Regulation and modification of peripheral circadian molecular clocks in 13-lined ground squirrels during hibernation.

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Regulation and modification of peripheral circadian molecular clocks in 13-lined ground squirrels during hibernation.

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Abstract

During winter, hibernators are able to conserve energy during times of limited resources through the virtual cessation of energetically expensive processes that are thought to be intrinsic to the cell in homeostasis. During prolonged hibernation, these mammals, such as the 13-lined ground squirrel (*Ictidomys tridecemlineatus*), shut down the bulk of transcription and translation in order to preserve resources yet still require the expression of subsets of genes to assist with the challenges encountered during hibernation. Hibernators provide a unique opportunity for examining the dynamics of circadian clock activation in a system that requires the selection of groups of transcripts against a backdrop of suppressed cellular activity. This research shows that peripheral circadian clocks are regulated and have adapted to function in a tissue-specific manner that is congruent with the tissues functions during hibernation.

In addition, substantial transcriptional and post-transcriptional machineries are required to endure deep torpor and low body temperature, including increased regulation over genomic activity by epigenetic enzymes. Both RNA adenosine and protein arginine methylation act to regulate activity within the circadian clock via epigenetic mechanisms and provide novel opportunities to uncover information about the post-translational modifications used during hibernation. RNA N6-methyladenosine (m6A) dynamics were maintained during hibernation and levels of m6A were increased on mRNA transcripts during torpor in liver. Responses by protein arginine methyltransferase (PRMT) enzymes were tissue-specific and within liver and white adipose, revealed responses that characterized metabolic reprogramming, whereas skeletal muscle PRMT activity was centered around transcriptional regulation. This research suggests that dynamic epigenetic

modifications provide a mechanism for maintaining translation of selected groups of necessary transcripts during hibernation, including core circadian clock genes, against a backdrop of stunted transcript processing. These data also provide evidence that the circadian clock is an important and integral regulator of peripheral tissues within the mammalian hibernation phenotype.

Acknowledgments

I wish to thank everyone who has helped me get to this point.

But I have the space to be a bit more specific – so here goes:

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List of Abbreviations

13LGS ..... 13-lined ground squirrel
4E-BP ..... Eukaryotic translation initiation factor 4E-binding protein 1
ACTA1 ..... Actin, alpha skeletal muscle
ADAR ..... Double-stranded RNA-specific adenosine deaminase
aDMA ..... Asymmetric dimethylation
AKT ..... RAC-alpha serine/threonine-protein kinase
AlkB ..... Alpha-ketoglutarate-dependent dioxygenase AlkB
ALKBH5 ..... AlkB Homolog 5
ANOVA ..... Analysis of variance
APS ..... Ammonium persulfate
ARNTL ..... Aryl hydrocarbon receptor nuclear translocator-like protein 1
ATP ..... Adenosine triphosphate
BAT ..... Brown adipose tissue
BCL ..... B-cell lymphoma 2
BCL2L1 ..... Bcl-2-like 1
bHLH ..... basic helix-loop-helix domain
BMAL1 ..... Brain and Muscle ARNT-Like 1
cAMP ..... Cyclic adenosine monophosphate
CBP ..... CREB binding protein
CCG ..... Clock-controlled gene
cDNA ..... Complementary DNA
CK1 ..... Casein kinase 1
CLOCK ..... Circadian Locomotor Output Cycles Kaput
Cq ..... Quantitation cycle
CREB ..... cAMP response element binding protein
CRY ..... Cryptochrome
Ct ..... Cycle threshold
D-box ..... [Vitamin] D-binding element
DEC1 ..... Deleted in esophageal cancer 1

DNA ..... Deoxyribonucleic acid
DTT ..... Dithiothreitol
E-box ..... Enhancer box
EA ..... Early arousal
EC ..... Euthermic control
ECL ..... Enhanced chemiluminescence
EDTA ..... Ethylenediamine tetra-acetic acid
EGTA ..... Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
eIF ..... Eukaryotic initiation factor
ELISA ..... Enzyme-linked immunosorbent assay
EN ..... Entry to torpor
FABP ..... Fatty acid binding protein
Fos ..... FBJ murine osteosarcoma viral oncogene
FTO ..... Fat mass and obesity-associated protein
GAPDH ..... Glyceraldehyde 3-phosphate dehydrogenase
HDAC ..... Histone deacetylase
HRP ..... Horseradish peroxidase
HSP ..... Heat shock protein
IA ..... Interbout arousal
IgG ..... Immunoglobulin G
JMJD6 ..... Jumonji-Domain Containing 6 protein
KMT ..... Lysine methyltransferase
L:D ..... Light-dark photoperiod
LT ..... Late torpor
m6A N6-methyladenosine
m7G 7-Methylguanosine
MAPK ..... Mitogen-activated protein kinase
METTL ..... Methytransferase like
miRNA ..... micro-RNA
MMA ..... Monomethylation
mRNA ..... Messenger RNA

MyoD ..... Myogenic differentiation 1
ncRNA ..... Noncoding RNA
NF-κB..... Nuclear Factor kappa-light-chain-enhancer of activated B cells
NR1D1 ..... Nuclear receptor subfamily 1, group D, member 1
NR1D2 ..... Nuclear receptor subfamily 1, group D, member 2
OD ..... Optical density
PAGE ..... Polyacrylamide gel electrophoresis
PAI-1 ..... Plasminogen activator inhibitor-1
PAR bZIP ..... Proline and acidic amino acid-rich basic leucine zipper
PAS ..... Per-Arnt-Sim domain
PBS..... Phosphate-buffered Saline
PCR ..... Polymerase chain reaction
PDK..... Pyruvate dehydrogenase kinase
PER ..... Period
PMSF ..... Phenylmethylsulfonyl fluoride
PPAR..... Peroxisome proliferator-activated receptor
PRMT ..... Protein arginine methyltransferase
PTL..... Pancreatic triacylglycerol lipase
PTM ..... Post-translational modification
PVDF ..... Polyvinylidene fluoride [membrane]
RAR ..... Retinoic acid receptor
RNA ..... Ribonucleic acid
RORA..... RAR Related Orphan Receptor A
RPP..... Reversible protein phosphorylation
rRNA ..... Ribosomal RNA
RT..... Reverse transcription
RT-PCR..... Reverse transcription PCR
RT-qPCR..... Quantitative reverse transcription PCR
SCN ..... Suprachiasmatic nucleus
sDMA ..... Symmetric dimethylation
SDS ..... Sodium dodecyl sulfate

SEM ..... Standard error of the mean
Sin3A ..... SIN3 Transcription Regulator Family Member A
SMAD ..... Small mothers against decapentaplegic
SSC ..... Saline sodium citrate
T_b ..... Body temperature
TBP ..... TATA-binding protein
TBST ..... Tris-buffered Saline with Tween-20
TGFβ ..... Transforming growth factor beta
TMB ..... 3,3′,5,5′-Tetramethylbenzidine
tRNA ..... Transfer-RNA
UCP1 ..... Uncoupling protein 1
UTR ..... Untranslated region
WAT ..... White adipose tissue
WT1 ..... Wilms tumor protein
WTAP ..... WT1 Associated Protein
YTH ..... YT521-B homology
YTHDC ..... YTH domain containing protein
YTHDF ..... YTH domain family protein

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Chapter 1

General Introduction

Introduction

Time is a central feature of anyone's life, and the same may be said for almost any organism. Time or more specifically, the earth's rotation on its axis and around the sun, and the resulting changes in daylight, temperature, and seasons that this causes, gives rise to an interesting feature of animal physiology and behaviour – the occurrence of both circadian and circannual rhythms that regulate diverse aspects of metabolism on a daily or seasonal basis. The circadian rhythm (from the Latin circa, about; diem, day) is the name given to physiological rhythms that occur on a daily basis, not just sleep-wake cycles but also body temperature (Tb), heart rate or blood pressure, and cognitive abilities, to name a few. Even childbirth or cardiac arrest are more likely to occur at certain times of day than others in humans. Indeed, most of our body's physiological or biochemical parameters show rhythmic variability over the 24-hour day, and this fact is taught to young medical students early on in their career, indicating its real importance (Kreitzman and Foster 2011). It is important to note that the daily physiological rhythms that we experience, occur in anticipation of, and not as a result of circadian rhythm changes, i.e., shifting one's circadian rhythm results in modulated physiological cycles that require a certain catch-up period giving rise to the colloquially-termed 'jet-lag.' The endogenicity of circadian rhythms in daily activity cycles was first commented on by Curt Richter in 1922, although Colin Pittendriegh is often credited with being the first to spur the systematic study of biological rhythm generation (Pittendrigh 1993). In the following years, chronobiological research explored biological rhythms in a plethora of animals showing that (i) many biological processes show close to 24-hour rhythms, (ii) these rhythms will persist under constant conditions, (iii) it is necessary for rhythms to

synchronize with the astronomical day [entrainment], and (iv) unlike many biochemical reactions, environmental changes in temperature do not noticeably affect the 'speed' [period] of biological rhythms.

In mammals, the control of many cyclical behaviours is centered in the suprachiasmatic nucleus (SCN), a small area of the anterior hypothalamus which receives information about the environment from sensory receptors, especially photoreceptors within the retina, and entrains [synchronizes] activity rhythms within single SCN neurons to the solar day. Efferent connections from the SCN influence endocrine (e.g., melatonin and arginine vasopressin) and neuronal signaling, ultimately synchronizing peripheral oscillators located throughout the body in most visceral organs and in several separate locations within the brain (Brown and Azzi 2013). The generation of behavioural rhythms may be exemplified by the roles of neuropeptides or humoral factors released by SCN efferents in regulating behaviour. For instance, transforming growth factor-alpha (TGF-alpha) acts on hypothalamic receptors to inhibit locomotor activity in response to light in nocturnal species, while prokineticin-2 (PK2) is regulated by circadian clock-controlled transcription and acts on receptors during the day to also decrease locomotor activity (Kramer et al. 2001; Cheng et al. 2002). Through separate diffusible factors regulating behaviours synergistically and being responsive to different environmental inputs, integration of information about physiological state is allowed.

Mammalian Hibernation

As winter approaches some small mammals display a remarkable phenotypic shift, lowering their metabolic rate and entering a state of torpor to survive seasonal stresses including extreme cold and limited food availability. This altered physiological

state, known as hibernation, is displayed annually by many animals that inhabit extreme climates, particularly at high latitudes or altitudes. The involvement of circadian controls in regulating adaptations to environmental stresses, such as those encountered by small mammals that overwinter in harsh climates, has been studied in a variety of systems and species making it clear that core molecular clock machinery must also be involved in the cellular responses to hibernation. Many animals show pronounced circannual rhythms that govern their behaviour and metabolism across the seasons of the year, influencing or defining the timing of major events including reproduction, migration, hibernation, etc.

Hibernation (Figure 1.1) is exhibited by diverse species ranging from temperate zones to the high Arctic in the Northern Hemisphere, and conversely in most southerly parts of Australia and Africa. Through the concerted effects of major physiological and biochemical changes, many small mammals can sink into a profound state of torpor, allowing their body temperature to fall to near ambient, and survive solely on stored body fats for days or weeks at a time. It has been estimated that hibernators can potentially conserve about 90% of the energy that they would normally require to remain active and euthermic all winter (Wang and Wolowyk 1988). Therefore, hibernation serves as a drastic mechanism for surviving harsh winter conditions in small mammals.

Molecular regulation of hibernation

For the thirteen-lined ground squirrel (13LGS), *Ictidomys tridecemlineatus*, preparation for hibernation begins when animals enter a phase of hyperphagia in early August during which they accumulate enough fat to increase body weight by around 50%. Pre-hibernation mass gain is accomplished through increased food consumption during the late summer and fall seasons, and is linked to increased circulating insulin

concentrations following its release from the pancreas (Boyer *et al*., 1993; Boyer and Barnes, 1999; Florant *et al*., 1990). Insulin then binds membrane receptors initiating a signaling cascade which ultimately promotes glycogen synthesis and deposition within the liver and muscle, as well as lipid synthesis and triglyceride production. During entry into torpid states, insulin serves to increase the activity of lipogenic enzymes and proteins that deposit fat into white adipose tissue (WAT) stores and build up metabolic fuel stores (Woods and Porte, 1978). For example, protein levels and enzyme activities of lipoprotein lipase, fatty acid synthase, and diacylglycerol acetyltransferase are all elevated during ground squirrel seasonal mass gain (Mostafa *et al*., 1993; Wang *et al*., 1997). In response to increased adipose stores and increased WAT cell size (Dark, 2005; Otis *et al*., 2011), digestive satiety signals (i.e., leptin) are released by adipocytes. While leptin typically has the effect of suppressing appetite and enhancing lipid oxidation, interestingly, its anorexigenic effects are not experienced by hibernators during the seasonal pre-hibernation mass gain (Florant and Healy, 2012; Healy and Florant, 2012). Therefore, while levels of serum insulin increase during the fall, typical of any hyperphagic state, a resistance to satiety signals is also experienced in hibernators that ultimately allows for increases in WAT mass that give the 13LGS adequate fuel supplies through lipid catabolism to last through the hibernation fast (Schwartz *et al*., 2015; Wu *et al*., 2013).

During the shift to a hypometabolic state, the majority of carbohydrate metabolism is suspended during hibernation and instead lipolytic enzyme activity is upregulated in order to ensure that a majority of the hibernator's energy demands are met by lipid oxidation derived from built-up adipose stores (Hittel and Storey, 2001; Storey

and Storey, 2010). Similarly, increased levels of pancreatic triacylglycerol lipase (PTL) stimulate lipolysis via the breakdown of circulating adiposomes. Levels of fatty acid binding protein, fatty acid transporter, and enzymes involved in ketone production such as hydroxymethylglutaryl-CoA synthase, are also increased and support the switch to increased reliance on the beta-oxidation pathway (Epperson *et al*., 2010a). Similarly, levels of pyruvate dehydrogenase kinase isozyme 4, which inhibits glycolysis through reversible protein phosphorylation (RPP) of pyruvate dehydrogenase, are upregulated within muscle, heart, liver and WAT tissues (Andrews *et al*., 1998).

Due to increased fatty acid catabolism, and concurrent decreases in glycolysis, blood glucose levels fall to an annual minimum during winter (Buck and Barnes, 1999), in turn causing reductions in insulin levels (Bauman *et al*., 1987; Woods and Porte, 1978). Interestingly, increased levels of glucagon, expected to occur in the face of lowered circulating glucose levels, are not seen during mammalian hibernation (Bauman *et al*., 1987; Hoo-Paris *et al*., 1985); however, a shift in the plasma glucagon to insulin ratio does occur which favors the effects of glucagon and in turn, poises catabolism towards the breakdown of glycogen through the activation of WAT lipolytic enzymes (i.e., hormone sensitive lipase), and inhibition of pyruvate kinase (Dark, 2005; Wilson *et al*., 1992). In fact, the shift towards fatty acid catabolism is so extensive that even the breakdown of glycogen is inhibited following decreases in activating-RPP of glycogen phosphorylase (Storey, 1987; 1997). Clearly, multiple systems ensure that during torpor in the 13LGS, glycolysis is suppressed while metabolic fuel requirements are fulfilled almost entirely via lipid oxidation.

Small mammalian hibernator species descend into torpor in either a facultative or obligate nature. Hibernation occurs in several groups of mammals, including elephant shrews, rodents, bats, several marsupials and even some primates, however the most commonly used model hibernators are the ground squirrel family. The thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*, is an obligate hibernator on an annual cycle, displaying the traits mentioned above each year. Obligate hibernators, like the 13-lined ground squirrel (13LGS) and other species in the *Sciuridae* family enter and arouse from hibernation on an annual cycle regardless of environmental cues (Hut et al. 2014; Pengelley and Asmundson 1969; Kondo et al. 2006).

Torpor and arousals

During torpor in the 13LGS, characteristics of mammalian life are significantly decreased for days at a time – e.g., for animals hibernating at a T_b near 0°C (as compared with T_b of 37°C) breathing rates are reduced from 100-200 breaths/min to 4-6 breaths/min, and heart rates drop from 200-300 beats/min to 3-5 beats/min (Boyer and Barnes, 1999; Nedergaard *et al*., 1990; Refinetti, 1996; Storey, 2010). These physiological changes enable the animal's metabolic rate to plummet often to just 2-4% of resting summer values (Carey *et al*., 2003).

Decreased insulin levels further inhibit glucose uptake in peripheral tissues via decreased stimulation of glucose transporter type 4 (Tessier and Storey, 2010; Wu *et al*., 2013). Glucose uptake is further inhibited by decreased phosphoinositide-3-kinase (PI3-K) mediated activation of the serine/threonine kinase Akt, resulting from decreased insulin binding to membrane receptors, which causes a significant regulatory shift within the liver and skeletal muscle tissues (Abnous *et al*., 2008; 2010). Furthermore, levels of

downstream targets of Akt, including mammalian target of rapamycin (mTOR) and tuberin (TSC2), are not changed during torpor but their activated forms are significantly suppressed during torpor – lowering rates of gene transcription and protein synthesis (Wu and Storey, 2012a).

Decreased rates of gene transcription and protein translation are common findings in studies done in a variety of hibernating mammals (Morin and Storey, 2009; Storey, 2003; Storey *et al*., 2010), including 13LGS (Frerichs *et al*., 1998; Morin and Storey, 2006; Tessier and Storey, 2014). Protein synthesis is a major consumer of a euthermic mammal's energy expenditure, and its downregulation during hibernation aids the conservation of fuel reserves for the most essential cellular tasks (Heldmaier *et al*., 2004). This suppression has been shown to occur in almost all 13LGS tissues during torpor, including brain, liver, kidney, brown adipose tissue (BAT) and digestive organs (Biggar and Storey, 2014; Hittel and Storey, 2002). Decreases in gene expression and protein synthesis are, however, not global over the entire genome but rather are specific to genes that are nonessential for the hibernator's switch between euthermia and torpor or the maintenance of either physiological state (Epperson *et al*., 2010a; 2010b; O'Hara *et al*., 1999). As an example, within the heart of hibernating 13LGS, the transcription factor myocyte enhancer factor 2 (MEF2) is upregulated and activated through RPP leading to increased levels of the cardioprotective proteins desmin and myomesin (Tessier and Storey, 2012). Similarly, changes in RPP of ribosomal initiation and elongation factors (Frerichs *et al*., 1998; van Breukelen and Martin, 2001), as well as proteins that lower messenger RNA (mRNA) turnover (i.e., poly(A) binding protein) and assist in stabilizing and folding existing proteins (i.e., heat-shock proteins) contribute to decreasing cellular

energy usage during hibernation (Fahlman *et al*., 2000; Knight *et al*., 2000; Wu *et al*., 2015). During hibernation, regulation over genome suppression is seen in combination with upregulation of certain genes whose products function in either cellular metabolism or preservation, and therefore an intricate level of selection over biological pathways is clearly required.

Intermittent arousals from torpor occur regularly over the hibernation season (Fons *et al*., 1997; Lovegrove *et al*., 1999; Mzilikazi *et al*., 2002; Wang, 1979) and, as such, mammals must have a way to reversibly return their bodies to euthermic T_b values and facilitate the necessary increases in cellular metabolism that will increase T_b above ambient temperature (T_a). Increased reliance on carbohydrate oxidation is seen during the brief interbout arousal periods and disruption of rewarming is seen when animals are given an inhibitor of glycolysis but not when given an inhibitor of lipid oxidation (Dark and Miller, 1997; Karpovich *et al*., 2009). Furthermore, levels of mitochondrial respiration are significantly increased as are activity measurements of succinate dehydrogenase during arousal from torpor, as compared to torpid animals, when measured in either liver or muscle (Armstrong and Staples, 2010; Brown *et al*., 2013). Increased cellular respiration during arousal shows that the favourability of lipid oxidation over carbohydrate oxidation is limited only to the torpid stages of hibernation. Rewarming from torpor and required metabolism during interbout arousals account for more than half of the use of a hibernator's entire winter-time fuel store (French, 1985; Wang, 1979).

As would be expected following a sudden reversal of hypometabolism, mammalian hibernators must also find ways to protect tissues in the face of enormous

increases in oxygen consumption and free-radical generation, as well as increased carbohydrate consumption following uncoupled cellular respiration supporting thermogenesis in BAT and shivering thermogenesis in skeletal muscle (Carey *et al*., 2003; Kloner *et al*., 1998; Meyer *et al*., 2012). Increased levels of antioxidant defences are observed during arousal from hibernation including heme oxygenase 1, and associated effector proteins including nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and MAF BZIP Transcription Factor G (MafG), in the liver, kidney, brain and heart of aroused 13LGSs (Ni and Storey, 2010). Antioxidant defences stemming from the transcriptional activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are also significantly increased in skeletal muscle tissue from 13LGSs transitioning to the arousal phase of the torpor/arousal cycle (Allan and Storey, 2012; Morin *et al*., 2008; Vucetic *et al*., 2013). In a similar fashion, anti-apoptotic protein expression is increased within a variety of tissues throughout the torpor cycle, as compared to summer euthermic animals (Fleck and Carey, 2005; Logan *et al*., 2016a; Rouble *et al*., 2013), as is heat shock protein expression (Feder and Hofmann, 1999; Storey and Storey, 2011; Vermillion *et al*., 2015).

Thanks to multiple cytoprotective mechanisms that are upregulated during arousal, 13LGSs may survive multiple bouts of torpor and subsequent arousal via protection from the harmful consequences of decreased tissue use (i.e., muscle atrophy), free-radical generation, as well as shifts in metabolic fuel or energy requirements and metabolite buildup or depletion. Since mammalian hibernators have these protective mechanisms, their abilities to defend themselves from cellular stresses that are atypical of normal mammalian life are of great interest to human medical research – especially with

regards to obesity and diabetes (Kirchner *et al*., 2013; Sookoian and Pirola, 2013; Wu *et al*., 2013), aging and longevity (Storey and Storey, 2004a; Wu and Storey, 2016) and neurodegenerative damage or diseases (Drew *et al*., 2007; Logan *et al*., 2016b; Wood, 2015).

Circadian rhythmicity during hibernation

In order for the 13LGS to progress through the phases of the torpor/arousal cycle, control over a variety of cellular processes is required in the form of genomic control over transcription, protein synthesis, as well as covalent modification of metabolic enzymes and structural proteins (as previously described).

Contemporary analyses of the persistence of circadian rhythms during hibernation have been limited. Circadian rhythmicity is controlled by a master circadian clock formed by neurons within the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. Interestingly, cellular activity within the SCN is increased in torpid animals compared to euthermic animals and similarly the area shows increased glucose uptake during hibernation in contrast to the majority of brain areas (Kilduff et al. 1990; Bratincsák et al. 2007). An early hypothesis suggested that torpor duration results from normal circadian functioning under conditions that slow its progression including reduced ambient and body temperatures. The idea that low T_b and impaired temperature compensation of the circadian system may extend the cycle experienced by hibernating mammals may be compelling, but it is not substantiated by persistent, temperature-compensated rhythms within the SCN in hibernators even though torpor bout duration shifts with reduced T_b (Twente et al. 1977; Grahn et al. 1994; Malan 2010). Studies of the regulation of torpor bout duration by the SCN showed that fully-lesioned and arrhythmic ground squirrels

would present torpor-arousal cycles with different durations, and that changing the rhythm within the SCN does not change the duration of torpor-bouts (Ruby et al. 1996; Oklejewicz et al. 2001). Finally, the persistence of circadian rhythms during prolonged torpor is indicated in species whose arousals remain entrained to a time of day and that periodically emerge from their hibernaculum, such as pygmy possums (Körtner et al. 1998; Turner and Geiser 2017) and foraging bats (Park et al. 2000; Hope and Jones 2013), but not in a number of rodent species whose arousals commence at seemingly random times of day (Oklejewicz et al. 2001; Hut et al. 2002; Russell et al. 2010). It may be that opportunistic hibernators, who take advantage of favorable conditions during the hibernation season have a greater need for entrained arousals. By contrast, most rodent hibernators typically dwell in deep burrows and are not exposed to astronomical and environmental cues, and generally don't eat over the winter season; hence, they have lost this sort of rhythmicity. The insignificance of daily cues among most rodents that hibernate in deep burrows is further indicated by the lack of rhythmic melatonin levels or reduced pineal size during hibernation (Stanton et al. 1986; Florant et al. 1984; Vaněcek et al. 1984), a lack of rhythmic clock gene transcription in the SCNs (as seen in European hamsters, *Cricetus cricetus* L. and Arctic ground squirrels, *Urocitellus parryii*) (Revel et al. 2007; Ikeno et al. 2017), and the absence of circadian rhythms of T_b in Arctic ground squirrels (Williams, Radonich, et al. 2017; Williams, Barnes, et al. 2017). These results clearly show that the circadian clock is inhibited during torpor in these rodent hibernators, but the question of whether these adaptation changes extend to the peripheral oscillators is still unanswered.

Molecular regulation of the circadian clock

In mammals, the molecular circadian clock is based around a transcription factor heterodimer complex: Brain and Muscle ARNT-Like 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK), as shown in Figure 1.2. BMAL1 and CLOCK are basic helix-loop-helix-PAS domain containing transcription factors which can bind E-box regulatory elements to stimulate transcription. Importantly, the canonical downstream targets of the BMAL1/CLOCK complex are *Per* and *Cry* genes that encode the inhibitory proteins PER1, PER2, PER3, CRY1 and CRY2, and can block BMAL1/CLOCK from activating transcription (i.e., of *Per* or *Cry*) (Gekakis et al. 1998; Shearman et al. 2000). Transcriptional activation by BMAL1/CLOCK occurs during the daytime, whereas PER and CRY proteins are transcribed and translated so that their expression peaks in the evening and night hours. Translocation of PER or CRY proteins occurs via their interaction with one another and a nuclear import receptor via their nuclear localization signals (Lee et al. 2015; Miyazaki et al. 2001), and is stimulated by their accumulation in the cytoplasm as the day progresses. Cytoplasmic accumulation of PER protein is the rate-limiting step of circadian clock 'ticking,' before translocation into the nucleus as part of the PER/CRY complex, where it may block BMAL1/CLOCK-controlled gene activation after 12 or so hours (Lee et al. 2001; Vielhaber et al. 2001). The degradation of PER and CRY proteins is mediated by their phosphorylation via serine/threonine kinases casein kinase 1δ (CK1δ) or CK1ε, which activate binding by E3 ubiquitin ligase complexes and targeting the proteins for proteasomal degradation either within the cytoplasm or nucleus (Gallego and Virshup 2007). The half-lives of PER and CRY proteins are short in the presence of phosphorylation (Eide et al. 2005) and so

transcriptional repression by the nuclear PER/CRY complex is soon relieved allowing BMAL1/CLOCK to bind and activate transcription, beginning the cycle anew.

Circadian activation of gene transcription is, of course, not limited to control over its own feedback repression. The BMAL1/CLOCK complex also drives transcription of two nuclear receptor families. The Rev-ErbA nuclear receptors (Rev-ErbAα and Rev-ErbAβ, encoded by *NR1D1* and *NR1D2*, respectively) are activated by BMAL1/CLOCK binding to an E-box element in the promotor of Rev-ErbA genes, allowing for a second transcriptional feedback loop. By acting on RevDR2 and retinoic acid-related orphan receptor (ROR)-binding elements (ROREs) in the promoter sequences of the *CLOCK* and *BMAL1* genes, Rev-ErbA receptor proteins can repress transcription of *CLOCK* and *BMAL1* when transcriptional activation of *NR1D1* or *NR1D2* [by BMAL1/CLOCK] is highest and the receptors accumulate in the nucleus (Preitner et al. 2002). A third class of proteins belonging to the BMAL1/CLOCK complex are known simply as clock-controlled genes (CCGs) and may have wide-reaching and numerous effects throughout the cell. The three best known of members of this class are the proline and acidic-rich (PAR) domain transcription factors: D-box binding protein (DBP); thryotroph-embryonic factor (TEF); and hepatic leukemia factor (HLF); all of which interact with D-box elements in the promotor regions of target genes (Mitsui et al. 2001). By acting on D-box elements within gene promoter regions these transcription factors can drive the output of multiple other CCGs and also serve somewhat as tissue-specific factors of circadian output. For instance, the RAR-related orphan receptor (*ROR*) transcription factor genes contain a D-box element in their promoter regions. The ROR transcription factors, RORα and RORβ, also act on ROREs in gene promoter regions and serve as a circadian output

oscillator by controlling the transcriptional activation of CCGs, including BMAL1 (Ueda et al. 2005; J. Lee et al. 2016). By acting on the promoter regions of several transcription factors, the BMAL1/CLOCK complex clearly controls the rhythmic output of numerous CCGs and integrates tissue-specificity into the regulation of CCGs.

Interactions between CCGs and core circadian clock members within gene promoter or enhancer regions produce phases of peak transcriptional activation for CCGs depending on the DNA binding elements present within the regulatory region of each CCG. For instance, binding of the BMAL1/CLOCK complex to E-box elements is anti-phasic (i.e., separated by 12 hours) to the repressor PER/CRY complex binding whereas DBP binding to D-box elements is directly preceded by the binding of a repressor protein, nuclear-factor interleukin-3 (NFIL-3) (Panda et al. 2002; Rey et al. 2011). The combination of DNA binding elements within genic regions further allows for co-operative or interfering interactions between CCGs, serving to extend or restrict transcriptional activation, respectively.

mRNA Adenosine Methylation

In comparison to the circadian regulation of, and by, methylation of DNA or proteins, RNA methylation is nearly uncharted territory. The most studied RNA methylation modification, aside from the structural m7G-cap placed on nascent mRNA molecules, is modification of N6 on adenosine residues (m6A). Introduced through the use of DEAE-cellulose (borate) chromatography (Desrosiers et al. 1974), RNA m6A modifications (Figure 1.3) have been shown to affect nearly all aspects of the mRNA life-cycle including stability, processing and splicing, translational activity and subcelllar localization (Meyer and Jaffrey 2014). Mapping of m6A to specific RNA molecules

within the transcriptome has further enhanced our understanding of the modification's dynamic and regulatory nature. The majority of m6A modifications placed upon mRNA are deposited within the untranslated regions (UTR) adjacent to the coding region (Meyer et al. 2015), whereas m6A located within coding regions makes up only about 35% of all m6A modifications (Mao et al. 2019). Moreover, m6A-mapping studies, accomplished through m6A-RNA immunoprecipitation followed by sequencing the resulting transcripts, has revealed that m6A is enriched selectively within certain transcripts, and is typically constitutive across the transcriptome within the UTR and near stop codons (Meyer et al. 2012; Dominissini et al. 2012; Schwartz et al. 2014). This modification can also be a dynamic modification following cellular stressors or changes in the cellular environment (Ke et al. 2017; Geula et al. 2015).

It is important to understand the control of mRNA methylation that occurs in response to environmental changes since it can be said to have regulatory influences over all the genetic output of a cell. For example, novel work by Fustin et al. (2013) investigated the ratio of AdoMet to its inhibitory by-product and found that circadian cycle duration was altered in both cultured cells and in mice upon manipulation of global methylation using an inhibitor, 3-deazaadenosine (DAA). Treatment with 3-DAA was associated with reduced m6A content within exons of *Per1, Per2, Per3, Dbp* and *Nr1d1* and nuclear retention of *PER2* mRNA due to a significant delay in its half-life. To validate these results as due to downregulated mRNA methylation, the authors used *METTL3* siRNA to reduce catalytic subunit activity in the mRNA methylation complex that, in turn, reduced the number of m6A modifications on circadian transcripts. Similarly, overexpression of METTL3 protein accelerated the nuclear exit of *Per2* and

BMAL1 mRNA. These results provide a fine demonstration of the regulatory power possessed by the mRNA methyltransferase complex over mRNA processing that is relevant not to just circadian rhythms but to countless other cellular events (Hastings 2013). It is unsurprising that nearly all epigenetic mechanisms, including [epitranscriptomic] mRNA methylation, show regulatory crosstalk with the circadian clock's functioning.

Protein Arginine Methylation

Methylation of arginine residues on histones has been shown to play a role in the remodelling of chromatin and regulate protein dynamics and functioning in several cellular pathways, including the circadian clock. Arginine is well positioned to interact with other biological molecules, since it has five potential hydrogen bond donors and has been shown to form predictable, defined, and frequent interactions with RNA, DNA and proteins (Luscombe et al. 2001; Najbauer et al. 1993; Hughes and Waters 2006). Methylation of arginine (Figure 1.4) may also change the likelihood of a protein binding to certain interacting partners by increasing the protein's affinity for certain molecular features including aromatic residues (Sprangers et al. 2003). Since methylation replaces available hydrogen bond donor atoms, it can alter the interactions of arginine residues with various binding partners (Pahlich et al. 2006) and, indeed, the discovery of this modification has been shown to be increasingly relevant to cellular dynamics.

Arginine methylation of histone proteins is directly modulated by the circadian clock. Na et al. (2012) showed that the type II PRMT5 methyltransferase enzyme is a binding partner of CRY1 and that this interaction is rhythmic and dependent on CRY1 accumulation. The authors then showed that, upon binding the CLOCK/BMAL1 complex

while bound to CRY1, levels of arginine-3 on histone H4 (H4R3) were increased in a rhythmic fashion (i.e., correlated to rhythmic circadian output), whereas histone acetylation was reduced. Finally, the authors showed that these mechanisms were absent in synchronized cells transfected with *PRMT5* shRNA. Given the far-reaching effects that PRMT enzymes may have that indirectly affect circadian regulation, as well as the direct mechanism detailed here, it is clear these enzymes are important for the regulation and temporal organization of chromatin modifications and architecture.

Objectives and Hypothesis

The research reported in this thesis is an investigation of the molecular circadian clock and its regulators in the 13LGS in the hopes of understanding their relevance to torpor-arousal cycles in an obligate hibernator as well as post-translational and post-transcriptional modifications that contribute to the functioning of circadian rhythmicity at the molecular level. Through characterization of the circadian pathway's expression and post-transcriptional and post-translational regulators during hibernation, an increased understanding of circadian clock functioning in hibernation as well the discovery of tissue-specific adaptations at such times is achieved.

Mammalian hibernators are subject to substantial genomic regulation as they transition to and from the torpid state, therefore my Ph.D. thesis explores three avenues of transcriptional and translational control. The core circadian clock, RNA adenosine methylation and protein arginine methylation are each able to influence either RNA and protein processing, or both, in order to help a hibernator adapt to and overcome the stresses of prolonged cycles of torpor and arousal. This research was achieved through characterization of the molecular responses to torpor-arousal cycles by both the circadian

clock (Chapter 2) and the enzymes controlling some of the post-transcriptional (Chapter 3) or post-translational modifications (Chapter 4) that affect the activity of the canonical circadian transcription-translation feedback loop. These studies focused on liver, WAT and skeletal muscle tissues of 13LGS and involved analysis of ground squirrels sampled at five different points on the torpor-arousal time course, allowing investigation of changes that are made while entering torpor as well as during arousal from torpor. Liver and WAT were chosen for their established roles in lipid metabolism and the maintenance of homeostasis during hibernation whereas skeletal muscle was chosen for its almost complete cessation of activity during torpor and for its importance in rewarming the animal via shivering thermogenesis during interbout arousals. Each chapter deepens our understanding of the control required for successful hibernation using novel approaches.

Objective 1: Expression and Activity of the Molecular Circadian Clock

As mentioned, many rhythmic behaviors are inhibited during torpor as the animal's metabolic rate falls to less than 10% of resting euthermic rate and only the most essential cellular tasks are sustained. This likely involves control over and from the core circadian clock within peripheral tissues. Previous studies completed in other small hibernating mammals have evaluated the existence of rhythmic physiological responses as well as responses by the neural circadian clock to hibernation and determined that most outward appearance of circadian rhythmicity is diminished or absent (Körtner and Geiser 2000). This thesis greatly extends the analysis of the circadian clock by focusing on the expression and activity of core clock components and CCGs involved in regulating homeostatic mechanisms within peripheral cells/tissues of the body.

Hypothesis 1: The molecular circadian clock responds to global metabolic rate depression during torpor cycles by contributing to decreased cellular transcriptional and translational activity.

Chapter 2 tests this hypothesis by assessing changes in the relative transcript and protein levels of canonical molecular circadian clock members as well as their protein regulators to examine the tissue-specific responses and implications of their roles during hibernation. Levels of 10 circadian clock transcripts were analyzed using qRT-PCR to examine the differential transcription induced by hibernation. Secondly, nuclear transcription-factor binding activity to circadian-clock response elements (E-box) was analyzed via transcription-factor ELISAs to determine if the core transcription factor CLOCK shows differential DNA binding during torpor. Western blot analysis of proteins involved in regulating the circadian clock were completed for BMAL1, CRY2, PER2, and CK1δ/ε.

Objective 2: Response of mRNA methyltransferases to suppressed transcriptional activity

The mechanisms controlling post-translational modifications during hibernation are unknown to date. On the other hand, it is known that during hibernation a complex selection of gene activity is required since selected pathways are enhanced while the majority of genomic activity is depressed. This clearly necessitates a role for molecular controls that are able to shut down or activate gene products that are produced and preserved until required or expressed at drastically lower amounts. The METTL RNA methyltransferases (METTL3, METT14), as well as their regulator WTAP, control the deposition of m6A on RNA molecules whereas eraser proteins (FTO and ALKBH5) are

in charge of their removal and influence m6A dynamics. Furthermore, reader proteins (YTHDF1/3, YTHDC2, eIF3d) bind to and exert control over the processing and stability of RNA molecules, and mRNA in particular.

Hypothesis 2: Adenosine methylation provides a means of reducing transcriptional rates through modification of mRNA and other types of RNA during torpor. Relative levels of proteins in control of depositing or removing adenosine methylation, as well as m6A reader proteins, accommodate increased m6A dynamics across cycles of torpor and arousal.

Chapter 3 tests this hypothesis by assessing total methyl-adenosine levels using an m6A ELISA procedure to assess global differences in RNA and mRNA methylation during torpor. Protein levels of mRNA methyltransferases and levels of m6A binding partners, were analyzed using Western blotting or ELISAs in search of responses to, or preceding, the inhibition of transcriptional and translational activity during torpor and arousal.

Objective 3: Arginine methylation and its role in chromatin remodeling

Arginine methyltransferase proteins such as PRMT1-7, play crucial roles in the deposition of PTMs and epigenetic marks in all tissues and in response to almost all cellular environmental changes. Importantly, these enzymes are able to methylate arginine residues on histones H3 and H4 which can influence global levels of transcription, genomic reorganization and the recruitment of transcription factors or structural proteins to the genome-chromatin assembly. Notably, dimethylation of R2 and R8 on histone H3, or R3 on histone H4 is repressive over genomic activity. Moreover, the dimethylation abilities of PRMT enzymes allow their categorization (Figure 1. 4) into

those enzymes that are able to methylate already modified guanidine groups (Type I) from those that are only able to methylate unmodified guanidine groups on arginine (Type II).

Hypothesis 3: Arginine methylation provides a means of reducing transcriptional rates through modification of histone proteins and is involved in modifying the activity of non-histone proteins during torpor. These responses are then reversed during arousal.

Chapter 4 tests this hypothesis by performing Western blot measurements of arginine methyltransferase enzymes to provide an indication of tissue reliance on arginine methylation for manipulating chromatin binding site accessibility or protein activity during hibernation. Furthermore, antibodies specific for dimethyl-arginine histone residues indicative of this PTM's role in manipulating chromatin accessibility were used in Western blotting to determine if the aforementioned residues are differentially regulated across torpor-arousal cycles. The activity levels of PRMT enzymes was assayed using an enzyme activity ELISA specific to Type-II enzymes, including PRMT5 and 7.

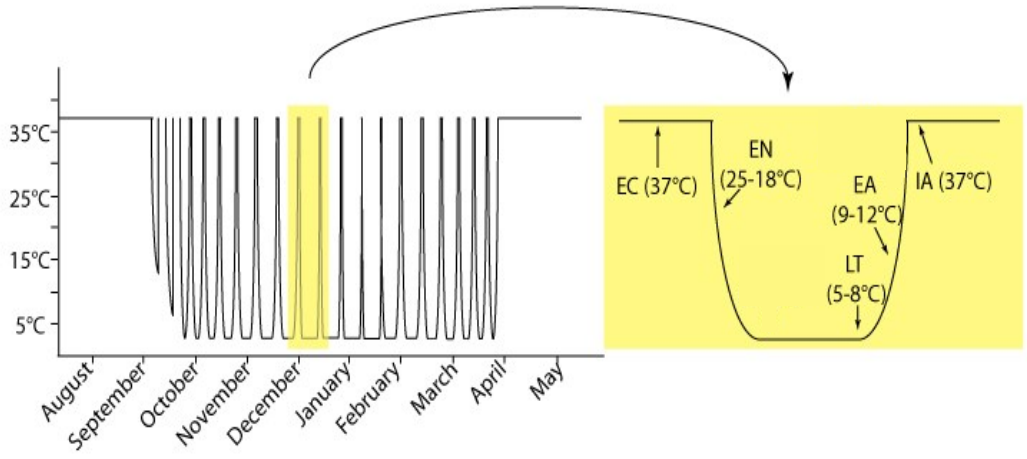


Figure 1.1: A representative image of the hibernation season of 13-lined ground squirrels displaying the relationship between hibernating body temperature and time. The hibernation season typically begins in late September with animals entering shallow torpor periods known as 'test-drops'. Torpor bouts lengthen over time as body temperature drops to lower levels and begin to shorten again as the hibernation season ends, seemingly without external cues in laboratory environments (animals held at constant 5°C in a cold room). A comparable pattern occurs in nature, associated with progressive lowering of ambient temperature (Ta) as autumn-winter progresses and a shortening of torpor bouts with spring warming. Each cycle is characterized by specific phases: EC, euthermic in the cold room (control animals); EN, entrance into torpor (approximately 12h); LT, late torpor (>5 consecutive days in a torpor bout); EA, early arousal (duration ~3h); and IA, interbout arousal (within 24h of returning to euthermic physiological conditions). Image modified from Tessier et al. (2016).

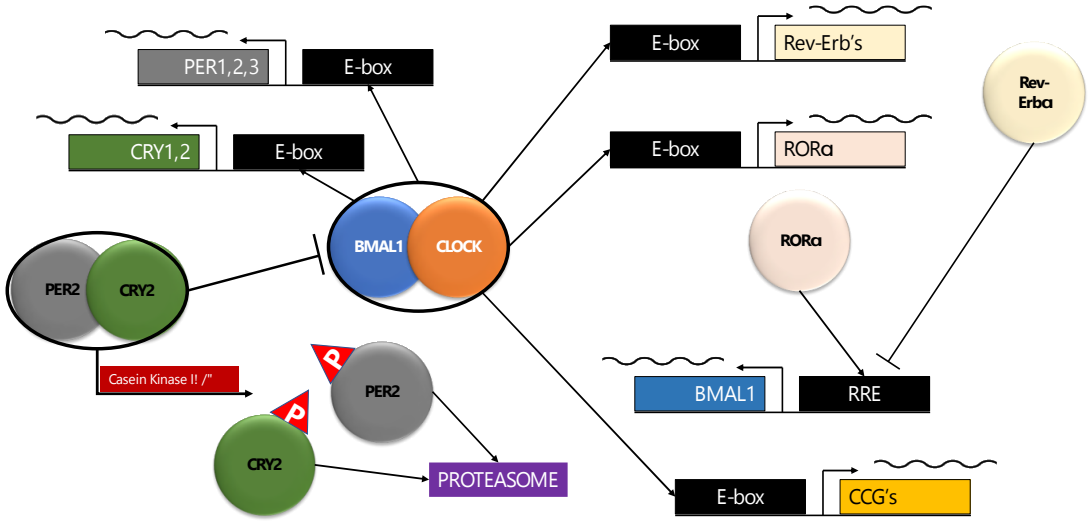


Figure 1.2: The circadian transcription/translation feedback loop in mammals is focused around the transcriptional activity of the BMAL1/CLOCK complex. This core transcription factor complex acts on the E-box element of the *Period* and *Cryptochrome* genes, whose products inhibit the transcription by BMAL1 and CLOCK. Genes for the inhibitor proteins Rev-ErbAα, Rev-ErbAβ and the activator protein RORα are also regulated by an E-box, creating a secondary regulatory loop controlling the transcription of BMAL1. Period and Cryptochrome degradation is mediated by their interaction with Casein Kinases, which phosphorylate both proteins (red triangles) and lead to shunting them into E3 ubiquitin ligase pathways. Interlocked transcriptional feedback loops whose products inhibit their activators produces a cyclic pattern of transcription and translation. In this way transcriptional regulation of downstream and tissue-specific rhythmic clock-controlled genes (CCGs) is possible. Figure modified from Buhr et al. (2013).

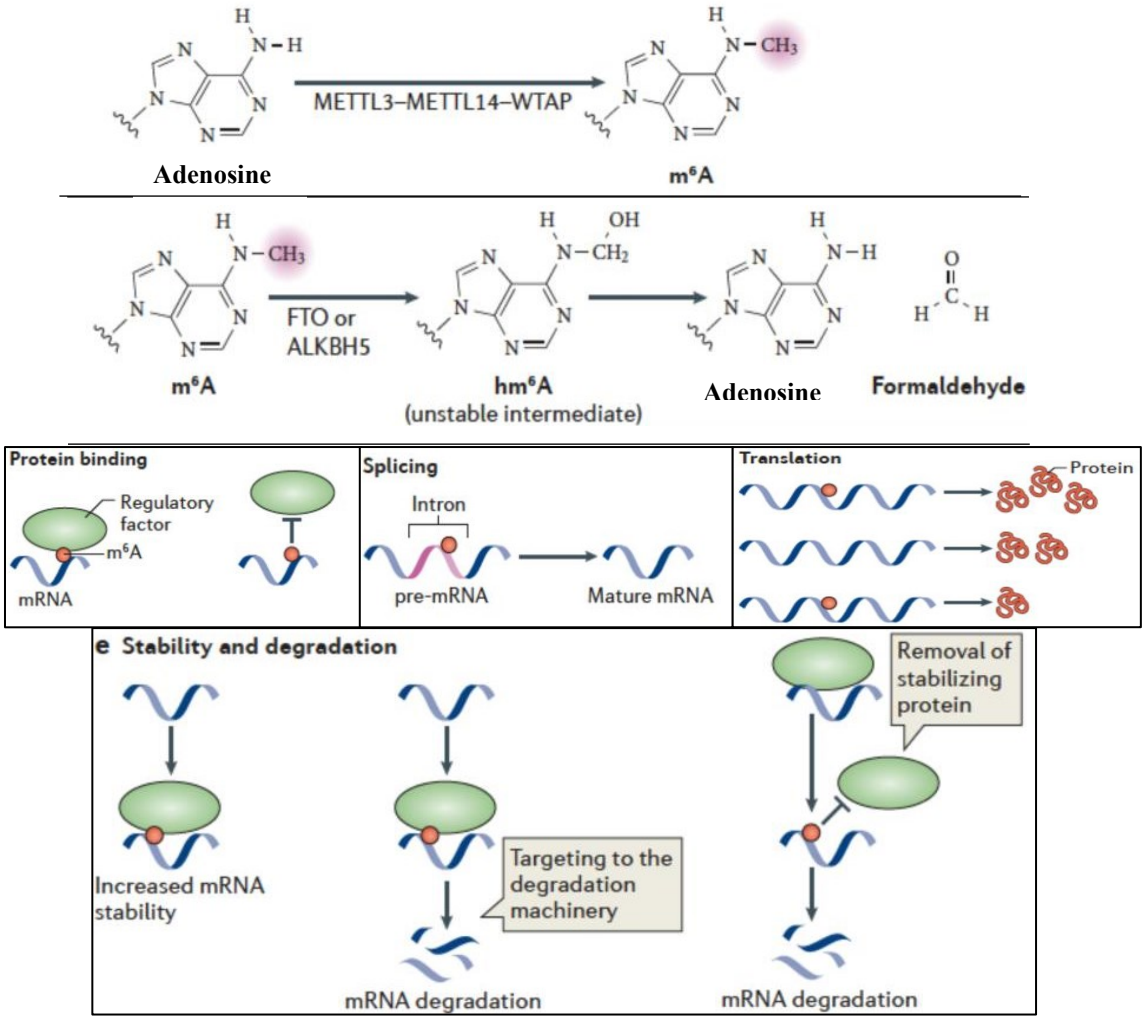


Figure 1.3: Top two panels: Modification reactions for adenosine methylation (top) by the METTL3, METTL14, and WTAP enzymatic complex, and demethylation (bottom) by either FTO or ALKBH5 demethylases. m6A modifications interfere with base triples by removing a free proton that may have bound a nucleic acid via Hoogsteen base pairing, while H-bonds that follow Watson-Crick base pairing will be unaffected. Bottom panels: Four mechanisms that allow mRNA-methylation to interfere with typical functions of mRNA, or their interactions with either catalytic, stabilizing or degradative proteins. Methylation of m6A may facilitate or block interactions with proteins, either in exon or intronic regions, or interactions with ribosomes leading to changes in translation efficiency. Image modified from Meyer et al. (2014).

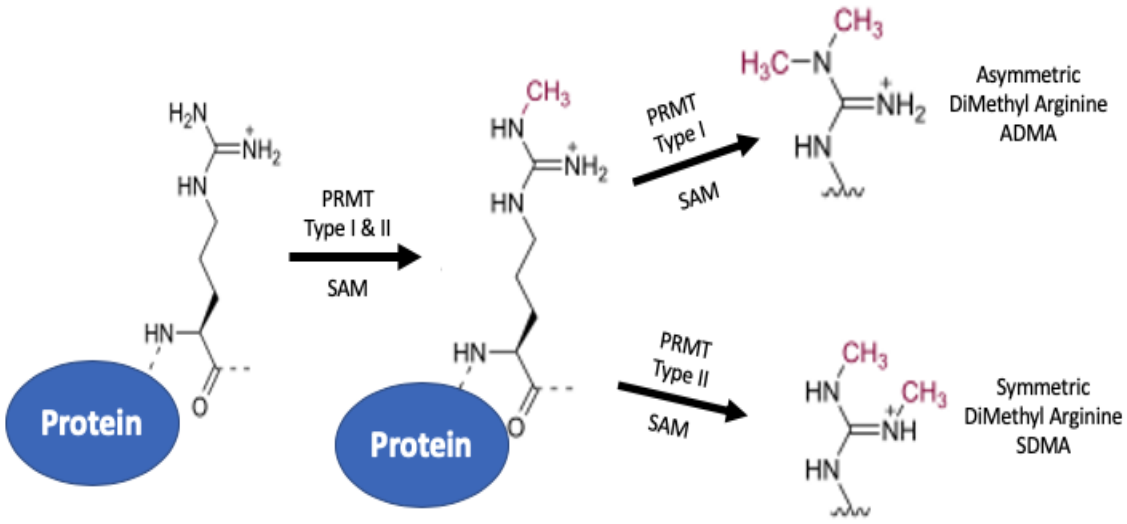


Figure 1.4: Arginine methylation by PRMTs catalyze the transfer of a methyl group to the side chain of arginine residues, thereby generating mono-methylarginine. Addition of another methyl group to the same guanidine group generates an asymmetric di-methyl-arginine, a reaction catalyzed by type I PRMTs, whereas addition of a second methyl group to another guanidine group generates symmetric di-methyl-arginine, which is catalyzed by type II PRMTs. Figure modified from Basso et al. (2015).

Chapter 2

Goin' down slow: Peripheral circadian gene activity is altered during hibernation in the thirteen-lined ground squirrel

Introduction

Over the course of a year, many animals fundamentally shift major phenotypic traits to best adapt to the changing environments they reside in. These changes, brought about and shaped by contextual clues that indicate seasonality from environmental conditions, allow these animals to meet changing demands in their environment (i.e., drought, food shortage), usually by introducing a helpful trait or removing unnecessary or harmful traits during stressful seasons (Visser et al. 2010; Schwartz et al. 2013; Boyce 1979). Hibernation in small mammals describes a predictable adaptation to environmental conditions that include cold temperatures and reduced resource supplies, and allows animals to survive these conditions through the almost complete arrest of unnecessary metabolic processes and descent into a hypometabolic state (Humphries et al. 2003; Heldmaier et al. 2004). By entering and using hibernation, small mammals can reduce their global metabolism by more than 90%, while decreasing cardiopulmonary rates to fewer than a dozen beats or breaths per minute and allowing body temperature (T_b) to fall to near ambient values (Wang and Wolowyk 1988; Nedergaard et al. 1990). Through major physiological and biochemical changes via reductions in the activity of nearly all cellular functions, hibernating mammals can sink into a state of torpor and survive solely on stored body fuels (chiefly lipids) for days or weeks at a time, interrupted by brief (~24 h) arousal periods where body temperature (T_b) returns to the euthermic level (Boyer and Barnes 1999).

The predictability of a small mammal's requirement to adapt to such stressful conditions (i.e. on a seasonal basis) have caused numerous researchers to investigate the extent to which endogenous timing mechanisms contribute to hibernation (for review, see Williams et al. 2014). Many animals show pronounced circannual rhythms that govern

their behaviour and metabolism across the seasons of the year, influencing major events including weight gain, hibernation, and reproduction, as well as molecular rhythms (Kondo et al. 2006). Further, these circannual clocks have an integral role in the persistence and sequence of seasonal life-cycle events, as shown in hibernating ground squirrels that continue characteristic behaviors even when removed from their natural environment and its accompanying external cues (Pengelley et al. 1976). For hibernators that store their fuel chiefly as fats for the duration of hibernation, preparation begins in early August as animals enter a phase of hyperphagia during which they accumulate enough fat to increase body weight by around 50%. Once peak body weight is reached after 1-2 months, the animals retreat into their burrows (a cool and dark hibernaculum) and soon begin hibernation, usually by late September (Mrosovsky and Fisher 1970; Dark 2005). Most hibernators, first go through multiple shallow bouts of torpor, known as a 'test-drops' that serve to shift their metabolic profile. These gradually deepen and lengthen until a rhythm is established typically accompanied by the animal's descent into their hibernacula but occurring also while exposed to ambient room temperatures in the lab (Wang 1973; Kisser and Goodwin 2012; Vaughan et al. 2006). This pattern gives rise to the "two switch" theory of mammalian hibernation - the first switch occurring as summer turns to autumn/winter, allowing shallow descents into heterothermy, and enabling test-drops to occur. A second switch is then responsible for alternating entries into torpor or arousal and is necessary for extended periods of torpor to take place (Russell et al. 2010; Serkova et al. 2007).

During the winter, metabolic rate inhibition, lowered rates of breathing and heartbeat and T_b set-point lead to the 'flipping' of the second switch and a steep drop in

body temperature as the animal enters the torpid phase of true hibernation (Jastroch et al. 2016; Malan 1973). It is during this phase that the most significant reductions in metabolic rate and physiological parameters take place, as energetically-expensive cellular processes (i.e., aerobic metabolism, transcription/translation, ion pumping, among others) become inhibited and the animal's cells redirect fuel sources to maintain regulatory abilities and cytoprotective or homeostatic mechanisms (Grabek, Martin, et al. 2015; Melvin and Andrews 2009). Hibernating mammals show characteristic, extended periods of torpor during which time body temperature can decrease to only a few degrees above 0°C. These torpor bouts typically last between three and seven days for ground squirrels and are interspersed with shorter [arousal] periods when body temperature returns to euthermic values. A switch in fuel utilization is also displayed. Hibernating animals primarily utilize lipids as fuels; these are retrieved from white adipose tissue (WAT) and delivered via the blood to organs that use aerobic lipid oxidation to generate ATP. To survive the long winter season only on resources stored within their bodies, hibernating mammals must also attenuate energy-intensive processes to minimal levels. The animal's metabolic rate may decrease to just 1-5% of euthermic resting metabolism, and physiological parameters such as respiration rate and heartbeat are also greatly reduced. Among hibernators in deep torpor, common metabolic responses have also been identified including: i) extensive protein post-translational modifications (PTMs) that provide a reversible switch to turn enzyme/protein activities down or off during torpor, as well as suppress processes such as global or gene-specific transcription/translation by mechanisms such as global histone-modifications or specific PTMs on transcription factors (Storey 2015; Tessier, Zhang, et al. 2017); ii) the selected upregulation of some

genes and proteins including stress-responsive transcription factors, cell-preservation factors, and antioxidant defenses in preparation for a surge in ROS production during rewarming (Eddy et al. 2006; Mamady and Storey 2008; Morin and Storey 2009; Rouble et al. 2013); and iii) differential expression of microRNA molecules and their downstream influences on mRNA translation (Frigault et al. 2017; Biggar and Storey 2017). All of these contribute to regulating the cellular environment during long-term hypometabolic states.

Given that circadian clock mechanisms are driven by transcription/translation feedback loops (Koike et al. 2012), and that both transcription and translation are globally suppressed during hibernation, save for selective genes which remain active during torpor (Hittel and Storey 2002; Tessier and Storey 2014), it is clear that the core molecular clock machinery must also be implicated in the cellular responses to hibernation. In mammals, the molecular circadian clock is based around a transcription factor heterodimer complex: Brain and Muscle ARNT-Like 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK), which are basic helix-loop-helix-PAS domain containing transcription factors that bind E-box regulatory elements on chromosomes to stimulate transcription. Importantly, the canonical downstream targets of the BMAL1/CLOCK complex are Per and Cry genes that encode the inhibitory proteins PER1, PER2, PER3, CRY1 and CRY2. PER and CRY proteins also bind E-box elements and can block BMAL1/CLOCK from activating transcription (i.e., of Per or Cry) (Gekakis et al. 1998; Shearman et al. 2000). Transcriptional activation by BMAL1/CLOCK occurs during the active period, whereas PER and CRY proteins are transcribed and translated so that their expression peaks during the resting phase.

Degradation of PER and CRY proteins is mediated by their phosphorylation via the serine/threonine kinases casein kinase 1δ (CK1δ) or CK1ε, which activate binding by E3 ubiquitin ligase complexes and target the proteins for proteasomal degradation within the cytoplasm or nucleus (Gallego and Virshup 2007). The half-lives of PER and CRY proteins are short in the presence of phosphorylation (Eide et al. 2005) and so transcriptional repression by the nuclear PER/CRY complex is soon relieved allowing BMAL1/CLOCK to bind and activate transcription, beginning the cycle anew. By acting on E-box elements within gene promoter regions these transcription factors can drive the output of multiple other protein feedback loops and can even regulate the activation of tissue-specific factors in a circadian fashion within peripheral tissues (Mitsui et al. 2001; Rey et al. 2011). Peripheral or 'slave' oscillators integrate circadian information from the 'master' circadian clock in the brain with physiological signals and serve to maintain and coordinate metabolism, appetite and activity in a rhythmic fashion. The physical separation of peripheral oscillators throughout the body allows for the generation of independent daily rhythms to influence the rhythmic signals generated by other peripheral oscillators. Multiple circadian oscillators exist throughout the body and core molecular clock machinery is expressed and active in nearly all cells and tissues as demonstrated by the generation of circadian rhythmicity in explanted rodent tissue cell cultures both before and after SCN ablation (Yamazaki et al. 2000; Yoo et al. 2004), although these rhythms typically degrade after a few days without regular input. Circadian rhythmicity within peripheral oscillators allows tissues to control their local environments and the genetic activity needed for regulating physiology or behaviour (Balsalobre et al. 1998; Yagita et al. 2001). Given the complexity and number of systems

regulated by homeostatic mechanisms, the hierarchical and multi-oscillatory nature of peripheral circadian rhythms is well-suited to its role.

The present study tested the hypothesis that the molecular circadian clock responds to global metabolic rate depression during torpor cycles and contributes to decreased cellular transcription and translation activity. This was achieved by characterizing the responses of the molecular circadian-clock within peripheral organs (liver, WAT, skeletal muscle) across torpor-arousal cycles. It is likely that during hibernation decreased transcriptional and translational activities influence the expression or activity of the molecular circadian clock, either due to altered transcription and translation of proteins involved in the required clock mechanisms, or proteins that influence the clock. The involvement of peripheral circadian clock mechanisms in regulating adaptations to environmental stresses has been shown in a variety of systems and species (van der Veen et al. 2017; Dumbell et al. 2016), but has never been explored in the context of hibernation, although it is likely that core molecular clock machinery is involved in the cellular responses required for mammalian torpor. The 13-lined ground squirrel (13LGS), *Ictidomys tridecemlineatus*, engages in hibernation to survive prolonged periods of resource scarcity during the winter and has been a commonly studied model organism for understanding the adaptations required for, and consequences of, prolonged hypometabolism. By examining changes in the protein levels of core molecular clock proteins or mRNA levels of downstream clock-controlled genes (CCGs), activity and turnover of the molecular clock may be analyzed during torpor and arousal. Activity levels of the molecular clock can be further gauged by assaying the DNA-binding potential of CLOCK during hibernation within peripheral tissues in order to

assess the potential for changes in downstream transcriptional activity. While it was hypothesized that, due to global changes in transcription and translation, circadian clock activity would be downregulated during hibernation, it was instead found that circadian clock activity is persistent during torpor.

Methods

Animal and Experimental Procedures

Wild 13LGS weighing at least 150 g were caught in summer by a trapper (TLS Research, Bloomingdale, IL) licensed by the US Department of Agriculture and were transported to the National Institute of Neurological Diseases and Stroke (NIH, Bethesda, MD, USA) where they were housed and cared for as described previously (Cai et al. 2004). The NINDS animal care and use committee (ACUC) approved all holding and experimental procedures (protocol number ASP 1223-05). Briefly, squirrels were housed individually in plastic shoebox cages in a vivarium with an ambient temperature of 21°C, a 12:12 L:D photoperiod, and were provided with standard rodent diet and water *ad libitum* until they gained sufficient lipid stores to enter hibernation. Well before experimental work began, squirrels were briefly anesthetized with 5% isofluorane and fitted with a telemetry device (IPTT-200; Bio Medic Data Systems, Seaford, DE) implanted subcutaneously to collect body temperature (T_b) measurements. The animals were moved into a hibernaculum for the months of November to March with an ambient temperature of 4-6°C and 60% humidity under 24-hour darkness. A photographic red safelight (3–5 lx) was used when necessary and the chamber was only accessible through a darkened anteroom.

Hibernation in ground squirrels is identifiable by minimal activity, a curled body position and near-ambient T_b. Animals were sampled at several points over the torpor-arousal cycle (as determined by changes in T_b and requiring quick removal from the hibernaculum, anesthesia and decapitation within two minutes. Whole organ samples were dissected and immediately frozen in liquid nitrogen and then shipped to Carleton University on dry ice, where tissues were immediately stored at -80°C until required. Samples were collected from animals at different stages: (i) euthermic in the cold room (EC) animals showed euthermic physiology (i.e., stable euthermic body T_b and normal metabolic rate) for at least 72 hours; (ii) entrance into torpor (EN) animals showed a declining T_b between 12-31°C; (iii) late torpid (LT) animals displayed a stable T_b of 5-8°C for longer than 72 h; (iv) early arousal (EA) animals displayed a persistent rising T_b between 9-12°C and more than 60 breaths per min; (v) interbout arousal (IA) animals had naturally returned to euthermic conditions for less than 24 h after a prolonged torpor bout.

RNA isolation, cDNA synthesis, and primers

Total RNA was isolated from liver of control and torpid squirrels as described previously (Hadj-Moussa and Storey 2018). Oligo-dT primer ligation and reverse transcription were performed as described previously (Hoyeck et al. 2017). Thirteen-lined ground squirrel mRNA sequences were identified by aligning CCGs against the ground squirrel genome to find orthologous mRNA sequences using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers for 13-lined ground squirrel genes were designed for *BMAL1*, *CLOCK*, *CRY1*, *CRY2*, *PER1*, *PER3*, *RORA*, *NR1D1*, *NR1D2* and *DEC1*. For a complete list of all the primer sequences refer to Appendix C.

Relative mRNA quantification and data analysis

Liver RT-qPCR reactions were performed as previously described (Hoyeck et al. 2017) following MIQE guidelines (Bustin et al. 2009) and using a BioRad CFX Detection System (Mississauga, ON, Canada). Post-run melt-curve analyses were performed to ensure the amplification of a single product. Reactions that amplified multiple products were not used. Relative mRNA expression levels were calculated using the comparative ΔΔCq method (Rao et al. 2013). Gene expression was standardized against a single reference gene (TBP in liver, ACTA1 in WAT, and BCL2L1 in muscle).

Soluble protein extract preparation

Frozen samples of liver, visceral WAT, and hind leg muscle tissue from four individual animals at each time point of the torpor-arousal cycle were crushed and homogenized as described previously (Watts and Storey 2019) in ice-cold homogenizing buffer (20 mM HEPES pH 7.8, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate) using a Polytron PT 10-35 homogenizer with sonication (Kinematica; #11010104); dilutions were 1:3 (w/v) in the case of WAT and 1:5 (w/v) for liver and muscle. Protein concentrations were standardized to 10 μg/μL before storage at −80°C. A portion of the extracts were removed and prepared for Western blotting, as described below, whereas other portions were immediately stored at -80C for use in other assays.

Western Immunoblotting

SDS-polyacrylamide gel electrophoresis and immunoblotting were accomplished as previously described (Watts and Storey 2019). Aliquots of total soluble protein extracts (10 μg/μL) were mixed 1:1 v/v with 2X SDS loading buffer (100 mM Tris-base H 6.8,

4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, 10% v:v 2-mercaptoethanol) and boiled for 10 min giving final standardized sample concentrations of 5 μg/μL that were then stored at −40°C until use. 20 μg of protein sample (which was adjusted as required in keeping with the linear dynamic range of the antibody reaction) was loaded on SDS-polyacrylamide gels and run at 180 V for 45-180 min. Transfer onto a 0.45 μm PVDF membrane was accomplished using target-specific conditions (typically 1.0 A for 5-15 min) in the Trans-Blot® Turbo™ Transfer System (Bio-Rad; Mississauga, ON, Canada).

Membranes were blocked using skim milk powder (0.5-5.0% w:v) dissolved in Tris-buffered saline with Tween (TBST; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% v:v Tween-20) for 30 min. Primary antibodies for BMAL1 (#A302-616A), PER2 (#A303-109A), CRY2 (#A302-615A), CK1δ (#A302-136A) and CK1ε (#A302-135A) were purchased from Bethyl Laboratories (Montgomery, TX) and are polyclonal antibodies raised in rabbit. Epitopes for all antibodies used in this study were available from the producer. These antibodies, which typically target human proteins, were confirmed to recognize the orthologous ground squirrel proteins using the NCBI protein database to assemble protein alignments of the human and squirrel proteins. Primary antibodies were diluted 1:1000 v/v in TBST (to 0.1 ng/nl) and then incubated with membranes on a rocker at 4°C overnight. Membranes were then washed before probing with HRP-linked affinity purified anti-rabbit IgG secondary antibody (BioShop; Burlington, ON, Canada; #APA007P.2). Specific immunoreactive bands were visualized via enhanced chemiluminescence (ECL; 1:1 hydrogen peroxide and luminol) using a Chemi-Genius Bioimager (Syngene, Frederick, MD). All antibodies cross-reacted with

bands on the immunoblots at the expected subunit molecular weight of their respective protein. Chemiluminescent band density in each lane was quantified using GeneTools (version 4.02, Syngene, Frederick, MD). Relative band density was then determined by standardizing all values to the first control sample that was arbitrarily set to one. Membranes were then stained using Coomassie blue (0.25% w:v Coomassie brilliant blue, 7.5% v:v acetic acid, 50% methanol) to visualize total proteins in each lane. To account for possible minor variations in sample loading between different lanes, the densities of immunoreactive bands corresponding to the protein of interest were standardized against the summed intensity of a group of Coomassie stained bands in the same lane that were well separated from the immunoreactive band of interest (Eaton et al. 2013); the same group of bands was used for all trials with a given antibody.

DNA-protein interaction ELISA

An ELISA was performed as described previously (Y. Zhang and Storey 2016) to determine the protein binding ability of CLOCK protein. Biotinylated probe oligonucleotides (CLOCK 5'- Biotin-AGTATTTAGCCACCGTGACAGTGTAAG-3'; and compliment 5'-CTTACACTGTCACGTGGCTAAATACT-3') were based on probes designed for the DNA binding elements of CLOCK, i.e., the canonical E-box sequence (CACGTG) surrounded by a 10 bp of flanking sequence (Gillessen et al. 2017). The probes were diluted in water and annealed at 94°C before dilution in 1X PBS. Double-stranded probe was then added to streptavidin-coated wells on a microplate, and the excess washed away in wash buffer (1X PBS containing 0.1% Tween-20).

Test trials were run with negative controls containing no probe, no protein or no primary antibody added, and the ELISA was optimized so that negative control wells

showed < 50% absorbance relative to positive (i.e., sample) wells. In order to assay CLOCK-DNA interactions, 15.5 µg of soluble protein extract was added to each well in the case of liver and muscle, while 30 µg was added for WAT, all in the presence of transcription factor binding buffer (10 mM HEPES, 50 mM KCl, 0.5 mM EDTA, 3 mM $MgCl_2$,10%v/v glycerol, 0.5 mg/mL bovine serum albumin, 0.05 % NP-40, 0.5 mM DTT, 20 pg/µL salmon sperm DNA, 44 mM NaCl, pH 7.9), and incubated for 90 minutes at room temperature. Diluted primary antibody (1:500) was then added (60 µL/well) and incubated for 1 hour. A primary antibody specific for mammalian CLOCK (#Y058186) was purchased from Applied Biological Materials (Richmond, BC, Canada) and used for the primary antibody incubations. Assay wells were rinsed three times with wash buffer before incubation with diluted anti-rabbit secondary antibodies (1:1000, 60µL/well) for 1 hour. The wells were again rinsed three times with wash buffer before TMB application for 15 minutes and then stopping with 1M HCl. Absorbance was measured at 450 nm (reference wavelength of 655 nm) using a Multiskan spectrophotometer.

Statistical testing

Fold change values were calculated by comparing the difference between a particular time-point, which has been standardized to the control value, and the appropriate control value. For example, the fold change of EN compared to EC for any target protein is expressed as EN/EC, where a fold change > 1 represents an increase, and a fold decrease is expressed as the percentage decrease and represents a fold change <1. All numerical data are expressed as mean ± SEM (n = 4 samples from independent animals). Statistical analysis of differences between experimental hibernation time-points was performed using a one-way ANOVA and post-hoc Tukey tests or a Student's *t*-test

using RBioPlot statistical package (J. Zhang and Storey 2016) with $p<0.05$ accepted as significant.

Results and Discussion

In all cells, circadian rhythmicity is dependent on regulated oscillations of the expression and activity of a number of core circadian clock proteins. At the center of the molecular circadian clock is the CLOCK and BMAL1 protein heterodimer that activates gene transcription by binding to CCGs that possess an E-box in their promoter region. In addition to numerous transcription factors and regulatory proteins, CLOCK/BMAL1 also act upon the *Cry* and *Per* genes that repress the activation of CCGs by binding to and repressing the CLOCK/BMAL1 complex in a rhythmic fashion that gives rise to the cyclic nature of the circadian molecular pathway (Chiou et al. 2016; For review, see Takahashi 2017). The transcription of *BMAL1* is regulated by other CCGs, specifically via ROR*α* binding to a ROR-response element in the *Bmal1* promoter region or inhibition by one of the REV-ERB proteins (J. Lee et al. 2016). Finally, an additional layer of complexity emerges from the activation of other transcription factors by CLOCK/BMAL1 which act upon D-boxes, E-boxes, ROR-elements or cAMP response elements to participate in the regulation of rhythmically expressed genes (Ueda et al. 2005). The actions of other accessory proteins are similarly required to regulate and keep the clock temporally regulated, including DEC1, an inhibitory binding partner of BMAL1 (Honma et al. 2002), and CK1δ/ε, that catalyze both the translocation of cytoplasmic PER/CRY protein heterodimers as well as the degradation of PER monomers (Zheng et al. 2014; Eng et al. 2017).

Circadian clock and CCG transcript abundance was investigated in three ground squirrel tissues comparing EC and LT to determine whether activity within the core

molecular clock is inhibited during deep torpor. Figures 2.1-2.3 show that during LT in liver, WAT and muscle, respectively, the majority of CCG transcripts were maintained at levels comparable with control (EC) animals. Within liver, in fact, all measured transcripts were unchanged from controls, showing that at least with respect to the 10 transcripts studied, transcriptional activity within the core molecular clock is maintained during torpor. Importantly, the transcript levels of *CRY* and *PER* in LT were comparable to those in control animals in both liver and WAT tissues, whereas transcription of NR1D1 in WAT was increased 3.2-fold over EC levels (Figures 2.1-2.2), and hence downstream activation of CCGs by CLOCK is likely maintained in both. On the other hand, *BMAL1* and *NR1D1* transcript levels in skeletal muscle were significantly heightened by 4.8- and 2.7-fold over EC levels, respectively, whereas levels of *PER1* were reduced to 37% of EC values (Figure 2.3). This pattern in muscle is not surprising since a reduction in the level of one of either CRY or PER proteins would release the inhibition imposed upon CLOCK and BMAL1 and allow activation of CCG transcription. CLOCK expression was also increased significantly 3.2-fold within muscle tissue, likely owing to the increased oxidation of lipids within muscle during torpor (Chen and Yang 2014). Notably, similar results were seen in the SCN of hibernating European hamsters where *PER2* fell to levels that were comparable with the active period in euthermic hamsters (Revel et al. 2007). Similar results were also observed within the SCN of Arctic ground squirrels in the case of *PER1* and *PER2* transcript levels (Ikeno et al. 2017).

With respect to circadian transcription, the CLOCK and BMAL1 protein heterodimer activates transcription of *Per* and *Cry* genes, as well numerous other

transcription factor genes that are involved in regulating several other networks including metabolic, immunological, and endocrine signaling (For review, see Bozek et al. 2009). For instance, ROR*α* transcript levels were maintained during LT in all three tissues; ROR*α* protein inhibits the activation of PPARγ downstream genes via interactions with HDAC3 that deacetylates histone lysine residues in the vicinity of PPARγ promoters (Kim et al. 2017). Previous work by our group has shown that in 13LGS muscle tissue, total HDAC activity significantly increased during LT with a resulting reduction in acetylation of lysine 23 on histone H3 (Morin and Storey 2006) and that acetylation of lysine 9 was diminished in WAT (Rouble et al. 2018). If levels of *RORA* transcripts are indeed indicative of maintained levels of ROR*α* protein during hibernation, it is likely that this mechanism contributes to regulating the activation of lipid homeostasis by PPARγ. Notably, ROR*α* deficient animals were shown to have increased thermogenic capacity, due to increased uncoupling protein 1 (UCP1) activity in both WAT and BAT (Monnier et al. 2018). Whereas increased WAT 'browning' in theory could be helpful to an animal during arousal, the effects of uncoupled energy generation in WAT would likely be deleterious to a hibernator given their reliance on lipids that need to be rationed to sustain life over the full winter season. Interestingly, *NR1D1* transcript abundance (which translates to REV-ERB*α*) was heightened by 3.2-fold above EC levels in WAT, and 2.7-fold in muscle. REV-ERB proteins interfere with activation of the *Bmal1* gene, as well as *CLOCK* (Crumbley and Burris 2011). However, transcript levels of both genes were unchanged during LT in WAT and rose significantly in muscle (by 4.8-fold in the case of *BMAL1* and 3.2-fold for *CLOCK*). Therefore, increased levels of *NR1D1*, and potentially REV-ERB*α*, are not likely to be involved in inhibiting circadian transcript

activation during hibernation. Instead, REV-ERBα may play a role in the differential regulation of lipid homeostasis genes, including plasminogen activator inhibitor-1 (PAI-1) that becomes unnecessary during hibernation (Wang et al. 2006; Bonis et al. 2019).

Following observations that the majority of circadian and CCG transcripts maintain their abundance levels during LT, the relative levels of CLOCK DNA binding were explored to determine if differences in the protein level or robustness of CLOCK occur during hibernation. Despite the strong increase in *CLOCK* transcript levels in muscle, no difference in CLOCK DNA binding was seen during LT, with a similar result for liver (Figure 2.4). On the other hand, CLOCK DNA binding increased 1.4-fold above EC levels in WAT. The mechanisms for and functional consequence of increased CLOCK DNA binding during hibernation are unexplored to date, since studies of circadian rhythmicity during hibernation so far have been limited to circadian activity within the brain and therefore have focused on expression of the neural activity marker, c-FOS, as evidence of circadian clock activity. Analogously however, activity within WAT during torpor is maintained as evidenced increased pyruvate dehydrogenase kinase isoform 4 (PDK4) and pancreatic triacylglycerol lipase (PTL) protein levels that inhibit the entry of pyruvate into the mitochondria, and promote increased lipid catabolism, respectively (Andrews 2004). One study that assessed the network of protein interactions of CCGs found numerous interactions with proteins involved in fatty acid, amino acid, and carbohydrate metabolism during the active period when *NPAS2* (which is paralogous to CLOCK) and *BMAL1* levels peaked (Christou et al. 2019). Interestingly, a circadian rhythm in fatty acid metabolism (i.e., regulation of lipid degradation pathways) was predicted by their study, wherein metabolic reactions are restricted to certain times, likely

to prevent the reaction from occurring at times when the necessary enzymes or cofactors are not expressed or active. During LT, *CLOCK* and *BMAL1* expression, as well as all investigated CCGs were maintained at EC levels (Figure 2.2), except for *NR1D1,* which as mentioned previously, plays an integral role in regulating lipid metabolism in WAT. The mechanisms that control increased CLOCK DNA binding in WAT during LT may rely on the presence of co-activators, such as BMAL1, or on post-translational modifications to CLOCK, including phosphorylation by AKT which negatively impacts CLOCK activity (Luciano et al. 2018). In fact AKT levels within WAT are significantly reduced during hibernation in 13LGS (Eddy and Storey 2003), making it possible that reduced levels of CLOCK phosphorylation by AKT lead to the increased DNA binding activity observed in this study. Protein phosphorylation is a tool frequently employed by hibernators to allow for the rapid and reversible modifications of protein activity (Storey 2010), and therefore are a likely regulator of circadian protein activity during hibernation.

Finally, circadian protein levels were investigated at five timepoints over the torpor-arousal cycle, to grasp the effects of the differential expression and CLOCK-DNA binding results observed earlier. Within liver, results were as would be expected given that cellular activity is more-or-less maintained during hibernation, since liver is the central processing hub for lipid mobilization and metabolism (Dark 2005; Epperson et al. 2010). BMAL1 protein levels were maintained during all stages of torpor and early arousal (EA) but were reduced to 34% of EC levels during interbout arousal (IA) (Figure 2.5). Notably, IA is the time when squirrels revert back to a dependence on carbohydrates for fuel and T_b returns to euthermic values. Additionally, PER2 levels fell to 22% and 19% during torpor entry (EN) and LT, respectively, but returned to control euthermic

levels during EA. Given the significantly reduced amounts of PER protein, it is not surprising that the protein abundance of CK1δ was elevated 2.9-fold above EC levels in LT, whereas at every other timepoint CK1δ and CK1ε were maintained at EC levels. On the other hand, CRY2 was maintained at euthermic levels during EN and was significantly increased 1.9-fold over EC levels in LT. The fact that CRY2 levels were maintained or even raised is intriguing since without PER2 as a binding partner, the only role for CRY2 within the circadian pathway is AMPK-mediated phosphorylation and ubiquitination. Furthermore, hepatic AMPK activity is increased during hibernation in 13LGS (Lanaspa et al. 2015), which would increase the likelihood that CRY2 be destined for degradation, unless it had some necessary role within the tissue. In fact, studies with human liver tissue show that *CRY2* mRNA is associated and correlated with hepatic triacylglyceride content and aids in limiting glucose production (Machicao et al. 2016). Whether or not CRY2 is specifically required for allowing the increased storage and throughput of lipids during hibernation is an interesting avenue that is yet to be explored. Lastly, an interesting question is posed by the results during IA specifically, when both BMAL1 and PER2 levels were significantly decreased, although, maintained levels of CRY2 likely point towards inhibited circadian clock output during IA. Given that while aroused, the animal is both in constant darkness and asleep (Strijkstra and Daan 1997) it may be that peripheral circadian clocks need to be 'reset' to realign with potential outputs from the master SCN clock during arousal, following the prolonged depression of neural temperature and circadian clock activity within the SCN that occurs during a torpor bout (Malan 2010; Malan et al. 2018).

Lipid metabolism and mobilization is dependent on efficient regulation of the protein networks involved within WAT, and so it is not surprising that levels of core circadian clock proteins which are intricately linked to lipid metabolism, are also tightly regulated during hibernation (Figure 6). During EN, all investigated proteins were maintained at levels that were comparable to EC control animals, whereas during LT, the levels of CRY2 and PER2 were significantly raised by 1.9-fold and 3.8-fold above EC levels, respectively. On the other hand, levels of CK1δ were diminished to 57% of EC values in LT, likely due to its redundancy in function with CK1ε. Levels of BMAL1 and CRY2 were elevated in EA, 1.6-fold and 2.6-fold above levels seen during EC, respectively, as were both CK1δ/ε by 1.5-fold and 3.5-fold, respectively. PER2 protein levels, on the other hand, fell back to levels comparable with EC. During IA, all proteins returned to levels that were not statistically different from protein levels in EC. Based on these results, it is clear that activity within the core circadian clock is maintained, given the shared patterns of protein expression that arise out of the tension between transcription factor and inhibitor activity (Christou et al. 2019). In essence, the results appear to show that levels of molecular clock proteins share a pattern of expression and abundance, leading to turnover and ultimately activation of the involved proteins.

Analysis of clock protein abundance in skeletal muscle depict a different cellular environment than the one described for liver. During EN and LT, BMAL1 was maintained at EC levels, and the inhibitory heterodimer proteins also showed maintained or increased levels (Figure 2.7). PER2 protein levels increased by 2.8- and 2.7-fold above levels in EC animals during EN and LT, respectively, and were still above EC levels in EA, although not significantly. Similarly, CK1δ, necessary for the phosphorylation-

mediated nuclear translocation of the PER/CRY heterodimer, was elevated in EN by 3.5-fold above EC levels but fell back to control levels during LT. However, CK1δ protein levels increased again during EA (by 2.8-fold above EC levels), and then increased a third time during IA, by 8.6-fold above EC (and 3.1-fold above EA values). In this case, it is clear that the inhibitory arm of the circadian clock is dominant and active in inhibiting circadian output within skeletal muscle. Indeed, the loss of circadian activity is likely intensified during EA and IA by the strong reduction in BMAL1 levels to less than 32% of EC values. Inhibited circadian output during hibernation within skeletal muscle is unsurprising given that this tissue is largely inactive during hibernation, aside from during arousals when skeletal muscle is important in generating heat through shivering thermogenesis (Cotton and Harlow 2009). At the same time, muscle tissue must protect itself from the potential negative consequences of disuse that typically lead to atrophy in non-hibernators (including humans). Indeed, hibernators show little to no atrophy, even though levels of myostatin, the main regulator of muscle mass, showed no increase during hibernation in 13LGS (Brooks et al. 2011). Important to the current study, the circadian network includes proteins that also act to maintain muscle tissue mass, requiring the complex interplay of CCG products (Chatterjee and Ma 2016). Studies of CLOCK or BMAL1 knockout mice have shown blunted responses by the MyoD transcription factor and disrupted myofilament organization (Andrews et al. 2010), whereas MyoD mRNA and protein increased within skeletal muscle during EA in 13LGS (Tessier and Storey 2010). Increased MyoD production despite reduced levels of BMAL1 during EA and IA may represent an adaptation by hibernators to the specific needs of skeletal muscle during arousal from hibernation. Alternately, this may simply represent a

'fine-tuning' of the clock for optimal cytoprotective functioning. This is certainly worth investigation, given that MyoD contains an E-box within its promoter region.

As measurements of circadian rhythmicity have become more robust, studies of hibernating mammals have recently begun to turn back to the question of circadian activity, as measured within the brain or through T_b rhythmicity observations. Typically, the results of these studies observe that circadian behavior, as measured by T_b rhythmicity (Williams, Radonich, et al. 2017; Hut et al. 2002) or transcription of core molecular clock genes within the SCN (Ikeno et al. 2017; Revel et al. 2007) is diminished during hibernation. Notably, a study by Ruby et al., (Ruby et al. 2002) provided a mechanistic link by measuring T_b rhythmicity in SCN-ablated ground squirrels for 2.5 years and found that rhythmic T_b was lost and was not recovered in ground squirrels kept on a 12 hr light/dark cycle, and that this loss was not dependent on hibernation. Importantly however, both wild-type and SCN-ablated animals had synchronized torpor entry times, whereas arousal from torpor remained desynchronized across the groups, showing that some element of temporal regulation over hibernation (i.e., the mechanism that controlled torpor entry) remains even following ablation of the master circadian pacemaker. At the same time, the SCN must certainly impose a degree of regulation over hibernation, since SCN-ablated animals showed increased numbers of torpor bouts, longer arousal durations, as well as more frequent and shorter torpor bouts, as compared with control animals (Ruby et al. 1996). Future studies that seek to understand the degree to which peripheral oscillators play a role in regulating not just the temporal control of hibernation, but also the necessary protein networks and endocrinological systems linking the SCN to peripheral tissues, will be helpful in uncovering additional roles for the core

molecular clock, as well as the adaptations that hibernators employ to successfully survive extreme winter environments.

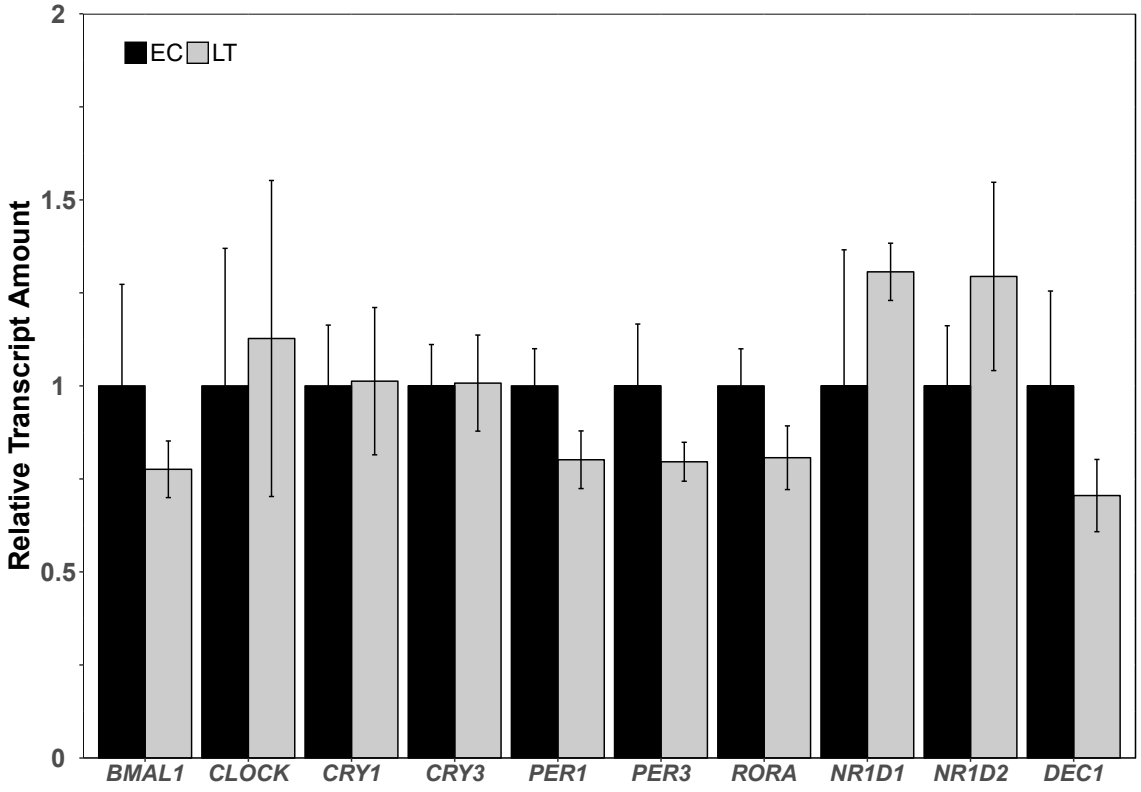


Figure 2.1: Expression of circadian and CCG mRNAs in liver as evaluated by RT-qPCR in euthermic control (EC) and late torpor (LT) hibernating 13-lined ground squirrels. Relative expression of the indicated mRNAs was standardized to the expression of *TBP* amplified from the same sample. Relative mRNA expression is expressed relative to the EC value which was set to 1.0. Data are displayed as mean ± SEM ($n = 4$ independent trials) and were analyzed using Students *t*-test (with a $p < 0.05$ cutoff). Significant differences from EC are shown with an asterisk (*) above the appropriate column.

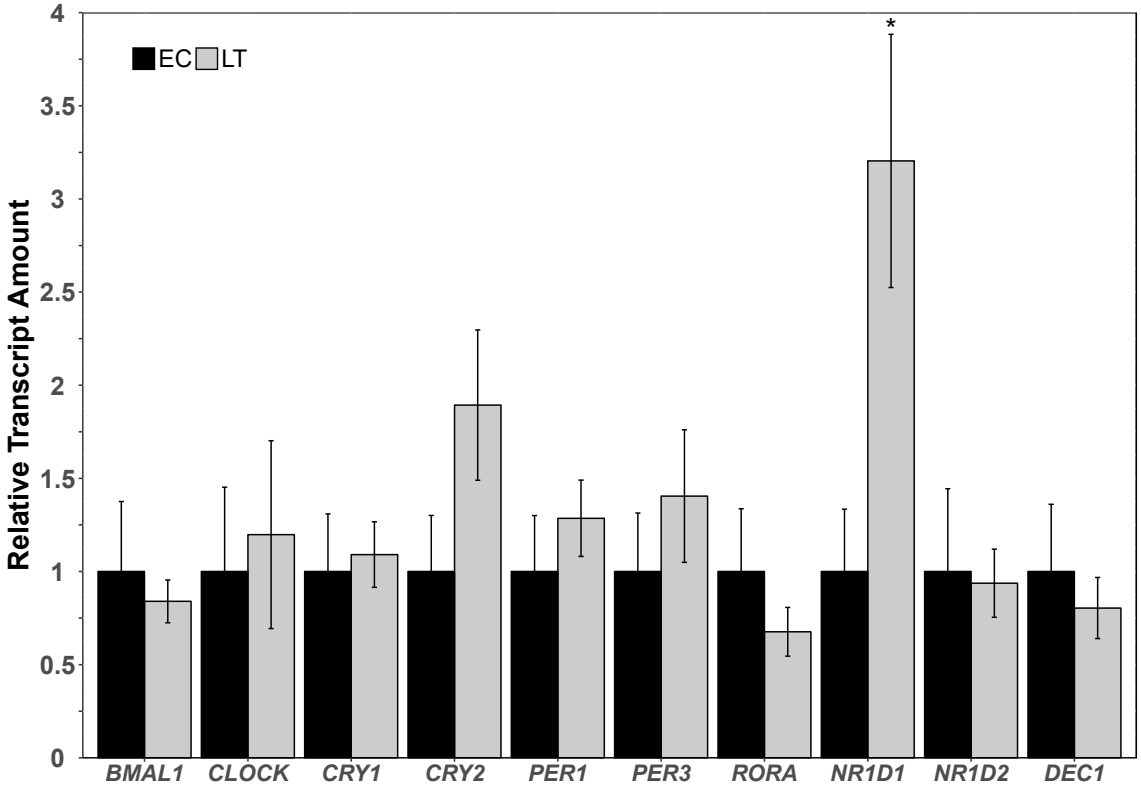


Figure 2.2: Expression of circadian and CCG mRNAs in white adipose tissue standardized to the expression of *ACTA1* amplified from the same sample. All other information is as in Figure 2.1.

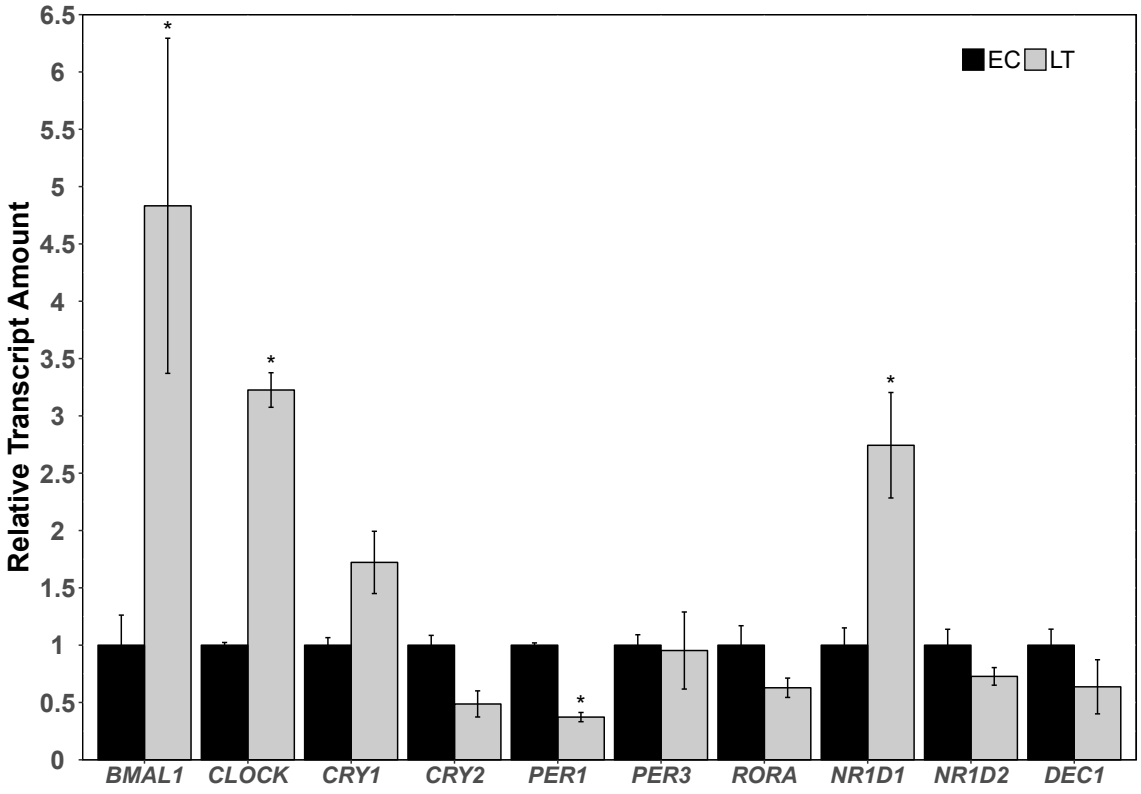


Figure 2.3: Expression of circadian and CCG mRNAs in skeletal muscle standardized to the expression of *BCL2L1* amplified from the same sample. All other information is as in Figure 2.1.

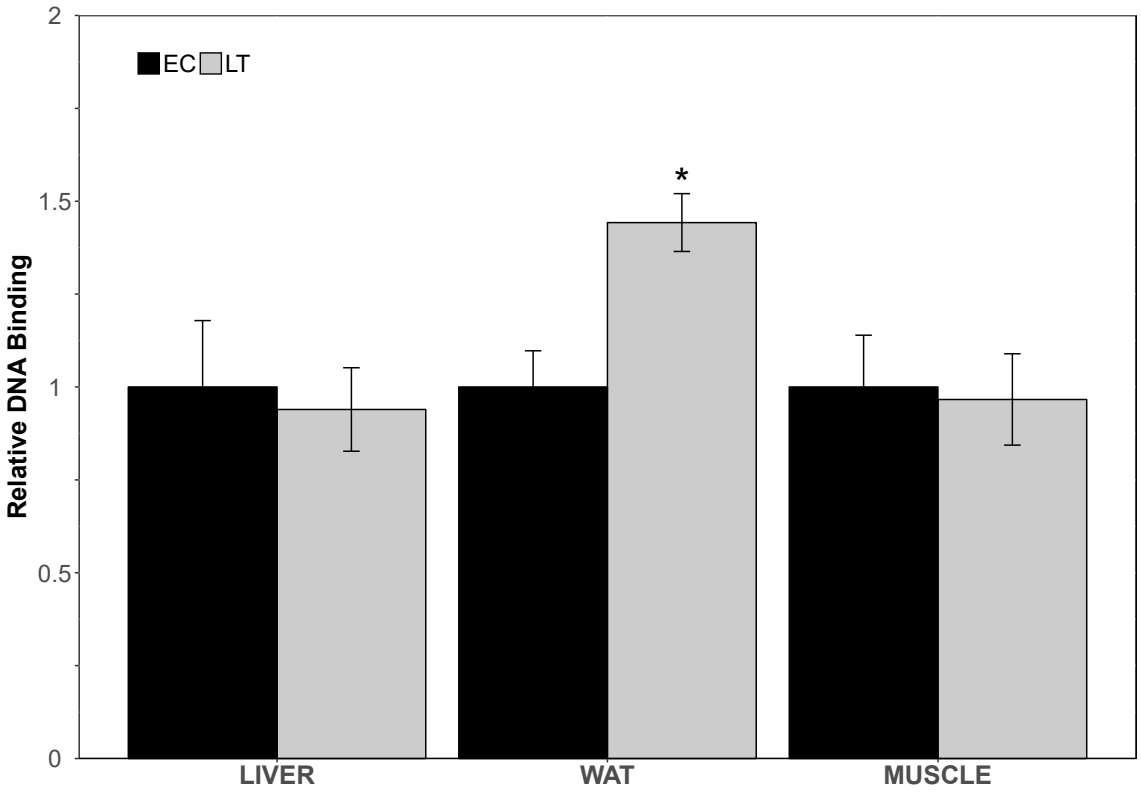


Figure 2.4: Changes in binding by the CLOCK transcription factor to a DNA-binding element designed for the E-box consensus sequence in liver, white adipose tissue (WAT) and skeletal muscle in EC and LT animals. DNA-protein interaction ELISA absorbance readings were corrected by subtracting the negative controls containing no protein, and values were expressed relative to EC. Data are displayed as mean ± SEM ($n = 4$ independent trials) and were analyzed using Students *t*-test (with a $p < 0.05$ cutoff). Significant differences from EC are shown with an asterisk (*) above the appropriate column.

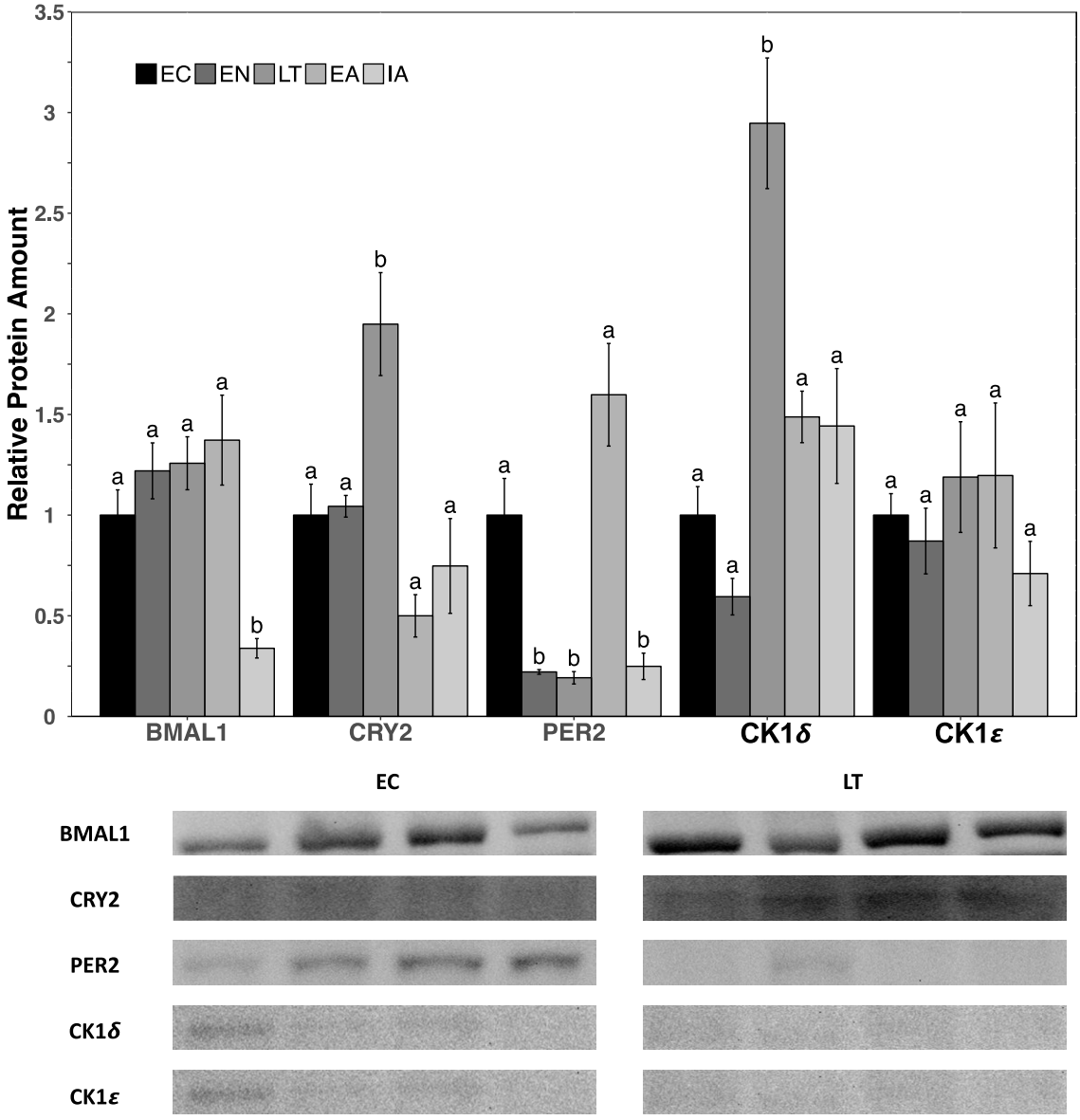


Figure 2.5: Relative total protein levels of core circadian clock proteins in liver of hibernating 13-lined ground squirrels. Sampling points are euthermic in the cold room (EC), entrance into torpor (EN), late torpor (LT), late arousal (LA), and interbout arousal (IA). Data are displayed as mean ± SEM (n = 4 independent trials) and were analyzed using ANOVA followed by a Tukey post hoc test and labeled so that different letters denote values that are significantly different from each other ($p < 0.05$). Representative immunoblot images below the histogram are for the EC and LT timepoints.

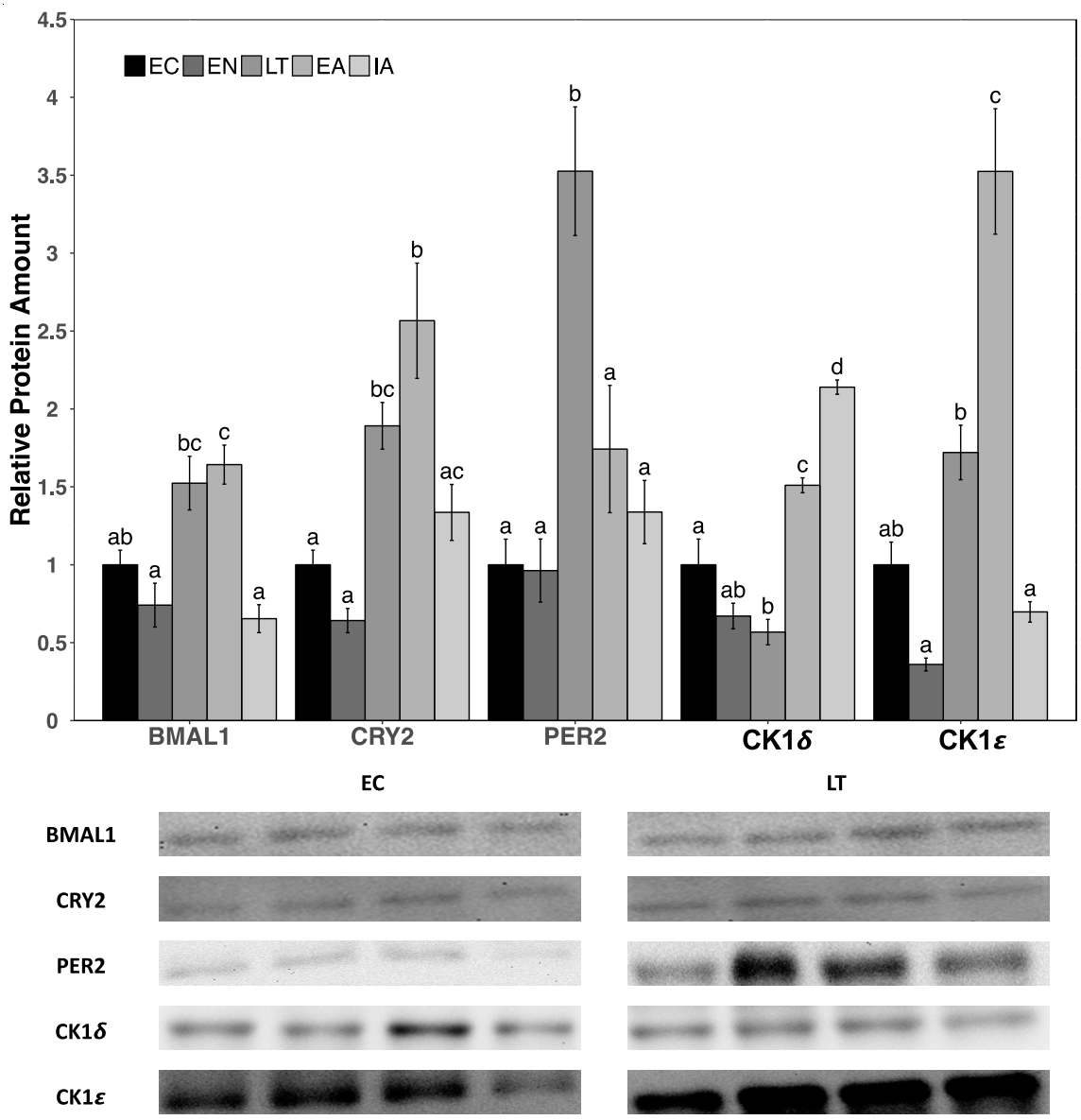


Figure 2.6: Relative total protein levels of core circadian clock proteins in white adipose tissue of hibernating 13-lined ground squirrels. All other information is as in Figure 2.5.

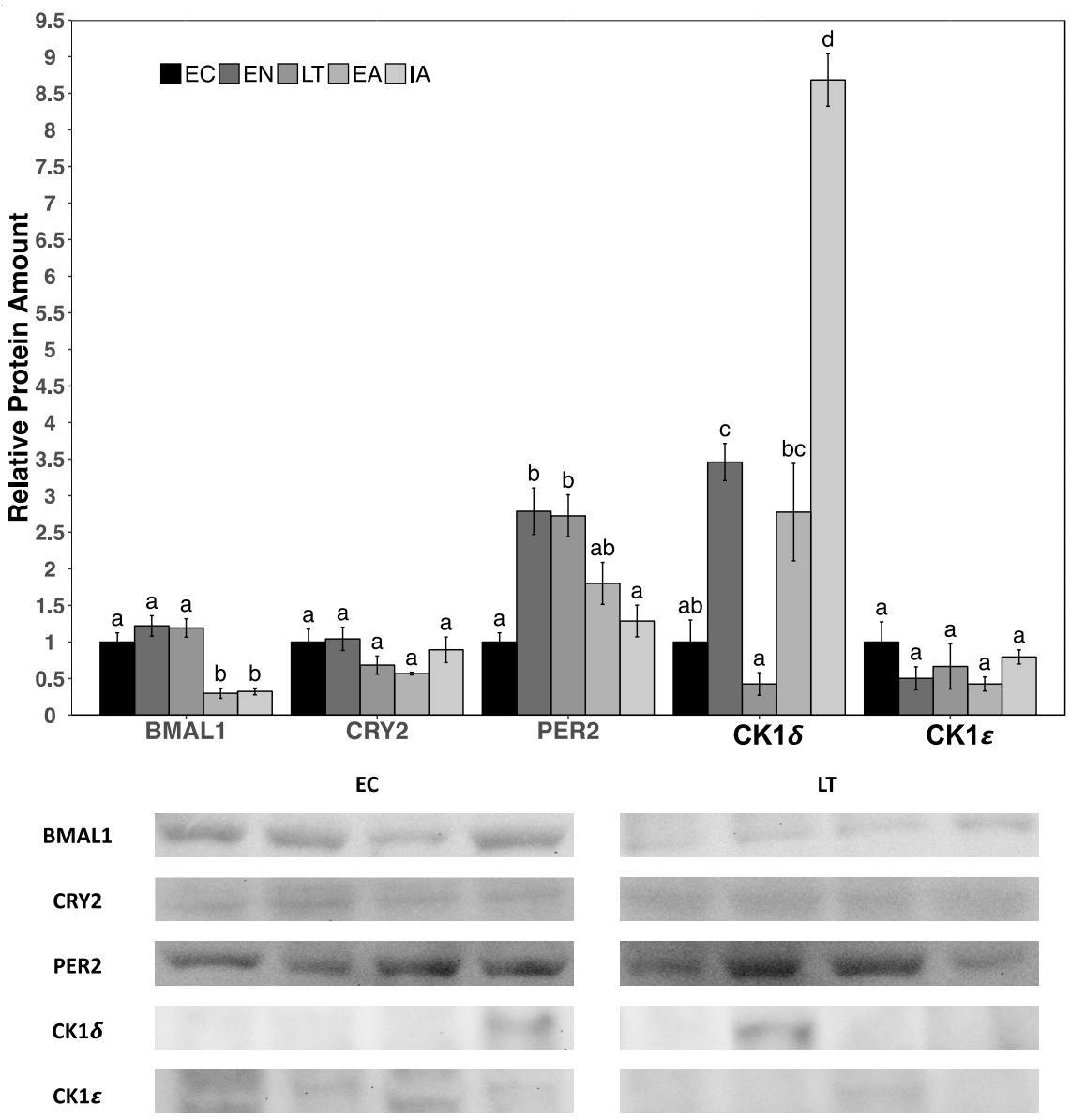


Figure 2.7: Relative total protein levels of core circadian clock proteins in skeletal muscle of hibernating 13-lined ground squirrels. All other information is as in Figure 2.5.

Chapter 3

RNA methylation dynamics are maintained during hibernation in the thirteen-lined ground squirrel

Introduction

Many mammals show pronounced circannual rhythms that govern their behaviour and metabolism across the seasons of the year, influencing or defining the timing of major events including reproduction, migration, hibernation, etc. As winter approaches some small mammals display a remarkable phenotypic shift, lowering their metabolic rate and entering a state of torpor to survive seasonal stresses including extreme cold and limited food availability. This altered physiological state, known as hibernation, is exhibited annually by diverse species that inhabit extreme climates, ranging from temperate zones to the high Arctic in the Northern Hemisphere, and oppositely in most southerly parts of Australia and Africa. Through the concerted effects of major physiological and biochemical changes, these small mammals can sink into a state of torpor, allowing their body temperature to fall to near ambient, and survive solely on stored body fats for days or weeks at a time. Therefore, hibernation serves as an important mechanism for surviving harsh winter conditions in small mammals. It has been estimated that these animals, by employing hibernation, can potentially conserve about 90% of the energy that they would normally require to remain active and euthermic all winter (Wang and Wolowyk 1988).

Among hibernators in deep torpor, common metabolic responses have been identified including: i) extensive protein post-translational modifications (PTMs) that provide a reversible switch to turn enzyme/protein activities down or off during torpor, as well as heavily suppressing transcription by placing PTMs on histones and specific transcription factors (Storey 2015; Tessier, Luu, et al. 2017; Tessier, Zhang, et al. 2017); ii) the selected upregulation of some genes or proteins including stress-responsive transcription factors, cell-preservation factors, and antioxidant defenses in preparation for a surge in ROS production during rewarming (Eddy et al. 2006; Mamady and Storey 2008; Morin and Storey 2009;

Rouble et al. 2013); and iii) differential expression of microRNA molecules and influences on mRNA translation (Frigault et al. 2017; Biggar and Storey 2017). These mechanisms all contribute to regulating the cellular environment during long-term hypometabolic states.

It is well established that gene expression is regulated in response to environmental change, and that this regulation may be imposed at various levels that are integrated with each other (Maniatis and Reed 2002; Orphanides and Reinberg 2002; Proudfoot et al. 2002; McKee and Silver 2007). Briefly, gene expression regulation can be divided into three main categories: (i) transcriptional control, (ii) post-transcriptional control, and (iii) post-translational control. While the first and last of these categories have been explored deeply, a burgeoning interest in post-transcriptional control has been insightful for our understanding of the regulation of hypometabolic states. A new player in this area is methylation of adenosine (m6A) that is an important PTM regulator of RNA transcripts. m6A RNA methylation is targeted to a GAC consensus sequence, especially near the stop codon and in linear regions but less-so in loops or bulge RNA structures. Methylation has increasingly been associated with multiple effects specifically altering mRNA stability and translational activity. For instance, localization of m6A to intron areas on pre-mRNA transcripts suggest its role in splicing control (Ping et al. 2014), whereas altered translational activity of methylated transcripts suggests that these modifications interact with other factors when needed (Heilman et al. 1996; Karikó et al. 2008). Furthermore, altered transcript stability among m6A-bearing mRNAs is likely a result of m6A binding to or blocking of mRNA stabilizing proteins from interacting with the transcript (Wang et al. 2014; Wang and He 2014). The manipulation of mRNA by methylation can therefore have far-reaching effects, and as such it has been shown to modify the expression of thousands of genes in almost every cellular pathway through altered transcript stability (Berulava et al. 2013). The most studied of these modifications, adenosine methylation (m6A) on nitrogen-6 of the basic group is

enriched near the stop codons and in the 5' UTR region (Meyer et al. 2012), and shows tissue-specific differences (Dominissini et al. 2012).Thus, RNA methylation clearly serves an important role in metabolic control by acting as a gatekeeper of transcript processing and translation.

RNA adenosine residue methylation is catalyzed by a very large multiprotein complex (approximately 1 MDa in size) containing methyltransferase-like 3 and 14 (METTL3, METTL14) enzymes and their required regulatory subunit Wilms Tumor 1 Associated Protein (WTAP). Notably, WTAP knockdown blocks the localization of the RNA methyltransferase complex to nuclear speckles (a major site of mRNA splicing), although mRNA methylation readily occurs within the nucleus or cytoplasm of wild type cells (Ping et al. 2014) and obviously will have different roles depending on the modified transcript's location.. Demethylation of mRNA is also possible, through the activities of fat mass and obesity-associated protein (FTO) or AlkB homologue 5 (ALKBH5) and their activities are necessary for effective mRNA processing and metabolism (Jia et al. 2011; Bartosovic et al. 2017; Kang et al. 2018).

The downstream effects of m6A methylation are regulated mainly via transcript binding to m6A reader proteins, typically proteins containing a YTH (YT521-B homology) domain. Just about every aspect of RNA metabolism is modulated by the presence of m6A modifications where effects are conferred to the transcript by m6A reader proteins; for example, sub-nuclear localization, splicing, nuclear export, and transcript stability and degradation are all modified by the presence of reader proteins binding m6A and altering these processes (for review see Dai et al. 2018). Furthermore, gene expression can ultimately be affected by this layer of regulation through the promotion of translation or transcript instability when m6A-bound reader proteins recruit transcripts to the ribosome or for deadenylation, respectively (Du et al. 2016; Wang et al. 2015). The regulation of downstream

functions in each individual m6A reader protein has yet to be determined, however m6A reader protein binding to transcripts is clearly an effective method for rapidly altering translation efficiency.

Similarly, m6A modifications can recruit the eukaryotic initiation factor 3D (eIF3d), which is remarkable for its ability to form a ribosome on transcripts that lack an m7G 5' cap and in the absence of eIF4 (Meyer et al. 2015). Normally, eukaryotic translation begins with the recruitment of the 43S pre-initiation complex to the 5' cap by eIF4F and allowing 48S initiation complex formation. The presence of an m6A modification within the 5' UTR is enough to allow for translation-competent 48S complexes to form however, in a way that is not dependent on eIF4F binding the 5' cap. This activity is elicited by eIF3D which binds the 5' UTR m6A-modification and allows the participation of transcripts lacking an m7G cap in forming the 48S complex (de la Parra et al. 2018; Meyer et al. 2015). In this way, the specific transcripts that are poised for translation may be dynamically altered by changing the proportion of transcripts that contain m6A modifications in their 5' UTR (Shah et al. 2016; J. Zhou et al. 2015) and can serve important roles as cells undergo rapid changes – as during heat shock for instance.

By testing the hypothesis that adenosine methylation provides a means of reducing transcriptional rates, greater understanding of the role of post-transcriptional regulations in controlling hibernation is to be gained by assessing the functioning of the m6A modification enzymes and related proteins, as well as the amount of modification occurring in a hypometabolic state. Using the 13-lined ground squirrels (*Ictidomys tridecemlineatus*; 13LGS) as the model hibernator, the present study assesses the relative protein levels of adenosine-methyltransferases (METTL3 and METTL14) and their regulator, WTAP, via Western immunoblotting, as well as relative protein levels of the demethylase enzymes FTO and ALKBH5, and reader proteins YTHDF1, YTHDC2 and YTHDF3 in liver, white adipose

tissue (WAT) and skeletal muscle of ground squirrels at multiple points over the torpor-arousal cycle. eIF3D responses are also analyzed using an ELISA. Characterization of relative adenosine demethylation activity over the torpor-arousal cycles was also assessed using a commercial ELISA-like activity assay to quantify demethylase enzymatic activity. Relative protein levels and enzymatic activity give a broad sense of the role that adenosine methylation has on the cell's ability to preserve mRNA transcripts until required during torpor-arousal cycles. In a similar fashion, analysis of total amounts of m6A using an immuno-dot blotting procedure was used to probe RNA samples for global differences in tissue m6A levels between euthermic and torpid states.

Methods

Animal and Experimental Procedures

Wild 13-lined ground squirrel (*Ictidomys tridecemlineatus*) weighing at least 150 g, were caught by a trapper (TLS Research, Bloomingdale, IL) licensed by the US Department of Agriculture, and transported to a National Institute of Health facility, where their care has been described previously (Cai et al. 2004). NINDS animal care and use committee (ACUC) approved all holding and experimental procedures were followed (protocol number ASP 1223-05). Briefly, squirrels were housed individually in plastic shoebox cages in a vivarium with an ambient temperature of 21°C, a 12:12 L:D photoperiod, and were fed standard rodent diet. Animals were anesthetized with 5% isofluorane and fitted with a telemetry device (IPTT-200; Bio Medic Data Systems, Seaford, DE) implanted subcutaneously for T_b measurements before experimental work began. 13LGS were moved to a hibernaculum for the months of November to March with an ambient temperature of 4-6°C and 60% humidity under 24-hour darkness. A

photographic red safelight (3–5 lx) was used when necessary and the animal's chamber was only accessible through a darkened anteroom.

Hibernation in ground squirrels is identifiable by minimal activity, a curled body position and near-ambient T_b. Animals were sampled at several points over the torpor-arousal cycle (as determined by changes in T_b), requiring quick removal from the hibernaculum, anesthetization and decapitation within two minutes. Whole organ samples were dissected and immediately frozen in liquid nitrogen and then shipped to Carleton University on dry ice, where tissues were immediately stored at -80°C until required. Samples were collected from: (i) euthermic in the cold room (EC) animals showed euthermic physiology (i.e., stable euthermic body T_b and normal metabolic rate) for at least 72 hours; (ii) entrance into torpor (EN) animals showed a declining T_b between 12-31°C; (iii) late torpid (LT) animals displayed a stable T_b of 5-8°C for longer than 72 h; (iv) early arousal (EA) animals displayed a persistent rising T_b between 9-12°C and more than 60 breaths per min; (v) interbout arousal (IA) animals had naturally returned to euthermic conditions for less than 24 h after a prolonged torpor bout.

Nucleic acid extraction and m6A quantification

Total RNA was isolated from samples of frozen liver, visceral WAT, and hind leg muscle using Trizol (Invitrogen; Cat# 15596-018) and homogenized with a Polytron PT 1200 homogenizer (Kinematica; #11010026) as per the manufacturer's instructions. Assessment of RNA contamination was done by measuring the 260/280 nm ratio (> 1.8) using a Take3 micro-volume quantification plate (BioTek). Additionally, mRNA was isolated from the RNA pool in some of the liver samples using the NEBNext® Poly(A)

mRNA Magnetic Isolation Module (New England Biolabs; #E7490) according to the manufacturer's instructions and assessed as described above.

To compare the relative amounts of m6A, the EpiQuik m6A RNA Methylation Quantification Kit (Epigentek; #P-9005) was used. RNA amounts of up to 5 µg were appropriate for the final load amounts chosen for the assays (0.2 µg in liver, 0.l µg in skeletal muscle and 0.5 µg in WAT). Assays were run according to manufacturer's instructions, while all other instructions are as described for the m6A demethylase activity assays. OD values were interpolated to an m6A concentration according to a reproduced standard curve of ΔOD/[m6A].

Soluble protein extract preparation

Frozen liver, visceral WAT, and hind leg muscle tissue from four individual animals at each time point of the torpor-arousal cycle were crushed and homogenized as described previously (Watts and Storey 2019) in ice-cold homogenizing buffer using a Polytron PT 10-35 homogenizer with sonication (Kinematica; #11010104); dilutions were 1:3 (w/v) in the case of WAT and 1:5 (w/v) for liver and muscle. Protein concentrations were standardized to 10 µg/µL before storage at −80°C. A portion of the extracts were removed and prepared for Western blotting, as described below, whereas other portions were immediately stored at -80C for use in other assays.

Western Immunoblotting

SDS-polyacrylamide gel electrophoresis and immunoblotting were accomplished as previously described (Watts and Storey 2019). Aliquots of total soluble protein extracts (10 µg/µL) were mixed 1:1 v/v with 2X SDS loading buffer (100 mM Tris-base H 6.8, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, 10% v:v 2-

mercaptoethanol) and boiled for 10 min giving final standardized sample concentrations of 5 μg/μL that were then stored at −40°C until use. 20 μg of protein sample (which was adjusted as required in keeping with the linear dynamic range of the antibody reaction) was loaded on SDS-polyacrylamide gels and run at 180 V for 45-180 min. Transfer onto a 0.45 μm PVDF membrane was accomplished using target-specific conditions (typically 1.0 A for 5-15 min) in the Trans-Blot® Turbo™ Transfer System (Bio-Rad; Mississauga, ON, Canada).

Membranes were blocked using skim milk powder (0.5-5.0% w:v) dissolved in Tris-buffered saline with Tween (TBST; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% v:v Tween-20) for 30 min. All primary antibodies were polyclonal and raised in rabbit. METTL3/MT-A70 (cat#A301-567A) and WTAP (#A301-435A) were purchased from Bethyl Laboratories (Montgomery, TX). Antibodies for YTHDF1 (#A13260), YTHDC2 (#A15004) and YTHDF3 (#A8395) were from ABclonal (Woburn, MA). The primary antibodies for METTL14 (#abx123810), ALKBH5 (#abx125516) and FTO (#abx125855) were purchased from Abbexa (Cambridge, UK). Epitopes for all antibodies used in this study were available from the producer. These antibodies, which typically target human proteins, were confirmed to recognize the orthologous ground squirrel proteins using the NCBI protein database to assemble protein alignments of the human and squirrel proteins. Primary antibodies were diluted 1:1000 v/v in TBST and then incubated with membranes on a rocker at 4°C overnight. Membranes were then washed before probing with HRP-linked affinity purified anti-rabbit IgG secondary antibody (BioShop; Burlington, ON, Canada; #APA007P.2).

Specific immunoreactive bands were visualized via enhanced chemiluminescence (ECL; 1:1 hydrogen peroxide and luminol) using a Chemi-Genius Bioimager (Syngene, Frederick, MD). All antibodies cross-reacted with bands on the immunoblots at the expected subunit molecular weight of each protein. Chemiluminescent band density in each lane was quantified using GeneTools (version 4.02, Syngene, Frederick, MD). Relative band density was then determined by standardizing all values to the first control sample that was arbitrarily set to one. Membranes were then stained using Coomassie blue (0.25% w:v Coomassie brilliant blue, 7.5% v:v acetic acid, 50% methanol) to visualize total proteins in each lane. To account for possible minor variations in sample loading between different lanes, the densities of immunoreactive bands corresponding to the protein of interest were standardized against the summed intensity of a group of Coomassie stained bands in the same lane that were well separated from the immunoreactive band of interest (Eaton et al. 2013).

Demethylase activity assay

To determine the relative activity of m6A demethylases in control, torpid and aroused ground squirrels, the Epigenase m6A Demethylase Activity/Inhibition Assay Kit from Epigentek (Farmingdale, NY; #P-9013) was employed to measure m6A demethylation (KMT) activity.

Total soluble protein extracts from animals sampled from EC, EN, LT, and IA time-points (N=4) were standardized to a concentration of 10 μg/μL. A standard curve and dilution curve were created before data collection to demonstrate that protein amounts below 80 μg did not produce signal saturation and were appropriate for the final load amounts chosen for the assays (30 μg in liver, 75 μg in WAT and 40 μg in skeletal

muscle). Assays were run according to manufacturer's instructions. Absorbances were measured at 450 nm using a PowerWave HT microplate spectrophotometer (BioTek, Winooski, VT) and Gen5 Microplate Reader and Imager Software (Version 1.09; BioTek). To calculate m6A demethylation activity, OD values were manipulated using a manufacturer-provided formula and the ΔOD/ng un-demethylated m6A, from the reproduced standard curve. KMT enzyme activity was expressed as nanograms of un-demethylated m6A remaining per hour per mg protein (ng/h/mg).

ELISA of eIF3d protein amount

To compare the relative amounts of eIF3D, the Human Eukaryotic Translation Initiation Factor 3 Subunit D (EIF3D) ELISA Kit (Abbexa; #abx387095) was used. Protein amounts of up to 5 μg were appropriate for the final load amounts chosen for the assays (0.2 μg in liver, 0.l μg in skeletal muscle and 0.5 μg in WAT). Assays were run according to manufacturer's instructions, while all other instructions were as described for the m6A demethylase activity assays. OD values were interpolated to an eIF3D concentration according to a reproduced standard curve of ΔOD/[eIF3D].

Statistical testing

Fold change values were calculated by comparing the difference between a particular time-point, which has been standardized to the control value, and the appropriate control value. For example, the fold change of EN compared to EC for any target protein is expressed as EN/EC, where a fold change > 1 represents an increase, and a fold decrease is expressed as the percentage decrease and represents a fold change <1. All numerical data are expressed as mean ± SEM (n = 4 samples from independent animals). Statistical analysis of differences between experimental hibernation time-points

was performed using a one-way ANOVA and post-hoc Tukey tests or a Student's t-test using RBioPlot statistical package (J. Zhang and Storey 2016) with $p<0.05$ accepted as significant.

Results

Levels of m6A are significantly altered during torpor

Antibody-mediated capture of m6A-laden transcripts was achieved from total RNA extracts of liver, WAT and skeletal muscle (Figure 3.1). It was determined that the percentage of m6A RNA modification increased in WAT by 5.5-fold during late torpor (LT) as compared with the euthermic in the cold room condition (EC) but decreased in muscle (to 59% of EC values) during LT. Total RNA extracted from liver did not show a change in m6A RNA during hibernation, but when mRNA specifically was extracted from the total pool of liver RNA, a decrease in the percentage of m6A RNA modification was observed within the mRNA pool from the LT condition, a reduction of 38% as compared with EC values.

Relative levels of enzymes involved in m6A-deposition and removal are regulated across the torpor-arousal cycle

The relative protein levels of several widely studied enzymes involved in methyl-RNA deposition (METTL3, METTL14, WTAP), removal (FTO, ALKBH5) or interactions (YTHDF1, YTHDC2, YTHDF3, eIF3d) were assessed in liver, WAT, and skeletal muscle of 13LGS across the torpor-arousal cycle. Preliminary analysis confirmed that each of the commercial mammalian antibodies used bound to protein at the expected molecular mass for the target.

In liver, METTL3 protein levels were unaffected during entrance into torpor (EN) or during LT as compared with EC values. However, METTL3 decreased significantly during the early arousal (EA) phase after torpor, falling to 41% in comparison to EC values (Figure 3.2). Levels of liver METTL3 then recovered strongly during interbout arousal (IA) to a level 2.39-fold greater than EC levels. METTL3 levels in WAT showed a different pattern with no significant difference from the EC value over the EN, EA and IA phases, but a large increase occurred during LT, 4.9-fold greater than EC levels. Skeletal muscle METTL3 levels were unchanged compared with EC in three phases but showed a significant decrease to just 31% of EC values during EA. The second writer enzyme, METTL14, displayed a different pattern over torpor/arousal. In liver, a significant increase occurred during LT, 1.6-fold greater than EC levels, but then fell during arousal (IA) to just 43% of EC values. In WAT, METTL14 also showed a significant increase during LT, 1.7-fold greater than EC, although this followed a strong significant decrease during entry to torpor (EN) when METTL14 levels in WAT fell to just 30% of EC levels. METTL14 levels in skeletal muscle showed a similar pattern as in liver; levels also increased during LT, 1.9-fold greater than EC, and then decreased during IA to just 11% of EC values. Lastly, the protein levels of WTAP in liver were constant over EN and LT phases compared to EC but decreased significantly during both arousal stages (EA and IA) to about 33% as compared to the earlier stages of torpor (EN or LT). On the other hand, WTAP levels in both WAT and skeletal muscle showed strong significant 4.4-fold and 2.2-fold increases during LT, respectively as compared to EC. WTAP levels were not significantly different from EC at any of the other timepoints in either tissue.

Relative protein levels of RNA removal enzymes are displayed in Figure 3.3 over the same timepoints of the torpor-arousal cycle. When compared to EC, levels of FTO in liver significantly decreased upon EN, and remained low at 56% of the EC value during LT and then rose back to values not significantly different from EC during arousal. Levels of FTO in WAT also fell significantly during EN to just 38% compared to EC, although protein levels rebounded during LT. However, during arousal FTO protein again fell significantly, and was as low as 29% of the EC value during IA. Skeletal muscle FTO showed a largely opposite response to that of liver and WAT. Skeletal muscle FTO levels did not change significantly during both EN and LT, but rose during the arousal stages, 2.2-fold above EC during EA and 4.1-fold above EC during IA (as well as 1.8-fold above EA levels). The second RNA-demethylase, ALKBH5, showed a significant increase during EN in liver, 1.2-fold over EC levels, before falling to values not significantly different from EC during arousal (EA, IA). In WAT, ALKBH5 levels were not significantly different with respect to EC over the EN, LT and EA timepoints, but ALKBH5 levels were significantly reduced during IA to just 20% of LT values. In skeletal muscle, levels of ALKBH5 remained constant over four timepoints but rose strongly during IA to values 7.7-fold greater than EC.

Levels of m6A demethylase activity are decreased or unchanged during hibernation

Total RNA m6A demethylase activity was assessed at four timepoints: EC, EN, LT and IA. Use of a bound m6A-bearing substrate allows a way to quantify the total activities of m6A demethylases that can bind to and demethylate the substrate, producing an immuno-signal that is proportional to the amount of remaining m6A. With this method, the measured m6A demethylase activity in tissues of control (EC) animals was

determined to be 42.97 ± 4.1 ng/min/mg, 6.22 ± 0.36 ng/min/mg, and 31.57 ± 4.86 ng/min/mg in liver, WAT, and muscle, respectively. Demethylase activities at the EN, LT and IA timepoints were then expressed relative to these EC values which were standardized to 1.0 (Figure 3.4).

Within liver, a significant decrease in m6A demethylase activity was observed during LT (a reduction to 35% of EC activity) and during IA (46% of EC activity). A decrease in demethylase activity was also observed in WAT, although this occurred during EN (44% of EC activity). On the other hand, no significant changes in m6A demethylase activity occurred in skeletal muscle over the torpor-arousal cycle.

Proteins involved in binding m6A are regulated across the torpor-arousal cycle

Three YTH domain-containing proteins involved in recognizing m6A modifications were quantified and the effects of the torpor/arousal cycle on these proteins are shown in Figure 3.5. YTHDF1 in liver showed a pattern that was largely constant over most of the torpor-arousal cycle, save for during EA when levels rose to 1.7-fold higher than EC. However, in both WAT and skeletal muscle, YTHDF1 levels rose significantly during EN and remained significantly higher than EC in LT; levels of YTHDF1 increased in the torpid period (EN, LT) by between 1.8 and 2.2-fold and in WAT and between 5.3 and 6.4-fold skeletal muscle. Levels of YTHDF1 in both WAT and skeletal muscle then fell back to euthermic values during the arousal from torpor. Levels of YTHDF1 continued to fall in WAT during IA when values for YTHDF1 were only 36% of EC values. Turning to YTHDC2, protein levels within liver were significantly lower during both LT and IA (reduced to 65% and 41%, respectively, as compared to EC values). In WAT, YTHDC2 levels were reduced in transitory periods

(EN and EA) to 44% and 60% of EC values, respectively, but lowest levels (28% compared to EC) occurred when squirrels were fully aroused during IA. In skeletal muscle, however, YTHDC2 levels increased significantly in EN, to 3.7-fold above EC levels but were not significantly different from EC values at other points of the torpor-arousal cycle. Finally, levels of YTHDF3 in liver increased strongly beginning in EN and remained elevated during LT and EA by 2.7 and 4-fold, respectively in comparison to EC values. On the other hand, levels of YTHDF3 in WAT were significantly reduced during EN to 30% of EC levels, but were re-established in LT, before falling again during EA and IA to 37% and 28% of EC levels, respectively. Skeletal muscle values for YTHDF3 showed no significant changes until IA, when levels rose 2.5-fold above EC values.

Finally, protein levels of eIF3d were analyzed using an ELISA to assay changes in this mRNA-binding protein involved in non-canonical translation initiation (Figure 3.6). Levels of eIF3d in liver rose significantly during EN to 1.3-fold higher than EC levels but were reduced again during LT and IA. Tissue levels of eIF3d were unchanged in WAT over the topor-arousal course. However, in skeletal muscle, levels of eIF3d rose significantly during LT and IA, by 1.8-fold and 2.2-fold, respectively, compared to EC eIF3d levels.

Discussion

The mechanisms that regulate posttranscriptional processing of genes mRNA transcripts in order to allow cells to adapt to stress signals are complex and still poorly defined. In particular, little is known about RNA dynamics during mammalian hibernation although the regulatory adaptations that control gene transcription and protein synthesis, the processes that come before and after RNA processing, respectively, have

received much attention (Storey 2015). The role of microRNA in determining the fate of mRNA transcripts has also been well studied in recent years. However, within the hibernation field, little or no attention has been given to mRNA itself and the impact of direct modifications of mRNA molecules as a factor in their control over the torpor/arousal cycles of hibernation. Methylation of adenosine residues (m6A) is a well-known post-translational modification of mRNA transcripts with strong regulatory impact on the fate of mRNA molecules. Understanding how functionally related proteins control m6A modification dynamics is a key missing part to our full understanding of the molecular mechanisms controlling the characteristic changes in gene expression that occur during hibernation. The question of RNA dynamics during hibernation is a complicated one, since the typical assumption about the genome of a torpid animal is that it is largely quiescent and inactive (van Breukelen and Martin 2002). In reality, however, parts of the genome must remain active during torpor in a bid to maintain key homeostatic and cytoprotective functions at a level that is capable of greater fuel-savings when compared to active, euthermic animals (for review, see Morin and Storey 2009). To date, the changes in genomic activity that occur during hibernation have, in large part, been linked to the activities of epigenetic programs that regulate post-translational modifications on the histone proteins that guard DNA (Tessier, Luu, et al. 2017; Biggar and Storey 2014). While much attention has been given to genomic reorganization during hibernation, the regulation of RNAs has been much less studied. One recent study showed that RNAs present within neural tissue of hibernating 13LGS are subject to transcript-specific regulation in the form of A-to-I editing by the ADAR family of deaminases (Riemondy et al. 2018) and that this mechanism contributes to increased RNA stability at the low body temperatures during torpor by avoiding double-stranded

RNA formation. In another study, increased polyadenylation of the C-terminal ends of mRNAs (a stabilizing modification) was observed to take place on a group of transcripts that are important to heat production by brown adipose tissue (Grabek, Diniz Behn, et al. 2015), likely by increasing the translational activation of modified transcripts. Certainly, a mechanism allowing the torpid genome greater flexibility in modifying the stability or the transcription and translation of related groups of RNA species during hibernation would be advantageous.

The results of the present study show that during hibernation in ground squirrels, the dynamic nature of RNA is further modulated by the deposition of methylation modifications on adenosine residues during torpor. Global levels of m6A-modified RNA were significantly altered in both WAT (strongly increased) and skeletal muscle (decreased), and specific m6A modifications on mRNA molecules were significantly decreased in liver (Figure 3.1). Since m6A modifications decreased on liver mRNA, and on global RNA in skeletal muscle, a role for this posttranslational modification in reducing the translational activity of most RNA species in these two tissues is suggested, which can be achieved via modifications of mRNA or other RNA species. However, the opposite was true of RNA species in WAT that showed greatly elevated m6A levels during late torpor (LT). Whether the purpose of a single m6A-modification is structural or functional appears to depend on the placement of that modification on each individual transcript (For review, see Meyer 2019), however this is beyond the scope of the present study. Briefly, m6A modifications present within the 5'UTR of gene transcripts typically increase translational activity or stability by increasing cap-binding to alternative translation initiation factors (i.e., eIF3d), whereas m6A modifications within the 3'UTR

increase transcription efficiency and alternative polyadenylation (Zhou et al. 2018; Yue et al. 2018). For instance, m6A modifications in the 5'UTR of *hsp70* can enable translation initiation independent of other necessary post-transcriptional modifications in times of cell stress (J. Zhou et al. 2015). On the other hand, m6A modifications within the coding region are largely subject to the functioning of reader proteins which have either activating or inhibiting effects on a transcript's activity depending on the function of the reader proteins (i.e., YTH family proteins) (Mao et al. 2019; Du et al. 2016). Clearly then, a more detailed picture of the dynamics underpinning m6A modifications during hibernation is required to fully understand the meaning of the present results.

Assessing the protein levels of m6A writers provides an understanding of the important enzymes that control m6A deposition and the potential for RNA methylation activity. The majority of transcripts bearing m6A sites are modified by an RNA methyltransferase complex containing a METTL3-METTL14 heterodimer, whereby METTL3 provides the enzymatic component and METTL14 provides allosteric regulation (X. Wang et al. 2016; Geula et al. 2015). The roles of both complex members are crucial to the deposition of m6A modifications onto almost all RNA species, as the number of m6A residues catalyzed by other writer enzymes is comparatively very small. Other complex members, such as WTAP, provide a selection mechanism for the methyltransferase complex, by preferentially binding and presenting certain transcripts for modification over others (Ping et al. 2014; Liu et al. 2014). For instance, WTAP is able to recruit METTL3-METTL14 to sites of active transcription by binding transcription factors bound to specific promoters. In 13LGS, levels of the METTL3-METTL14 heterodimer appear to be maintained in all three tissues during the torpor

phase of the hibernation cycle, since only during EN in WAT does the level of either member, in this case METTL14, decrease significantly in comparison to euthermic (EC) protein levels (Figure 3.2). Furthermore, during arousal from torpor, in both liver and skeletal muscle, levels of METTL3 are significantly reduced in EA and the same is true of METTL14 in IA. Apparently, the regulation of the core RNA methyltransferase complex is remarkably consistent within skeletal muscle and in liver, whereas WAT seems to be under the effects of a different tissue-specific regulatory mechanism. Since the METTL3-METTL14 complex requires the synergistic activities of both members (Śledź and Jinek 2016; P. Wang et al. 2016), it is likely that a decrease in m6A site creation occurs when either member is significantly reduced; this is the case during EN in WAT and arousal in both liver and skeletal muscle. Relative protein levels of WTAP are regulated in a congruent pattern within WAT and skeletal muscle, whereas WTAP is significantly decreased in liver during both arousal periods. Both WTAP and METTL14 have intrinsic RNA-binding activities (Liu et al. 2014; X. Wang et al. 2016), and so increased levels of either complex member may serve to increase the number of m6A sites on certain groups of transcripts over others. Within human stem cells WTAP has been shown to preferentially modify SMAD2/3-induced transcripts via interactions with METTL3-METTL14 in response to TGFβ signaling (Bertero et al. 2018). This interaction could prove to be important within hibernation as the TGFβ/SMAD signaling pathway is known to be intricately regulated in a tissue specific manner during hibernation (Wu and Storey 2018; Brooks et al. 2011; Talaei et al. 2011), and is a worthwhile candidate for future studies seeking to identify the transcript-specific nature of m6A modifications during 13LGS hibernation.

Eraser proteins control the demethylation of RNA transcripts and contribute to the dynamics of m6A sites, although increased protein expression of these enzymes appears to be limited to specific stress-relevant conditions (Zhao et al. 2017; Darnell et al. 2018). For both FTO and ALKBH5, ferrous iron and α-ketoglutarate are needed as co-factors to oxidize the *N*-methyl group of m6A to a hydroxymethyl group. Depletion of FTO or ALKBH5 leads to increased levels of m6A on RNA species (Li et al. 2017; Zheng et al. 2013). Protein levels of FTO were reduced in liver during torpor and across the torpor-arousal cycle in WAT except during LT (Figure 3.3). However, ALKBH5 protein levels were maintained at euthermic levels in torpor and EA, and in fact were significantly increased during EN in liver. By contrast, levels of both FTO and ALKBH5 in skeletal muscle were maintained during torpor and were increased during arousal, with respect to EC animals. When comparing the results for the relative protein levels with the data on RNA demethylase activity (Figure 3.4), the data for liver show a significant decrease in demethylase activity during arousal which is not explained by the changes in the protein levels of FTO and ALKBH5. Furthermore, significantly decreased levels of FTO and ALKBH5 protein in WAT during IA were not reflected in the constant levels of demethylase activity. It could be that m6A dynamics are less required during arousal as cells re-establish typical mechanisms for transcript selection (Wu and Storey 2012; Tessier and Storey 2014). Importantly, ALKBH5 and FTO are substrates for serine and tyrosine phosphorylation, as well as lysine acetylation and these posttranslational modifications have the potential to effect changes in demethylase activity and downstream RNA interactions (Choudhary et al. 2009; Lin et al. 2014; Aik et al. 2014). For instance, phosphorylation of FTO prevents its polyubiquitination and leads to altered

expression levels (Lin et al. 2014), whereas another group posited that FTO interacts with calcium/calmodulin-dependent protein kinase 2 leading to increased phosphorylation activity on CREB and altered levels of CREB-downstream mRNA (Faulds et al. 2018). Mammalian hibernation employs post-translational modifications (PTMs) to rapidly and reversibly alter enzyme/protein activity on a number of substrates (Morin and Storey 2009). Since PTMs on FTO or ALKBH5 can change their activity levels, it is possible that even though protein levels of demethylase enzymes were maintained in liver during hibernation, the activities of these proteins could be reduced during arousal; the opposite could be true in WAT or muscle.

In order to fully appreciate the downstream consequences for mRNA molecules targeted for N6-A methylation, levels of m6A reader proteins must be analyzed. Proteins that specifically recognize and bind m6A control the fate of methylated mRNA by interacting with other protein factors that recognize the RNA sequence or structure in order for downstream processing to be regulated (Dominissini et al. 2012). In vertebrates, the role of YTHDF1, YTHDC2 or YTHDF3 following methylated-mRNA binding is to act in the cytosol to increase the translation of the bound molecule by facilitating ribosomal loading, as is the case for YTHDF1 and YTHDF3 (Shi et al. 2017), or possibly by acting as an RNA helicase, as has been proposed for YTHDC2 (Hsu et al. 2017). For these reasons, changes in protein levels of m6A readers during hibernation likely indicate changes in the transcripts being selected for translation. Indeed, the transcriptome of the 13LGS changes drastically during hibernation (Andrews 2019) and leads to major changes to physiology as compared with the euthermic state. In assessing the changes in protein levels of YTHDF1 and YTHDF3 (Figure 3.5), it becomes evident that an opposite

pattern emerges in liver and muscle where each protein is significantly increased during hibernation or are maintained at euthermic levels. YTHDF1 is significantly increased within liver during EA, however the same is true in muscle during EN and LT. This is interesting as YTHDF1 is significantly increased in each tissue at the times that the tissue is thought to be relatively inactive during the torpor-arousal cycle; By the same token, YTHDF3 levels were significantly increased at the times when tissues are comparatively active: throughout the heterothermic period in liver (i.e., during EN, LT and to a certain extent in EA), and during IA in muscle. Liver and muscle tissues become active at different phases of hibernation: muscle activity is known to increase during the arousal period to enable shivering-thermogenesis, whereas the liver proteome is known to shift to favor and actively metabolize lipids while in torpor (for reviews, see Tessier and Storey 2016; Lang-Ouellette et al. 2014). Additionally, YTHDF1 is significantly increased in WAT during both phases of torpor that YTHDF1 levels in muscle are elevated (EN and LT), pointing to a role in the mobilization and metabolism of fats that actively occur while the animal is torpid. During IA, both YTHDF1 and YTHDF3 are significantly reduced in WAT, when the preference for lipid-based metabolism is reversed (Wu et al. 2013). Notably, adipogenesis within intramuscular adipocytes is promoted through a mechanism dependent on the methylation of mitochondrial carrier homology 2 (*MTCH2*) mRNA and its binding by YTHDF1 (Jiang et al. 2019), showing that mRNA binding proteins can play an important role in regulating lipid metabolism during normal physiology. Finally, while the exact mRNAs targeted by YTHDC2 are as yet unknown, it has been shown that m6A localized to the mRNA coding region can regulate the formation of mRNA post-secondary structures and surprisingly can increase transcription

via reliance on YTHDC2 (Mao et al. 2019). This makes it possible that the significant changes in protein levels of YTHDC2 which occur during IA in all three tissues are indicative of changes to the m6A modifications being deposited within the coding regions of mRNAs required at that time. In fact, the effects of temperature on mRNA interactions has been predicted to change significantly during torpor (Hadj-Moussa and Storey 2018), adding to the proposition that an RNA helicase would aid the selection of mRNAs for processing.

The fact that these m6A reader proteins are significantly increased only at specific times of the torpor-arousal cycle in any tissue is important, and mirrors increases in transcription factors that are typically active at only selected timepoints during the torpor-arousal cycle (Srere et al. 1992). If these transcription factors, whose role is to favor the transcription of a group of transcripts when active, also interact with an RNA methyltransferase, then those transcripts could be selected by the m6A readers which are most abundant. While the literature is still unclear on how a YTH protein can mediate different functions, the question remains of why either reader needs to be more abundant during hibernation. It could be that this need arises from differences in the processing, stability and metabolism required of the bound transcripts (Shi et al. 2017), given the differences in cells within a tissue that require increased output at a certain timepoint within the torpor-arousal cycle (i.e., intricate regulation of mRNA fate and the need for non-canonical mechanisms of translation initiation) (Pan and van Breukelen 2011; Tessier and Storey 2014).

In addition, eIF3 is a known m6A reader and enables m6A-dependent recruitment and formation of the ribosomal 48S complex in a non-canonical mechanism for initiating

translation, and notably this occurred even in the absence of group 4 eIFs (Meyer et al. 2015). The fact that eIF3d, as a subunit of, eIF3 allows ribosomal formation without the requirement for an eIF4 cap-binding complex is interesting in this context, as the formation of the eIF4 complex is known to be disrupted by eIF4E hyperphosphorylation, and significantly increased levels of 4E-binding protein-1 (4E-BP) and activating phosphorylation during torpor in the 13LGS (van Breukelen et al. 2004; Frerichs et al. 1998). Through recognition of m6A within the 5'UTR, eIF3 is able to initiate translation independent of eIF4E recruitment, using a cap-recognition mechanism by eIF3d (A. S. Lee et al. 2016; Coots et al. 2017). When protein levels of eIF3d were assayed, they were seen to be maintained in both liver and WAT with a significant increase during EN in liver, making it possible that eIF3d participates in cap-dependent initiation during torpor (Figure 3.6). Levels of eIF3d were also significantly increased during IA in muscle and likely contribute to translation initiation in this context, given the need for protein turnover in IA (Tessier and Storey 2016), but also surprisingly during LT, when hibernator muscle is not thought of to be active. An eIF3d-dependent mechanism for cap-recognition operates as an alternate form of translation initiation that operates independent of the eIF4E-cap recognition mechanism and facilitates the translation of approximately 20% of mRNAs when eIF4 is disrupted (de la Parra et al. 2018). Within muscle tissue during LT, 4E-BP is hypophosphorylated suggesting increased binding to eIF4 and inactivation of eIF4-dependent translation initiation (Wu and Storey 2012). Given the ubiquity and redundancy of an eIF3-dependent mechanism for cap-recognition and translation initiation, it follows that increased levels of eIF3d in muscle during LT

may be required for the translation of necessary (e.g., cytoprotective) transcripts while canonical mechanisms for translation initiation are inhibited.

It is clear from these results that RNA methylation is maintained during hibernation in ground squirrels within the three tissues investigated. In a majority of cases, protein levels of RNA writers, readers and erasers were seen to be at euthermic values, or were elevated from those levels, indicating their potential requirement for hibernation to take place in a coordinated and efficient manner. Hibernation is a reversible hypometabolic state that requires numerous changes to the transcriptome and proteome at specific times. The mechanisms available to a hibernator doubtless include all manner of regulation, and surely include RNA editing in the form of methylation modifications. Future research would be wise to include sequencing of m6A on transcripts during hibernation in order to further investigate the roles of proteins that recognize, and process essential RNA species and transcripts needed for cellular adaptation, as well as to uncover novel regulatory control points that control and enable mammalian hibernation.

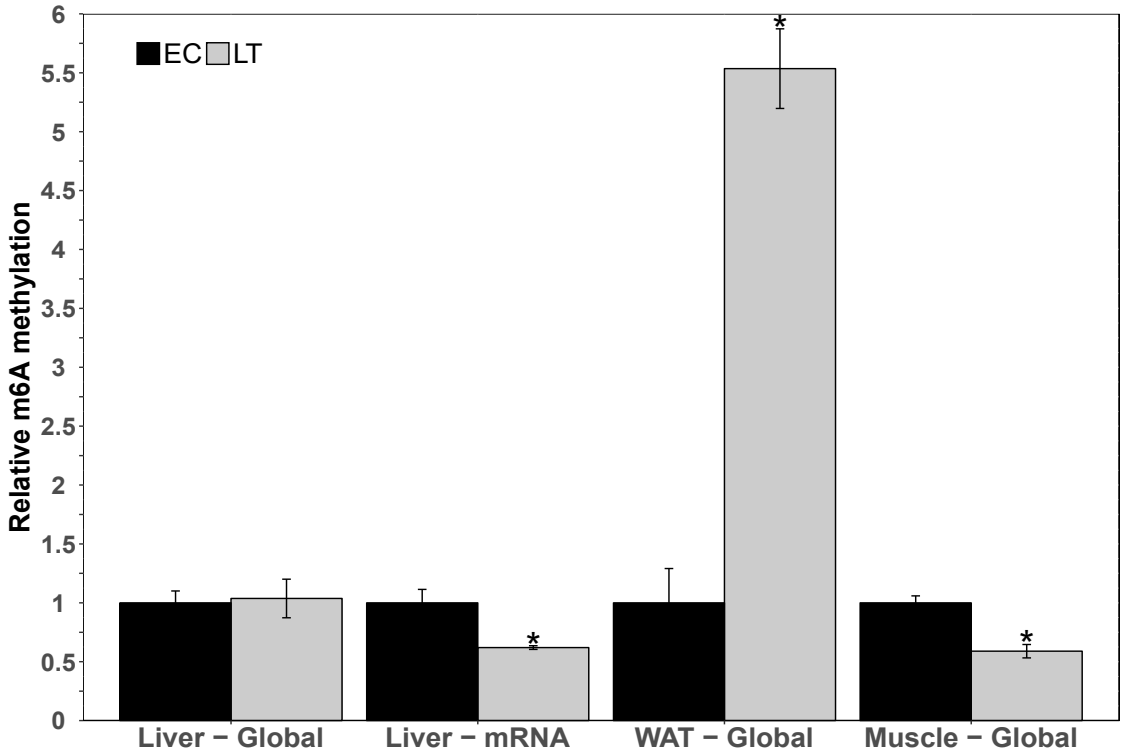


Figure 3.1: Relative levels of N6-methyladenosine (m6A) in total RNA extracts (global) from liver, WAT and skeletal muscle tissues, comparing ground squirrels in euthermic control (EC) and late torpor (LT) conditions. For liver, the mRNA fraction was further isolated from the global RNA pool and m6A levels within this subset are also shown. Histogram shows the relative mean levels of m6A ± SEM (n = 4 independent RNA isolations from different animals). Data were analyzed using the Student's t-test ($p < 0.05$) and labeled with an asterisk shown above the LT bar when the result was statistically significant.

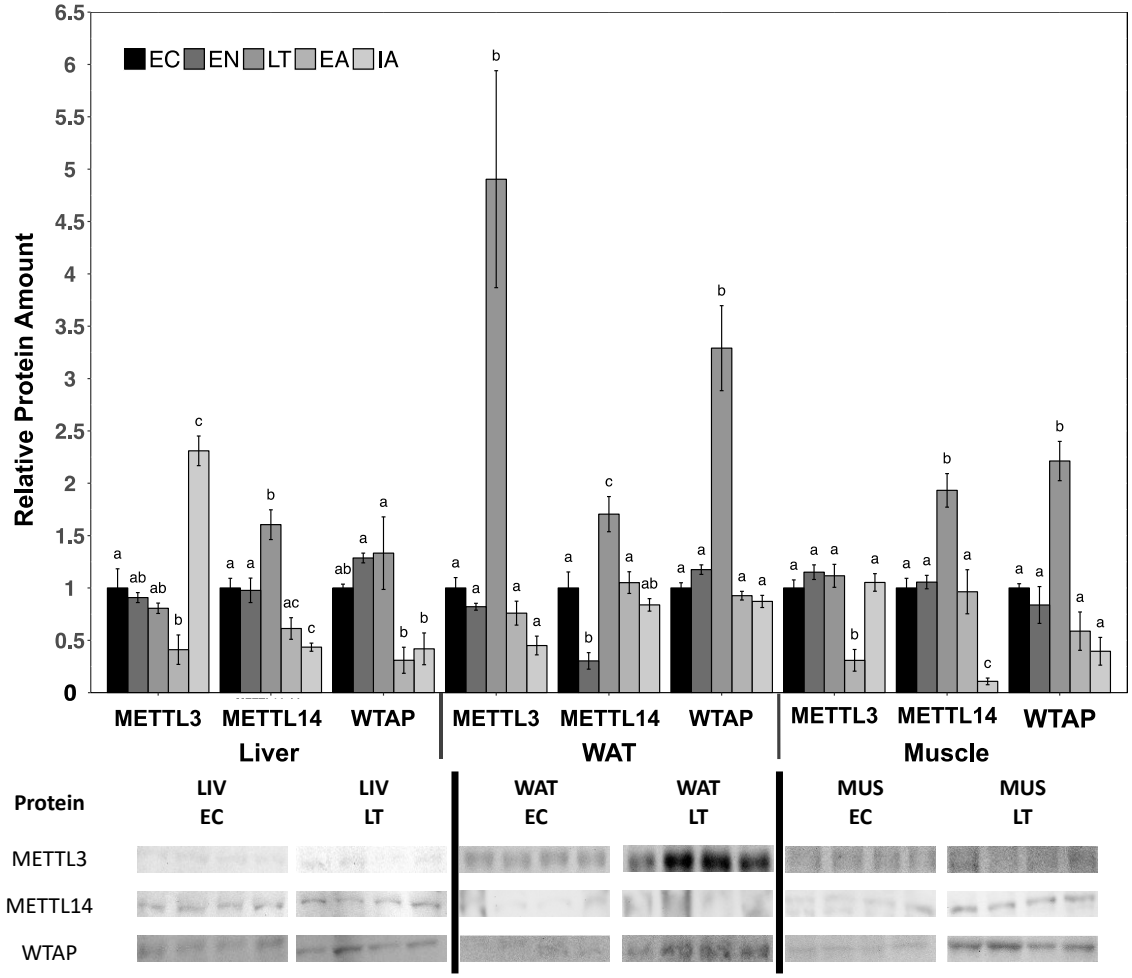


Figure 3.2: Relative total protein levels of m6A writer proteins in liver, white adipose (WAT) and skeletal muscle of hibernating 13-lined ground squirrels. Sampling points are euthermic in the cold room (EC), entrance into torpor (EN), late torpor (LT), late arousal (LA), and interbout arousal (IA) and used 20 μg of protein per lane. Histograms show mean standardized band densities ± SEM (n = 4 independent trials from different animals). Data were analyzed using ANOVA followed by a Tukey post hoc test and labeled so that different letters denote values that are significantly different from each other ($p < 0.05$). Representative immunoblot images below the histogram are for the EC and LT timepoints.

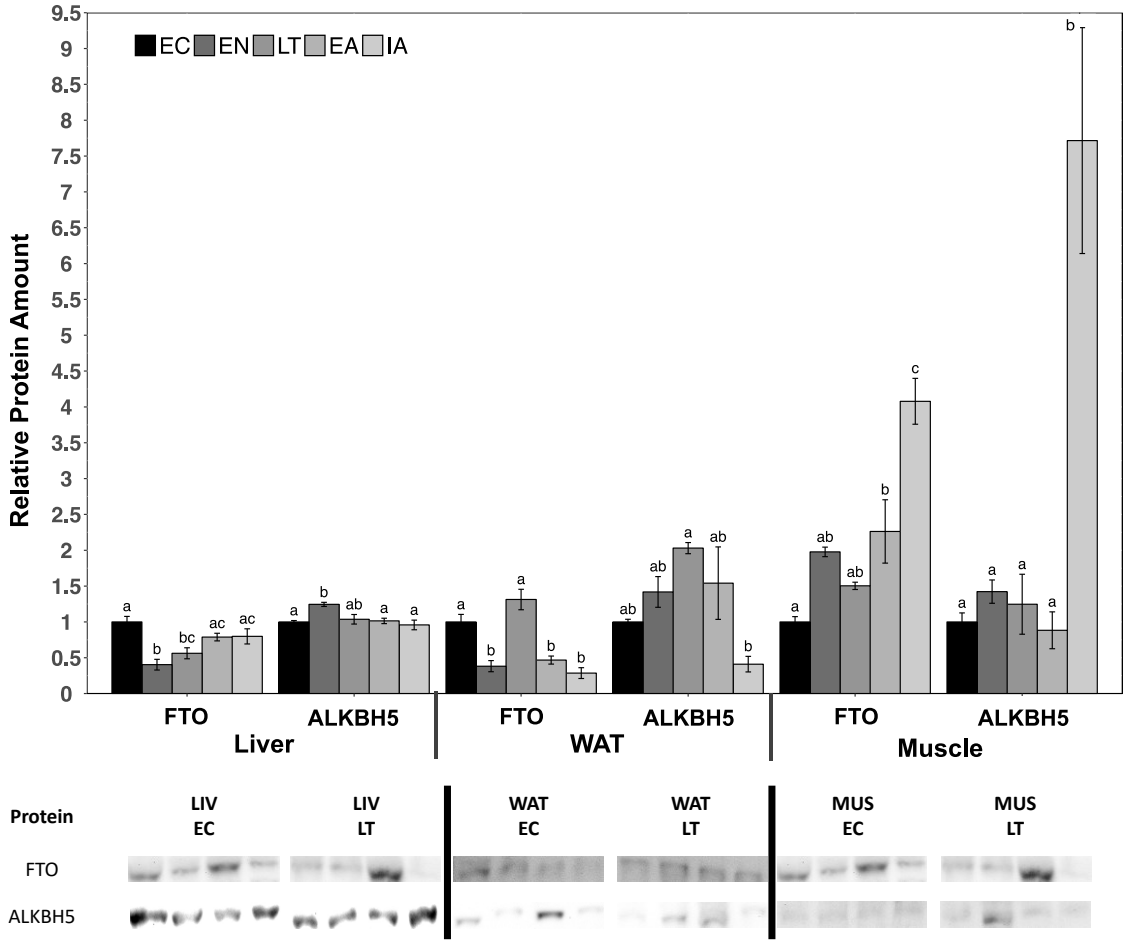


Figure 3.3: Relative total protein levels of m6A eraser proteins in three tissues of hibernating 13-lined ground squirrels. All other information is as in Figure 3.2.

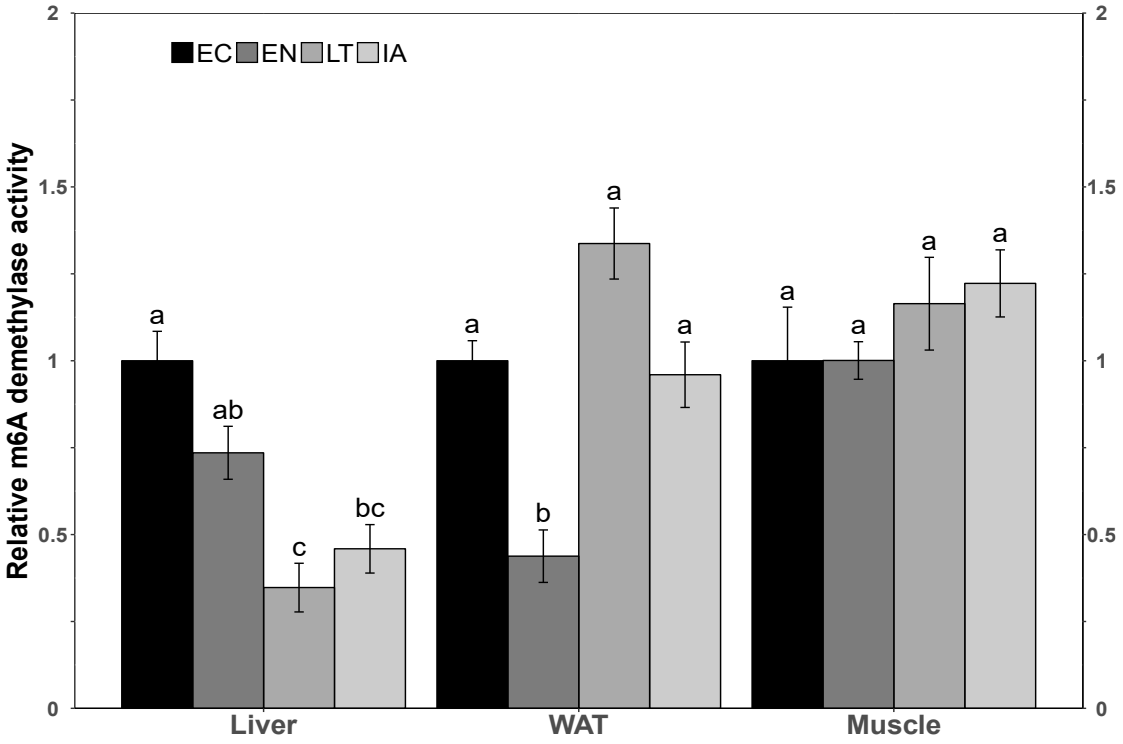


Figure 3.4: Relative levels of m6A demethylase activity over 4 time points of the torpor-arousal cycle in hibernating 13-lined ground squirrels. Histogram shows the relative mean level of m6A demethylated per hour per mg protein ($ng \cdot h^{-1}\ mg^{-1}$) ± SEM ($n = 4$ independent protein isolations from different animals). All other information is same as in Figure 3.2.

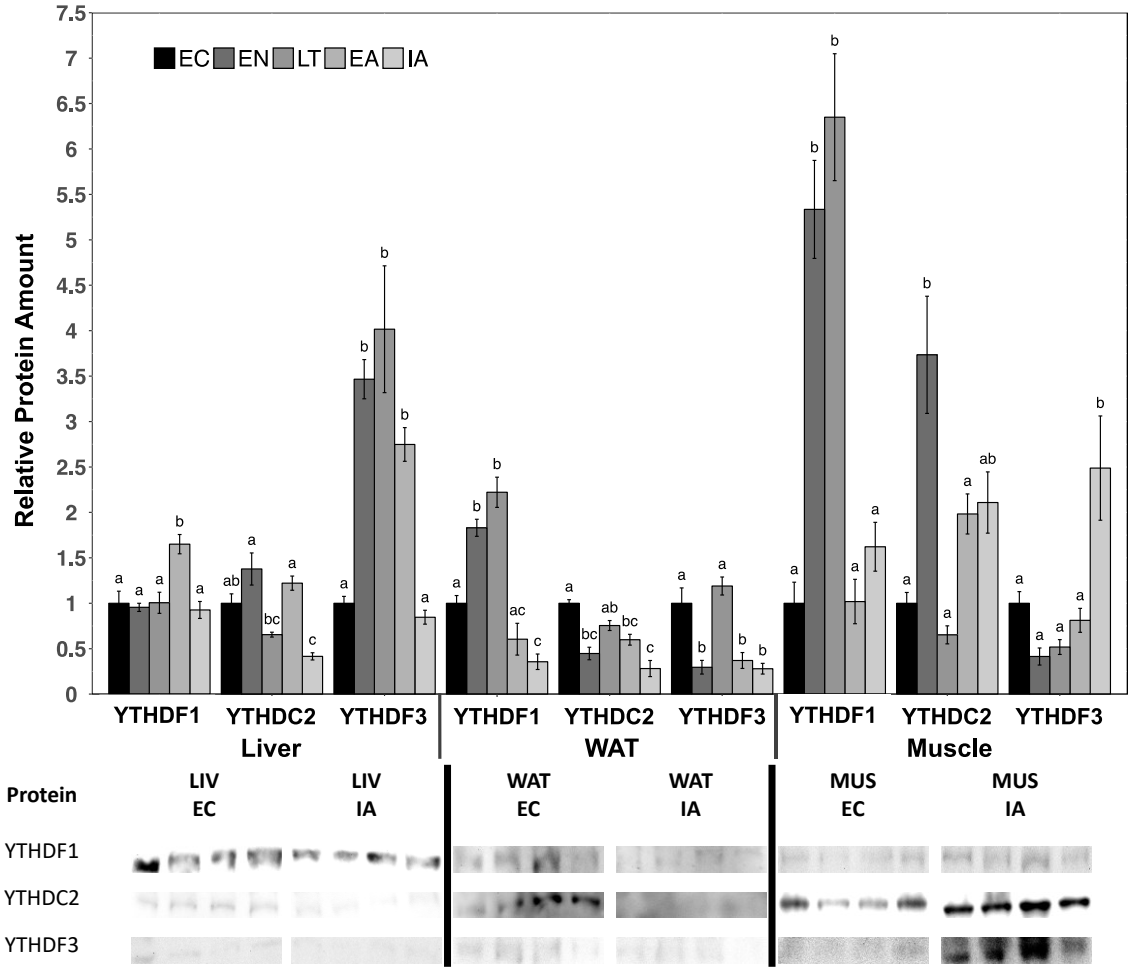


Figure 3.5: Relative total protein levels of m6A reader proteins in three tissues of hibernating 13-lined ground squirrels. Blots images compare EC and interbout arousal (IA) time points. All other information is as in figure 3.2.

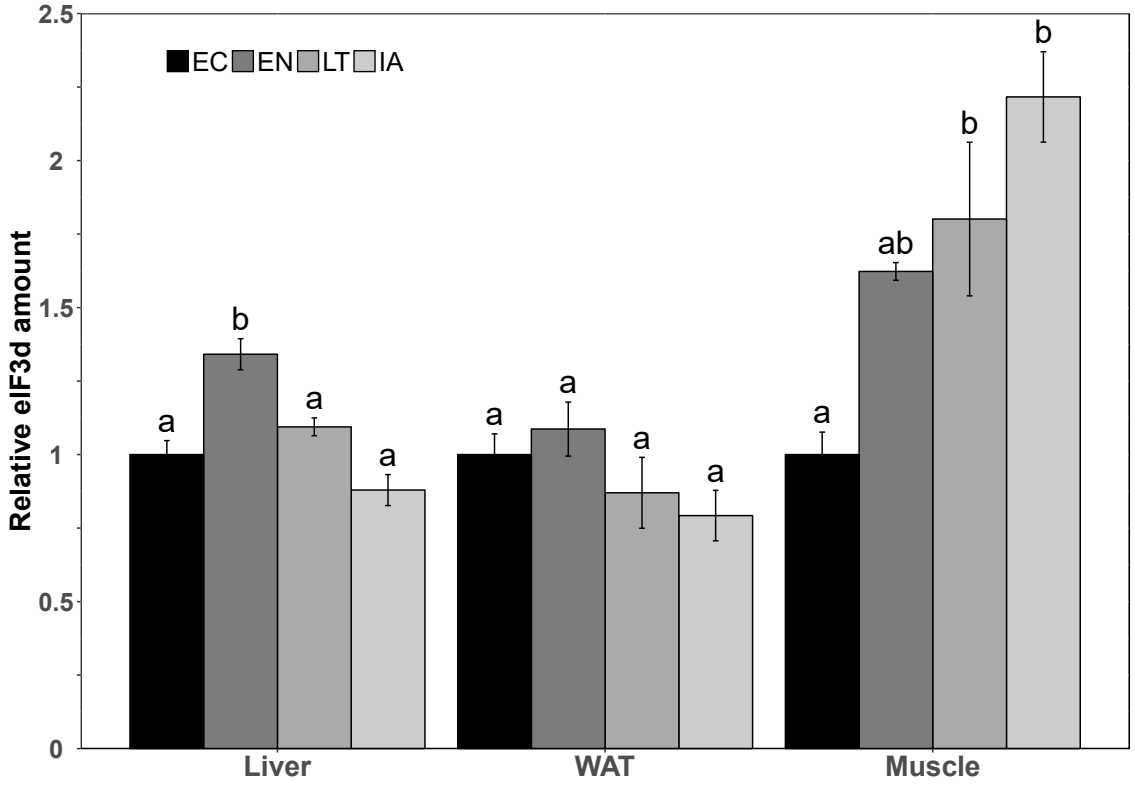


Figure 3.6: Relative levels of eukaryotic translation initiation factor 3, subunit D (eIF3d) over the torpor-arousal cycle in hibernating 13-lined ground squirrels. Histogram shows the relative mean level eIF3d ± SEM (n = 4 independent protein isolations from different animals). All other information is same as in Figure 3.2.

Chapter 4

Arginine methylation produces differential regulation of epigenetic marks during hibernation in the thirteen-lined ground squirrel

Introduction

Some sciurid rodents such as the 13-lined ground squirrel (13LGS, *Ictidomys tridecemlineatus*) engage in hibernation to survive prolonged periods of resource scarcity (e.g., winter). In mammals, hibernation is characterized by prolonged periods of hypometabolism (i.e., torpor) wherein global reductions in fuel and energy requirements reduce the resting metabolic rate to <5% of euthermic (i.e., summer) values (Ruf and Geiser 2015). Attenuation of nearly all cellular functions may be achieved via passive suppression through lowering the hypothalamic thermal set-point, combined with active suppression imposed upon many biochemical pathways. During the hibernation season, the squirrels undergo long periods of deep torpor that are interspersed with brief arousal periods where body temperature (T_b) returns to the euthermic level (Carey et al. 2003), indicating that the mechanisms involved in controlling torpor/arousal must be readily reversible. For these reasons, the 13LGS has become a major model organism for investigating the molecular controls of natural mammalian hibernation.

The majority of 13LGS tissues show evidence of strong metabolic rate depression during torpor, allowing them to survive hibernation through increased regulation over protein production and protein/enzyme activity which provides intricate selection of necessary, tissue-specific cytoprotective biochemical pathways (for review, see Storey and Storey 2010). This feat is achieved, in large part, through the programmed use of post-translational modifications (PTMs), which effect a pattern of down-regulation over protein production that rapidly and reversibly modulates target proteins during torpor and arousal. For example, selected gene transcription patterns are achieved through acetylation and phosphorylation of activated transcription factors during torpor, and

allow greater regulatory plasticity over the genome (Morin and Storey 2009; Srere et al. 1992).

Similarly, changes to PTMs on histone proteins are a crucial factor in the regulation imposed on protein production during hibernation. Methylation of arginine residues on histones has been shown to play a role in the remodelling of chromatin and regulates aspects of protein dynamics and functioning in several cellular pathways. Arginine is well positioned to interact with other biological molecules, since the residue has five potential hydrogen bond donors and has been shown to form predictable, defined, and frequent interactions with RNA, DNA and proteins (Luscombe et al. 2001; Najbauer et al. 1993; Hughes and Waters 2006). In addition, methylation of arginine may also increase the likelihood of a protein binding to certain interacting partners by increasing a protein's affinity for selected molecular features including aromatic residues (Sprangers et al. 2003). Since methylation replaces available hydrogen bond donor atoms, it can thereby alter the interactions of arginine residues with various binding partners (Pahlich et al. 2006) and, indeed, the discovery of this modification has been shown to be increasingly relevant to cellular dynamics.

Arginine methylation is catalyzed by protein arginine methyltransferase (PRMT) enzymes that remove a methyl-group from a donor molecule [S-adenosyl-L-methionine, AdoMet] and place the modification onto terminal nitrogen atoms of arginine residues. In mammals this can occur on two of the three nitrogen atoms within the guanidinium group. Arginine methylation comes in three forms, depending on the enzyme that places the modification: monomethylarginine (MMA), symmetric dimethylarginine (sDMA) and asymmetric dimethylarginine (aDMA). These are named for the number of methyl groups

placed on the arginine and their placement on either one or both terminal nitrogen atoms (Wolf 2009; Bachand 2007). This difference in enzymatic products separates PRMT enzymes into two groups based on their ability to place MMA as well as either aDMA (type I PRMTs) or sDMA (type II PRMTs) modifications. Seven PRMT enzymes have been found (PRMT1-7) in mammals, of which PRMT5 and PRMT7 are the only type II enzymes. Arginine methylation has also been shown to be reversible via the demethylase activity of Jumonji-Domain Containing 6 protein (JMJD6) (Chang et al. 2007), although this mechanism is still hotly debated (Blanc and Richard 2017). Protein arginine methylation has major implications for the cell's transcriptional regulatory capacity through actions on several binding partners.

When placed upon histone proteins, arginine methylation acts as a stable and reversible epigenetic modification, that can regulate gene transcription by altering the degree of chromatin relaxation (activating) or condensation (inhibitory). The PRMT4 enzyme is perhaps the best characterized and is associated with the recruitment of nuclear receptor transcription factors to DNA due to methylation of histone H3 on R2, -17 and -26 (Schurter et al. 2001; Lee et al. 2002). Activation following arginine methylation and nuclear receptor binding is further increased via synergistic effects of histone acetylation. PRMT1-3 seemingly act via similar mechanisms (e.g., H4R3 methylation) to activate gene transcription in cooperation with other histone modifications and are thus characterized as transcription coactivators (Lee et al. 2005). PRMT5, on the other hand, was associated with repression of gene activity, in association with the co-repressor proteins histone deacetylase 2 (HDAC2) and Sin3A or with chromatin-remodelling factors following methylation of histone H4 on R8 (Pal et al. 2003; Pal et al. 2004). By

modifying histone proteins, the PRMT enzymes alter the chromatin accessibility and transcriptional activation of nearby genes.

The present study aims to understand the role played by arginine protein methylation in mammalian hibernation and metabolic rate depression. It is thought that arginine methylation provides a means of reducing transcriptional rates through modification of histone proteins and is involved in modifying the activity of non-histone proteins during torpor. Therefore, the protein levels of several PRMT enzymes that play a role in chromatin condensation and relaxation were analyzed along with total type-II arginine methyltransferase activity, and changes in methylarginine modifications on histone proteins over the torpor-arousal cycle. These results allow for conclusions to be made about the roles that arginine methylation may provide in regulating hibernation.

Methods

Animal Preparations

As described by Cai et al. (2004), 13LGS were caught in the wild between the months of August and September in Illinois by a trapper licensed by the US Department of Agriculture. Squirrels were transported to a National Institute of Health facility where they were quarantined during antiparasitic treatment. NINDS animal care and use committee (ACUC) approved animal housing and experimental procedures were followed (protocol number ASP 1223-05). Squirrels were housed individually in plastic shoebox cages in a vivarium with an ambient temperature of 21°C and a 12:12 h L:D photoperiod. Animals were fed standard rodent diet. Weight gain before hibernation served as a cue for subcutaneous implantation of telemetry devices for T_b measurements (IPTT-200; Bio Medic Data Systems, Seaford, DE) while the squirrels were anesthetized with 5% isofluorane. Animals were housed in a hibernaculum during the months of

November to March with an ambient temperature of 4-6°C and 60% humidity under 24-hour darkness. A photographic red safelight (3–5 lx) was used when necessary and the animal's chamber was only accessible through a darkened anteroom.

Hibernation in 13LGS is identifiable by a near-ambient T_b, minimal activity and curled body position. Animals were sampled at several points over the torpor-arousal cycle, requiring quick removal from the hibernaculum, anesthetization and decapitation within two minutes. Whole organ samples were dissected and immediately frozen in liquid nitrogen for subsequent transport to Carleton University on dry ice. Tissue samples were then stored at -80°C until required.

Total Soluble Protein Extraction

Frozen liver, visceral white adipose tissue (WAT), and hind leg skeletal muscle tissue from four individual animals at each time point of the torpor-arousal cycle were crushed and homogenized as described previously (Watts and Storey 2019) in ice-cold homogenizing buffer using a Polytron PT 10-35 homogenizer with sonication (Kinematica; #11010104); dilutions were 1:3 (w/v) in the case of WAT and 1:5 (w/v) for liver and muscle. Protein concentrations were stored at -80°C and standardized to 10 μg/μL before use.

Arginine Methyltransferase Assay

Arginine methyltransferase activity was examined in total soluble protein extracts using the Epigenase PRMT Methyltransferase (Type II-specific) Activity/Inhibition Assay Kit (Epigentek, Cat# P-3088) according to manufacturer's instructions. A standard curve was first produced to find the optimal amount of input material required (10 μg protein for liver or WAT, and 30 protein μg for muscle). The assay involves addition of protein samples to wells coated with a substrate compatible with type-II PRMT enzymes.

The modified, methylated products were detected using the provided primary and secondary antibodies before exposure using a developing solution. Methylated products are proportional to the absorbance measured in a microplate spectrophotometer at a wavelength of 450nm. Specific methyltransferase activity is reported as ng of methylarginine produced per hour per mg of protein sample added.

Western Blotting

SDS-polyacrylamide gel electrophoresis and immunoblotting were accomplished as previously described (Watts and Storey 2019), using total soluble protein extracts standardized to 5 µg/µL by mixing 1:1 v/v with 2X SDS loading buffer (100 mM Tris-base H 6.8, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, 10% v:v 2-mercaptoethanol). 20 µg of protein sample (which was adjusted as required in keeping with the linear dynamic range of the antibody reaction) was loaded on SDS-polyacrylamide gels and run at 180 V for ~60 min and transferred onto a 0.45 µm PVDF membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad; Mississauga, ON, Canada). Membranes were blocked using skim milk powder (1.0-3.0% w:v) dissolved in Tris-buffered saline with Tween (TBST; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% v:v Tween-20) for 30 min. Primary antibodies were diluted 1:1000 v/v in TBST and then incubated with membranes at 4°C overnight. Primary antibodies for PRMT1 (#sc-166963), PRMT2 (#sc-393254), and PRMT7 (#sc-376077) were purchased from Santa-Cruz Biotechnology (Dallas, TX); these were monoclonal and raised in mice. The primary antibodies for PRMT3 (#A302-526A), PRMT4 (#A300-420A), and PRMT5 (#A300-850A) were purchased from Bethyl Laboratories (Montgomery, TX), and antibodies targeting methylarginine-modified histones, H3R2me^{2}a (#MBS9402172), H3R8me^{2}s (#MBS9607605), and H4R3me^{2}s (#MBS126222) were purchased from

MyBioSource (San Diego, CA); these antibodies were polyclonal and raised in rabbit. Epitopes for all antibodies used in this study are available from the producer. Membranes were probed with HRP-linked affinity purified anti-rabbit IgG secondary antibody (#APA007P) or with HRP-linked affinity purified anti-mouse IgG secondary antibody (#APA005P) purchased from BioShop (Burlington, ON, Canada). Specific immunoreactive bands were visualized via enhanced chemiluminescence (ECL; 1:1 hydrogen peroxide and luminol).

To account for potential minor variations in sample loading between different lanes, the densities of immunoreactive bands corresponding to the protein of interest were standardized against the summed intensity of a group of Coomassie stained bands in the same lane, these bands being consistently present across all lanes and well-separated from the immunoband of interest (Eaton et al. 2013). Coomassie blue-stained membranes (0.25% w:v Coomassie brilliant blue, 7.5% v:v acetic acid, 50% methanol) allowed visualization of the total protein loaded, which was constant across all lanes.

Quantification and Statistics

Chemiluminescent band density was quantified using GeneTools (version 4.02, Syngene, Frederick, MD). Relative band density was determined by standardizing all values to the first control sample that was arbitrarily set to one.

Fold change values were calculated by comparing the difference between a particular time-point, which as standardized against the control value, and the appropriate control value. For example, the fold change of EN compared to EC for any target protein is expressed as EN/EC, where a fold change > 1 represents an increase, while a fold decrease is expressed as the percentage decrease and represents a fold change <1. All numerical data are expressed as mean ± SEM (n = 4 samples from independent animals).

Statistical analysis of differences between experimental hibernation time-points was performed using a one-way ANOVA and post-hoc Tukey tests or a Student's t-test using RBioPlot statistical package (J. Zhang and Storey 2016) with $p < 0.05$ accepted as significant.

Results

The relative protein levels of six widely studied methyltransferase enzymes (PRMT1, 2, 3, 4, 5, 7) involved in regulating chromatin condensation were assessed in skeletal muscle and liver of 13LGS across five time points of the torpor-arousal cycle. Samples were collected from (i) euthermic in the cold room (EC) animals which showed euthermic physiology (i.e., stable euthermic body T_b and normal metabolic rate) for at least 72 hours; (ii) entrance into torpor (EN) animals which showed a declining T_b between 12-31°C; (iii) late torpor (LT) animals that displayed 5-8°C T_b for longer than 72 hours; (iv) early arousal (EA) animals that had begun to rewarm (persistent rising T_b between 9-12°C) and displaying more than 60 breaths per minute; and (v) interbout aroused (IA) animals that had naturally returned to euthermic conditions for no more than 24 hours after a prolonged torpor bout. Preliminary analysis confirmed that each of the commercial antibodies used for western blotting reacted with an immunoblot band at the at the expected molecular mass of each monomeric PRMT.

Type-II PRMT methylation activity was assessed in liver, white adipose (WAT) and skeletal muscle of squirrels sampled from EC, EN, LT and IA conditions. Active type-II PRMTs transfer a methyl group to methylate a substrate that is detected by a specific antibody. Using this method, the measured PRMT methylation activity in control (EC) animals was determined to be 53.4 ± 3.7 ng·min^{-1}·mg^{-1}, 29 ± 5.2 ng·min^{-1}·mg^{-1} and 50.8 ± 5.2 ng·min^{-1}·mg^{-1} in liver, white adipose, and muscle tissues, respectively.

Methylation activity in the EN, LT and IA timepoints are expressed as fold change values relative to these EC values, which were standardized to 1.0 (Figure 4.1). Methyltransferase activity was increased significantly in liver during IA, 2.3-fold above levels seen in EC, and a comparable result was seen in muscle tissue, where PRMT activity was 2.0-fold above EC levels. Similarly, in both liver and muscle, methyltransferase activity did not change significantly from EC levels during either of the torpor timepoints (i.e., EN and LT). On the other hand, methyltransferase activity in WAT was not significantly different from euthermic levels across hibernation time-course, but a significant change in activity was seen between EN and LT time points when levels increased by 2.5-fold in LT as compared with the EN value that was just 44% of EC levels.

Liver PRMT protein levels were observed to change significantly over the torpor-arousal cycle (Figure 4.2). PRMT1 levels decreased by 45% upon torpor entrance, but re-established protein levels that were comparable to control EC animals during LT. These levels were maintained across EA and IA timepoints. Levels of PRMT2 were maintained throughout EN and LT, as was also true for protein levels of PRMT4 and for PRMT5 throughout the torpor phases. However, levels of PRMT2 rose sharply during arousal, values in EA increasing by 2.3-fold over control levels in EA. The opposite was seen for PRMT4, which decreased to 45% of EC levels during EA, and then to 39% during IA. Protein levels of PRMT3 and 7 both increased significantly compared to EC values during EN, by 1.4-fold and 2.2-fold for PRMT3 and 7, respectively, and this increase was maintained in LT, at 1.4-fold and 2.3-fold for PRMT3 and 7, respectively.

In WAT, the protein levels of PRMT1, 2, 4 and 7, (i.e., all except for PRMT3 and PRMT5), were observed to decrease significantly during EN, to between 15% and 39% (which was the case for PRMT2 and 7, respectively) (Figure 4.3). Subsequently, values returned to control EC levels in LT, or exceeded them as was the case for PRMT7 that rose to levels that were 2.5-fold above EC levels (a 6.5-fold increase over EN levels). On the other hand, levels of PRMT3 were maintained during EN, but decreased to just 7% of EC levels in LT, whereas levels of PRMT5 were not significantly different than EC values in both EN and LT. As opposed to the decreases observed during EN, protein levels of all PRMTs, except PRMT3, returned to values not significantly different from controls (EC) at both arousal timepoints. Levels of PRMT3, on the other hand, continued to be significantly reduced in both EA and IA at values ~40% of EC levels.

PRMT protein levels were also investigated in skeletal muscle tissue (Figure 4.4) where it was seen that PRMT1 levels decreased significantly in LT to 34% of EC levels, and remain decreased in EA at 45% of EC levels. Levels of PRMT1 enzyme were seen to be comparable to control protein levels in both EN and IA. Interestingly, PRMT2 also showed a decrease in enzyme levels in LT (49% of EC levels), but were then elevated 2.6-fold above EC levels in EA (representing a 5.3-fold increase over levels in the preceding LT timepoint), and continued to rise, showing a 3.8-fold increase over EC levels in IA. Similarly, levels of PRMT3 and PRMT5 showed a significant decrease in protein levels in LT, to 34% and 32% of EC levels, respectively, however their protein levels then rose during EA and were returned to values comparable to EC controls during IA. Remarkably, both PRMT4 and PRMT7 showed an increasing trend during EN, which was significant in the case of PRMT7 (increased 1.8-fold above EC levels). Levels of

PRMT7 then fell back to EC levels by LT and remained low until IA when protein levels were elevated 3.3-fold above EC levels (and 1.8-fold above EN levels). Levels of PRMT4 on the other hand, continued to increase and were raised significantly in LT by 1.6-fold over EC levels. This was reversed during arousal when PRMT4 levels decreased to 40% of EC levels during EA, and remained reduced in IA compared to the earlier peaks during torpor but were not significantly different as compared to control animals.

The relative levels of methylated arginine residues present on histone proteins under EC, LT and IA conditions in the three tissues was also investigated by immunoblotting (Figure 4.5) by targeting the asymmetric demethylated R2 and symmetric demethylated R8 on histone H3 (H3R2me^{2}a and H3R8me^{2}s), as well as symmetric demethylated R3 on histone H4 (H4R3me^{2}s). In liver, H3R2me^{2}a showed significantly greater methylation in IA compared to EC (a 2.3-fold increase), whereas methylation of H4R3me^{2}s decreased significantly during LT (to 29% of EC levels). The pattern of methylation seen in WAT was almost completely opposite to that seen in liver; in WAT methylation of H3R2me^{2}a was significantly reduced during LT (to 54% of H3R2me^{2}a levels in EC), whereas methylation of H4R3me^{2}s increased significantly during LT, 1.8-fold above EC levels. H3R8me^{2}s levels also rose significantly in WAT to values at least 2.4-fold over EC levels during both LT and IA. Finally, skeletal muscle levels of H3R2me^{2}a were elevated 1.6-fold above EC levels during LT, but fell back to control levels during arousal. On the other hand, LT levels of both H3R8me^{2}s and H4R3me^{2}s were reduced to 7% and 33% of control levels, respectively. H3R8me^{2}s remained at a reduced level (30%) compared to EC during IA, whereas levels of H4R3me^{2}s were significantly elevated 2.1-fold above EC levels during arousal.

Discussion

Studies have previously investigated the protein levels and activity of various DNA or protein methyltransferase enzymes during hibernation of 13-lined ground squirrels (13LGS), but this is the first study to examine the arginine methyltransferase enzymes themselves and, as such, this study provides support for PRMT enzymes having a regulatory role in the metabolic control of mammalian hibernation. The significant, tissue-specific, changes in PRMT protein levels and methylarginine residues on histone proteins seen over the torpor-arousal cycle demonstrate that PRMT enzymes are modulated in their downstream regulatory abilities during hibernation, as posited by previous studies of methylation activity during hibernation (Morin and Storey 2009). Reversible methylation of cytosine residues on DNA, long thought to be a mechanism for widespread genomic changes, was significantly altered in the muscle of hibernating 13LGS and likely contributes to the dynamic nature of DNA methylation across the seasons (Alvarado et al. 2015), and to a somewhat maintained level of transcriptional activity in skeletal muscle during hibernation (Allan and Storey 2012; Malatesta et al. 2009). Similarly, changes in posttranslational methylation modifications to proteins provide rapid and reversible control over diverse metabolic functions in cells. A previous analysis of lysine methyltransferase protein levels and histone PTMs by our lab found that lysine methylation is potentially an important regulator of the mammalian hibernation phenotype in both liver and muscle (Watts and Storey 2019). Results from this study contribute to our understanding of how changes to protein methylation and the "histone code" control the adjustments in metabolic and genomic activity necessary for proper and efficient hibernation.

In adjusting to hibernation, tissues such as liver and WAT must switch to a prolonged fasting mode and regulate lipid metabolism, which is favored over carbohydrate as the fuel supporting torpor (Dark 2005). PTMs have long been known to be commonly employed in these tissues during hibernation in order to force shifts in protein/enzyme activity, stability, or localization (Grabek, Martin, et al. 2015; Storey 1987). As such, the role of methylation as a dynamic and stable protein modification is worth investigating. In both liver and WAT, type-II PRMT activity levels were consistent with control levels throughout torpor but were heightened strongly during IA in liver (Figure 4.1). Both PRMT5 and PRMT7 are type-II PRMT enzymes responsible for symmetric dimethylation of histones at H3R8 and H4R3. Protein levels of PRMT5 were maintained across hibernation in liver whereas PRMT7 levels showed a significant increase in both stages of torpor (Figure 4.2). H3R8me2s, which was also maintained at control levels within liver during hibernation (Figure 4.5), is the product of PRMT5 activity and possibly plays a role in the prevention of oxidation following increased lipid catabolism via necessary interactions with Oxidation Resistance gene 1 (OXR1A) that promotes H3R8 methylation and prevents the development of fatty liver disease (Yang et al. 2020). The fact that neither PRMT5 or PRMT7 protein levels corresponded to the decrease in H4R3me2s within liver, make it likely that a non-histone protein serves as a more favorable substrate during torpor (Wei et al. 2014). Further, methylation of H4R3me2s is be inhibited by the presence of other neighboring histone modifications that are increased in liver during torpor, such as H3K27 (Watts and Storey 2019; Liu et al. 2020). Interestingly, the increase in type-II methylation occurred at the same time that levels of H3R2me2a were also significantly increased in liver, pointing to the

involvement of PRMT enzymes in shifting metabolism back towards carbohydrate metabolism during interbout arousal through interactions with histone proteins. Dimethylation of H3R2 by PRMT6 is typically localized to regions of repressed transcriptional activity and interferes with the modification of nearby lysine residues available for methylation or acetylation (Guccione et al. 2007; Bouchard et al. 2018). Our previous analysis (Watts and Storey 2019) showed that monomethylation of H3K4, an inherently repressive modification that serves as the scaffold for activating marks (Cheng et al. 2014), is kept at control levels or significantly increased during torpor. Whereas determination of whether localization of these marks on the genome is targeted to enhancer or promoter regions is outside the scope of the present study, it is evident that the interaction of histone modifications serves integral roles in the metabolic shift that takes place during hibernation. Unfortunately, PRMT6 did not produce quantifiable protein abundance results in the tissues investigated, however significant increases in H3R2me2a are enough to conclude that the enzyme is active in liver.

A similar story may be inferred from the data in WAT, as PRMT activity and protein levels (with the exception of PRMT3 and PRMT7) were, unchanged from at EC control levels between LT and IA (Figures 4.1, 4.3). Indications of the downstream significance of these conserved protein activity and abundance levels are found in the heightened levels of both H3R8me2s and H4R3me2s during LT and IA (Figure 4.5). PRMT5 can di-methylate either of these modifications, and interestingly has been found to interact with mitochondrial biogenesis and peroxisomal lipid metabolism via regulation of PPARα and PGC-1α. In mice fed a high-fat diet, PRMT5 levels were increased in liver, whereas hepatic PRMT5 knock-down led to increased levels of

phosphatidylinositol 3-kinase/AKT signaling, but these changes were not seen in WAT or other tissues that are metabolically-relevant in hibernation (Huang et al. 2018). Given the difference in cellular and biochemical responses between a hibernating mammal and other mammals in terms of lipid-metabolism, specifically in regards to PPARα (El Kebbaj et al. 2009; Han et al. 2015), it is remarkable that PRMT5 levels were maintained in both liver and WAT. However, the question remains as to why H3R8me2s and H4R3me2s increase only within WAT, despite significant increases in PRMT5 within both WAT and liver. Levels of activated AKT in liver are known to decrease during torpor and increase during arousal despite maintained PRMT5 levels, and in contrast to mice fed a high-fat diet (McMullen and Hallenbeck 2010; Wei et al. 2012), raising the possibility that this correlation may be less relevant in hibernators. While the mechanism underlying the correlation between AKT activation and PRMT5 abundance is still unclear, their links to PPAR signaling and the active role that PPAR proteins play in hibernation, makes investigating the methylation status of proteins that regulate these pathways, such as mTOR, warranted. Localization of PRMT5 bound to the WAT genome has previously been carried out with adipocytes undergoing adipogenesis, where it was determined that the enzyme acts on histone proteins to regulate the binding of chromatin-remodeling enzymes and activate sequences downstream of a PRMT5 binding site (LeBlanc et al. 2012). Given that WAT undergoes extensive remodelling in the pre-hibernation season and during hibernation in at least one hibernating species (Chayama et al. 2018), it is very likely that maintained levels of PRMT5 in WAT are required for genomic reorganization, as is evident from the large increase in WAT H3R8me2s and H4R3me2s (Figure 5).

Meanwhile, muscle type-II PRMT activities indicate that much of the enzyme activity by PRMT5 and PRMT7 occurs during IA (Figure 4.1), but in contrast to liver tissue, is targeted towards H4R3me2s (Figure 4.5). Increased levels of H4R3me2s typically localize to areas of DNA damage (Karkhanis et al. 2012) and its significant increase during arousal in muscle likely represents the hibernators remarkable ability to avoid genomic instability during hibernation by repairing the DNA when the tissue is able to during arousal (Yancey 2018). Furthermore, while PRMT7 levels show maintained or significantly increased levels across torpor, levels of PRMT5 decreased in LT and remained somewhat reduced throughout arousal (Figure 4.4), explaining the decrease in levels of its product, H3R8me2s, during hibernation. However, in a similar vein to the role of type-II methylation in liver, the role of PRMT7 methylation may combat increased levels of oxidative metabolism occurring in muscle during arousal as the proteome shifts and the tissue becomes more active as shivering thermogenesis is triggered to rewarm the animal (Hindle et al. 2011). PRMT7 depletion within muscle affects levels of PGC-1α and inhibits its interaction with p38 MAPK, leading to reduced endurance and energy expenditure (Jeong et al. 2016). Thus, increased levels of PRMT7 during arousal could accommodate the protection of muscle tissue through activation of the endurance exercise pathway during hibernation (Xu et al. 2013). Finally, given that none of the PRMT proteins studied showed increased levels during LT, drastic reductions in symmetric dimethylation of both H3R8 and H4R3 are more likely to be a result of antagonism by other histone modifications than by asymmetric methylation of these residues by a different PRMT enzyme (Guccione et al. 2007; Liu et al. 2020).

While the analysis of type-II PRMT activity and products are certainly instructive to our understanding of the biochemical and molecular responses of cells to hibernation, analysis of PRMT protein levels during hibernation can also be useful for increasing our understanding of the purpose of arginine methylation during hibernation. This is true given that differential gene expression during hibernation represents the expenditure of resources at a time when there is increased pressure for conservation (Srere et al. 1992). Therefore, levels of PRMT1, 2, 3, and 4 were also investigated to assess arginine methylation writer enzymes for tissue-specific responses during torpor and arousal.

Both PRMT1 and PRMT4 (CARM1) are known to methylate arginine residues on histone tails and are typically located in areas surrounding activated gene transcription. Specifically PRMT1 acts on H4R3 and H2AR3 while PRMT4 acts upon H3R17 and R26, and either enzyme can dimethylate these residues but only in an asymmetric form (Daujat et al. 2002; Huang et al. 2005). Through their interactions with histone targets, both PRMT1 and PRMT4 are transcriptional co-activators responsible for recruiting chromatin modifiers following their recruitment to the genome by various transcription factors (Bedford and Richard 2005). Upon entry to torpor, protein levels of PRMT4 were maintained in liver whereas PRMT1 protein levels fell (Figure 4.2), however this trend was reversed in arousal as PRMT1 levels climbed to return to EC values. Protein levels of both enzymes are maintained in WAT, except during EN when they were strongly reduced by about 70% (Figure 4.3). PRMT1 is important for the maintenance and deposition of histone modifications. Through interactions with histone H4, its product (H4R3me2a), is influential over surrounding lysine methylation or acetylation modifications on neighbouring histone tails and allows PRMT1 to activate transcription

(Huang et al. 2005). The fact that PRMT1 is maintained between LT and IA in both liver and WAT is evidence of the active roles that these tissues have in regulating lipid metabolism during hibernation, and the role that epigenetics plays in supporting this task (Storey 2015). Notably, PRMT1 levels decreased during LT and EA in skeletal muscle (Figure 4.4), and perhaps influenced the histone code by releasing the inhibition over H3K9 and H3K27 methylation which previous work observed to rise significantly during LT in skeletal muscle (Watts and Storey 2019). The role of PRMT4 may be significant in the regulation of lipid metabolism due to interactions with both PPARγ and GAPDH. By acting as a co-activator of PPARγ (Yadav et al. 2008), PRMT4 is likely to be influential in maintaining energy balance during torpor in liver and arousal in WAT (Eddy et al. 2005). On the other hand, PRMT4 methylation of GAPDH is important in signaling cellular glucose levels and suppresses GAPDH activity during glucose starvation (X.-Y. Zhong et al. 2018; Bell et al. 2014). This interaction could be especially relevant in skeletal muscle during torpor, since predicted levels of PRMT4 rise following depletion of glucose in muscle tissue (Wu et al. 2013). By acting as gene co-activators or through non-histone interactions, both PRMT1 and PRMT4 are able to influence the regulation of both lipid and carbohydrate metabolism, and these interactions are likely to be important during hibernation.

Finally, the enzymes PRMT2 and PRMT3 are responsible for histone modifications typically found in areas surrounding activate gene transcription, namely asymmetric dimethylation of H3R8 and H4R3, respectively (Dong et al. 2018; Min et al. 2019), and are likely responsible for methylating non-histone proteins as well. Significant elevations in PRMT2 in both liver and skeletal muscle, and control levels in WAT, likely serve to

influence the regulation of damaged cell turnover during arousals by promoting apoptosis in affected cells (Ganesh et al. 2006) via activation of the NF-kB pathway during hibernation (Hadj-Moussa et al. 2020). PRMT3 levels were elevated only in liver, whereas both WAT and muscle showed significant decreases in PRMT3 protein levels LT and reduced levels as well in EA. When overexpressed, PRMT3 is associated with the increased expression of lipogenic proteins within liver and a high fat diet is known to increase PRMT3 levels in liver of animal models (Kim et al. 2015). It seems probable that the role of PRMT3 is associated with the increased lipid metabolism occurring in liver during torpor, given its significant increase in both EN and LT. The roles of both PRMT2 and PRMT3 are certainly also involved in regulating the metabolic needs of the animal during hibernation, as their suitability for this role is clear given their known roles and protein interactions.

In summary, arginine methylation plays an important role in controlling the vast reorganization of metabolic regulation which is required in hibernation. Moreover, these mechanisms are likely necessary for proper and efficient hibernation due to their influence over cell and organ-specific responses. Arginine methylation certainly contributes to altering and adapting the cellular environment to support hibernation through multiple mechanisms as described.

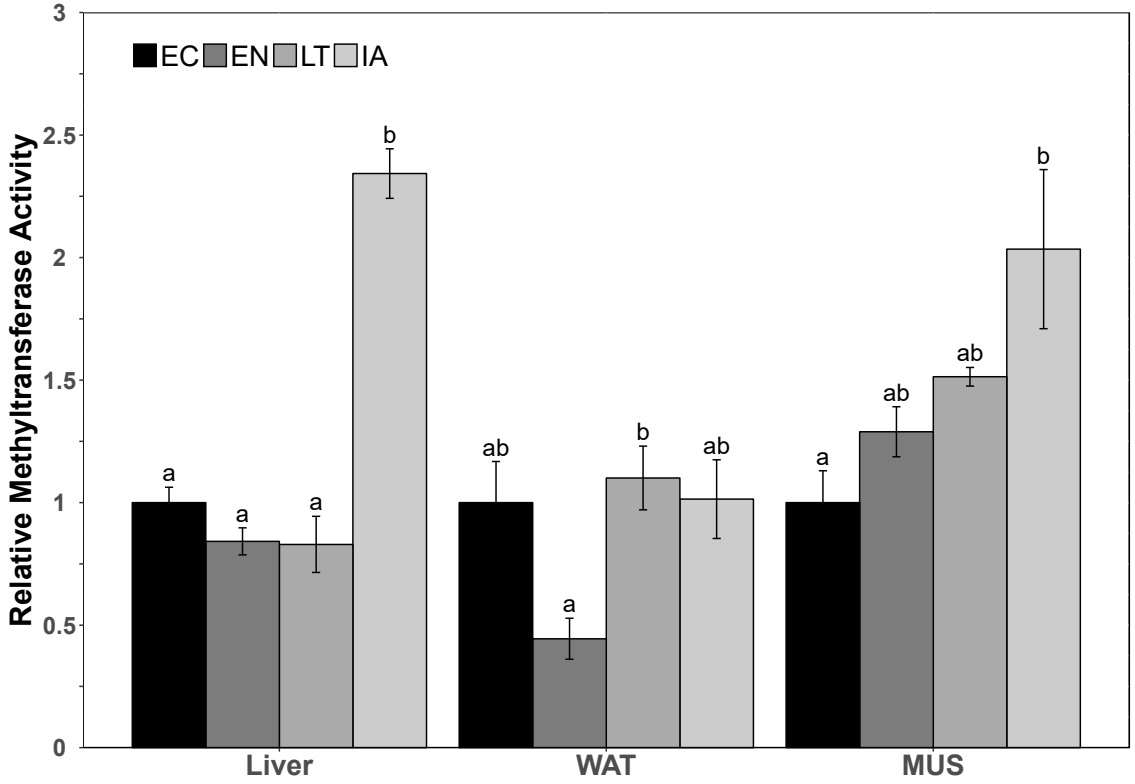


Figure 4.1: Relative Type II-specific PRMT methyltransferase activity in total soluble protein extracts of liver, white adipose tissue, and skeletal muscle at selected time points of the torpor-arousal cycle. Sampling points are euthermic in the cold room (EC), entrance into torpor (EN), late torpor (LT), and interbout arousal (IA). Histograms show the mean amount of methyllysine produced per hour per mg protein ($ng \cdot min^{-1} \cdot mg^{-1}$) ± SEM, $n = 4$. Different letters denote values that are significantly different from each other ($p < 0.05$).

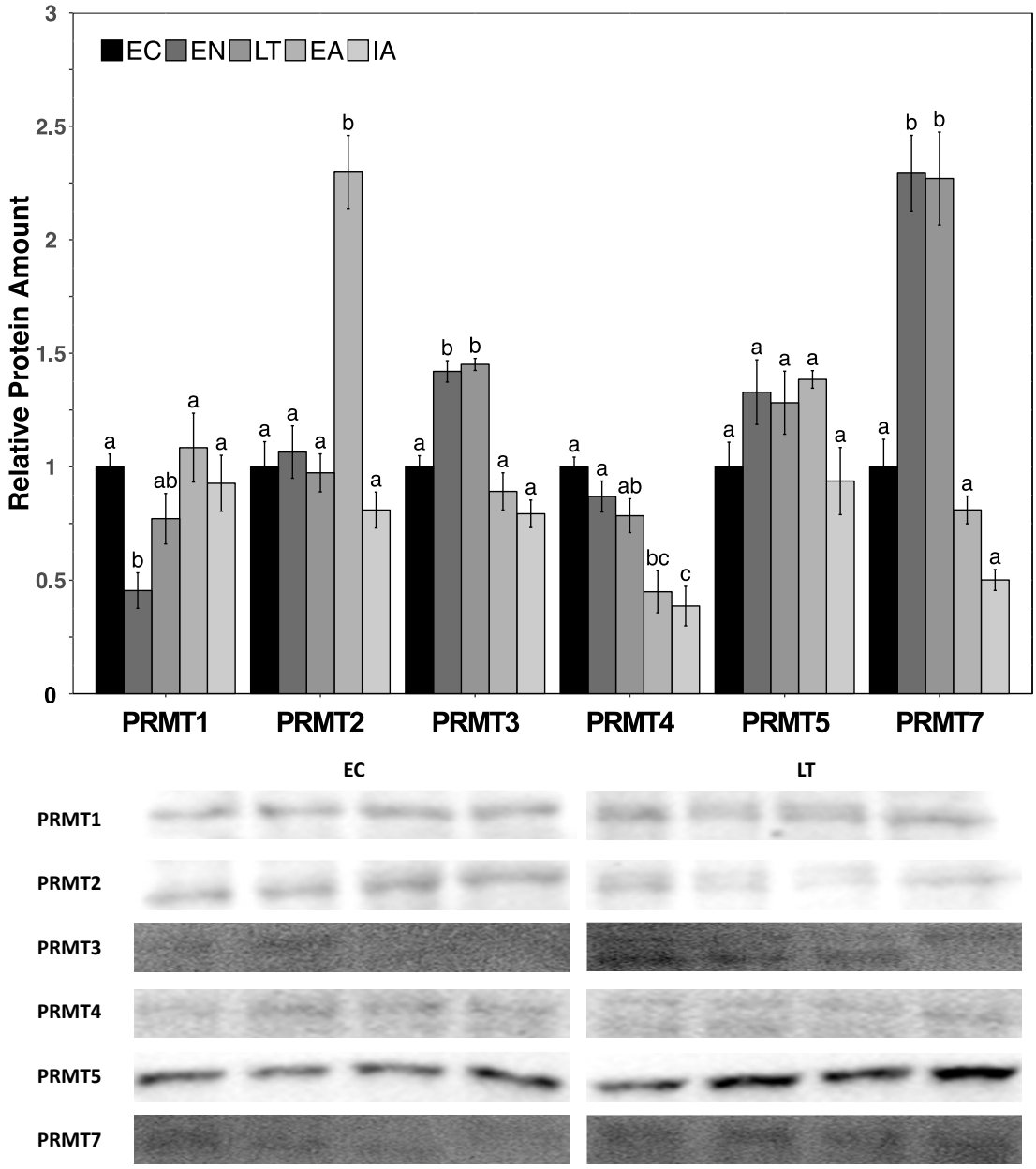


Figure 4.2: Relative protein abundance of PRMT enzymes in liver over the torpor-arousal cycle along with representative immunoblot protein bands for EC and LT time points. Sampling points are euthermic in the cold room (EC), entrance into torpor (EN), late torpor (LT), early arousal (EA), and interbout arousal (IA). Representative immunoblots each show n =4 lanes for EC on the left and LT on the right, as labeled above. Histograms show mean standardized band densities (± SEM, n = 4 independent trials on tissue samples from different animals). Different letters denote values that are significantly different from each other as assessed by ANOVA ($p < 0.05$).

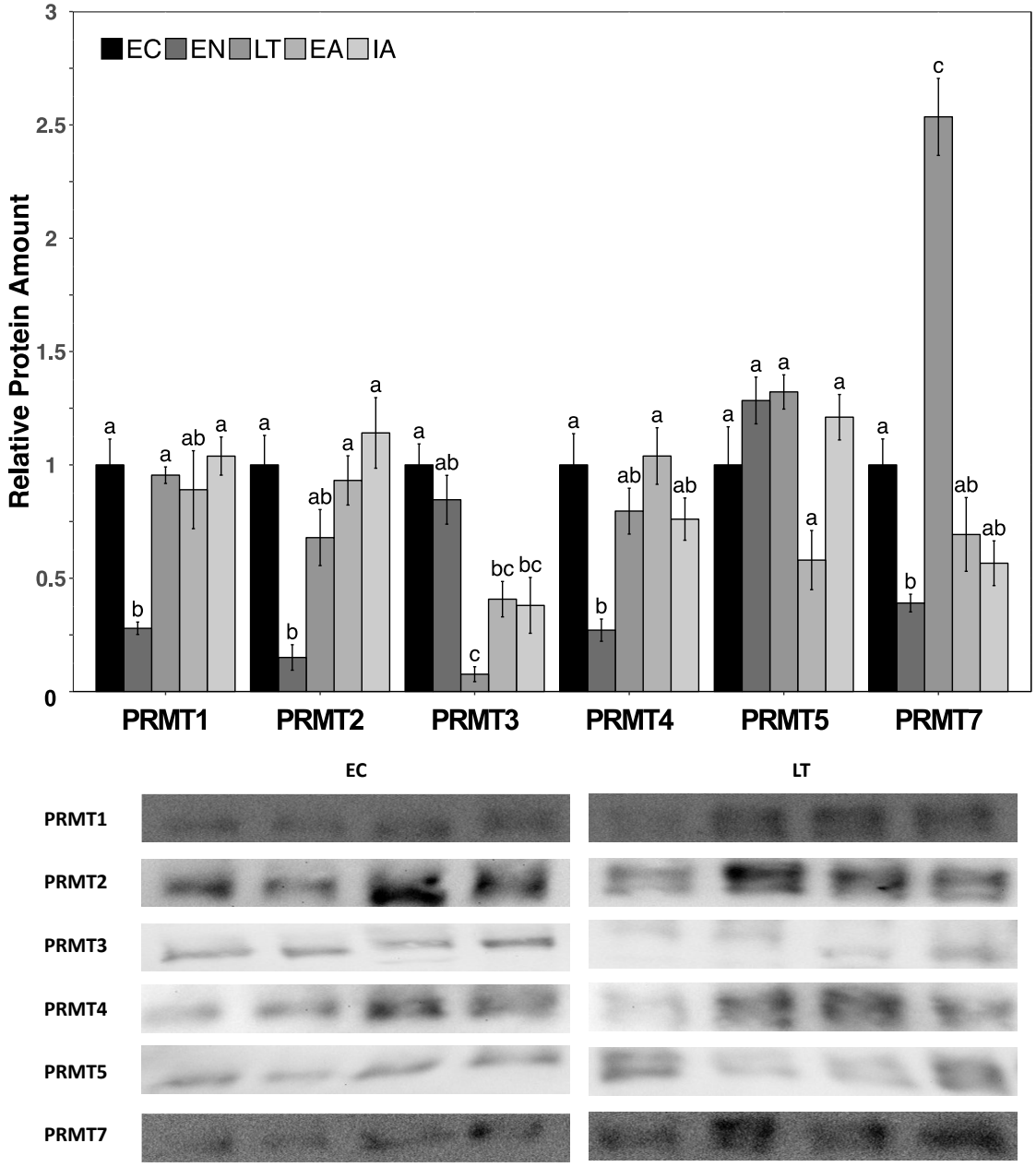


Figure 4.3: Relative protein abundance of PRMT enzymes in white adipose tissue over the torpor-arousal cycle along with representative immunoblot protein bands for EC and LT time points. All other information is as in figure 4.2.

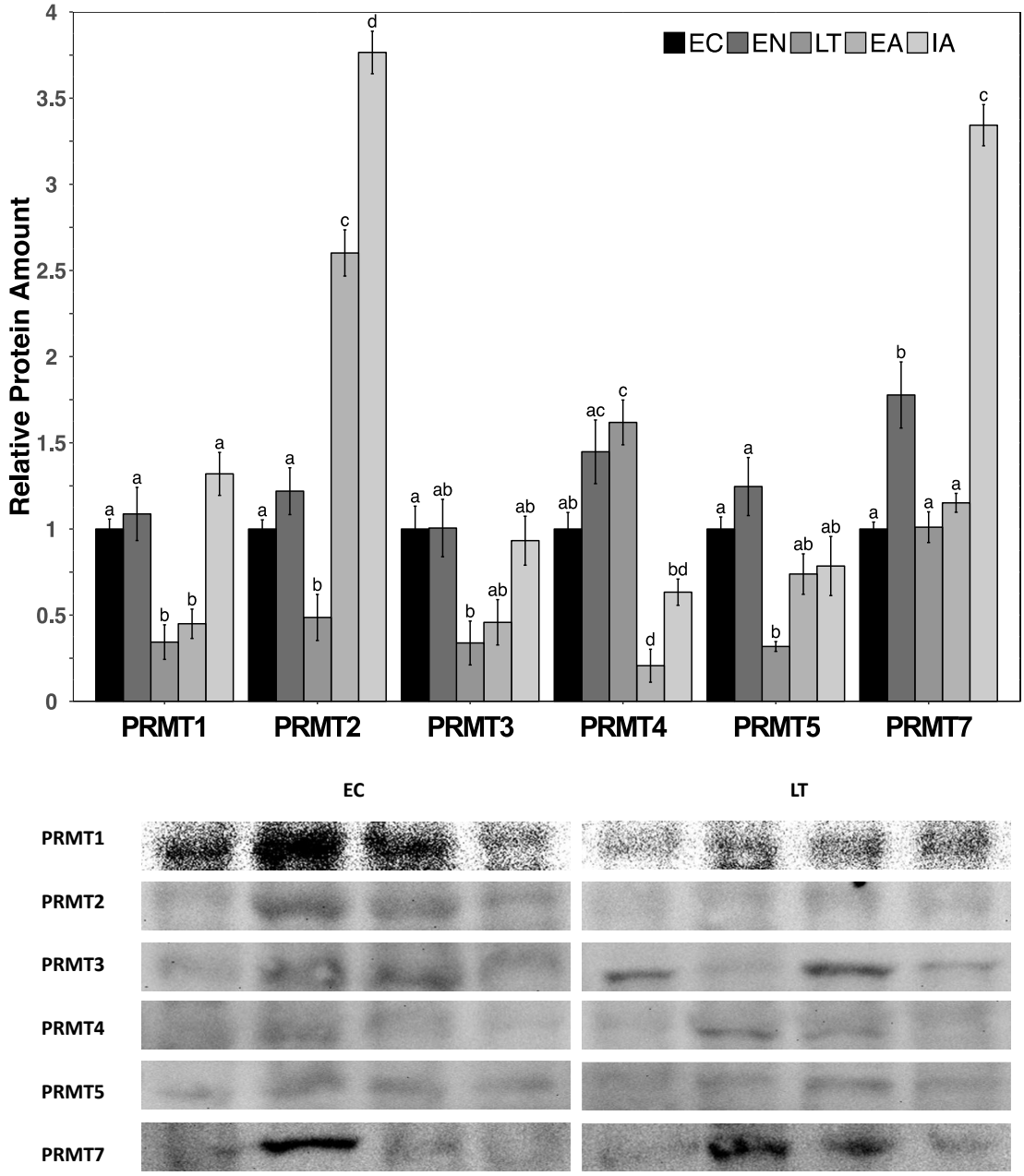


Figure 4.4: Relative protein abundance of PRMT enzymes in skeletal muscle over the torpor-arousal cycle along with representative immunoblot protein bands for EC and LT time points. All other information is as in figure 4.2.

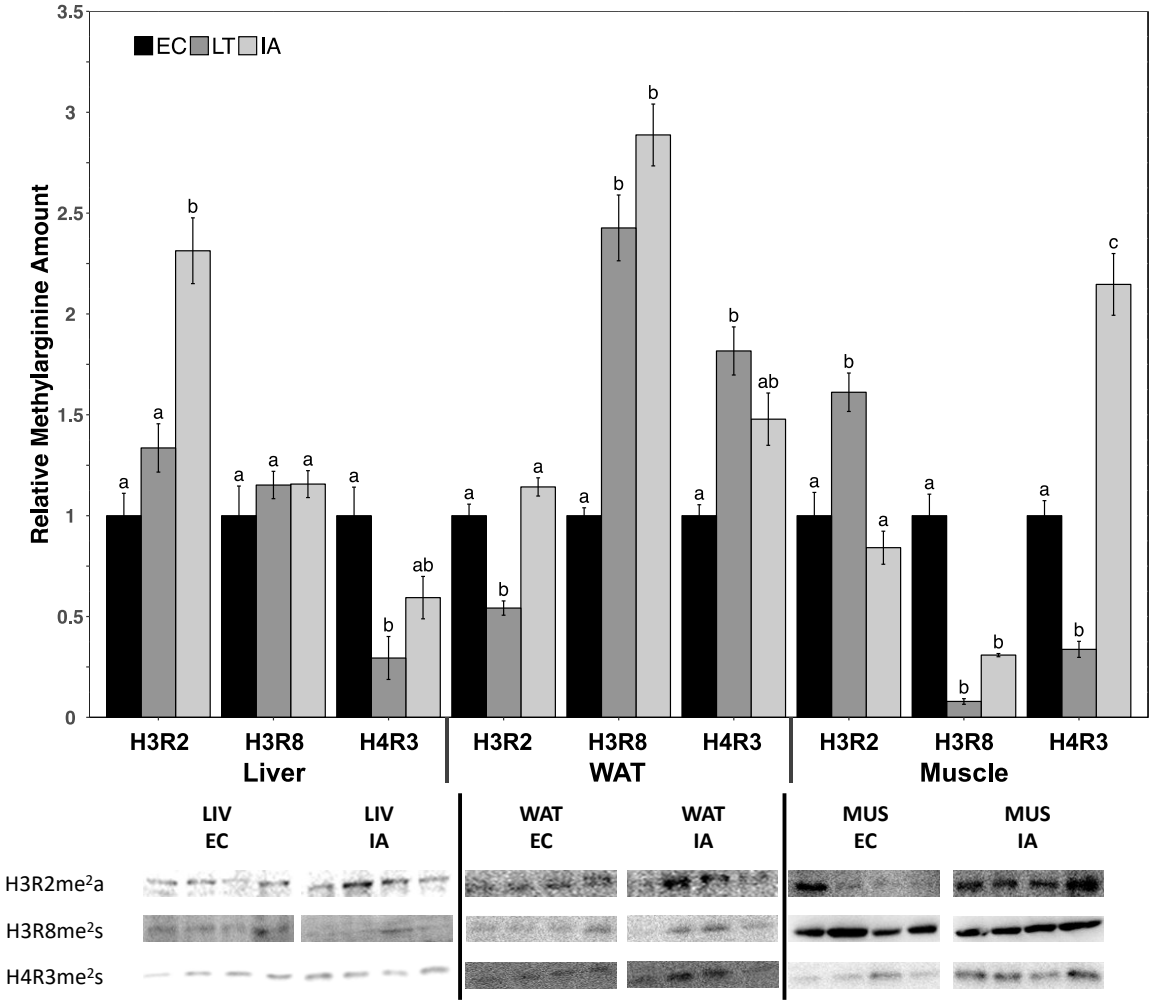


Figure 4.5: Relative amounts of histone H3 showing asymmetric dimethylated R2 (H3R2me^{2}a) and symmetric dimethylated R8 (H3R8me^{2}s), as well as histone H4 showing symmetric dimethylated R3 (H4R3me^{2}s) in liver, WAT, and skeletal muscle over the torpor-arousal cycle. Representative immunoblots each show *n* =4 lanes for EC on the left and IA on the right, as labeled above. All other information is as in figure 4.1.

Chapter 5

General Discussion

Two themes of mammalian hibernation biology are explored within this thesis: i) the dependence of hibernators on rhythmic activation of gene transcription and activation, and ii) the use and repurposing of epigenetic mechanisms for adapting and overcoming massive changes in cellular activity by adding an additional layer of control over protein expression and activation. Obviously, these two themes are interlinked, and so both aid our understanding of the complexity involved in successfully employing hibernation to overcome winter-time stresses.

In an effort to save as much energy as possible during winter and survive prolonged resource scarcity coupled with increased energetic demands, the 13-lined ground squirrel (13LGS, *Ictidomys tridecemlineatus*) undergoes huge phenotypic plasticity in an effort to conserve stored fuels for necessary purposes. In turn, the vast majority of metabolic functions are turned down or turned off, whereas regulatory demands which are intertwined with survival are prioritized and upregulated. As an example, thermogenic and fatty acid catabolism pathways, crucial for the hibernator's survival, were seen to be prioritized in all cases studied within this thesis. By modulating the requirements for gene activation by transcription factors, chromatin remodeling enzymes, and post-transcriptional modification enzymes, cells can increase the transcriptional activity of required genes groups.

Preparation for an almost complete dependence on lipid fuels during the winter is first achieved in late summer to early autumn, when ground squirrels double their body weight on average and increase their fat mass by up to 4-fold (Sheriff et al. 2013). This leads to large increases in white adipose tissue (WAT) stores and alters the number, shape, size and function of cells as depots increase in triglyceride levels for use during

hibernation (Chayama et al. 2018). During torpor, WAT and liver support vital functions by mobilizing fat stores as Tb falls to ambient temperatures during torpor throughout the hibernation season. Within liver, levels of long-chain fatty-acid transport proteins such as apolipoprotein A1 as well as lipid catabolism and mobilization pathways are selectively activated, in addition to proteins involved in supporting and maintaining protein stability and folding (Epperson et al. 2010). For these reasons, dietary lipids impact the efficacy and success of hibernation as a survival strategy – animals that consume larger amounts of polyunsaturated fatty-acids (PUFAs), and especially n-6 class PUFAs hibernate more successfully due to these lipids intrinsic ability to maintain membrane fluidity and promote energy savings (Munro and Thomas 2004; Jastroch et al. 2016).

As opposed to the active roles of liver and WAT in lipid metabolism during torpor, transcriptional suppression and global decreases in metabolic rate define the environment within skeletal muscle tissue. Overall energy expenditure within muscle tissue during torpor is modulated through reductions in non-essential cellular processes such as new protein synthesis and cell division, whereas cytoprotective and chaperone proteins remain activated to accommodate shivering thermogenesis that is an important component of rewarming during arousals as well as for emergence from hibernation in the spring (Hindle et al. 2011). Remarkably, hibernators are able to fend off and negate the effects of prolonged muscle inactivity, showing preserved muscle morphology and mass upon re-emergence and maintained muscle strength (Cotton 2016), mainly due to increased stability and preservation of existing cellular proteins while in torpor.

Although this is not the first study to assess and uncover changes in the mechanisms controlling metabolic and cytoprotective pathways in hibernator cells, only a

select few have touched on the roles of peripheral circadian oscillators and their modifications when an organism undergoes suppressed metabolism events (i.e., translational and transcriptional arrest, regulation of post-transcriptional and post-translational modifications, etc). The aim of my thesis was to investigate regulatory patterns of signaling pathways that have previously been shown to contribute to the rhythmic output of a cell, based on previous studies in other mammalian models. The thesis aimed to address two goals: 1. Conservation of regulatory pathways in ground squirrels, and 2. Differential regulation of these pathways in energy conservation. To address these goals, I undertook a molecular approach, utilizing a variety of tools to characterize genomic and proteomic regulation of peripheral circadian clock pathways, as well as assessing structure and function of key enzyme regulators of the molecular clock.

Metabolism Interacts with the Molecular Circadian Clock

In Chapter 2 the regulation of key components of the molecular circadian clock were assessed during torpor, focusing on the BMAL1/CLOCK complex due to its direct link in regulating protein synthesis. Emphasis was placed on comparing the regulatory patterns observed in each of liver, WAT and muscle tissues due to their differing activities during hibernation. The levels of nearly all clock components were present at the transcript level in all three tissues, and levels of only one transcript, PER1 in muscle, was observed to decrease. Similarly, CLOCK DNA-binding activity was maintained in the investigated tissues showing significant increases in WAT during late torpor (LT). The results in all tissues suggested that circadian mechanisms remain intact in these peripheral tissues during hibernation. However, the circadian transcription/translation feedback loop likely swings towards members of the activating arm (i.e.,

CLOCK/BMAL1 are more active) within liver and WAT, whereas the opposite is true in muscle tissue, where PER2 and CRY2 inhibition seemed to dominate. This pattern of regulation suggests a state of maintenance of protein transcription in liver and WAT, but transcriptional decreases in skeletal muscle during torpor.

It is perhaps not surprising that major integrators of cellular energy information affect transcriptional activity within both the activating and repressive arms of the circadian clock transcription/translation feedback loop. Integration of metabolic sensor elements into the regulatory DNA regions of the *Per* genes, such as cAMP Response Elements (CREs), allows signals of low cellular fuel availability to activate the repressive arm of the core transcription/translation feedback loop so that rhythmic pathway activation becomes muted after entrainment. In an elegant experiment performed by Vollmers et al. (2009) it was shown that phosphorylation of CRE Binding protein (CREB) coincides and is in phase with periods of fasting in animals lacking circadian rhythms. CREB activation of *Per1* and *Per2* transcription, attenuates downstream CCG activation when the animal is under adverse metabolic conditions by repressing circadian activation in numerous pathways. Similarly, increased levels of 5'AMP-activated protein kinase (AMPK) activity are correlated with nutrient/energy levels within most tissues, and alter CRY protein rhythmicity and localization, as well as CCG activation within peripheral oscillators (Lamia et al. 2009; Vieira et al. 2008). Clearly, signaling of rhythmic behavioural and environmental changes, including feeding, has direct effects on gene expression controlled by the circadian clock and provides additional regulatory layers on important cellular events.

The Peroxisome Proliferator-Activated Receptor (PPAR) family of transcription factors are another class of CCGs that show daily changes in expression levels in a number of peripheral tissues (Lemberger et al. 1996; Yang et al. 2006; Gachon et al. 2011). There are three PPAR isoforms in mammals [PPARα/δ/γ] which share largely similar structural and functional homology, but differ in their tissue expressions (Desvergne and Wahli 1999). PPARs bind several endogenous ligands including lipids and are recruited to PPAR response elements, PPREs, after binding retinoid X receptors (RXRs) to control and activate downstream gene transcription of targets that most prominently serve to regulate lipid metabolism and adipogenesis (Wu et al. 2009). The integration of the PPAR family into the circadian clock is significant and reciprocal, meaning that many genes activated by PPAR family members show circadian variation, but also that modulation of PPAR transcriptional activity feeds back to alter the transcriptional activity of core circadian clock genes (Chen and Yang 2014; Canaple et al. 2006). For example, expression of PPRE-regulated genes in the intestine and in fibroblasts, were modulated in a manner that correlated to the expression of BMAL1 (Inoue et al. 2005; Oishi et al. 2005) through interactions with an E-box element within an intron of the *PPARα* gene. On the other hand, mice fed a PPARα agonist showed advanced circadian locomotor activity and core molecular clock gene expression in peripheral tissues due to action on a PPRE within the *BMAL1* promoter region, but this does not affect gene expression within the SCN (Canaple et al. 2006; Shirai et al. 2007). Notably, levels of PPARγ, which have been assessed previously in 13LGS, show increased levels in liver and WAT, and correlate with protein levels of BMAL1 during torpor (Eddy et al. 2005). While similar findings have already shown a relationship

between PPARγ and the control of rhythmic protein levels in peripheral oscillators or rhythmic physiological parameters (Yang et al. 2012; Wang et al. 2008), the same connectedness may not be true for PPARδ. Whereas PPARδ does not show a direct link to transcriptional activation of the circadian clock, it is the main target for a liver-specific microRNA that is regulated by the oscillating Rev-ErbAα repressor. By adding a layer of circadian regulation over lipid metabolism in the liver, PPARδ can show variations in diurnal expression and affect fatty acid metabolism in the liver (Liu et al. 2013; Gatfield et al. 2009). Finally, any one of the PPAR family transcription factors can be a binding partner for the CLOCK transcription factor and in this way inhibit CLOCK's interaction with BMAL1 (McNamara et al. 2001). Since PPARs play an intricate role as regulators of circadian lipid metabolism, it is likely that changes in rhythmic metabolic homeostasis, such as those that occur during hibernation (McGinnis and Young 2016; Wu et al. 2013), will alter both PPAR and circadian clock protein levels.

Modifications of Circadian Clock Molecules

Both torpor and circadian rhythmicity are dynamic processes that must be rapidly controlled in a coordinated manner. PTMs serve as an indispensable mechanism for quickly and effectively altering cellular activities via changes to the status of covalent modifications on pathway regulatory proteins/enzymes. Given the already noted importance of PTMs in the regulation of torpor-arousal cycles, their massive effects on biochemical pathway activity and their ubiquity throughout the cell make their investigation during hibernation warranted. PTMs modify numerous cellular regulators of transcription/translation, protein-protein or protein-transcript interactions and protein or transcript stability or localization (Lee and Stallcup 2009; Di Lorenzo and Bedford 2011;

Ma et al. 2017; Engel and Chen 2018) and play a major role in regulating protein turnover and activity in almost all molecular pathways, including the circadian clock. The repressive complex of the core clock is modified by several mechanisms, such as the balance between kinase (CK1δ or CK1ε) and phosphatase (phosphatase 1, PP1) activities (Lee et al. 2011) as well as ubiquitination by the β-TrCP E3 ubiquitin ligase complex (M. Zhou et al. 2015). An inactivating mutation in the gene encoding CK1δ lengthens the circadian cycle by increasing the stability of PER1/2 as a result of attenuating phosphorylation-dependent ubiquitination (Etchegaray et al. 2009; Meng et al. 2010), in contrast to CK1ε which regulates the nuclear entry of the repressive complex (Vielhaber et al. 2000; Eide and Virshup 2001). The CRY1/2 or BMAL1 proteins may also be phosphorylated by casein kinase 1 enzymes, following kinase association with PER1/2/3 (Akashi et al. 2002; Eide et al. 2002). Phosphorylated PER2 proteins bound to CRY are actually stabilized by inhibiting PER ubiquitination (Lee et al. 2001). While the inhibitory phase of the transcriptional loop is active, phosphorylated CRY/PER complexes within the nucleus bind the BMAL1/CLOCK complex (which remains bound to the DNA) and serve as a scaffold for hyperphosphorylation of the complex members by CK1δ or CK1ε, forming the transcriptionally repressive ‘timesome’ complex (Reppert and Weaver 2002). In this way, rhythmic binding of the repressors for the DNA-anchored transcriptional activator pushes the circadian clock into the night phase of the cycle.

Acetylation of BMAL1 is a second major PTM mechanism that regulates transcriptional activity of the core clock complex. A second role for the CLOCK transcription factor is as an activator of its own binding partner via inherent acetyltransferase activity. Hirayama et al. (2007) showed that a conserved lysine residue,

K537 in mice, is acetylated in the liver and that this modification occurs in preparation for, and is necessary for, binding of the repressive complex. By further showing that mutant BMAL1 lacking the conserved lysine residue is unable to drive rhythmic transcription of *PER2*, the authors showed that this modification is essential for effective circadian transcription/translation feedback.

While numerous PTMs modify the circadian clock, epigenetic mechanisms, so-called for their ability to transfer through the germline, also play a major role in the regulation of cellular timing. For instance, the acetyltransferase activity of CLOCK may acetylate histone H3 on lys9 and lys14 residues, and the deacetylase sirtuin 1 regulates circadian functioning by associating with the core clock complex (Doi et al. 2006; Nakahata et al. 2008; Asher et al. 2008). The coordinated activities of core circadian proteins and their abilities to recruit and bind chromatin remodelling enzymes has been noted to play an intricate role in the regulation of rhythmic gene transcription within multiple pathways and cell types (Ripperger and Schibler 2006; Yeung et al. 2018). Undoubtedly, the clock's ability to recognize and place epigenetic modifications is crucial to its functioning and depends on the actions of histone-modification mechanisms.

Maintenance of RNA methylation dynamics during torpor

Regulatory crosstalk between the deposition of m6A methylation with the circadian clock has so far been established in only a limited number of systems. As mentioned in Chapter 1, some circadian-controlled gene products contain m6A sites, and moreover, inhibiting m6A methylation prolongs the circadian clock period through uncoupled rhythms of *BMAL1* and *PER2* production (Fustin et al. 2013). At least one of the mechanisms responsible for modulating the circadian clock period depends on m6A

methylation of circadian mRNA products. In addition to *PER2*, *CK1δ* mRNA, that codes for one of the critical kinases responsible for translocation of the PER/CRY inhibitory complex, also contains sites for m6A modification which inhibit its translation. Modulation of the abundance of m6A deposited within the coding regions of *CK1δ* can modulate circadian clock period by regulating the amount of CK1δ and ultimately phosphorylation of PER2 proteins (Fustin et al. 2018). The deposition of m6A also affects proteins that regulate the circadian clock, such as PPARα (discussed above) which controls *Bmal1* gene activity via PPAR-response elements in the upstream promoter region. Following modification of PPARα by METTL3, and binding by YTHDF2, the transcription factor is recruited to the genome where it can affect and alter the production of numerous gene products (X. Zhong et al. 2018). Importantly, this last result reveals not only the interconnectedness of the circadian and epigenetic mechanisms, but also the link between both pathways and the regulation of metabolism following changes in the cellular environment.

Chapter 3 examined the global levels of m6A within the transcriptome, where it was seen that m6A shows differential abundance during torpor – increasing in WAT and decreasing in skeletal muscle. Whereas liver showed no changes in RNA methylation, m6A levels did show decreases when assessing levels within mRNA selectively. When comparing levels of m6A writer enzymes, all tissues appeared to show maintenance of m6A deposition during torpor although to different degrees in each tissue.

Writer, eraser and reader enzymes show rhythmicity in their gene expression, and similarly, in the deposition of m6A (X. Zhong et al. 2018). Within 13LGS, levels of one of the two m6A eraser enzymes were decreased in both liver and WAT, whereas both

were maintained in skeletal muscle. These results, as well as observations of reduced m6A removal activity during LT in liver and EN in WAT, appear to mirror the maintenance of transcriptional activity within these tissues in the context of molecular circadian clock activity. On the other hand, skeletal muscle levels of eraser proteins and activity indicate a paucity that is apparent in circadian transcriptional activity during torpor. Through crosstalk with the circadian pathway and metabolism, mRNA methylation is able to impact the homeostatic balance of multiple metabolic cellular functions. Precisely how m6A methylation coordinates circadian clock functioning and cellular metabolism is currently still debated.

Overall, chapters 2 and 3 indicate the continued production or preservation of selected groups of gene transcripts during torpor-arousal cycles within three central tissues of the 13LGS. The regulatory control over genomic activity inherent in post-transcriptional modifications likely functions in co-operation with the regulatory abilities of gene activators through recruitment to the genome, or localization of transcripts towards processing or degradation sites within the cell. Future studies of hibernation or circadian rhythmicity would be wise to include investigations of m6A deposition and reader enzyme complexes when studying apparent transcriptional activity. Furthermore, the localization of m6A within the coding regions of selected transcripts during torpor, as investigated by me-RIP-seq (Meyer 2019; Saletore et al. 2012), would allow a greater understanding of the prioritization of gene products within the phenotypically plastic cell and during the observed physiological changes inherent in hibernation.

Modulation of protein arginine methylation during torpor

Contemporary biological research, especially within the context of organisms adapting to environmental changes, has shown a sustained interest in seeking to understand epigenetic mechanisms as well as the underlying "histone code" (Keating and El-Osta 2015; Storey 2015). Epigenetic mechanisms regulate levels of genomic activity on a global scale via chromatin remodeling but can also act selectively on specific genes or proteins via recruitment or inhibition of transcription factor-DNA binding. In particular, histone methylation that is thought to be a relatively neutral modification when compared to the much larger or charged PTMs (i.e., acetylation, phosphorylation), is a key regulator of gene expression through co-operativity with methyl binding domains and effects on the deposition or removal of modifications on neighbouring amino acids (Miao and Natarajan 2005). Chapter 4 surveyed the landscape of arginine methylation on a global level during hibernation by employing assays of type-II specific arginine methyltransferase activity in three tissues which showed maintenance during torpor, and significant elevations during IA in both liver and muscle tissue, whereas WAT showed reduced methylation activity during torpor. These changes were substantiated by levels of PRMT enzymes that in many cases mirrored the levels of type-II specific methyltransferase activity, as measured by Western blotting. While measurements of methylation activity within liver and muscle appeared congruent, the downstream effects on histone methylation differed between the two tissues during hibernation, with liver showing increases in symmetric dimethylation of H3R8, whereas muscle tissue showed the same increased modification but targeted to H4R3 residues instead. The differential regulation of histone methylation during hibernation can have multitude effects as

discussed in Chapter 4 and previously (Tessier, Luu, et al. 2017; Watts and Storey 2019) and, in this case, seem to indicate differences in the levels of activity or cellular damage that occur in these tissues during torpor. Additionally, relative levels of PRMT enzymes and downstream histone modifications in WAT indicate the importance of maintaining lipid catabolism via apparent upregulation of PPAR-related pathways.

Similar to arginine methylation of histone proteins, non-histone protein methylation may have wide-reaching effects on cellular mechanisms. For instance, methylation of the chromatin binding protein high-mobility group protein A1 (HMGA1a) by PRMT1, -3 or -6 is associated with blocking its association with DNA and activating apoptosis (Sgarra et al. 2006; Zou et al. 2007). With regard to transcriptional control, PRMT4 also plays an interesting role by both activating transcription (as mentioned earlier) via histone methylation while also blocking transcription activation by CREB. Activation of genes by CREB, relies on a coactivator, CREB binding partner (CBP) and CBP may be methylated on a conserved arginine residue by PRMT4. This inhibits its interaction with CREB and thereby leads to repression of CREB-mediated gene transcription (Xu et al. 2001). Hence, by affecting protein interacting partners and enzymatic activity, arginine methylation clearly has numerous effects throughout the cell.

Both arginine methylation and the circadian clock are regulated by metabolic signaling and cellular energy levels, a pattern which is evidenced in both Chapters 2 and 4. In particular, PRMT3 and PRMT5 that have integral links with lipid metabolism (Huang et al. 2018; Hsu et al. 2019) showed similar patterns of protein abundance with BMAL1 protein across hibernation. As previously mentioned, PRMT5 can methylate the CRY1 protein and is rhythmically recruited to the *Per1* gene promoter to increase levels

of H4R3 dimethylation at times when the inhibitory arm of the circadian transcription/translation feedback loop is dominant (Na et al. 2012). Interestingly, H4R3me2 was significantly increased in muscle tissue, where results from Chapter 2 indicated activation of the inhibitory circadian proteins, whereas the opposite occurred in liver where significant decreases in H4R3me2 were seen. For these reasons, future studies of arginine methylation research in the context of hibernation must include measurements of methylarginine localization within the genome, that could potentially be done by ChIP-seq using antibodies targeted towards methylarginine histone-residues (Kirmizis et al. 2007), PRMT enzymes (Ma et al. 2001), or methyl-binding proteins containing Tudor domain (Sprangers et al. 2003). Furthermore, measurements of arginine methylation readers and erasers, responsible for carrying out the downstream effects of arginine methylation and removing methylation modifications, respectively, would be of interest in understanding the dynamics of epigenetic or cytoplasmic mechanisms during environmental adaptation. While the full landscape of methylation-specific binding domains and the enzymes responsible for arginine demethylation are still an area of current research (Blanc and Richard 2017; Biggar and Li 2015), these proteins clearly would play an integral role in the mechanisms discussed in this thesis.

Outlook

This thesis aimed to characterize the regulatory processes of circadian related metabolic or epigenetic functions in response to hibernation in ground squirrels. The components of the circadian clock pathway were found to be differentially regulated in each of liver, WAT and skeletal muscle during torpor. Similarly, m6A methylation by METTL enzymes, as well as mechanisms controlling protein arginine methylation

dynamics displayed tissue specific modes of regulation, functioning to maintain cellular activity within liver and WAT, while increasing cellular paucity within skeletal muscle tissue. The dependence of complex cellular pathways on energy levels as shown in other hibernating models or in cells exposed to changes in their environment, leads one to expect that energy status and metabolic functions are important to hibernators and deserve investigation. This relationship is a fundamental principle of how animals adapt to various types of environments via metabolic reprogramming, not only under winter stress conditions but also in various metabolic diseases or dysregulations.

While the work within this thesis is comprehensive of the details contained, these experiments may be supplemented in future through use of larger sample sizes, more complex cell sorting mechanisms or lab-models of torpor regulation. This thesis makes clear that the 13LGS is a natural model of hibernation which clearly makes use of the mechanisms detailed in this work, opening the door to further experiments that manipulate these pathways to uncover and understand the implications and consequences of the manipulation. For example, in this work tissue organization and complexity is reduced in various biochemical assays used whereas cell- and gene-specific changes are clearly at play.

This thesis provides evidence of transcriptional activation occurring despite the metabolic rate of hibernating animals being reduced by >90% and vast numbers of pathways being inhibited during torpor. These results reinforce the idea that a hypometabolic state should be considered an actively regulated process wherein coordination of metabolic and molecular controls contribute to a state of regulated dormancy. Just as remarkable, however, is the animal's ability to emerge from

hibernation and arouse back to euthermic Tb and metabolic rate – providing evidence of the reversibility of circadian and PTM systems in regulating hibernation, as well as numerous other systems likely to be required during hibernation. Whereas further evidence remains to be gathered about the biological cues that induce entry into hibernation, it is undoubtable that circadian regulation over gene activity, as well as its related controls, are involved. The involvement of circadian rhythmicity of cellular activity in inducing hibernation is certainly only one question that still needs to be answered in hibernation research. The resulting research will enlighten future scientists seeking to appreciate this incredible natural model of phenotypic plasticity.

Appendix A: Circadian controlled gene primer sequences

The primer sequences (purchased from Integrated DNA Technologies, Inc.) were as follows:

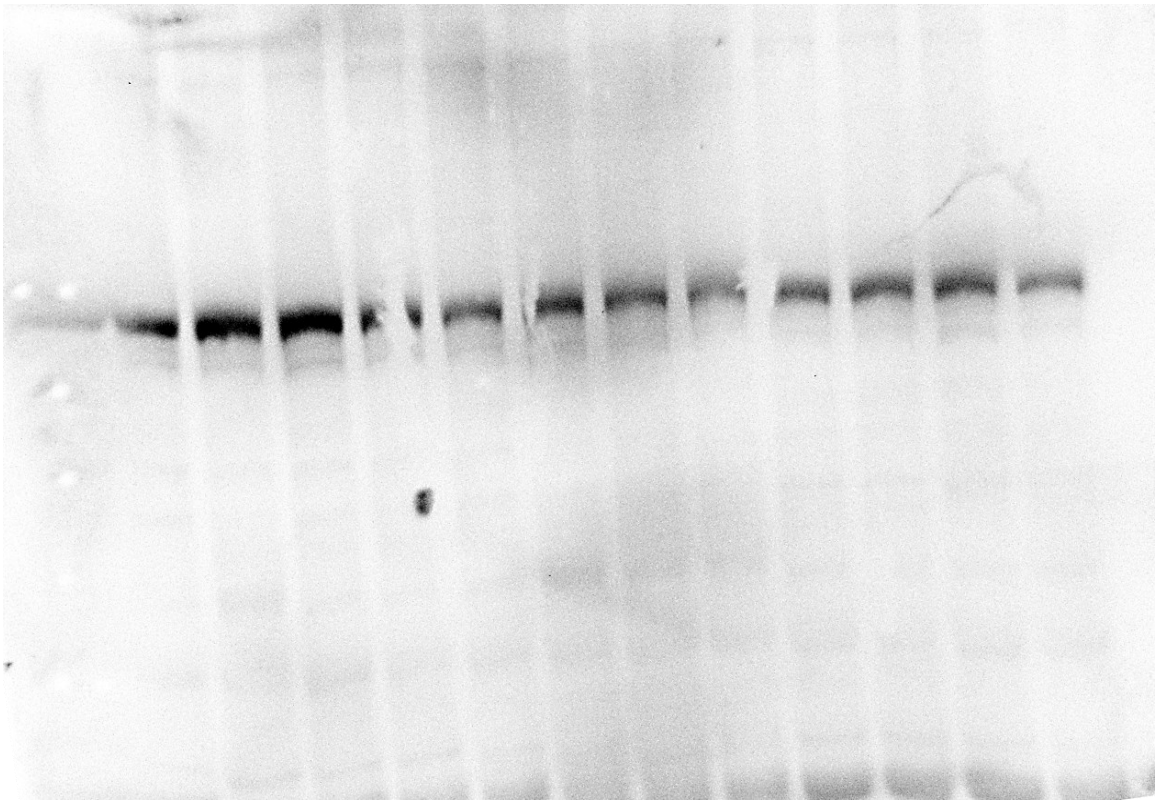
Itr BMAL	forward 1	5' -	TGACTACCAAGAAAGCATGGACA	- 3'
Itr BMAL	reverse 1	5' -	ACTGTGAGCTTCCCTTGCAT	- 3'
Itr CLOK	forward 1	5' -	GGGTTGGTGGAAGAAGATGAC	- 3'
Itr CLOK	reverse 1	5' -	AGCATTACCAGGAAGCATGGAT	- 3'
Itr CRY1	forward 1	5' -	CATGGGGTATGCACCTGGAG	- 3'
Itr CRY1	reverse 1	5' -	AGGTGAGTTTGCTGACTGTCT	- 3'
Itr CRY2	forward 2	5' -	AGGTCTCATGGGGTATGCAC	- 3'
Itr CRY2	reverse 2	5' -	CCATGGAGCTTCTTCTTCCACT	- 3'
Itr PER1	forward 2	5' -	AGCCAGATTGGTGGAGGTAAC	- 3'
Itr PER1	reverse 2	5' -	CTGACTGCTGCCAGAACGC	- 3'
Itr PER3	forward 1	5' -	CCTAAGCCACCTGTGTCCAG	- 3'
Itr PER3	reverse 2	5' -	GATACGGGGGTTGTGCAAGA	- 3'
Itr RORA	forward 1	5' -	ACCTACATCGATGGGCACAC	- 3'
Itr RORA	reverse 1	5' -	AGAGGGCTGTATGTCCAGGT	- 3'
Itr NRD1	forward 1	5' -	TCTCGAGATGCTGTGCGTTT	- 3'
Itr NRD1	reverse 1	5' -	CTGCTCAACTGGTTGTTGGC	- 3'
Itr NRD2	forward 1	5' -	TGTCAGCAGTGTCGCTTCAA	- 3'
Itr NRD2	reverse 1	5' -	TACGACCAAACCGAACAGCG	- 3'
Itr DEC1	forward 1	5' -	GTCATCCAGCGGACATTTGC	- 3'
Itr DEC1	reverse 1	5' -	GAAGTACGGCTGCTCATTGC	- 3'

Primers were designed using NCBI Primer Designer to amplify all known isoforms of CCG transcripts.

Appendix B: Western Blot Considerations

The antibodies used for PTMs and m6A are likely to give robust data owing to the known epitope sequence and the company's specificity guarantees. All epitope sequences were aligned with the homologous sequence in 13LGS to assess identity, with all primary antibodies showing at least 85% identity in epitope sequence.

Representative western blot images were shown to display to the reader the results of an optimization procedure which accomplished the blocking of non-specific bands and the dilution of primary and secondary antibodies to keep ECL signals within the linear dynamic range. A representative Western blot image is shown below.



Appendix C: Communications at scientific meetings

Oral Presentations

"*Investigation of the Molecular Clock Circadian During Hibernation in the Thirteen-Lined Ground Squirrel,*" at the 10th International Congress of Comparative Physiology and Biochemistry, 2019 August, Ottawa, ON

"*Genes of the undead: hibernation and death display different gene profiles*" at the 10th International Congress of Comparative Physiology and Biochemistry, 2019 August, Ottawa, ON

"*Lysine Methylation and Methyltransferase Enzymes in Mammalian Hibernation*," at the 13th Ottawa-Carleton Institute of Biology Symposium, 2016 May, Ottawa, ON

"*Hibernation in the 13-Lined Ground Squirrel, (I. tridecemlineatus), Involves Differences In Lysine Methyltransferases That Modulate Transcription*," at the 12th Ottawa-Carleton Institute of Biology Symposium, 2016 April, Ottawa, ON

Poster Presentations

Watts, A., Storey, K.B. *m^6A Methylation Modulates Transcriptional Activity during Hibernation in a Small Mammal, the 13-Lined Ground Squirrel.* Poster presented at: 10th International Congress of Comparative Physiology and Biochemistry, 2019 Aug; Ottawa, ON.

Watts, A., Storey, K.B. *m^6A Methylation Modulates Transcriptional Activity during Hibernation in a Small Mammal, the 13-Lined Ground Squirrel.* Poster presented at: TREnD – Toronto RNA Enthusiasts' Day, 2019 July; Toronto, ON.

Hadj-Moussa‡, H., Watts‡, A., Storey, K.B. *Genes of the Undead: Hibernation and Death display different Gene Profiles*. Poster presented at: Young Researchers' Brain Health Research Day, 2019 May; Ottawa, ON.

Watts, A., Storey, K.B. *m^6A Methylation Modulates Transcriptional Activity in a Small Mammalian Hibernator.* Poster presented at: 21st Chemistry and Biochemistry Graduate Research Conference, 2018 Nov; Montreal, PQ.

Watts, A., Storey, K.B. *Lysine methylation provides epigenetic control over cellular processes during hibernation in the thirteen-lined ground squirrel.* Poster presented at: Lung Development, Injury and Repair, Gordon Research Conference, 2017 Aug; New London, NH.

Watts, A., Storey, K.B. *Lysine methylation provides epigenetic control over cellular processes during hibernation in the thirteen-lined ground squirrel.* Poster presented at: The Summit on Organ Banking through Converging Technologies, 2017 Aug; Boston, MA.

Watts, A., Storey, K.B. *Lysine Methylation Regulates Transcriptional Control during Hibernation in the Thirteen-Lined Ground Squirrel*. Poster presented at: 60th Canadian Society for Molecular Biosciences, 2017 May; Ottawa, ON.

Watts, A., Storey, K.B. *Lysine Methylation Regulates Transcriptional Control during Hibernation in Ictidomys tridecemlineatus.* Poster presented at: CRYO2016 – 53rd Meeting of the Society for Cryobiology, 2016 July; Ottawa, ON.

Watts, A., Storey, K.B. *Differences in Lysine Methylation and Associated Methyltransferase Enzymes in Mammalian Hibernation in the Thirteen-Lined Ground Squirrel, Ictidomys tridecemlineatus.* Poster presented at: 13th Ottawa-Carleton Institute of Biology Symposium, 2016 May; Ottawa, ON.

Watts, A., Storey, K.B. *Regulation of Lysine Methyltransferases and Lysine Methylation during Torpor cycle in 13-Lined Ground Squirrel, Ictidomys tridecemlineatus.* Poster presented at: 18th Chemistry and Biochemistry Graduate Research Conference, 2015 Nov; Montreal, PQ.

Watts, A., Storey, K.B. *Hibernation in the 13-Lined Ground Squirrel, Ictidomys tridecemlineatus, Involves Differences in Lysine Methyltransferases that Modulate Transcription.* Poster presented at: 12th Ottawa-Carleton Institute of Biology Symposium, 2015 Apr; Ottawa, ON.

Appendix D: Publication List

Research Articles Published

Logan, S.M., Watts, A.J., Posautz, A., et al. 2020. The ratio of linoleic and linolenic acid in the pre-hibernation diet influences NFκB signaling in garden dormice during torpor. *Frontiers in molecular biosciences* 7, p. 97. doi: 10.3389/fmolb.2020.00097

Hadj-Moussa‡, H., Watts‡, A., Storey, K.B. (2019) Genes of the undead: hibernation and death display different gene profiles. *FEBS Letters*. **593**: 527-532. doi: 10.1002/1873-3468.13338

Watts, A., Storey, K.B. (2019) Hibernation impacts lysine methylation dynamics in the 13-lined ground squirrel, *Ictidomys tridecemlineatus*. *Journal of Experimental Zoology Part A*. **331**: 234-244. doi: 10.1002/jez.2259

Watts, A., Storey, K.B. (2016) Lysine methylation regulates transcriptional control during hibernation in *Ictidomys tridecemlineatus*. *Cryobiology*. **73**: 435. doi: 10.1016/j.cryobiol.2016.09.140

MacKay, H., Charbonneau, V.R., St-Onge, V., Murray, E., Watts, A., Wellman, M.K., Abizaid, A. (2016) Rats with a truncated ghrelin receptor (GHSR) do not respond to ghrelin, and show reduced intake of palatable, high-calorie food. *Physiology & Behavior.* **163**:88-96. doi: 10.1016/j.physbeh.2016.04.048

King, S.J., Rodrigues, T., Watts, A., Murray, E., Abizaid, A. (2016) Investigation of a Role for Ghrelin Signaling in Binge-Like Feeding in Mice Under Limited Access to High-Fat Diet. *Neuroscience.* **319**:233-45 doi: 10.1016/j.neuroscience.2016.01.004

St. Onge, V.; Watts, A.; Abizaid, A. (2015) Ghrelin enhances cue-induced bar pressing for high fat food. *Hormones and Behavior*. **78**:141-9 doi: 10.1016/j.yhbeh.2015.

Book Chapters Published

Logan, S.M., Watts, A.J., and Storey, K.B. (2018) Brain dead: the dynamic neuroendocrinological adaptations during hypometabolism in mammalian hibernators. In: Ludwig, M. and Levkowitz, G. eds. Model animals in

neuroendocrinology: from worm to mouse to man. Chichester, UK: John Wiley & Sons Ltd, pp. 207–231.

Research Articles in Preparation

Watts, A.J., Logan, S.M., Kübber-Heiss, A., et al. Regulation of Peroxisomal Lipid Metabolism Pathway during Torpor in the Garden Dormouse, *Eliomys quercinus*.

Watts, A.J. Childers, C., Storey, K.B. Regulation of Signal Transduction Pathways during Torpor within the Grey Mouse Lemur Lung.

Watts, A.J. and Storey, K.B. Goin' down slow: Peripheral circadian gene activity is altered during hibernation in the thirteen-lined ground squirrel.

Watts, A.J. and Storey, K.B. RNA methylation dynamics are maintained during mammalian hibernation**.**

Watts, A.J. and Storey, K.B. Arginine methylation produces differential regulation of epigenetic marks during hibernation in the 13-lined ground squirrel.

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