue-specific in our animals as well (Maegawa et al., 2010).

In general, DNMT1 is considered the main DNMT expressed in adulthood (and somatic cells) and is responsible for maintaining DNA methylation therefore, it is considered the largest contributor to DNMT activity. Overall, liver DNMT activity was comparatively higher in the young and lower in old dwarf mice compared to age-matched controls (Figure 6). The pattern of activity in dwarf mice may be significantly affected by the contribution DNMT3a since DNMT1 protein is present at markedly lower levels in dwarf mice. Increased DNMT activity has been widely associated with hepatocellular carcinoma, loss of GNMT expression, and methyl-deficient diets (Y. Wang et al., 2011). However, tumor initiation and progression are controlled by the magnitude and timing of DNMT protein expression changes that can cause substantial alterations in the disease

phenotype (Gao et al., 2011; Kinney et al., 2010; Yamada et al., 2005). A diminished level of DNMT activity concomitant with high GNMT expression in dwarf mice during old age might delay cancer and aging in dwarf mice in comparison to wild type mice. It is also possible that DNMT activity is under greater inhibition in the liver of 24-month-old dwarf mice due to higher SAH levels. Nonetheless, in young dwarf animals, increased DNMT3a may override SAH inhibition and GNMT competition for SAM in the liver allowing greater DNMT activity. From another perspective, early life over-expression of DNMT3a (before and during 3 months of age) may set up a more stable methylation pattern for many genes which are maintained and become larger in magnitude with age, as some research demonstrates, regardless of DNMT protein or activity changes later on (Takasugi, 2011).

Global methylation was also examined in Ames dwarf mice to determine the impact DNMT expression may have on the global DNA methylation landscape (Figure 7). According to current literature, increased DNMT activity in the liver due to methyl-deficient diets can lead to global DNA hypomethylation, whereas, in hepatocellular carcinoma cells, the administration of glucose and insulin can increase DNMT activity and global methylation. The induction of GNMT expression has opposite effects (Chiang et al., 2009; Pogribny et al., 2004; Wang et al., 2011). Ames dwarf mice retain high insulin sensitivity, high GNMT expression, and low levels of DNMT1 throughout aging, making it difficult to predict whether these mice would show any difference in global methylation in the liver. In addition,

through earlier work, we found no differences in DNA methylation between 18-month-old Ames dwarf and wild type mice (Uthus & Brown-Borg, 2003). In this cross-sectional study, DNA methylation in Ames mice was similar or higher than that observed in wild type mice at all three ages (Figure 9; Armstrong, et al., 2013). Wild type mice exhibited a u-shaped pattern in global DNA methylation comparable to their DNMT transcription; however, overall DNA methylation in dwarf mice remained relatively stable. Consistent with the earlier study using 18 month old mice, the older 24-month-old groups did not show any difference between genotype. Also our methylation results showing approximately 16% more methylation in dwarf mice at 12 months of age was consistent with Snell dwarf global methylation measurements at 9-12 months of age (Armstrong et al., 2013; Vitvitsky et al., 2013). Snell dwarf mice are phenotypically identical to the Ames dwarf but have a single nucleotide mutation in *pit-1*. This gene is a transcript acting downstream of *prop-1* (the mutation in Ames dwarf mice) which is also responsible for the development of somatotropes, lactotropes and thyrotropes in the anterior pituitary. Snell dwarf mice also do not secrete thyroxin stimulating hormone, prolactin, or growth hormone, and live longer than their wild type counterparts similar to dwarf mice (Bartke et al., 2001). Therefore, the similarity of global methylation between the two dwarf populations at similar ages are strong validation for our results, and suggests that these results are independent of background strain.

We did not see a linear progression in hypomethylation in this particular strain of mice, which has a heterogenous background, contrary to many laboratory strains. In a widely cited landmark study, C57Bl/6 mouse liver was shown to lose DNA methylation with age using a linear model (Wilson, Smith, Mag, & Cutler, 1987). However, in that same study older mice showed large variations in methylation measurements after 20 months of age. In addition, another report within the same timeframe found a u-shaped pattern of global methylation using a different technique in the same strain of mice indicating that methylation in the liver does not necessarily decrease in a linear fashion with age (Singhal et al., 1987). Despite the contradiction in studies that may be due to murine strain background, the main finding of this study is that dwarf mice appear resistant to age-related methylation changes due to age compared to wild type mice.

DNMT1 is required for the activity of *de novo* methylation of repetitive elements that make up a large part of murine and human DNA (Liang et al., 2002). This, as stated earlier, is important to the maintenance of genetic stability that occurs with retroviral transcription and possible transposition. We investigated whether the genotype differences discovered in our study contribute to epigenetic stability as postulated (Figure 8). Also, studying retroviral element methylation may give a different picture of the methylation landscape since LTRs and LINEs are found in gene poor regions (rich in A-T sequences) and SINEs are found in gene rich regions (higher frequency of C-G sequences) (Tomilin, 2008). Many of these repeats are expressed during embryonic development and

reduced in expression during induced differentiation (Martens et al., 2005; Tomilin, 2008). This indicates they could play a regulatory role even though the functions are unknown. Interestingly, we found differences in the methylation status of IAP-LTR, LINE-1 and SINE B2 between dwarf and wild type mice due to age and/or genotype (some differences were nearly statistically significant). We also found transcription of all four retroviral elements to be affected by age but found genotype differences only in SINE B1 transcription. Particular transcripts of the IAP retroviral element have been known for some time to increase with aging in mice and become hypomethylated with age in different mouse strains and from methyl-deficient diets (Dupressoir, Puech, & Heidmann, 1995; Mays-Hoopes, Brown, & Huang, 1983; Pogribny et al., 2009). We found that the IAP-LTR of wild type mice was hypomethylated with age relative to the Ames dwarf, and remained less methylated with age. Interestingly, dwarf mice regain some methylation at 24 months of age. Age and genotype are independent factors that significantly influence the methylation of this transposable element. Transcription of IAP-LTR, which by earlier studies was thought to be induced by hypomethylation, did not correlate with methylation loss or gain. In fact, we observed the u-shaped transcription expression remarkably similar to DNMT mRNA expression in all of our retroviral elements for both wild type and dwarf mice. Transcription of IAP-LTR was higher in dwarf mice at 3 months of age, though not statistically significant, and was definitively influenced by age for both genotypes.

LINE-1 is the most abundant transposable element in the murine and human genome (Han, Szak, & Boeke, 2004; Waterston et al., 2002). Increased LINE-1 expression or hypomethylation with age have been noted in several studies across many species and disease states including rats, mice, humans, hepatocellular carcinomas, and methyl deficient diets (Asada et al., 2006; Pogribny et al., 2009; Takai, Yagi, Habib, Sugimura, & Ushijima, 2000). LINE-1 also plays a role in cellular senescence (Baker & Sedivy, 2013). However, age-related LINE-1 hypomethylation in human studies has had mixed results and more recent studies have found *Alu* repeats are better markers of age-related hypomethylation (Valentina Bollati et al., 2009; Langevin et al., 2011). LINE-1 hypomethylation in our Ames dwarf mice or their wild type controls did not show age-related statistical differences. Genotype differences come close to significance, with more hypomethylation being present in dwarf mice at 3 and 12 months of age using a methylation restriction PCR technique (McrBC-qPCR) (Figure 8). When LINE-1 was further investigated through methylation specific-PCR, in which more than one primer set can be designed for better coverage of the 5' UTR region, we found that one area from 150-324 base pairs of this region was significantly less methylated, at three months of age (Figure 9). Also this area of LINE-1 showed nearly equivalent methylation at 12 months of age, and more methylation at 24 months of age in dwarf mice. This validates the changes seen at 3 months in the dwarf mice through McrBC-qPCR. It appears for dwarf mice LINE-1 methylation is age-related possibly indicating better maintenance of methylation in gene poor regions in old age. LINE-1 transcription has been

observed to disrupt genome stability, transcription, and translation in other human and mouse cell types from aging, cellular senescence, or age-related diseases (De Cecco et al., 2013; Han et al., 2004; Muotri et al., 2010; Neidhart et al., 2000; Oricchio et al., 2007). However, as in IAP-LTR transcription, LINE-1 transcription did not appear to be regulated by methylation and changed with age similarly in dwarf and age-matched wild type controls. Our transcription data correlates with a recent publication that examined the transcription of interspersed repeats in C57Bl/6 liver at early and late ages (De Cecco, Criscione, Peterson, et al., 2013). However, LINE-1 and the other repetitive elements diminish transcription at 12 months of age regardless of genotype; this may be an indicator of an important period in adult development whose regulatory mechanisms are unknown.

Murine SINE B1 is homologous to the human *Alu* repeat which has been associated with aging. SINE B1 in the liver also loses methylation in rodents fed methyl-deficient diets. Its transcription can be increased during embryonic implantation, by retinoic acid induced differentiation, and cellular or physiological stress (Kalkkila et al., 2004; Li et al., 1999; Liu et al.,1995; Martens et al., 2005; Ohnishi et al., 2012; Tryndyak et al., 2006). A subtype of SINE B1 transcripts may also have gene insulator effects by cooperating or inducing repressive heterochromatin and forming chromatin barriers to transcription (Román, González-Rico, & Fernández-Salguero, 2011; Román et al., 2011). In humans, *Alu* repeats are thought to control transcription and RNA splicing and play a role in aging, however except for the study of *Alu* transcription in senescent adult

stem cells, to date transcriptional differences in *Alu* in human aging have yet to be defined. In our study, hypomethylation of SINE B1 did not differ by age or genotype (Figure 8). However, transcription was affected by both age and genotype, showing prominence in dwarf mice at 24 months of age. Wild type mice showed the highest transcription of SINE B1 at 3 months of age. In agreement, a recent study also found an increase of SINE B1 transcription in C56BL/6 mice at 24 months of age corresponding to our findings in dwarf mice (De Cecco, Criscione, Peterson, et al., 2013). C56BL/6 is one of the longest living strains among laboratory mice and this paper also noted that calorie restriction, a long studied and well-documented intervention that increases longevity in mice, decreased transcription of this SINE B1 as well as LINE-1. However, SINE B1 expression may be a positive response if it is increased in longevity models compared to shorter living models of mice.

Specific Aim 2- To examine growth hormone administration effects on DNA methylation mechanisms, and global DNA methylation.

Ames dwarf do not secrete three hormones, thyroxine stimulating hormone, prolactin, or growth hormone. However, many studies indicate that growth hormone is the hormone responsible for life span extension and increased cellular stress resistance, especially during the early part of adult development before physiological (reproductive) maturity (Panici et al., 2010). Some work has shown that GH can alter chromatin modifications on a specific predefined set of genes known to be affected by or regulate growth hormone signaling (Chia & Rotwein, 2010). Growth hormone administration to adipocytes

causes changes in the nuclear architecture forming punctate patterns in the nucleus indicating a rearrangement of chromatin (Piwien Pilipuk, Galigniana, & Schwartz, 2003). Liver tissue regeneration depends on growth hormone secretion more than cytokine secretion for cellular proliferation (Pennisi et al., 2004). However, the examination of GH's possible role on epigenetic methylation mechanisms, which are affected by the methionine pathway and SAM abundance in liver, is undefined and unique. Growth hormone regulates GNMT activity and, though not directly correlative, alterations in GNMT expression and activity do coincide with differences in DNMT expression, DNMT activity, and DNA methylation in Ames dwarf mouse liver. In the current study, increases in the transcription of DNMT1 and DNMT3b were noted upon one week of GH administration in dwarf liver (Figure 11). DNMT3a transcription, while still somewhat increased basally in dwarf liver at 6 months of age, showed no difference, therefore transcription of this gene may be influenced by another pathway downstream or through a related pathway separate from GH, such as the insulin pathway. Indeed, there is evidence that the induction of glucose and insulin pathways in hepatocellular carcinoma cells are able to induce large increases in DNMT3a mRNA but not DNMT1 or DNMT3b (Chiang et al., 2009). Glucose and insulin administration also have large effects on the transcription of many methionine enzymes such as GNMT and MAT along with the methionine salvage pathway and transulfuration enzymes. Both glucose uptake and insulin release are greatly influenced by GH signaling in liver (Brown-Borg, 2009; Louis et al., 2010; Masternak et al., 2010). The insulin pathway is also involved in

longevity (Bartke et al., 2013). Both young and old dwarf mice are naturally more insulin sensitive than their wild type counterparts (Bartke et al., 2001; Louis et al., 2010). It is also possible that differences in DNMT3a transcription do not show immediate effects by acute GH administration, but may show effects after many weeks of treatment. Interestingly, our DNMT hepatocyte data does not correspond to those found in rat primary hepatocytes (Vinken et al., 2010). However, both species and culture condition differences likely account for this discrepancy. For instance, DNMT1 is regulated by glucocorticoid receptor stimulation by dexamethasone and corticosterone (Yang et al., 2012). Rat hepatocyte culture protocols require these compounds for primary hepatocyte cultures contrary to murine protocols.

Measurements of relative protein quantities following *in vivo* and *in vitro* GH administration to GH-deficient dwarf mice displayed a marked increase in DNMT1 and decreased DNMT3a levels (Figures 12 and 13). A lower concentration of GH was required by dwarf hepatocytes to stimulate DNMT1 expression when compared to wild type cells suggesting increased sensitivity in the GH-deficient state. This supports earlier findings that dwarf liver is more sensitive to GH stimulation than wild type tissue in pathways related to stress response and cellular proliferation (Brown-Borg & Rakoczy, 2003; Miquet et al., 2010). As previously noted, wild type cells did not produce enough DNMT3a protein for us to detect by immunoblotting and may be a consequence of hepatocyte culture conditions.

We next examined whether GH alters global DNA methylation in dwarf mice. GH-treated dwarf mice showed significantly lower global DNA methylation compared to saline injected wild type and dwarf mice (Figure 7). This novel finding may be indicative of global chromatin changes and globally increased gene expression. Therefore determining the genomic location of these DNA methylation changes would provide insight on the magnitude of gene expression alterations and possible heterochromatic architectural changes between dwarf and wild type mice. We examined whether global methylation changes occurred in the most abundant and highly methylated repetitive element LINE-1, to look for changes in gene poor regions of the genome due to GH (Figure 9). Previous publication has shown, decreases in liver LINE-1 methylation are induced by methyl-deficient diets, which also show alterations in SAM and SAH abundance (Pogribny et al., 2009). However, in our study, no changes in DNA methylation were noted in the LINE-1 region either basally between dwarf or wild type mice at 4-6 months of age, or with the addition of growth hormone in the liver. This leads us to conclude that the majority of DNA hypomethylation related changes due to GH may be found in gene rich areas of expression or at repeat regions associated with these areas.

GH administration did not appear to have large effects on mRNA expression. In addition Ames dwarf mice had higher levels of DNMT1 transcription yet lower levels of protein, especially at 3 months of age. This suggested that DNMT1 was somehow being post-transcriptionally regulated. Recently a study discovered a novel pathway of DNMT1 post-transcriptional

regulation in hepatocellular carcinoma cells and mouse liver (Takata et al., 2013). They demonstrated a regulatory role for a DEAD-box helicase (DDX20) in decreasing DNMT1 levels post-transcriptionally via the regulation of microRNA-140-3p, which has some complementarity to the 3' untranslated region (UTR) of the DNMT1 mRNA sequence. According to miRBase, the miR-140-3p is highly conserved between species (Kozomara & Griffiths-Jones, 2014). DNMT1 negatively regulates the expression of metallothionein (MT) through promoter methylation. In human hepatocellular carcinoma lines knockdown of DDX20 decreases metallothionein expression (Takata et al., 2013).

DDX20 is also known as Gemin 3, and is a dead-box helicase that plays a major role in transcriptional repression through attachments to microRNAs and other proteins forming a ribonucleoprotein and guiding miRNA to the RNA induced silencing complex (RISC) (Van Kouwenhove et al., 2011; Wilker et al., 2010). DDX20 is involved in apoptosis and cellular proliferation, and is deficient or not functional in many diseases such as cancer, and motor neuron diseases such as spinal muscular atrophy (Guo, Zhang, Zhao, Li, & Li, 2011; Shpargel & Matera, 2005; X. Sun et al., 2010; van Kouwenhove et al., 2011; H. Yang et al., 2008). Increases of DDX20 transcription are observed in murine fibroblasts exposed to ultraviolet radiation acquiring consequent DNA lesions (Garinis et al., 2009). Interestingly, these irradiated fibroblasts also exhibit growth hormone and IGF signaling deficits reminiscent of Ames dwarf mice. DDX20 is definitely differentially expressed in dwarf mice at the protein level but not at the transcriptional level (Figure 16). As expected, increased levels of DDX20 protein

were seen in Ames dwarf mouse liver at 3, 12, and 24 months of age corresponding with our cross-sectional studies of decreased DNMT1 protein at all of these ages.

We next investigated whether miRNA 140-3p was basally overexpressed in dwarf mice, since DNMT1 protein was so diminished compared to wild type mice (Figure 17). Surprisingly, miRNA 140-3p levels were lower at three months of age in dwarf mice, and increased at 12 months of age, though not statistically significant at that age. When we investigated miRNA 140-3p at 24 months of age, we found significantly diminished levels. We observed that 5S rRNA levels were decreased (indicated by increased Ct counts in RT-PCR), which would indicate that 24-month-old dwarf mice have a lowered expression of this transcript. This is an unexpected finding and may have implications for overall translational function of dwarf liver cells. One possible explanation for this change is the overall decrease in total mRNA in aging mice which has been recently documented (De Cecco, Criscione, Peterson, et al., 2013). This may be a product of increased transcriptional expression of interspersed repeats. However, the only transcript we noted as being increased in dwarf mice at 24 months, compared to wild type mice was SINE B1. In many studies, transient induction of SINE B1 had no effect on 5S rRNA abundance and was used as a control, however, both are transcribed by RNA polymerase III (T. Li et al., 1999; Liu et al., 1995). Chronically induced SINE B1 transcription, could affect RNA polymerase III levels available for ribosomal RNA transcription, and thereby inhibit translational processes. However, there is evidence that *Alu* repeats (similar to SINE B1 transcripts in mice) can repress

translation by forming complexes with other proteins acting as *trans* regulatory factors (Häsler & Strub, 2006). In aging *Drosophila*, increased ribosomal RNA transcription is associated with the loss of heterochromatin, which might be differently affected in mammals, where there is a gain of heterochromatin in C56BL/6 mouse liver with aging (De Cecco, Criscione, Peterson, et al., 2013; Kreiling et al., 2011; Larson et al., 2012).

Metallothioneins are free radical scavengers and respond to many cellular stressors. Metallothioneins 1 and 2 are prevalent in most tissues and decreased protein expression is associated with hepatocellular carcinoma. Treatment with the DNMT inhibitor 5-azacytidine restored MT1 expression and tumor regression in rat hepatomas (Majumder et al., 2002). DDX20 and miRNA 140-3p purportedly suppressed DNMT1 translational repression and thereby increased metallothionein (MT) expression via loss of methylation in CpG regions of the MT gene in hepatocytes. Interestingly, it is documented that Ames dwarf mice exhibit increased basal expression of MT1 and MT2. We observed that liver MT1 expression is age-related decreasing in both dwarf and wild type mice after 3 months of age. However the expression of MT1 and MT2 is greatest in difference between wild type and Ames dwarf mice at 3 months of age. Therefore, since DNMT1 levels and methylation of the MT promoter drive mRNA expression, we studied the promoter regions of MT1 and 2 at 3 months of age in dwarf and wild type mouse liver (Figure 19). Metallothionein 1 showed less methylation in dwarf mice in an area of CpG methylation that resides between exons 1 and 2 of this gene, rather than transcription factor binding areas. Using a transcription factor prediction data base

for this area of the MT-1 gene (TFSearch), several potential transcription factors including GATA-2 and Ik-2, which are both implicated in controlling the senescence protein marker-30 in aging rat liver were found and could be potential regulators (Heinemeyer et al., 1998; Rath, Pandey et al., 2008). Normal metal response element transcription binding areas of MT1 were not differentially methylated in dwarf mice compared to wild type mice. However, methylation in these areas of transcription are induced by the presence of heavy metals such as zinc, ischemia, and hypothermia, MTF-1, and metal response elements (MREs) (Majumder et al., 2006; Park et al., 2013). In addition, preliminary qualitative analysis of whole genome bisulfite sequencing of young dwarf liver compared to wild type liver is consistent with dwarf mice exhibiting more methylation differences within gene and promoter regions (Brown-Borg and Adams, unpublished data). The MT2 gene was highly unmethylated and methylation-specific PCR provided no evidence of differential methylation in this region.

Since DNMT1 expression is increased by growth hormone administration, we thought perhaps that DDX20 would be decreased and miRNA 140-3p might show decreased expression, in line with what was seen in hepatocellular carcinoma cultures (Takata et al., 2013). Unexpectedly, we observed DDX20 protein expression significantly increased in the dwarf liver after one week of growth hormone administration (Figure 20). Previously, we showed that GH administration increased DNMT1 translational expression in dwarf mice; however, protein levels were not rescued to wild type levels. This increase in DDX20 may be an acute or transient response to increased DNMT1 expression

as part of a negative feedback loop disallowing a full rescue of DNMT1 to wild type levels. This lack of rescue of GH treated dwarf mice to wild type phenotypes has been observed in other studies as well. For instance, Ames dwarf mice did not reach body weights of wild type mice even when exposed to as much as 6 weeks of GH treatment, nor were wild type levels attained in the expression of proteins, or activation of pathways, regulated by GH such as IGF1 and phosphorylation of STAT3 respectively (Louis et al., 2010; Panici et al., 2010). Whether DDX20 expression modulates or associates with these pathways is currently unknown. Further study is required to elucidate the response of DDX20 expression to long-term GH administration. Interestingly, there was no change in miRNA 140-3p in response to GH treatment indicating that in normal liver miRNA 140-3p expression is either tightly controlled, or other pathways may influence its expression (Figure 21).

Growth hormone administration decreased global DNA methylation in dwarf mouse liver (Figure 14) and because there are significantly higher metallothionein mRNA expression levels, we investigated whether there were methylation changes in the metallothionein gene region. Changes in DNA methylation of this region at 6 months of age in dwarf liver were not significant. In addition there were no differences between methylation in MT1 methylation at base pairs covering the area between exons 1 and 2 in dwarf mice with the administration of growth hormone (Figure 22). The loss of statistical methylation difference in dwarf liver may be an age-related change that coincides with a large loss of MT1 transcription after 3 months of age (Figure 18).

CpG methylation was not significantly different in areas common to metal transcription factor 1 (MTF-1) or the family of metal response elements (MREs) probed in Figure 18 between young dwarf and wild type mice. However, GH administration greatly increased methylation in this area. This increase in methylation should decrease MT1 transcription and yet it does not. This indicates the methylation regulation of MT1 is more complex that just the up- or down-regulation of DNMT1 or global DNA methylation. Because the MT1 gene contains many transcription binding factor sites, the increased expression of MT1 due to GH administration may be at binding sites farther upstream from the transcriptional start site, such as sequences associated with STAT. GH administration is known to activate JAK/STAT signaling. STAT3 signaling has also been implicated in the increase of MT1 expression due to hypothermic protection against induced ischemia (Park et al., 2013). To address this question, we designed methylation specific primers for the binding sites of STAT and the STAT response element (which also is a response element for interleukin-6) within the first 150 base pairs of the MT1 gene (Figure 23). However, the results were inconclusive for any relative methylation changes in all groups, (wild type, dwarf, and dwarf treated with growth hormone) and the STAT binding regions all appeared to be highly unmethylated. More sensitive measures will have to be undertaken to probe these two methylation sites. There is also a possibility that because of the increase of methylation in MREs, GH treated dwarf mice may be more resistant to the heavy metal and

stress induction of MT1, causing a deficiency in the ability to scavenge free radicals and heavy metals decreasing oxidative stress resistance.

At any rate, GH associated changes in methylation of the MT1 gene do not appear to be simply a consequence of global methylation changes caused by increased DNMT1 as suggested in previous literature, but specific to regulatory areas of the MT1 gene and promoter regions. Metallothionein expression due to GH administration may be a transient response that may be down-regulated during long term GH treatment such as has been indicated in one study through a six-week treatment of GH in 7-month-old Ames dwarf mice (Swindell et al., 2010).

To summarize, in contrast to the findings of Takata et al (2013), in our mice, miRNA 140-3p expression does not appear to be the key factor in regulating DNMT1 expression. DDX20 protein levels however, still could be important to DNMT1 regulation if this pathway is a major regulator. High levels of DDX20 protein may still be regulatory by selectively bringing miR-140-3p to the RISC complex and therefore offset the much higher transcription of Ames dwarf mice as seen in our previous work, however, we feel it is unlikely for several reasons. Firstly, manipulation of DNMT1 expression through DDX20 could involve many different miRNA transcript partners. For instance, only one of many possible miRNA partners for DDX20 could include miR-148a, which has been predicted to negatively regulate DNMT1 and is involved in maintaining the differentiation of hepatocytes and repressing hepatocellular carcinoma malignancy (Denis et al., 2011; Gailhouste et al., 2013). miR- 148a

expression has been noted to change with age in murine tissues and is responsive to calorie restriction, which is a well-known intervention that increases life span in mice (Dhahbi et al., 2013). Also miR140-3p may be more important for tissues other than the liver for the suppression of other pathways such as IGF-1 (Yuan, Shen, Xue, & Fan, 2013). Other factors may regulate DNMT1 as well, and miRNA regulation may only be a minor player in regulation. For example, DNMT1 is also regulated in *cis* by unique RNA molecules expressed by gene regions (Ruscio et al, 2013). Several other factors have also been shown to modulate DNMT expression and activity and include PCNA, PKA, PKB (Akt), PKC, and GSK3β. GH treatment of dwarf mice increased liver phosphorylated Akt protein and phosphorylated GSK3β protein levels when compared to wild type mice or untreated dwarf mice (Masternak et al., 2010; Miquet et al., 2010; Panici et al., 2010). GSK3β regulates pAkt activity and can stabilize DNMT1 affecting its expression and activity (Sun et al., 2007). PI3K and pAkt are known to be involved in cell proliferation and aging. Since liver DNMT activity is higher in the dwarf at 3 months and lower at 24 months, there may be additional regulation by PKA, PKB (Akt), PKC, and other pathways where Ames dwarf mice exhibit increased responses to GH treatment in the liver. If the increase of DDX20 protein expression is significant in DNMT1 post-transcriptional regulation, more studies are needed to identify the ways in which DDX20 may be working with one or several miRNA transcripts to post-transcriptionally regulate DNMT1 transcription. Together, all of these pathways could modulate DNMT

expression and represent potential avenues of investigation for post-transcriptional regulation of DNMT in the liver of Ames dwarf mice and in aging studies, in general. Growth hormone deficiency in the Ames dwarf mouse may contribute to epigenetic stability by decreasing DNMT1 protein through a variety of aging-associated pathways, and increasing DNMT3a protein at young ages. We have data indicating differences between genotypes in global DNA methylation and preliminary results showing differences in specific gene regions (Brown-Borg, unpublished data). Furthermore, GH signaling may also be important to differences in tissues other than liver such as muscle and fat. The results of this study do not exclude potential IGF-1 effects on DNMT expression. DNMT1 also has DNA methylation independent actions that may be different in dwarf mice and contribute to cell cycle regulation (Milutinovic et al., 2004).

Specific Aim 3- to examine histone H3 and H4 markers associated with aging

Histone methyltransferases also use S-adenosylmethionine from the methionine pathway as a substrate. Therefore these methyltransferases may well be affected by methionine pathway alterations similar to GNMT and DNMTs. Methyl-deficient diets are known to cause alterations in SAM, methionine, and the expression of methionine enzymes (Pogribny et al., 2009). Alterations in the methionine pathway due to nutritional alterations, or enzyme loss, such as GNMT knockout mice, have been shown to prolong life (methionine restriction) or cause cancer (choline or folate deficient), an age-related disease, in mice (Cavuoto &

Fenech, 2012; Martínez-Chantar et al., 2008). These factors are also known to alter the DNA and histone methylation in mice (Davis & Uthus, 2004; Pogribny et al., 2009). As stated earlier, histone methylation has also been linked to aging in a variety of species (Figure 36). We found profound changes in histone abundance and distribution between Ames dwarf and wild type mice during our cross-sectional studies (Figures 24 and 25). First was the discovery that total H3 was not expressed similarly between wild type and dwarf mice in liver histone extracts. However when compared within genotype, dwarf mice showed more stable expression of this protein through all three age groups, while wild type mice showed a progressive increase in total histone H3 which led to differences in expression between the two genotypes at different ages (Figure 25). One of the first studies to note changes in histone H3 was in *Drosophila*, and showed that histone H3 decreases with age, which as stated earlier, suggests a loss of heterochromatin with age (Larson et al., 2012). In rodents however, heterochromatin increases with age as evidenced by studies in mice and increases in H4K20me3 in rat liver (De Cecco et al., 2013; Sarg et al., 2002). In addition, in mice exposed to early life stressors that trigger abnormal emotive behavior, total H3 is responsive to pharmaceuticals, showing this marker to be dynamic and impacted by environmental events and chemical interventions (Levine et al., 2012).

We studied one histone methylation marker associated with transcriptionally active areas of the genome, H3K4 tri-methylation (H3K4me3). Dwarf mice compared to wild type mice have altered total expression of this marker at 12 and 24 months. Gains of this marker are associated with aging in *c. elegans* (Greer et al.,

2011). In humans, losses or gains of this marker are associated with cancer progression and with hepatic steatosis (Blair & Yan, 2012; Jun, Kim, Hoang, & Lee, 2012). Of note, it has been shown that the loss of this H3K4me3 is associated with a higher activity of DNMT3a; this might be important as dwarf mice exhibit less DNMT3a protein and more H3K4me3 at 24 months of age (Kuzumaki et al., 2010b; Ooi et al., 2007). This could be associated with resisting the gain of methylation in gene regions during aging as our mice demonstrate less global methylation changes over all in whole genome bisulfite sequencing (Brown-Borg, unpublished data).

The facultative repressive marker histone H3K27me3, is also diminished in dwarf mice compared to age-matched wild type mice at 12 months of age, and increased at 24 months of age, similar to H3K4me3 abundance. When examined in proportion to total histone H3 expression however, less histone H3 is methylated at lysine 4 at 3 months of age (similar to H3K4me3). Since H3K27me3 is purportedly decreased in abundance with aging in *C. elegans*, and in cells from patients with Hutchinson–Gilford Progeria Syndrome (HGPS) it is interesting to note that dwarf mice resist this change at 24 months of age (S. Han & Brunet, 2012; Shumaker et al., 2006).

The constitutive heterochromatin marker H3K9me3 has also been found to be reduced with age in cells taken from patients with HGPS (Shumaker et al., 2006). However it increases with age in *Drosophila* (Han & Brunet, 2012). Dwarf mice had higher total levels of this marker at 3 months of age and similar levels at 12 and 24 months of age. However when normalized to total H3 protein at 12 months of age, H3K9me3 is more abundant in dwarf mice and equivalent at 3

and 24 months of age. Therefore, dwarf mice may be allocating most of the histone H3 resources into retaining areas of constitutive heterochromatin at this age. Our studies did not indicate that H3K9me3 was especially altered between the two genotypes at 24 months of age either in overall abundance or in total histone H3 distribution. Coupled with the high DNA methylation status in dwarf mice and the resistance to DNA methylation changes, it appears that areas of constitutive chromatin are well maintained in dwarf mice compared to wild type mice even with fluctuations in total histone H3. Locations of these markers may be more informative in determining their impact on the aging phenotype.

In a preliminary experiment, we noted some changes in methylation enzymes related to H3K9me3 and H3K4me3 in 3 month old dwarf mice compared to their wild type counterparts (Table 5). The lysine demethylase Kdm5 (also known as Jarid1b) is known to participate in cellular senescence, and its expression affects longevity in *c. elegans* and cancer in humans, making its expression important as a possible aging target. Also, the histone methyltransferase Setdb2 is a marker that hasn't been identified as an aging related protein yet, thus this may be a novel finding that warrants further investigation.

Specific Aim 4- To examine global DNA methylation and DNMT expression and growth hormone expression mutants.

For our last specific aim, we began investigations to validate some of the epigenetic methylation differences that we observed in Ames dwarf mice in other GH mutant mice. We focused on growth hormone receptor binding protein knockout mice that exhibit high growth hormone but lack the ability to respond to

it due to a mutated receptor. Global DNA methylation in these mice did not differ from their age-matched wild type counterparts, but an age-related effect was nearly significant (Figure 28). For mice overexpressing growth hormone however, differences were noted at 10 months of age, where aged GH Tg mice display ~50% higher levels of DNA methylation compared to age-matched wild type mice (Figure 32). This is interesting as these mice live only to approximately 12 months of age and express premature aging phenotypes by six months of age. It is possible that result may show they accumulate a large increase in heterochromatin at a younger age, which has been shown in normally aging mice. However, heterochromatin markers have yet to be studied in this model.

For the methylation assessment of interspersed repeats, some similarities and differences were noted in GHRKO, GH Tg, and dwarf mice when studied cross-sectionally. Both long-living GH signaling deficient mice (Ames, GHRKO) showed some resistance to the hypomethylation of IAP-LTR repeats, although at different ages (Figures 8 and 29). GH Tg mice however, displayed more hypomethylation at younger ages, while their wild type counterparts maintained similar levels of methylation (Figure 33). For wild type mice this could be expected since the wild type mice from our two other long-lived strains showed differences at 12 months of age. However, we may have missed potential changes in methylation as we didn't look past 12 months of age in the wild type controls of the GH Tg line. Excitingly, the hypomethylation of IAP-LTR may be a consistent marker for aging among many mouse strains, and such an early loss

of methylation in GH Tg mice for IAP-LTR may have huge implications for genetic instability and chromatin structure in these mice.

In addition, GHRKO mice express more IAP-LTR at 3 months of age than their wild type counterparts. Though it was not statistically significant, Ames dwarf mice showed an increase in transcription at this age as well. The GH Tg mice showed less IAP-LTR transcription at 10 months of age compared to their wild type counterparts where expression was unchanged with age. This altered transcription occurred at an age when the premature aging phenotype is already apparent and there is a large increase of genomic DNA methylation in these animals. This repeat appears to have some important function in relation to the aging epigenome and requires further investigation.

LINE-1 was more hypomethylated at 24 months of age in wild type mice than in the age-matched mutant GHRKO mice. The longer-lived mutant mouse was able to maintain consistent methylation of this repetitive element. Interestingly, methylation changes in this repeat in the wild type mice might be strain specific when they are compared to the wild type mice of the Ames dwarf strain. LINE-1 hypomethylation during aging has been somewhat controversial as mentioned earlier. LINE-1 hypomethylation is not always apparent in normally aging human cells, but is more apparent in cancer, where the knockdown of expression can abrogate cellular proliferation or metastases (Chalitchagorn et al., 2004; Oricchio et al., 2007; Takai et al., 2000). In GH Tg mice, LINE-1 hypomethylation does not change in mutant mice compared to a small elevation

at 6 months methylation loss in its wild type counterpart. The six month increase is surprising, and will have to be investigated in other mouse lines to see if this is consistently observed. The transcription of LINE-1 in both lines of dwarf mice is age-related regardless of genotype, however whereas Ames dwarf mice exhibit no genotypic differences in expression, GHRKO mice exhibit higher levels of expression. GH Tg mice exhibit lower amounts of transcription compared to wild type counterparts. This may be an important factor specific to growth hormone signaling. These results are somewhat contrary to the current literature where LINE-1 expression appears to be deleterious with age and promotes cancer. However, as LINE-1 is usually found in A-T regions in gene-poor regions, loss of expression in GH Tg mice might be a result of increased heterochromatin formation at an early age.

SINE B1 GH Tg mice did not show any significant changes in hypomethylation due to age or genotype. Only the GHRKO line appeared to have an interactive effect between age and genotype that altered expression of SINE B1. This is most likely a strain specific difference. SINE B1 transcription however, showed very interesting results. Just as its transcription was increased in dwarf mice at 24 months of age, its transcription was increased in GHRKO mutants at 12 and 24 months of age when compared to age-matched controls. In GH Tg mice, there was no genotype difference between the mice though it decreased with age. The fact that SINE B1 transcription is higher in two long-living mutants, and not in short-living GH Tg mice, raises the possibility that the transcription of this element may indeed be protective in aging, especially when expressed at

later ages. Further study will be required to assess whether GHRKO mice have reduced amounts of translation or RNA splicing differences at these older ages which could be protective mechanisms in the aging of these rodents.

For SINE B2, the rodent specific repeat, hypomethylation assays showed age-related differences in all three strains of mouse. In the GHRKO and Ames dwarf mouse lines the hypomethylation was increased with age in both wild type and mutant mice. Only the Ames dwarf showed resistance to hypomethylation of this repeat that may or may not be growth hormone associated. In GH Tg mice both the wild type and mutant mice gain methylation (lost hypomethylation) with age, however, since this cross-sectional study does not include wild type mice older than 10 months of age, hypomethylation of this repeat may yet occur. Age-related transcriptional differences were only seen in the Ames dwarf line, while there were genotype differences in GHRKO mice, and no differences between GH Tg and respective age-matched wild type mice. Differences in transcription appear to be strain specific for the mice overall.

Examination of DNMT transcription yielded fascinating results when comparing all three lines of mice. DNMT1 transcription was significantly increased in GHRKO mice compared to their wild type counterparts. At 12 months of age, DNMT3a was also 2-fold higher than wild type transcription levels. However, Ames dwarf mice showed greater transcription levels of DNMT1 and DNMT3a at 3 months of age. At first this seems as if it may not correlate, however, earlier reports from our lab showed that methionine pathway gene expression differences between GHRKO mice and wild type mice do occur until

12 months of age, and are not as large in magnitude as Ames dwarf mice (Brown-Borg et al., 2009). The 2-fold increase in DNMT1 and DNMT3a RNA expression observed at 12 months of age in GHRKO mice is mirrored by the two-fold increase in MAT1a and GNMT expression, two important enzymes in the production and regulation of SAM in the methionine pathway (Brown-Borg et al., 2009). This substantiates our finding that DNMT1 and DNMT3a transcription coincide with methionine pathway differences in both strains of long-living dwarf mice. DNMT1 transcription in both the GHRKO and GH Tg lines of mice decreased with age, in agreement with the literature that examined DNMT1 transcription in T cells mentioned earlier (Ray et al., 2006). Both GHRKO and GH Tg are laboratory inbred strains, as are the DNMT1 deficient mice used in the T cell study, which unlike the Ames dwarf mouse line which is bred on a heterogenous background. DNMT1 was not altered in transcription in GH Tg mice compared to wild type mice at any age. DNMT3a however did show genotypic differences, however the increase in DNMT3a expression did not approach the magnitude of change detected in the dwarf mouse lines. DNA methyltransferase 3b was not different between genotypes in GHRKO mutants or wild type mice at any age, reminiscent of the Ames dwarf line. In contrast, transcriptional expression of DNMT3b in GH Tg mice at 3 months of age was 2-fold higher compared to wild type controls. Age-related differences were noted between all three strains of mice however, DNMT3b mRNA increased with age in the Ames dwarf line, while both the GHRKO and growth hormone transgenic lines decreased with age, indicating strain specific differences. Interestingly,

DNMT3b differences in transcription of the GH Tg mice had confounding effects of age and genotype, which frequently reoccurs in all the mouse lines when studying these transcripts in a cross-sectional manner.

Interestingly, we found background genetics alters the wild type pattern of expression or hypomethylation, supporting the idea that epigenetic mechanisms are at least partially controlled through genetic background. However, there is plasticity in epigenetic expression that can be permanently altered by chronic or early life events. Strain differences in aging are well-documented and should be expected when comparing different mutant strains of mice (Yuan et al., 2011). However, when commonalities between diverse genetic background strains are discovered due to a lack of hormone signaling, the findings become an even more potent discovery.

A few other preliminary studies were accomplished to further elucidate the epigenetic characterization of the GHRKO and GH Tg mutants. Since GH modulates differences in DNMT expression of Ames dwarf mice, we decided to see if Insulin-like growth factor-1 administration would alter liver DNMT levels (Figure 31) in GHRKO mice. IGF-1 administration to Ames dwarf, GH Tg and age-matched wild type hepatocyte cultures induced significant differences of expression in enzymes responsible for oxidative stress response, including glutathione peroxidase, a transulfuration enzyme which is connected to the methionine pathway (Brown-Borg et al., 2002). IGF-1 also has feedback effects on growth hormone secretion, cellular proliferation, mediating many of the functions of GH, and is affected by the nutritional availability of methionine

(Bartke et al., 2013; Miller et al., 2005). IGF-1 receptors are not expressed in liver; however, IGF-1 is able to stimulate insulin receptors, illustrating some promiscuity in signaling. Interestingly, DNMT1 and DNMT3a mRNA expression were responsive to IGF-1 injections in the liver of GHRKO mice (Figure31). Surprisingly, in this genetic background, GHRKO mutant mice at 6 months of age showed decreased expression basal expression of DNMT3b that remained unchanged upon IGF-1 treatment. GH, IGF-1, and insulin signaling in the liver are interconnected. While DNMT1 showed no effects when insulin was applied to hepatocellular carcinoma cells, differences in insulin signaling between hepatocellular carcinoma cells and normal liver may have accounted for the differences (Chiang et al., 2009). The increase in DNMT3a is likely due to the ability of IGF-1 to influence insulin receptor signaling, similarly to what has been seen in hepatocellular carcinoma cells. Also IGF-1 signaling through insulin receptors had no effect on DNMT3b transcription, similar to the literature. However, IGF-1 has a much smaller affinity for insulin receptors than its own receptors, therefore applications or injections of insulin would be expected to produce more drastic alterations in these transcripts, and would be an excellent future study. More DNA methyltransferase protein studies will be forthcoming subject to tissue availability.

We also began preliminary studies based on tissue availability for the expression of DNMT proteins in transgenic mice (Figure 35). For this study, we expected DNMT1 protein to be higher in these mice since one week of GH administration in dwarf mice was able to increase DNMT1 protein. Though at 3

months of age DNMT1 protein appeared to be slightly increased, low samples sizes may have left any results statistically non-significant (n=5-6). However there was decreased expression in DNMT1 in 10 month old GH Tg mice compared to wild type counterparts. This coincided with a large increase in global methylation. Also DNMT3a protein at every age is significantly lower in expression in GH Tg mice, a dramatically different expressional profile than Ames dwarf mice, which showed an increased protein expression at 3 months of age. DNMT3b protein expression results are still forthcoming, as are replication of these studies with larger sample sizes when tissue availability allows. From this data, it appears DNMT3a expression is a vital enzyme to longevity and possibly delays hepatocellular tumorigenesis as predicted from methyl-deficient dietary studies (Pogribny et al., 2009). DNMT3b may play an opposing role in longevity based on the transcriptional studies of GH Tg mice, however further study of epigenetic methylation mechanisms of GHRKO and GH Tg mice are warranted.

In conclusion, it has been proposed that epigenetic mechanisms can contribute greatly to the aging phenotype and genetic instability. DNA methylation plays a significant role in development and likely in responses to early life events, either of which may avert or exacerbate the aging phenotype. However, due to the lack of GH signaling, Ames dwarf mice remain able to resist and delay many pathologies of aging such as the hepatocellular carcinoma found in their wild type siblings (Ikeno et al., 2003). GHRKO mice are also long-lived mutants, that exhibit GH signaling deficiency and showed some strikingly similar

features of DNMT mRNA expression and interspersed repeat methylation or transcription with Ames dwarf mice. Our study demonstrated that Ames dwarf mice, GHRKO, and GH Tg mice exhibited significant differences in DNMT expression that change with age. We also demonstrated that GH administration can have a significant impact on the methylation mechanisms and global DNA methylation of the Ames dwarf. Similarly, IGF-1 has some transcriptional effects on methylation enzymes for GHRKO mice as well. The differential expression of both *de novo* and maintenance DNMTs between Ames dwarf, GHRKO, and GH Tg mice, compared to their wild type counterparts affects the methylation of repetitive elements and specific genes and therefore contributes to epigenetic stability, or instability in the case of GH Tg mice. Also Ames dwarf mice showed differences in histone methylation that can offer further clues for chromatin structure and organization. Continuing studies of these proteins and enzymes in GH signaling mutant mice are extremely valuable tools to elucidate the changes of the epigenetic landscape during aging. These novel findings have initiated a new line of inquiry and contributed to our understanding of the mechanisms underlying the relationships between GH, longevity and epigenetic stability associated with aging and aging pathologies.

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