Dynamic changes in global and gene specific DNA methylation during hibernation in adult thirteen-lined ground squirrels, *Ictidomys tridecemlineatus*

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Abstract

Hibernating mammals conserve energy in the winter by undergoing prolonged bouts of torpor, interspersed with brief arousals back to euthermia. These bouts are accompanied with a suite of reversible physiological and biochemical changes; however, much remains to be discovered about the molecular mechanisms involved. Given the seasonal nature of hibernation, it stands to reason that underlying plastic epigenetic mechanisms should exist. One such form of epigenomic regulation involves the reversible modification of cytosine bases in DNA by methylation. DNA methylation is well-known to be a mechanism that confers upon DNA its cellular identity during differentiation in response to innate developmental cues. However, it has recently been hypothesized that DNA methylation also acts as a mechanism for adapting genome function to changing external environmental and experiential signals over different time scales, including during adulthood. Here, we tested the hypothesis that DNA methylation is altered during hibernation in adult wild animals. This study evaluated global changes in DNA methylation in response to hibernation in the liver and skeletal muscle of thirteen-lined ground squirrels along with changes in expression of DNA methyltransferases (DNMT1/3B) and methyl binding domain proteins (MBDs). A reduction in global DNA methylation occurred in muscle during torpor phases whereas significant changes in DNMTs and MBDs were seen in both tissues. We also report dynamic changes in DNA methylation in the promoter of the *myocyte enhancer factor 2C* (*mef2c*) gene, a candidate regulator of metabolism in skeletal muscle. Taken together, these data show that genomic DNA methylation is dynamic across torpor-arousal bouts during winter hibernation, consistent with a role for this regulatory mechanism in contributing to the hibernation phenotype.

Introduction

DNA methylation is a mechanism that confers upon identical genomes different chemical identities by covalently modifying cytosine bases at different positions in the genome (Razin and Riggs, 1980). This mechanism has been implicated in cellular differentiation for the last three decades and a large body of data has linked DNA methylation with regulation of transcription (Razin, 1998). DNA methylation is known to be dynamically altered during embryonic development (Lister et al., 2009; Razin and Szyf, 1984; Razin et al., 1984) resulting in tissue specific patterns of methylation that are maintained throughout life. However, the idea that this mechanism is not completely "fixed" during development and could also play a role in stably altering genome function in response to external environmental exposures after embryonic development and even in adults has recently been gaining support. Whereas there is an emerging body of evidence suggesting that exposure to social adversity early in life (Weaver et al., 2004), different diets (Bollati et al., 2007; Waterland and Jirtle, 2003), or toxins could alter DNA methylation, it is unclear whether these constitute stochastic responses to signals modulating DNA methylation enzymes or whether there is an organized programmed genomic response to distinct and predictable environmental signals. One of the most remarkable organized responses to predictable environmental change over seasons is mammalian hibernation. It provides a stark example of how the same adult genotype undergoes an organized, predictable and comprehensive but nevertheless dynamic phenotypic change. This provides a unique real-life model to test the hypothesis that there is a programmed DNA methylation response to the environment in adult animals.

Mammalian hibernation involves extended bouts of torpor during which core body temperature can drop to near 0ºC and metabolic energy expenditures can be reduced to as low as 1-5% of euthermic rates (Carey et al., 2003). The hibernation phenotype includes alterations to multiple behavioral, physiological, biochemical and molecular parameters. In thirteen-lined ground squirrels, *Ictidomys tridecemlineatus*, these changes include programmed down-regulation of most mRNA and protein expression accompanied with a suite of transcriptional (Morin and Storey, 2006; Srere et al., 1992), translational (Wu and Storey, 2012b) and posttranslational (Abnous et al., 2012; Morin and Storey, 2006) signatures consistent with quiescent cellular function (for review (Storey and Storey, 2010). In contrast, selected transcription factors have been shown to be activated during bouts of torpor and arousal indicating a complex interplay between gene repression, activation and metabolic

depression (Tessier and Storey, 2010; Tessier and Storey, 2012). Transcriptional differences occurring during hibernation are also expressed in a tissue-specific manner (Fujii et al., 2006). However, the exact regulatory mechanisms that mediate transcriptional activation and repression in these altered metabolic states are largely unknown, particularly at the level of global transcriptional controls (Hampton et al., 2011; Yan et al., 2008).

One form of genomic regulation, DNA methylation, controls gene function by the steric hindrance of transcriptional activators or recruitment of transcriptional repressors (Razin and Riggs, 1980; Siegfried et al., 1999). Additionally, recent evidence links DNA methylation with the regulation of seasonal behaviors (Alvarado et al., 2014) and responses to seasonal disruptions (Cao-Lei et al., 2014) in various species. However, dynamic changes in the methylome often reflect broader changes that cannot be solely accounted for by transcriptional activation or repression of protein-coding genes (Alvarado et al., 2013; Tajerian et al., 2013). DNA methylation is catalyzed by DNA methyltransferases that target either newly replicated hemi-methylated DNA (DNMT1) or unmethylated DNA to mediate *de novo* DNA methylation (DNMT3A/B). DNA methylation is often read by a family of methyl-binding domain proteins (MBDs) to mediate multiple functions of transcriptional repression and activation (Angrisano et al., 2006; Brown and Szyf, 2007; Ng et al., 1999). These regulators define a "methylation toolkit" that is largely conserved in the animal kingdom and is capable of regulating genomic function in response to a dynamic environment (Jurkowski and Jeltsch, 2011; Lee et al., 2010).

To date, the role of epigenetic modifications of DNA in mammalian hibernation has been limited to two studies that showed altered posttranslational modifications of histones and transcriptional repression (Morin and Storey, 2006) and aberrant genomic methylation in brown adipose tissue (Biggar and Storey, 2014). In addition, Fujii et al. (2006) analyzed the role of DNA methylation of the promoter region in regulating the expression of a liver gene in chipmunks that is specifically down-regulated in hibernation. Hypomethylation of a CpG dinucleotide at −161 within the promoter region in liver was linked with tissue-specific expression of the *HP-27* gene (the site was hypermethylated in kidney and heart) but methylation status at -161 and three other CpG dinucleotides did not change in a comparison of liver from hibernating and non-hibernating chipmunks (Fujii et al., 2006). Hence, to advance the field, the present study tests the hypothesis that DNA methylation dynamically responds to cyclic changes occurring over torpor-arousal bouts in adult mammals. Our analysis focused on liver and skeletal muscle. Liver is of central

importance to metabolic homeostasis of the whole animal including involvement in torpor-specific fuel metabolism (e.g. gluconeogenesis and ketone body synthesis to provide fuels for brain) whereas muscle, although inactive during most of a torpor bout, plays a key role in arousal by generating heat via shivering thermogenesis. Both tissues exhibit a range of transcriptional, translational and posttranscriptional changes during hibernation and metabolic depression (Abnous et al., 2008; Green et al., 1984; Mamady and Storey, 2008; Storey, 1987). Since the transcriptional and translational response in hibernation involves global changes we first evaluated genomic changes in DNA methylation and its conserved machinery. We then used a candidate gene approach to measure DNA methylation in a gene that plays a role in muscle metabolic regulation during hibernation, the transcription factor *myocyte enhancer factor 2C* (*mef2c*) (Tessier and Storey, 2010). *Mef2c* is a master transcription factor that plays roles in cellular differentiation including myogenesis, neurogenesis, development of the heart and the neural crest (Potthoff and Olson, 2007). In mammals, *mef2c* is predominantly expressed in the brain and skeletal muscle with a suggested role for the maintenance of the myocyte differentiated state (Breitbart et al., 1993).

Results

Global DNA methylation changes during hibernation

In order to evaluate whether global DNA methylation states responded to changes related to hibernation (FIG. 1A), we assayed global DNA methylation in *I. tridecemlineatus* using LUMA in two tissues; skeletal muscle and liver. In skeletal muscle, significant hypomethylation (an approximate 16% reduction, assuming 100% cutting by restriction enzymes) was observed in animals with cold body temperature (*T*b) values that were in late torpor, just beginning to arouse or in interbout (FIG. 1B) as compared with animals that were euthermic in the cold room. A query of the ground squirrel genome for HpaII/MspI sites (Ensemble release 66) indicated that the extent of changes in methylation that were observed in our study corresponded, at the very least, to 206573 differentially methylated CpG sites in the muscle genome. If we are to consider 16% of CGs outside of the CCGG restriction site this could represent the entire population of genomic CGs ranging close to~1.5 million CGs. Significant changes in global methylation state were observed in the liver between euthermic in the cold room and interbout (FIG.1E).

Altered expression of DNA methylation regulators accompany hibernation and seasonal change

Considering the changes in DNA methylation levels that occurred in muscle over torpor-arousal, we next investigated whether these changes were associated with overall changes in the expression of known DNA methylation regulators. We assessed changes in the mRNA transcript levels of DNA methyltransferases (*dnmt1*, *dnmt3B*) over the course of hibernation bouts. We observed that DNMT transcript levels in skeletal muscle did not change significantly over the torpor-arousal cycle (FIG. 1C,D). Thus, the observed reduction in global DNA methylation in muscle is not a consequence of a major change in DNMT transcription. However, in the liver where we saw no genomic changes in DNA methylation, we observed differences in *dnmt1* and *dnmt3B* expression between animals that were euthermic in the cold room versus those at the early torpor stage (FIG. 1F,G). Thus, changes in DNMT were not correlated with global changes in DNA methylation. Knowing that the DNA methylation signal is in part transduced by several proteins we measured mRNA levels of four methyl binding domain (MBD) proteins (*mbd1*, *mbd3*, *mbd3*, *mecp2*) over the hibernation stages. Expression of these regulators was altered both between tissues and across the torpor-arousal cycle (FIG. 2A-H). In muscle, a 14-fold increase was seen in *mecp2* mRNA levels between euthermic animals in the cold room and the early arousal stage (FIG.

2D). In liver, we observed significant increases in transcript levels between euthermic animals in the cold room and in early or late torpor for *mbd2* (FIG. 2F), and between euthermic animals and the early arousal stage for *mbd3* and *mecp2* (FIG. 2G,H). Although these changes in expression can't directly explain the changes in global DNA methylation observed in skeletal muscle, they do support the hypothesis that the DNA methylation machinery in this candidate screen is altered during different stages of hibernation.

Dynamic expression and promoter methylation of the candidate regulator of muscle function, *mef2c*, across hibernation.

We examined myocyte enhancer factor 2C *(mef2c*) since its expression has been shown to be altered during hibernation in a tissue specific manner (Bratincsak et al., 2007)(Tessier and Storey, 2010, 2012). In skeletal muscle the expression of the gene was dramatically reduced during the entering torpor associated with the entry phase where *T*b was decreasing towards the stable low *T*b value (Fig. 3A). Expression was slightly increased again in early torpor but remained low in late torpor and early arousal. In contrast, in the liver gene expression remained low across all torpor stages and was up-regulated only during the early arousal phase (Fig. 3B).

We then used bisulfite pyrosequencing of the *mef2c* promoter (FIG. 3E, F) in skeletal muscle and liver to follow its state of methylation over the different hibernation stages. The promoter region of *mef2c* was predicted from an alignment to the human *mef2c* promoter (Fig. 3C,D) and was confirmed by transient expression using a luciferase expression vector in HEK 293 cells (Fig. 3G). Several CpG sites upstream of the *mef2c* transcriptional start site (-297, -299, -321, -329, -331, -351 and -353) showed dynamic changes between two hibernation stages in skeletal muscle but not in liver (FIG. 3E, F). A large increase in the methylation state of all CG sites across the promoter region of skeletal muscle *mef2c* occurred as the animals entered torpor (Fig. 3E), which correlates with the sharp reduction in gene expression (Fig. 3A). However, the CG sites were demethylated again during early torpor but showed a slight remethylation in late torpor before methylation was reduced again. A minor demethylation was again apparent upon early arousal but did not correlate with induced gene expression. The *mef2c* gene was silenced in the liver through most of the stages of hibernation (Fig. 3B) however the promoter was methylated at the same level as in skeletal muscle (Fig. 3F) and the spike of *mef2c* expression during early arousal was not accompanied by a demethylation of the gene promoter. It is possible that a DNA methylation independent process silences the expression of this gene in the liver and is responsible for its induction

during early arousal for example the formation of RNA stress granules(Tessier and Storey, 2014). Alternately, this could indicate that this specific region is a muscle-specific promoter or enhancer that does not regulate liver-associated transcripts.

The above results suggested that DNA methylation might be involved in silencing *mef2c* promoter activity. Therefore, to investigate this further we analyzed whether methylation of the CG sites in the promoter would alter the ability of *mef2c* to direct transcription of a luciferase mRNA in a transient expression assay (Klug and Rehli, 2006). The *mef2c* promoter region was cloned into a CpG-less promoter construct in sense and antisense (as a negative control) directions. The plasmids were either *in vitro* methylated or mock methylated by the bacterial CG DNA methyltransferase *mSss1* (Nur et al., 1985). Since the vector lacks CG sites this will result in only methylation of the sites in the *mef2c* promoter and will thus recapitulate the situation *in vivo* where all of these sites were found to be methylated during entry into torpor (Fig. 3E). The plasmids were transfected into HEK293 cells and assayed after 48 hours. The unmethylated promoter in the sense orientation expressed higher levels of luciferase compared to the background levels expressed from the antisense promoter (FIG. 3G). Furthermore, methylation of the sense promoter reduced the level of expression to the background levels expressed from the antisense promoter. These results show that DNA methylation is capable of reducing the transcriptional activity of the *mef2c* promoter *in vitro* and further supports evidence of muscle-specific regulation of transcription *in vivo*.

Discussion

Epigenomic mechanisms such as DNA methylation are well-suited to serve as an interface between the DNA of a cell and changing environments, but the main question is whether there exists an organized and predicted response of the methylome to natural environmental changes. Our current understanding of epigenetic mechanisms is largely limited to studies in human pathology or embryogenesis. As a result, very little is known about the role of epigenetic modification in adults facing naturally dynamic environments. Hibernation is one of the most dramatic phenotypic responses to change in the climactic environment and could serve as an excellent paradigm for testing the response of the methylome to extreme changes in physiology. Hibernating mammals are capable of reducing their metabolic expenditures by 95-99% resulting in a very strong suppression of multiple core processes including respiration, heartbeat, transcription (Osborne et al., 2004), translation and the cell cycle (Frerichs et al., 1998; Wu and Storey, 2012a) over torpor bouts that can last for days or weeks. Considering the genetic similarity between seasonal hibernators and non-hibernators (Carey et al., 2003; Srere et al., 1992), this phenotype may not require novel genes, but rather the reconfiguration of the transcriptome. In this respect, seasonal hibernators may be reiterating a neonatal program in preparation for torpor; suggesting that the genetic potential for heterothermy that exists in all mammalian neonates is invoked in adult hibernators by the continued or renewed expression of selected genes that provide crucial for heterothermy (Harris et al., 2004).

In the present study, we show that DNA methylation, its effectors, and its targets can be altered in hibernating mammals over winter torpor-arousal cycles. We further show tissue-specific genomic DNA methylation changes that accompany the torpor-arousal cycle and uncoupled transcription of methyltransferases and differential regulation of MBD proteins. We observed that the differentially expressed *mef2c* transcript was a tissue-specific target of differential DNA methylation *in vivo* in during the hibernation cycle (Fig. 3), and that hypermethylation upon entrance into torpor is partly associated with reduced mRNA expression. The sites that are hypermethylated upon entrance into torpor could silence the *mefc2* promoter as determined by the promoter-luciferase assays (Fig. 3G). To our knowledge, this study is the first to demonstrate dynamic changes in genomic DNA methylation and its regulators in seasonal hibernating mammals. This study illustrates DNA methylation as a dynamic process in adult tissues that can be programmed to respond to distinct environmental cues. Although hibernation is a unique and extreme example of mammalian response to environment change, it provides a proof of principle that the genome

of adult mammals can respond to environmental stress with tissue-specific changes in DNA methylation. Thus, this study provides evidence in support of the hypothesis that dynamic DNA methylation programs are not limited to embryogenesis and that they can change in a predictable manner in response to external environmental and physiological signals.

Tissue-specific genomic methylation in during hibernation

Our data show that the response of DNA methylation is tissue-specific since liver and muscle altered their DNA methylation patterns differently during different phases of hibernation. In muscle, genomic DNA methylation decreased dramatically during late torpor, early arousal and interbout stages of hibernation (FIG. 1B) on a scale similar to that reported for global hypomethylation in cancer (Feinberg and Vogelstein, 1983; Lu et al., 1983) or autoimmune diseases (Cornacchia et al., 1988). In liver, genomic DNA methylation increased in animals during Interbout. It should be noted that the use of LUMA in our study is limited in only being able to assay absolute changes in genomic methylation. As a result we are unable to offer a comprehensive insight into which genes are differentially methylated, whether methylation is happening outside of a CpG context, or if a reconfigured methylome results in a zero sum change in genomic methylation. The exact role of the observed hypomethylation in muscle in controlling genome function is unknown since the extent of DNA demethylation is broader than what would be anticipated if only CpGs in regulatory regions and promoters were demethylated. This suggests that demethylation as a regulatory mechanism must involve wider genomic regions. At the very least, the genomic hypomethylation observed within the muscle represents a molecular rearrangement that is supported by the overall transcriptional activity observed in the skeletal muscle of hibernating mammals (Malatesta et al., 2009). This needs to be explored in the future with full genomic mapping of DNA methylation over different hibernation stages. Nevertheless, our study shows that global hypomethylation is a clear component of the altered physiological state of muscle associated with seasonal hibernation and it stands to reason that it has an important role in regulating genome function. Particularly interesting is the possibility that global DNA methylation may alter structural features of the genome (Espada et al., 2007) considering that altered nucleolus structure and aggregation of RNA binding proteins into subnuclear particles has been reported during hibernation (Malatesta et al., 2011; Tessier et al., 2014).

Uncoupled transcription of epigenetic regulators from genomic DNA methylation

Given the differential genomic methylation over the course of torpor-arousal bouts, we further investigated whether altered transcription of DNA methyltransferases also occurred as a possible mechanism for the observed changes in methylation. We focused our interpretation of the data to the window of time that defined the greatest changes in genomic methylation of skeletal muscle, ie. the stages from euthermic animals in the cold through to early arousal (FIG. 1A). Significantly altered expression of *Dnmt* transcripts was only observed in the liver (FIG. 1F,G), but the changes in expression did not associate with changes in genomic DNA methylation at different stages of hibernation. Furthermore, no significant changes in *Dnmt* expression were observed across the torpor-arousal cycle of hibernation in skeletal muscle (FIG. 1C,D) despite decreases in global DNA methylation (Fig. 1A). If a model of maintenance methylation is to be considered, this could further implicate trans-acting mechanisms known to regulate methylation such as posttranslational (Kang et al., 2001) or posttranscriptional (Garzon et al., 2009) suppression of DNA methyltransferases. However, given the scale and nature of changes observed in muscle, we propose mechanisms of hypomethylation that act independently of methyltransferase function through passive (Kagiwada et al., 2013) or active demethylation (Kohli and Zhang, 2013). In passive demethylation, muscle-specific protection from atrophy and wasting during hibernation (Lin et al., 2012) suggests the possibility of myosatellite rejuvenation of the cell pool in the absence of DNMTs, leading to genomic hypomethylation. We speculate this molecular regulation of the myocyte cell line based on previous reports where this tissue was shown to have the highest variability in genomic methylation(Horvath, 2013), suggesting a dynamic role in cell fate during self-renewal (Motohashi and Asakura, 2012). Similarly, active demethylation of the genome through a DNA demethylase could accompany hypomethylation. Currently, a *bona fide* DNA demethylase has yet to be fully characterized despite several lines of evidence in non-dividing cells and tissues within the brain (Bocker et al., 2011; Guo et al., 2011; Ma et al., 2009). In this respect, it has been suggested that DNA demethylation occurs through hydroxylation of methylcytosine mediated through complexes of the ten-eleven-translocation (TET) family of enzymes (Kohli and Zhang, 2013).

While changes in the transcription of DNMTs between euthermic and early torpor animals were not accompanied with global changes in DNA methylation in liver, their expression does not necessarily drive a genome-wide change in methylation. For example, many studies examining the localization of methyltransferases with respect to other repressive marks

demonstrate the targeted hypermethylation of a small subset of the genome (Jin et al., 2012). Additionally, limitations of LUMA would not be able to discern small changes that affect a targeted area of the genome. These DNMTs could likely serve a function in *de novo* methylation independent of DNA replication due to cell cycle arrest seen in the liver during bouts of torpor (Wu and Storey, 2012a). Previous studies have shown that the expression of *Dnmt3b* is mediated by the transcription factor BmaL with circadian rhythms in the liver known to underlie metabolic rate depression during hibernation in ground squirrels (Maekawa et al., 2012; Ruby et al., 2002).

The MBD family of proteins retains a conserved methyl binding domain motif that serves as a reader of the DNA methylation patterns and mediates the activities of DNA methylation (Nan et al., 1993). These proteins can be present in varying amounts that do not necessarily rely on the abundance of genomic methylation; for example, MBD2 and MBD4 are overexpressed in CD4+ T-cells that exhibit genomic hypomethylation in erythematosus lupus (Balada et al., 2007). MBDs can act in both repressor (Klose and Bird, 2006; Ng et al., 1999) and activator (Baubec et al., 2013; Bhattacharya et al., 1999; Brown and Szyf, 2007) roles. In this study we identified differential expression of MBD transcripts in both liver and muscle over torpor-arousal during hibernation. In muscle, *Mecp2* expression changed between euthermia and arousal (FIG. 2D). In the liver, *Mbd3* and *Mecp2* mRNA levels differed between animals that were euthermic in the cold room and those in early arousal. We speculate that *Mbd3* transcription may support the activation of rRNA, as seen in previous studies (Brown and Szyf, 2007), consistent with the need for rapid reactivation of liver ribosomal protein synthesis when animals arouse from bouts of torpor (Epperson et al., 2004; Fedorov et al., 2009). Additionally in muscle, MeCp2 has been reported to be involved in myogenesis since its role in forming pericentric heterochromatic centers is important for differentiation (Brero et al., 2005). However, these roles of MBDs in regulating changes in genomic function are intricate in many tissues (Gunther et al., 2013; Yasui et al., 2013) and will require additional studies to validate their actions within hibernation stages.

Tissue-specific methylation control of *Mef2c*

In addition to our genome wide screen, a candidate gene screen revealed a unique promoter-specific pattern of methylation that is dynamic during bouts of torpor. We focused on *mef2c* since previous studies showed that it is upregulated during torpor along with downstream targets GLUT4 and MyoD in skeletal muscle (Tessier and Storey, 2010; Tessier and Storey,

2012). *mef2c* was particularly interesting to focus on due to its activity as a transcription factor known to regulate many downstream targets of muscle development (Potthoff et al., 2007a). For example, *mef2c* has been shown to directly regulate myomesin and M-protein, known mediators of skeletal muscle function (Potthoff et al., 2007a). *Mef2c* deletion and overexpression was also shown to regulate activity-dependent fast to slow fiber transformation in skeletal muscle. Interestingly, the overexpression of *mef2c* can promote the slow fiber phenotype in skeletal muscle, enhancing endurance and enabling mice to run twice the distance of wild type littermates (Potthoff et al., 2007b). Given a tentative role in maintaining the myocyte state (Breitbart et al., 1993) and endurance, we speculate that these roles support each other in a transcriptional program that sustains the functionality of skeletal muscle during hibernation bouts. This would support the crucial role of skeletal muscle in shivering thermogenesis to rewarm animals during arousal and potentially minimize/avoid atrophy during torpor so that the aroused animal is prepared to resume euthermic life. Regarding DNA methylation, a previous study showed that pharmacologically induced hypomethylation in murine myocytes could enhance myogenesis (Montesano et al., 2013). Interestingly, induced transcripts in this study such as MyoD are known downstream targets of *mef2c* in *I. tridecemlineatus* during bouts of torpor (Tessier and Storey, 2010). Furthermore, methylation of Mef2 binding sites within gene promoters inhibited transcription factor binding (Palacios et al., 2010). We showed dynamic changes in DNA methylation of the *mef2C* gene during bouts of hibernation in muscle but not in the liver, while expression changes occurred in both tissues. It is important to note that methylation signatures and their function are tissue- and cell type-specific (Razin and Riggs, 1980). It is possible that cis-regulatory regions (enhancers and promoters) may constrain *mef2c* DNA demethylation to specific tissues (Andres et al., 1995). Although it is clear that in the muscle the region analyzed is not involved in silencing of the gene, our *in vitro* luciferase assay of the *Mef2c* promoter at the very least validates that DNA methylation is capable of inhibiting its transcriptional activity supporting our interpretation of muscle-specific *Mef2C* regulation.

Taken together, our data demonstrate that DNA methylation is dynamically altered during hibernation and that there can be an organized response by DNA methylation to environmental cues in adult tissues. However, it is still unknown what the physiological role of these DNA methylation events are in orchestrating the molecular changes seen in hibernating mammals over the torpor-arousal cycle. Additionally, considering the tissue-specific expression of epigenetic regulators, this study begs for more comprehensive studies aimed at understanding how genome wide changes in methylation affect specific gene

functions. To our knowledge our work reports, for the first time, differential DNA methylation (and its machinery) within torpor-arousal cycles in a mammalian hibernator. We also characterize, for the first time (*in vivo* and *in vitro*), promoter methylation of a gene that is differentially regulated in hibernation. This work demonstrates that DNA methylation is dynamic and capable of changing significantly at both genome-wide and gene-specific levels long after mammals have developed and reached maturity. Considering the time scales over which these changes occur, the data also provides a new, but limited, understanding of the chronicity in which DNA methylation can change in cycles (in a matter of days). Whereas these mechanisms have been classically studied in cancer and disease states, few studies have examined the roles that dynamic DNA methylation in adult tissues could play in natural biological phenomena, thus emphasizing its importance to other fields of organismal and ecological biology.

Materials and Methods

Animal Collection and hibernation protocol

Thirteen-lined ground squirrels, *I. tridecemlineatus*, were captured by a trapper licensed by the United States Department of Agriculture (TLS Research, Bartlett, IL, USA) using humane traps. Male squirrels were used for hibernation studies as previously described; temperature transponders implanted under the skin allowed body temperature (T_b) to be monitored to identify sampling times (McMullen and Hallenbeck, 2010). Studies took place within the natural hibernation season (January and February) in the northern hemisphere. For sampling, animals were anesthetized with isoflurane and decapitated within 2 min of removal from the hibernation chamber. Tissues were rapidly excised, flash frozen in liquid nitrogen, transported to their destination on dry ice and then stored at -80°C until use. All animal procedures were approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke (National Institutes of Health, Bethesda, MD, USA; animal protocol no. ASP 1223-05). Animals were sacrificed at the following seven stages of as illustrated in FIG. 1A (McMullen and Hallenbeck, 2010): euthermic at room temperature (A), euthermic in the cold room for 3 days (Tb 34-37ºC)(B), entrance into torpor with decreasing T_b (18ºC≤ Tb≤ 31ºC) (C), early torpor with core T_b of 5-7ºC for ~24 hours (D), late torpor with core T_b between 5-7ºC for at least 3 d (E), early arousal from torpor with T_b rising to at least 12ºC (F), interbout arousal with euthermic T_b re-established at 36-38ºC (G).

DNA/RNA extraction

Thigh hind leg skeletal muscle and liver were collected in biological replicates of n=7 for each sampling stage. Tissues were ground to a powder under liquid nitrogen and split into 300 mg aliquots for separate RNA and DNA extractions. Tissues were homogenized with a Tissue Tearor (Biospec, Bartlesville, OK, USA) in Trizol (Invitrogen) for RNA extraction and extracts prepared according to manufacturer's protocols (Invitrogen). Quantity and quality of RNA was determined using spectrophotometric analysis. DNA extractions were carried out in an extraction buffer (500 µl) containing Proteinase K (20 µl:20mg/µl; Roche, Basel, Switzerland) at 56ºC for 12 hours. Samples were then treated with RNAse A (50 U/mg; 30 min; Roche) and phenol chloroform 1:1 v:v. DNA was then pelleted with 70% ethanol and resuspended in 100 µl of buffer (10 mM Tris-HCl, 1 mM EDTA). Quantity and quality of DNA was determined using spectrophotometric analysis.

Bisulfite pyrosequencing

DNA was treated with sodium bisulfite as described previously (Clark et al., 1994) and primers were designed for a converted 136 bp fragment (Scaffold Name: JH393279.1; 7205500-7205635 according to SQUIRREL Ensembl Release 66) of the *mef2C* promoter. This promoter was predicted from an alignment of the fully annotated human *mef2C* gene with the *I. tridecemlineatus* homolog. Bisulfite treated *mef2C* promoter region PCRs were amplified using two rounds of PCR with outer and nested primers (see Table 1). Cycling conditions involved an initial step of 5 minutes at 95°C followed by 35 cycles of (95°C for 1 minute, 60°C for 2.5 minutes, and 72°C for 1 minute); the final step was a 5 minute hold at 72°C. PCR products were sequenced using the Biotage Pyrosequencer according to the manufacturer's protocol (Biotage, Uppsala, Sweden).

Expression analysis

For all samples, 500 ng of RNA was subjected to RT-PCR according to manufacturer's protocols (Roche) and quantified using quantitative PCR on the Lightcycler 480 (Roche,Indianapolis, IN, USA). Primers for all genes (see Table 1) were created across exon boundaries using Autoprime (Wrobel, 2004) and were validated with single peak melting curves following PCR and matching product sizes on agarose gels. G*apdh* was used as a reference gene for relative quantification as it was previously shown to have stable expression in across hibernation bouts in *I. tridecemlineatus* (Wu and Storey, 2012a; Wu and Storey, 2012b). Quantitative PCR was conducted with a pre-incubation at 95°C for 10 minutes followed by 45 cycles of [95°C for 10 seconds, 60°C for 10 seconds, 72°C for 10 sec] with a final hold of 10 minutes at 72°C.

Luminometric Methylation Assay (LUMA)

LUMA was used to assay global DNA methylation across the genome. Our method was based on that described by Karimi et al. (Karimi et al., 2006b) with minor modifications. LUMA involves the digestion of genomic DNA by methylation sensitive (HpaII) or insensitive (MspI) restriction enzymes in combination with an internal control restriction enzyme (EcoRI) to normalize DNA input. EcoRI (R0101), HpaII (R0171) and MspI (R0106) were all purchased from New England Biolabs. Samples were incubated (37°C, 4 hours) and then heat inactivated (80°C, 20 minutes). Digested genomic DNA (15 µl) was mixed with pyrosequencing annealing buffer (15 µl; Qiagen, Toronto, ON, Canada) and pyrosequenced according to manufacturer's guidelines (PyroMark 24; Biotage, Uppsala, Sweden). The

nucleotide dispensation order used was based on Karimi et al. (Karimi et al., 2006a; Karimi et al., 2006b). All samples were run in technical triplicates with biological replicates of seven. Data was then presented as an index of overall genomic methylation calculated as [(HpaII/EcoRI) /(MspI/EcorI)]. This is a measure of CCGGs that are demethylated divided by methylated CCGGs.

In vitro Luciferase Assay

Luciferase construct PCR primers (Table 1) were made to amplify the 136bp fragment of the *mef2c* promoter. BamHI and HindIII restriction sites were incorporated into primers in order to clone into the CpG-less pCpGl (Klug and Rehli, 2006) and cloned in 5' to 3' (sense) or 3' to 5' (antisense) orientation, respectively. The constructs were methylated in vitro with SssI CpG DNA methyltransferases (M0226L, New England Biolabs) as recommended by the manufacturer. Transfections were performed using calcium phosphate precipitation as described previously (Champagne et al., 2006) into HEK293 cells (CRL-1573, ATCC). Cells were harvested 48 hours after transfection and luciferase activity was assayed using the Luciferase Assay System (Promega).

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM), n = 7 independent samples from different animals. A one-way ANOVA was performed on each dataset followed by a Dunnett's test assigning euthermic in the cold room as control. Outliers were excluded based on Grubb's outlier tests on the data for a given stage of hibernation. Significance was set at $P<0.05$. Statistical analysis was undertaken using Prism (GraphPad Software Inc, San Diego, California).

List of symbols and abbreviations used

DNMT: DNA methyl transferase

MBD: Methyl binding domain

MeCP: Methyl CpG binding protein

Tb: body temperature

LUMA: Luminometric methylation assay

Mef2C: Myocyte enhancer factor 2C

Competing interests: The authors declare no competing interests.

Author Contributions: SA, KBS and MS designed the experiments. SA, TM, and SL performed experiments. SA, KBS, and MS analyzed the data. SA, KBS and MS wrote the manuscript.

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Figures

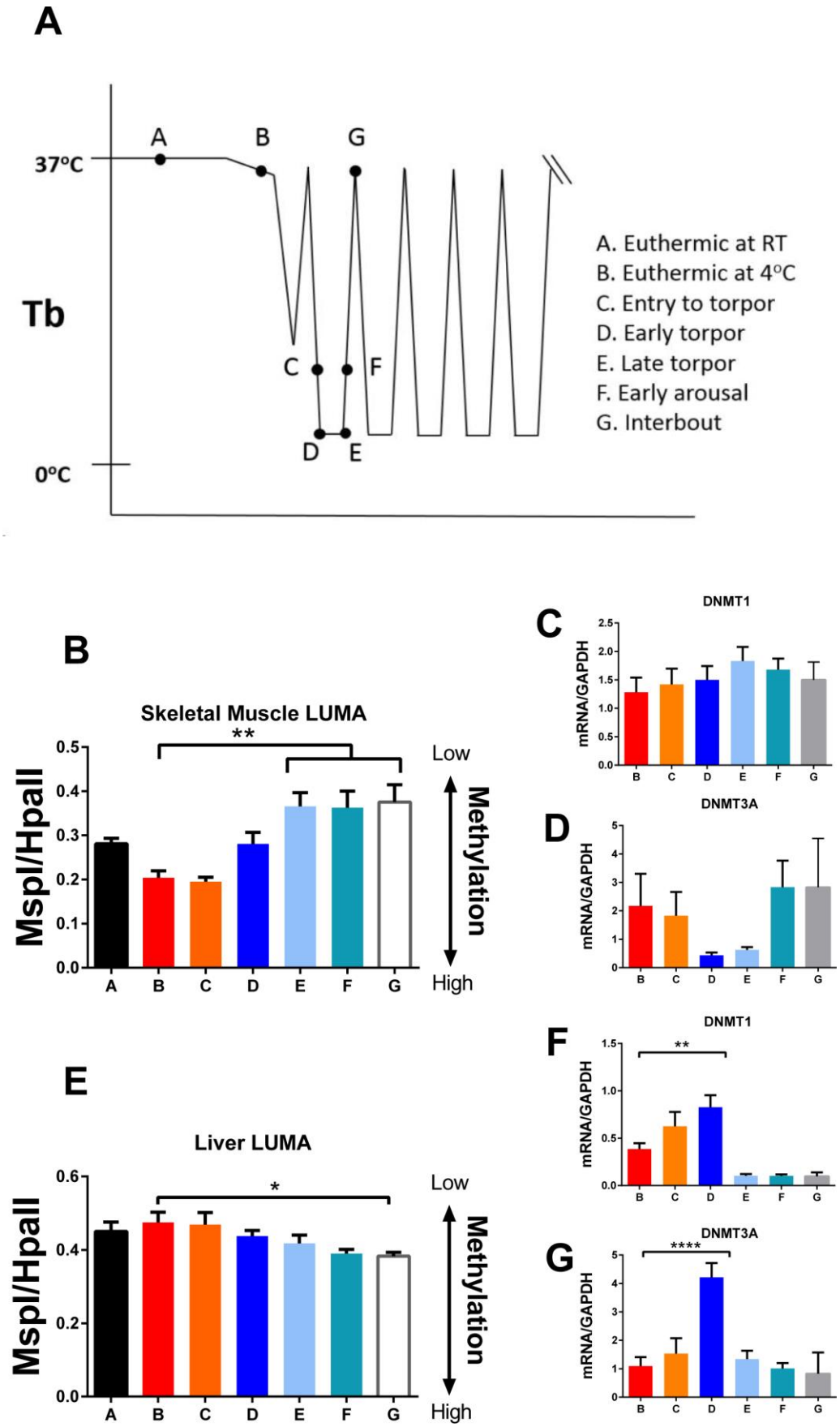

FIG. 1: **Levels of genomic DNA methylation over the torpor-arousal cycle of winter hibernation in thirteen-lined ground squirrels, *I. tridecemlineatus*.** (A) A scheme of body temperature variations over the course of torpor-arousal bouts showing the different sampling points used in this study. (B) Global DNA methylation levels across torpor-arousal in skeletal muscle and expression levels of *dnmt1* (C) and *dnmt3b* (D). (E) Global DNA methylation levels across torpor-arousal in liver and expression levels of *dnmt1* (F) and *dnmt3b* (G). Data in histograms are mean ± SEM, n = 7 independent samples from different animals. One-way ANOVA was carried out followed by a Dunnett's post-hoc test. * Significantly different from the control [(Euthermic in the Cold (EC)] value, * P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001.

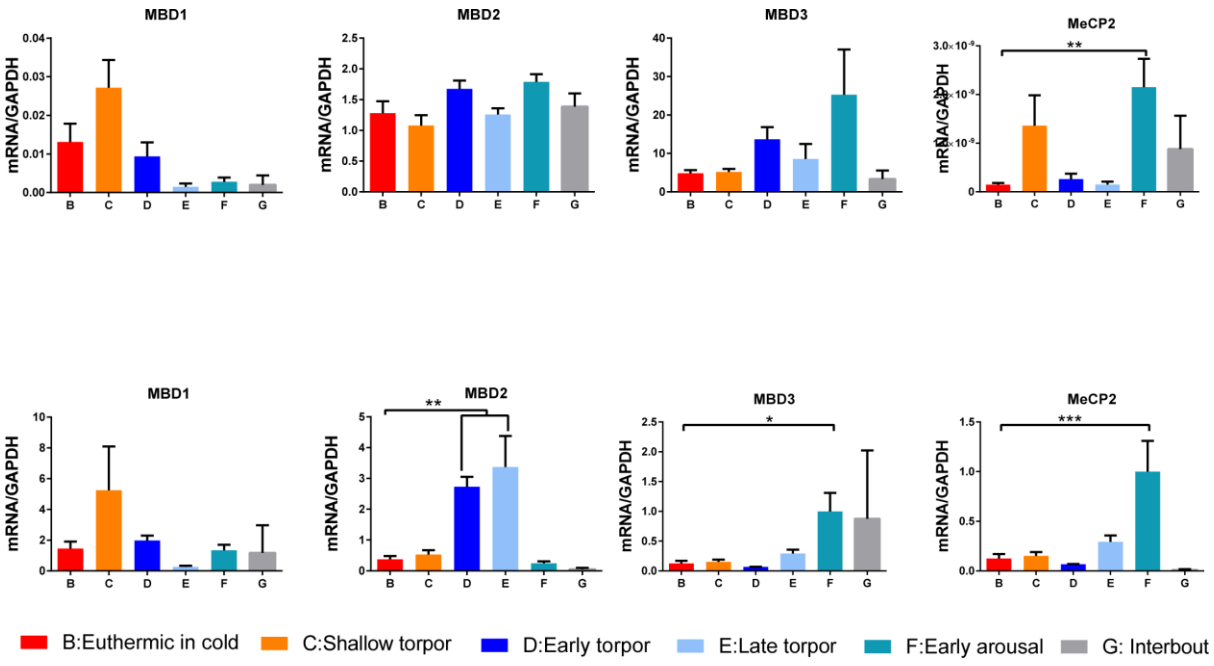

FIG. 2: **Differential expression of mRNA transcript levels of methyl binding domain proteins across the torpor-arousal cycle of hibernation.** Expression of *mbd1* (A), *mbd2* (B), *mbd3* (C), and *mecp2* (D) in skeletal muscle. Expression of *mbd1* (E), *mbd2* (F), *mbd3* (G), and *mecp2* (H) in liver. Other information as in Fig. 1. One-way ANOVA was carried out followed by a Dunnett's post-hoc test. * Significantly different from the control [(Euthermic in the Cold (EC)] value, * P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001.

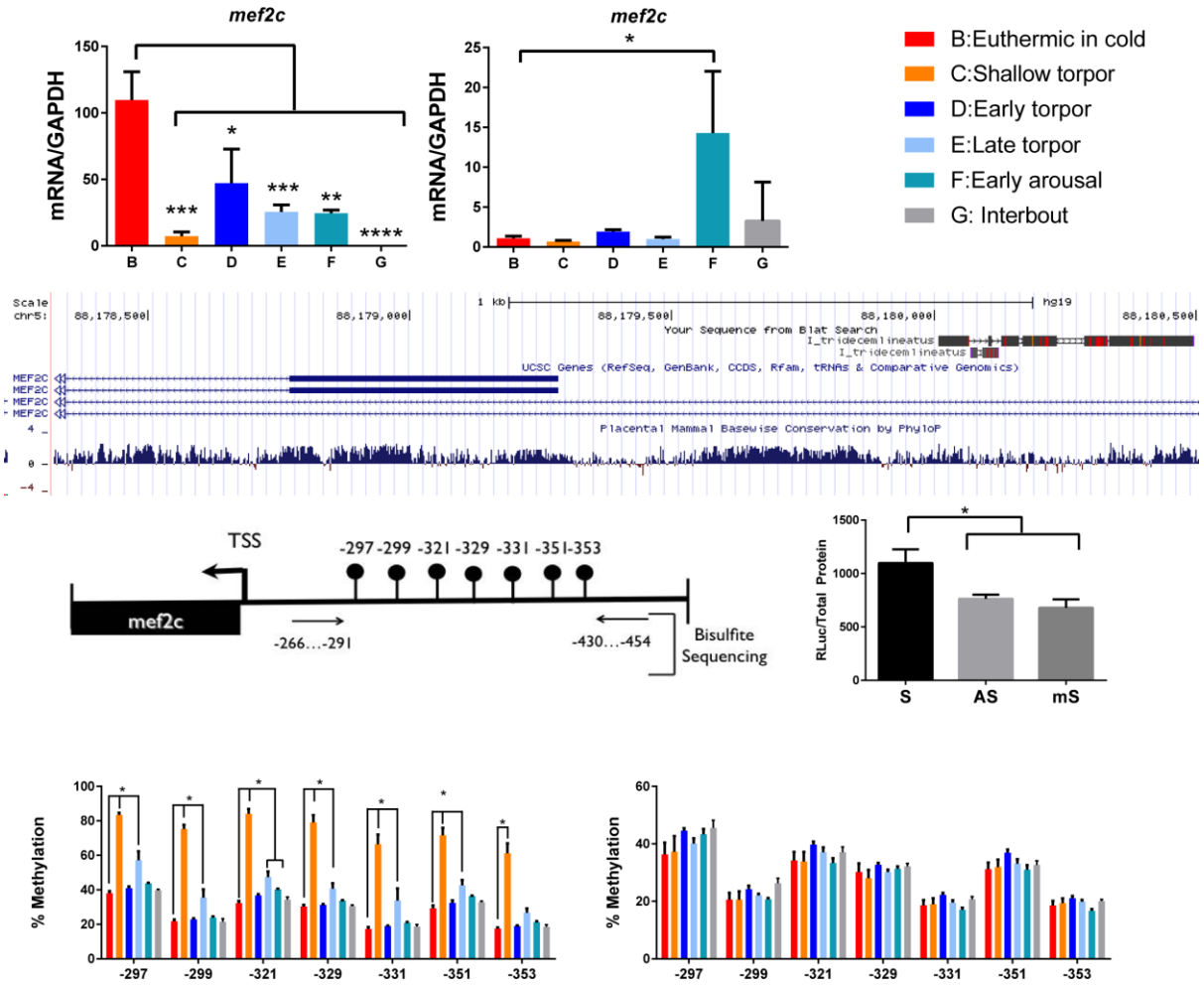

FIG. 3: **Dynamic methylation of the *mef2c* promoter at different hibernation stages.** Expression of *mef2C* mRNA measured in muscle (A) and liver (B). (C) Alignment of the *I. tridecemlineatus mef2c* promoter against the human *mef2c* promoter. (D) Physical map of the CpG dinucleotides in the *mef2c* region highlighted with balloons. Bisulfite map of DNA methylation in the *mef2c* promoter in muscle (E) and liver (F). (G) Luciferase reporter constructs containing the *mef2c* regulatory region in pCpGl in [S] sense, [AS] antisense and [mS] methylated sense directions were transfected into HEK293 cells and their ability to direct expression of a downstream coding sequence was determined by assaying luciferase activity in extracts of transfected cells. Other information as in Fig. 1. One-way ANOVA was carried out followed by a Dunnett's post-hoc test with Euthermic in the Cold Room (ECR) as our control.

* P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001.

Tables

Table 1: Primer sequences used for bisulfite sequencing, in vitro promoter assays and expression analyses.

Primer	Forward	Reverse
qpcr DNMT1	AGGCGGCTCAAAGACTTG	TCAATTTCTCCTTCACACACTC
qpcr DNMT3B	CTGCAAGCCCAGCAGTCC	GGACTTGGAAGCACTGTTGTTG
qpcr MBD1	GGAGACCCAAGAGGATGAG	AGCTGATTCCACAGTTCTCAC
qpcr MBD2	AAATAAGGGTAAACCAGACTTG	ACTTTGGTTACTGGTTGTTTG
qpcr MBD3	AACCAGGTCAAAGGCAAG	TTAAAGATGGACGCTGTCTG
qpcr MEF2C	CGTCTCCAGCTCTCTGCTCT	CAGCCTTGAAGTGCTTCTCC
bis mef2c	AATAGTTGTTTTTGTGATTTTTTTT	TTTAACCCTTCCACTTCTCAAAAC
seq mef2c	TTGTTTTTGTGATTTTTTTTTTTATT	
luc mef2c S	GGATCCTATAAAAGTCGGGATCTTTCCTCTG	AAGCTTTATATCTTTCGGTTCACTTTTTAGCC
luc mef2c AS	AAGCTTTATAAAAGTCGGGATCTTTCCTCTG	GGATCCTATATCTTTCGGTTCACTTTTTAGCC