Histone methylation in the freeze-tolerant wood frog, *Rana sylvatica*

By

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Abstract

The wood frog, *Rana sylvatica*, has developed numerous adaptations to survive days with up to 65% of its body fluid frozen. One such adaptation is to reduce their metabolic rate, employing only those processes needed to survive until temperatures rise. The establishment of this hypometabolic state is mediated by transcriptional regulation that is elicited in part by histone methylation, however this has yet to be explored in the context of metabolic rate depression and freeze tolerance. This thesis provides the first characterization of histone methyltransferases (HMTs) and the histone and non-histone proteins they methylate in the wood frog. Transcriptionally permissive histone residues (H3K4me1 and H3K27me1) were found to decrease during freezing in skeletal muscle while those that silence transcription (H3K9me3 and H3K36me2) were maintained, whereas differential levels of histone residues were seen in liver. These findings suggest a novel role for HMTs in freeze tolerance.

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List of Abbreviations

AAT	ADP/ATP translocase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	5’ adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
APS	Ammonium persulfate
ASH2L	ASH2 like histone lysine methyltransferase complex subunit
ATP	Adenosine triphosphate
cDNA	Complimentary DNA
CHD1	Chromo-ATPase/helicase-DNA binding domain 1
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP-sequencing
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EDTA	Ethylenediaminetetraacetic acid
EHMT2	Euchromatic histone-lysine N-methyltransferase 2
FBPase	Fructose 1,6-bisphosphatase
FR10	Freeze responsive protein fr10
FR47	Freeze responsive protein fr47
FXR	Farnesoid X receptor
G6P	Glucose 6-phosphate
G6Pase	Glucose 6-phosphatase
G6PDH	Glucose 6-phosphate dehydrogenase
H3K4me1	Monomethylated histone H3 lysine 4
H3K9me3	Trimethylated histone H3 lysine 9
H3K27me1	Monomethylated histone H3 lysine 27
H3K36me2	Dimethylated histone H3 lysine 36

H3K79	Histone H3 lysine 79
H3R17	Histone H3 arginine 17
H3R26	Histone H3 arginine 26
H4K20me1	Monomethylated histone H4 lysine 20
H4R3	Histone H4 arginine 3
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMT	Histone Methyltransferase
HP1	Heterochromatin protein 1
HRP	Horse radish peroxidase
INP	Ice nucleating protein
kDa	Kilodalton
LI16	Freeze responsive liver protein li16
LSD1	Lysine-specific demethylase 1
MRD	Metabolic rate depression
mRNA	Messenger RNA
nt	Nucleotide
OD	Optical density
p53BP1	p53 binding protein 1
p53K370me2	Dimethylated p53 lysine 370
p53K372me1	Monomethylated p53 lysine 372
PCR	Polymerase chain reaction
PMSF	Phenylmethane sulfonyl fluoride
PRMT	Protein arginine methyltransferase
PTM	Post-translational modification
PVA	Poly(vinyl alcohol)
PVDF	Polyvinylidene difluoride
RBBP5	Retinoblastoma binding protein 5

RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RPP	Reversible protein phosphorylation
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SET	Su(var)3–9, Enhancer of zeste, and Trithorax
SET8	SET domain containing (lysine methyltransferase) 8
SETD7	SET domain containing (lysine methyltransferase) 7
SMYD2	SET and MYND domain containing 2
SUMO	Small ubiquitin-like modifiers
SUV39H1	Suppressor of variegation 3-9 homolog 1
TBST	Tris-buffered saline with tween 20
TEMED	Tetramethylethylenediamine
TMB	3,3’,5,5’- Tetramethylbenzidine

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Chapter 1

General Introduction

1.1 Freezing stress and survival strategies

Exposure to freezing temperatures for long periods of time can lead to potentially lethal consequences for animals that do not have the proper adaptations for survival. When temperatures drop below 0°C, ice crystal formation creates stresses that most animals cannot survive. These stresses occur in both the intracellular and extracellular environment, and involve ice formation, ischemia, dehydration, and osmotic stress. Inside the cell, osmotic pressure increases drastically as water is incorporated into the ice crystal lattice (Storey 1990). This leads to not only an osmotic stress from increased concentrations of solutes, but also a dehydration stress as the amount of water needed for normal cellular operations decreases (Lee *et al.* 1992). Consequently, cell membranes shrink and rupture from compression and ice induced mechanical stress. At the tissue and organ level, ice crystal formation causes similar damage by tearing and puncturing tissues as more and more water is incorporated into the ice (Storey 1990). If these problems weren't enough, freezing of body fluids disrupts the most crucial of physiological processes; freezing of blood plasma prevents the heart from pumping, while the lungs become physically restricted and can no longer bring in oxygen. For the non-adapted, this damage is irreversible and leads to certain death (Mazur 1984; Storey and Storey 1988).

Freezing survival strategies can be grouped into two main categories: (1) freeze avoidance, and (2) freeze tolerance. Freeze avoidance is described as the prevention of body fluid freezing, which can be accomplished through a multitude of different methods. One of these methods is through seasonal migration to warmer climates, which is a successful method for animals with the capabilities of traversing long distances (e.g. migrating birds). For those animals without this capability, escaping to thermally

buffered environments provides the necessary conditions for surviving the winter (Storey 1997), an example of which are invertebrates residing in decaying wood during the winter (Baust 1976). A third method of freeze avoidance is through supercooling of the body fluids, used by many arthropods (Sømme 1982) and reptiles (Lowe *et al.* 1971) to survive the winter. Animals with this ability produce anti-freeze agents that lower the freezing point of the intra- and extracellular fluids sufficiently to avoid ice crystal formation (Lowe *et al.* 1971). The vast majority of animals living in cold climates fall under the category of freeze avoidance, however, some animals have evolved the opposite strategy for surviving sub-zero temperatures (Storey and Storey 1988). Instead of avoiding freezing altogether, they actively allow freezing of their body fluids and consequently have adaptations to deal with the above mentioned dangers.

Freeze tolerance has arisen in multiple different animal lineages, from insects and intertidal invertebrates (Aarset 1982; Ring 1982; Murphy 1983; Storey and Storey 1988), to reptiles and amphibians (Schmid 1982; Storey 1984; Storey 1990; Layne and Kefauver 1997). These different organisms share common adaptive mechanisms to survive freezing of bodily fluids. Firstly, ice nucleation is prompted through skin contact with ice from the environment, by ice nucleators such as bacteria on the skin or gut, or by the production of ice nucleating proteins (INPs) (Storey and Storey 1985a; Lee and Costanzo 1998; Zachariassen and Kristiansen 2000). The animal uses one or more of these methods to trigger ice nucleation at high sub-zero temperatures, close to the freezing point of their body fluids. Freezing near the equilibrium freezing point slows the rate of ice formation, which increases the amount of time the animal has to enact other freeze tolerance adaptations. Without the controlled use of ice nucleators, body fluids supercool, and

subsequently results in the rapid and unorganized formation of ice, causing much more damage than would otherwise have been accrued. The distribution and size of ice crystals is also controlled by INPs so that ice only forms extracellularly, which avoids higher levels of damage that intracellular ice may cause, such as mechanically rupturing plasma membranes (Storey and Storey 1988). As previously mentioned, pure water is drawn out of cells as it is incorporated into the growing ice crystals. To counteract damage to cell membranes by the decrease in cellular volume and increase in residing solute concentration, these animals produce cryoprotectants, such as glucose or glycerol, to maintain membrane fluidity during freezing and allow for the restoration of membrane function upon thawing (Schmid 1982; Storey and Storey 1984; Storey and Storey 1985b; Storey and Storey 1988; Košťál and Šimek 1995). These small molecules are transported and accumulated in cells of most tissues during periods of freezing. The incorporation of water into ice crystals creates an increase in solute concentration extracellularly, therefore increasing the osmotic pressure and drawing water out of cells. The accumulation of cryoprotectants within the cell counteracts this osmotic force, and helps maintain cell volume. Increasing the intracellular solute concentration through the buildup of cryoprotectants also has the effect of lower the freezing point of intracellular fluids, aiding INPs in segregating ice formation to extracellular fluid.

Although freeze tolerant animals have adaptations for avoiding mechanical damage from ice – INPs for excluding ice to extracellular cavities, and cryoprotectants for preserving membrane structure and function – they still must contend with the physiological challenges of body fluid freezing. As mentioned above, exposure to sub-zero temperatures results in the freezing of blood plasma and the physical interruption of

heart and lung function, which would have disastrous consequences for a species without the necessary adaptations. Freeze tolerant animals are not exempt from these stresses, but rather have evolved mechanisms for surviving without these basic physiological processes while frozen. At the onset of freezing the animal becomes ischemic (when an insufficient amount of blood is delivered to tissue and organs), and thus, cannot transport oxygen throughout its body. This anoxic state results in two consequences for the animal, (1) they must switch ATP production from aerobic oxidative phosphorylation to an anaerobic method of producing ATP, and (2) they are limited to a finite supply of energy resources, mostly in the form of glycogen, that must last an indeterminate amount of time until thawing occurs. The switch to anaerobic glycolysis while in a frozen state has been documented in multiple species through the measurement of accumulated lactate (Storey and Storey 1986; Storey *et al.* 1988; Churchill and Storey 1992b; Churchill and Storey 1992a; Packard and Packard 2004). This alternative to oxygen dependent ATP production is necessary when the blood plasma freezes and cellular oxygen levels are depleted and cannot be replenished. Oxygen is the terminal electron acceptor in the electron transport chain, and thus necessary for aerobic ATP production through oxidative phosphorylation. As will be discussed, freeze tolerant animals don't enter a true state of suspended animation as select tissue-specific metabolic activity is maintained throughout the period of freezing, and therefore continued, albeit low levels, of energy production is vital.

While frozen, not only is breathing halted, but the frozen state also prevents the intake of energy resources in the form of food. As a result, all energy production during the freezing period is derived from energy resources the animal has accumulated prior to freezing in the form of glycogen. If the euthermic rate of ATP usage were to be

maintained during freezing, the animal would quickly run out of the majority of glycogen stores that are needed for anaerobic production of ATP and would not survive long enough for temperatures to rise again. Freeze tolerant animals respond to this challenge by switching into a hypometabolic state for the duration of the freezing period by globally coordinated metabolic rate depression (MRD) (Storey and Storey 1988; Storey 1990). By reducing metabolic rate, these animals necessarily reduce the rate of energy expenditure, thus increasing the amount of time before the glycogen reserves run out. Although there are many energy intensive cellular processes, such as DNA replication, cell cycle progression, and ion motive ATPases, some of the most energetically consuming cellular processes are gene expression and protein synthesis. These two process use 1-10% of the total energy production of the cell (Rolfe and Brown 1997), thus, they are key targets for downregulation during MRD. As such previous studies have shown transcription and translation to be suppressed to fractional levels during hypometabolism (Land *et al.* 1993; Frerichs *et al.* 1998; Fuery *et al.* 1998). However, this is done in a selective manner as some pathways are utilized for surviving environmental stresses. Freeze tolerant animals employ specific genes that are necessary for survival, such as fibrinogen (Cai and Storey 1997a) and Freeze Responsive Protein 10 (Fr10) (Cai and Storey 1997b), which must be upregulated, while those that are unnecessary and energetically expensive, such as genes involved in cell cycle progression, are downregulated (Zhang and Storey 2012). This ability to suppress metabolism has been extensively characterized not only in freeze tolerant animals (Storey and Storey 1988; Storey 1990), but also in mammalian hibernators (Storey 1997; Heldmaier *et al.* 2004; Geiser 2004; Stieler *et al.* 2011), anoxia tolerant turtles (Storey and Storey 1990;

Hochachka *et al.* 1996), and estivating frogs and invertebrates (Storey and Storey 2012; Wu *et al.* 2013). The common theme amongst these different organisms is the ability to selectively downregulate the majority of metabolic pathways, while maintaining only those vital for survival. The alternative approach, general suppression of all metabolic output, is not a viable option as these animals have specific molecular and metabolic adaptations needed for survival.

The selective regulation needed to enter a hypometabolic state is accomplished by multiple mechanisms at all stages of gene expression starting at the transcriptional level. Regulation and modification of transcription factors have shown to be integral mediators of MRD (Storey and Storey 2004a; Eddy and Storey 2007). Under euthermic conditions, activated transcription factors bind to their respective DNA binding sequences upstream of the promoter regions of the target genes and recruit transcriptional machinery, allowing the gene to be expressed. During a state of hypometabolism, some of these transcription factors may be downregulated in expression or activation and thus, transcription does not proceed at full strength. Post-transcriptional control of MRD has mostly focused on microRNA (Morin *et al.* 2008; Biggar and Storey 2011; Biggar *et al.* 2012; Biggar and Storey 2012; Wu *et al.* 2013; Bansal *et al.* 2016; Wu *et al.* 2016). With the ability to either silence or degrade mature mRNA transcripts, microRNA can act quickly to suppress the energetically expensive process of protein translation (Bartel 2004; Filipowicz *et al.* 2008) with the possibility of storing mRNA in stress granules for when conditions improve (Kedersha and Anderson 2007). Proteins that have already been translated are also regulated once a hypometabolic state is established. If left unchecked these enzymes and signaling proteins can quickly use up valuable cellular resources, and

therefore, must be modified, segregated, or degraded to conserve energy at the biochemical level. One method for regulating these proteins is through post-translational modifications by various processes such as phosphorylation, acetylation, methylation, nitrosylation, and ubiquitination. The addition of these chemical groups can have various effects on the activity and function of the protein depending on which group is added and which amino-acid residue it is added to. For example, ubiquitination of proteins is known to be an integral part of protein degradation (Hershko and Ciechanover 1992). Post-translational modification of proteins has also been shown to be regulated in animals that undergo metabolic rate depression. Both transcription factors, such as p53 in the anoxia tolerant turtle, *Trachemys scripta elegans* (Zhang *et al.* 2013), and metabolic enzymes, such as creatine kinase in the freeze tolerant wood frog, *Rana sylvatica* (Dieni and Storey 2009) have been shown to be modified in response to environmental stressors.

This brief overview of the systematic regulatory mechanisms gives an idea of the complex coordination needed for each animal to survive an extended period of time in a hypometabolic state. Stressors from freezing temperatures create circumstances that these animals have adapted to survive. These adaptations involve a broad range of mechanisms from the application of INPs and cryoprotectants, to the regulation of energy utilization through the modification of signaling proteins and enzymes.

1.2 The freeze tolerant wood frog, *Rana sylvatica*

The most well-studied freeze tolerant vertebrate is the wood frog, *Rana sylvatica*. With a range that extends from Alaska to Ohio (**Fig. 1.1**), this anuran spends the winter

months frozen amongst the leaf litter of the boreal forest floor. Although air temperatures can reach -30°C, the temperatures under leaf cover and snow pack rarely drop below -5°C. The wood frog can survive up to 65% freezing of body fluids at these temperatures for days to weeks at a time (Lee *et al.* 1992), and recently individuals from an Alaskan population have been shown to survive freezing for an average of 193 days (Larson *et al.* 2014). The wood frog begins to freeze at high sub-zero temperatures (-0.5°C) like other freeze tolerant species, which allows the slow spread of ice and enough time to initiate freeze tolerant strategies. Ice nucleation begins with inoculation from environmental ice, which is followed up by non-specific nucleators in the gut (e.g. bacteria), and INPs in the blood to control the location and size of ice crystal formation. Multiple physiological and biochemical adaptation work in unison to provide the wood frog with the ability to survive in a frozen state for extended periods of time. The trigger to initialize these adaptation is not low temperature itself, but rather is the onset of ice nucleation (Storey and Storey 1985a). Upon ice nucleation, the liver begins to mobilize large quantities of glycogen in the form of glucose, which is used as a colligative organ and tissue cryoprotectant (Storey and Storey 1984; Storey 1984; Storey 1987a; Costanzo *et al.* 1993). Liver glucose transporter capacity increases leading up to the winter season so that the large quantities of glucose can be shuttled to every organ of the body in a rapid time frame (King *et al.* 1993; King *et al.* 1995). Previous studied have shown that euthermic frogs have plasma glucose levels of 1-2 mM which then increase to 250-300 mM during periods of freezing (Storey 1984; Storey and Storey 1986). Glucose production is maintained throughout the entire freezing period (Storey and Storey 1984) in the liver,

and it is not until the beginning of thawing that glucose production ceases, and is reversed to replenish glycogen stores.

As with other freeze tolerant species, freezing in the wood frog prevents the heart from beating, represses lung ventilation, and the blood from flowing, thus oxygen ceases to be transported to organs and tissues. The wood frog transitions from oxidative phosphorylation when oxygen is readily available to anaerobic glycolysis during freezing. This is evident by an increase in lactic acid formation (an end product of pyruvate being converted into lactate) in frozen frogs (Storey 1984). Throughout the duration of the freezing period, the wood frog relies on internal energy stores since they no longer have the ability to forage for food. The wood frog increases its chances of surviving the freezing period by creating a new equilibrium between ATP generation and ATP expenditure. Since its capacity to produce energy resources has diminished, its use of said energy resources also decreases. This is accomplished mainly through the downregulation of unnecessary metabolic pathways and the maintenance and/or upregulation of pathways that are needed for freezing survival, resulting in a hypometabolic state. Mechanisms of MRD and maintenance of a hypometabolic state have been widely characterized in the wood frog. Reversible protein phosphorylation (RPP) by proteins such as AMPK negatively regulates energy expensive cellular process during freezing in the wood frog (Greenway and Storey 2000; Rider *et al.* 2006; Zhang *et al.* 2013; Biggar *et al.* 2015), whereas metabolic enzymes are heavily regulated to reduce energy consuming reactions and switch to anaerobic glycolysis (Dieni and Storey 2008; Dieni and Storey 2009; Dieni and Storey 2011). Recently, the role of epigenetic

mechanisms has been examined in the context of metabolic rate depression and freeze tolerance.

1.3 Epigenetics and histone methylation

Epigenetics refers to functional changes to the genome without altering the DNA sequence of the genome itself (Wolffe and Matzke 1999). These changes influence the transcriptional state of genes, which in some cases can results in stable transgenerational phenotypic changes. Much of the research on epigenetics has focused on these transgenerational effects, however, these same mechanisms work on much shorter time scales in response to environmental input to dynamically regulated transcription. There are three main categories of epigenetic mechanisms: (1) DNA methylation, (2) microRNA regulation, and (3) histone modifications. DNA methylation is perhaps the most well characterized epigenetic mechanism. This type of methylation occurs on cytosine bases, most commonly where the methylated cytosine is next to a guanine nucleotide, known as a CpG site. CpG islands are regions of high CpG density, mostly found upstream of promoter regions, and are the gene regulatory unit for DNA methylation. When CpG islands are hypermethylated transcription of the associated gene is silenced. Methylated CpG islands physically interfere with binding of transcription factors and machinery to the DNA itself, and also recruit transcriptionally repressive proteins (Bogdanović and Veenstra 2009). DNA methyltransferase 3a and 3b (DNMT3a and DNMT3b) are the enzymes responsible for methylating unmethylated CpG sites, whereas DNMT1 preserves existing methylated DNA patterns by methylating

hemimethylated DNA after DNA replication. The result is a stable, reversible, and heritable modification to the genome with no change to the underlying code.

Recently microRNAs have emerged as a major player in the regulation of protein translation. MicroRNA are short (18-23 nt) non-coding RNA that integrate with RNA-induced silencing complexes (RISC) to regulate mRNA transcripts (Bartel 2004). The action microRNA-RISC performs on a given mRNA transcript depends on the complementarity between the microRNA and the mRNA (Nelson *et al.* 2003). Partial complementarity results in reversible silencing, whereas full complementarity leads to degradation of the transcript. This means that a single microRNA is capable of regulating multiple different mRNA transcripts, while a single mRNA transcript may be regulated by multiple different microRNAs. This results in a hugely complex regulatory network of RNA controlling RNA (Hon and Zhang 2007).

Similar to DNA, histones can be chemically modified to regulate gene transcription. The regulatory complexity of histone modification is enormous given that a single histone can be post-translationally modified in multiple ways, at multiple residues, and can be effected by cross-talk between modifications. Recent evidence has suggested that these epigenetic mechanisms can be crucial in the cellular response to environmental perturbation (Storey 2015). In the context of metabolic rate depression, DNA methylation, protein acetylation, and microRNA expression have all shown to be differentially regulated between control and hypometabolic states in a variety of animals such as hibernating ground squirrels (Morin *et al.* 2008; Biggar and Storey 2011; Biggar and Storey 2014; Rouble and Storey 2015; Wu *et al.* 2016), anoxic turtles (Krivoruchko and Storey 2010; Biggar and Storey 2012; Wijenayake and Storey 2016), dehydration

tolerant *Xenopus laevis* (Wu *et al.* 2013), freeze tolerant *Littorina littorea* (Biggar *et al.* 2012), and the wood frog (Bansal *et al.* 2016), leading to the idea that other epigenetic systems may be involved.

Unlike DNA methylation, histones can be modified in a variety of ways including acetylation, phosphorylation, ubiquitination, SUMOylation, and methylation (Berger 2002; Kouzarides 2007). Histone modification appear primarily on histone N-terminal tails, which when present create or modify binding sites on histones and surrounding DNA (Razin and Riggs 1980). The results of these modification include transitioning to euchromatin or heterochromatin (Bradbury 1992; Noma *et al.* 2001), recruiting transcriptional machinery, and interacting with epigenetic "readers", "writers", and "erasers" to alter other histone and DNA modifications (Ben-Porath and Cedar 2001; Lee and Zhang 2008; Jobe *et al.* 2012). The collective combination of modification type, and placement along the histone tails creates what is known as the "histone code", likely comprising innumerable distinct transcriptional outcomes. (Strahl and Allis 2000; Jenuwein and Allis 2001; Margueron *et al.* 2005).

One of the most well studied histone modification is histone methylation, and as a result has been found to be strongly linked with transcription control (Zhang and Reinberg 2001; Kouzarides 2002; Martin and Zhang 2005; Liu *et al.* 2010; Greer and Shi 2012). Both lysine and arginine residues can be methylated on histone tails (Sims *et al.* 2003; Di Lorenzo and Bedford 2011), however, histone lysine methylation has dominated the literature and is used primarily in decoding the function methylation plays in the histone code. Other histone modifications, such as histone acetylation, involve the addition of a charged chemical group to the histone, changing the interaction between the

histone and the negatively charged DNA. Methyl groups do not change the charge distribution of the histone, and thus, do not change the interaction with surrounding DNA. Instead, the addition of uncharged methyl groups creates binding sites for methyl-binding proteins (Lachner *et al.* 2001; Kim *et al.* 2006; Adams-Cioaba and Min 2009). The creation of these binding sites therefore allows histone methylation to have many functions, including transcription activation or suppression, depending on what proteins are recruited. Lysine methylation differs from most other modifications in that each lysine residue can be methylated multiple times, resulting in mono-, di-, and trimethylation (me1, me2, and me3 respectively). Methyl-binding domains on effector proteins are often specific to the degree of methylation on a given lysine residue (Sims and Reinberg 2006), thus, the combination of lysine position and degree of methylation on a histone tail can have a functionally distinct outcome within the nucleus. For instance, active transcription and euchromatin has been associated with monomethylation of lysine 4 and monomethylation of lysine 27 of histone H3 (H3K4me1 and H3K27me1 respectively) (Schneider *et al.* 2004; Martin and Zhang 2005), whereas the opposite function, gene silencing and heterochromatic regions are associated with trimethylation of lysine 9 and dimethylation of lysine 36 (H3K9me3 and H3K36me2 respectively) (Peters et al. 2002; Keogh et al. 2005; Youdell et al. 2008; Li et al. 2009; Hsia et al. 2010).

The enzymes that add methyl groups to histone lysine residues are histone methyltransferases (HMTs). Most HMTs contain the Su(var) 3–9, Enhancer of zeste, and Trithorax (SET) domain as their key catalytic feature. The SET domain uses donor methyl groups from S-adenosyl methionine (SAM) to methylate the ε-amino group of

lysine residues (Herz *et al.* 2013). Proteins such as SETD7, RBBP5, ASH2L, and SMYD2 either directly methylate, or are important parts of complexes that methylate H3K4 residues. SUV39H1 and EHMT2 both methylate H3K9, EHMT2 and SMYD2 methylate H3K27 and H3K36 respectively, while SET8 methylates H4K20 (**Fig. 1.2**). The role of histone methylation in metabolic rate depression and freeze tolerance has yet to be elucidated, and similar to other epigenetic processes (Krivoruchko and Storey 2010; Biggar and Storey 2014; Rouble and Storey 2015; Wijenayake and Storey 2016) it may regulate the entry and maintenance of a hypometabolic state.

1.4 Non-histone methylation

Histones are not the only proteins that can be methylated. HMTs often target non-histone proteins for methylation, giving them the ability to regulate the functions of a wide variety of signaling pathways and cellular processes (Biggar and Li 2015) such as gene transcription (Huang *et al.* 2007; Munro *et al.* 2010; Calnan *et al.* 2012), protein translation (Dhayalan *et al.* 2011), cell survival, apoptosis, and DNA repair (Kassner *et al.* 2013). The transcription factor p53 is one such protein that is regulated by lysine methylation. p53 is a well-studied transcription factor, known for its involvement in a high percentage of cancers (Agarwal *et al.* 1998), due to the broad range of cellular functions involving it. Previous studies have shown that p53 is heavily controlled by post-translational modifications such as phosphorylation, acetylation, and ubiquitination (Tibbetts *et al.* 1999; Barlev *et al.* 2001; Tang *et al.* 2008; Lee and Gu 2009). However, methylation of lysine residues regulating p53 has also been discovered (Chuikov *et al.* 2004; Huang *et al.* 2006; Shi *et al.* 2007). Lysine 370 and 372 both act to increase the

activity and stability of p53 when di- and monomethylated respectively (p53K370me2 and p53K372me1 respectively). Methyl-regulation of p53 is of particular interest in the context of this study due to the many functions p53 is involved in, including cell cycle arrest, DNA damage repair and apoptosis signaling, which have implications for freeze tolerance and MRD. Previous studies have shown that species that use MRD to survive unfavorable environmental conditions either increase levels of p53, or use activating post-translational modification in a tissue specific manner (Zhang *et al.* 2013; Biggar *et al.* 2015). Given that some downstream effects of p53 activation such as cell cycle suppression have been shown in the wood frog (Zhang and Storey 2012), then methylation and subsequent activation of p53 may be expected during freezing temperatures.

1.5 Objectives and hypotheses

1.5.1 ***Objective 1****: Profile the state of HMTs and their targets during the freeze-thaw cycle in skeletal muscle*

It has been demonstrated in previous studies that the wood frog's metabolism suppresses to survive periods of freezing. Skeletal muscle remains metabolically dormant during freezing, and thus mechanisms for metabolic rate depression are exemplified in

Hypothesis 1:

HMT expression and activity will be differentially regulated and will be reflected in the methyl-lysine residues they act on during freezing, and subsequent thaw in skeletal muscle.

this tissue. One of the top-down methods for conserving cellular energy is to regulate transcription by only transcribing genes vital for freezing survival. Since histone methylation has been tightly associated with transcriptional regulation, it stands to reason that the wood frog may use it as a method to enter and maintain a state of hypometabolism, thus allowing it to survive freezing temperatures.

Chapter 3 tests this hypothesis by measuring HMT protein levels, global HMT activity, and methyl-lysine residues on histones and non-histone proteins in skeletal muscle throughout the freeze-thaw cycle of the wood frog. The goal of this chapter is to create a profile of the changes that occur with respect to HMTs and their targets during freezing and thaw, and if there are commonalities between HMTs that methylate the same lysine residues in skeletal muscle. Specifically, the objective is addressed by measuring seven HMTs (SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8), HMT activity on H3K4, levels of methylated histone residues (H3K4me1, H3K9me3, H3K27me1, and H3K36me2), and non-histone targets (p53K370me2 and p53K372me1). Corresponding levels of HMTs, their activity, and the targets they methylated would indicate a coordinated role for HMTs in freezing tolerance and metabolic rate depression in skeletal muscle.

1.5.2 ***Objective 2****: Profile the state of HMTs and their targets during the freeze-thaw cycle in the liver*

The wood frog liver is unlike its skeletal muscle in the sense that it carries out an array of metabolically important processes during and after periods of freezing. Glucose is synthesized from liver glycogen to act as a cryoprotectant even while the wood frog is frozen solid, and beginning immediately during thawing, glycogen stores are replenished through glycogenesis and excess glucose is secreted. Although most liver processes are halted during freezing, the few that do remain active demonstrates the need for dynamic transcriptional regulation. Histone methylation provides a means for doing so, and thus, may be play a role in the liver during periods of freezing.

Hypothesis 2:

HMT protein levels and their corresponding activity and targets will be differentially regulated in the liver during the freeze-thaw cycle.

Chapter 4 tests this hypothesis by measuring HMT proteins levels, HMT activity on specific histone residues, and levels of methylated histone and non-histone proteins in the liver across the freeze-thaw cycle. By determining the relationship between HMTs and the targets they methylate in liver, I hope to elucidate the possible involvement that histone methylation plays during freezing survival. Since the targets measured in this chapter are the same targets in skeletal muscle, it stands to reason that the difference in results between the tissues will be a reflection of their remarkably different responses during periods of freezing.

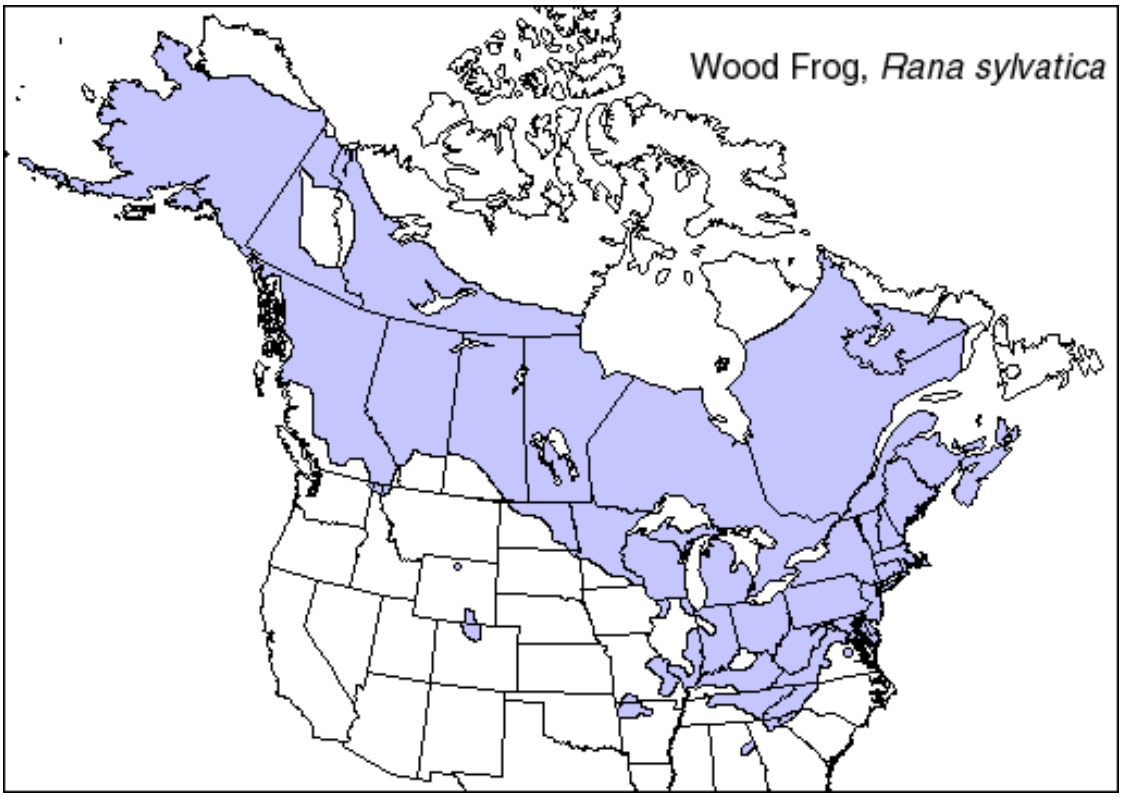

Figure 1.1: Geographical range of the wood frog, *Rana sylvatica*. Image from United States Geological Survey, hosted on Wikimedia Commons. Retrieved from https://en.wikipedia.org/wiki/File:Rana-sylvatica_Range.gif on May 18, 2016.

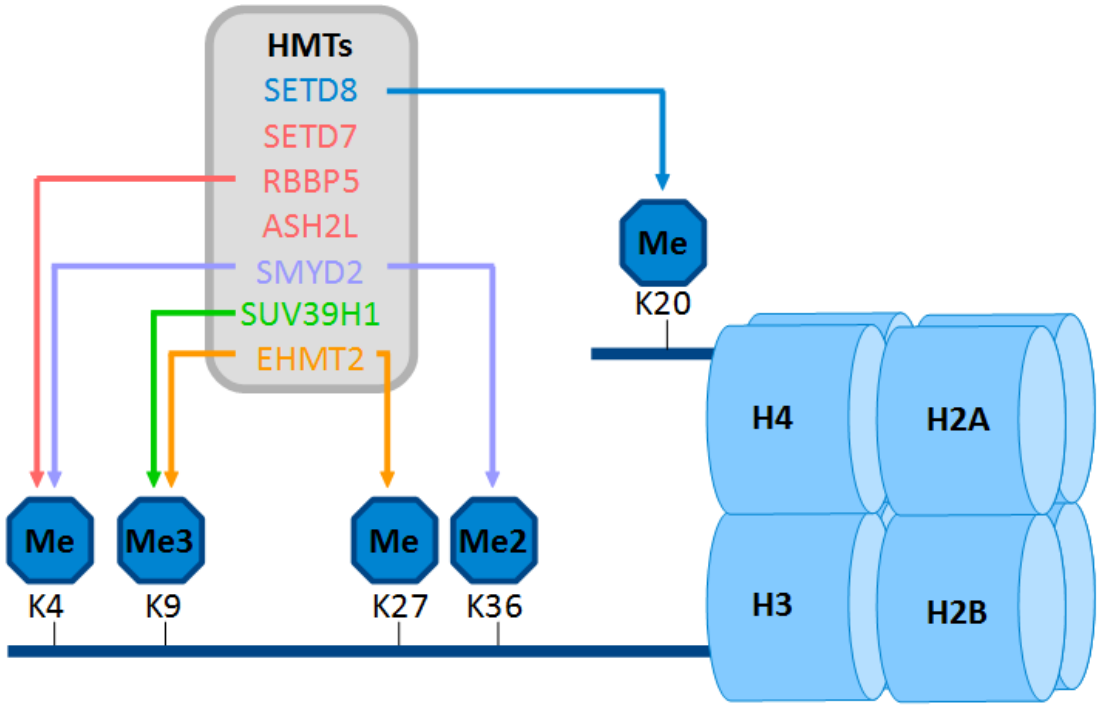

Figure 1.2: Histone lysine residues methylated by histone methyltransferases. Histone methyltransferases (HMTs) such as SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8 transfer methyl groups from S-adenosyl methionine (SAM) to recipient histone lysine residues. Each residue can accept up to three methyl groups (me1, me2, and me3 respectively).

Chapter 2

General Materials and Methods

2.1 Animal experiments

Male wood frogs (*Rana sylvatica*) were caught during the spring from breeding ponds in Ottawa, Ontario, kept in coolers on crushed ice from the edges of the ponds, and transported to Carleton University. All frogs were washed in tetracycline and then placed in plastic containers with sphagnum moss for one week in an incubator at 5°C. Control frogs were sampled from this condition after the one-week period.

For the frozen condition, frogs were placed in a plastic container with damp paper towel and put into an incubator at -4.0°C for 45 min in order to lower the frog's body temperature to below zero. The temperature was then raised to -2.5°C for 24 hours and frogs were randomly selected for sampling. The remaining frogs were placed back into an incubator at 5°C for 8 hours to allow thawing before sampling.

Control, 24 h frozen, and 8 h thaw frogs were euthanized by pithing, then skeletal muscle and liver tissues were excised and flash frozen in liquid N_2 for a total of n = 4-5 of each tissue per condition. All Tissues were then stored at -80°C for future use. All protocols were conducted with permission of the Carleton University Animal Care Committee and within the guidelines of the Canadian Council on Animal Care.

2.2 Total soluble protein extraction

Muscle and liver tissues from control, 24 h frozen and 8 h thaw conditions were weighed, then crushed with a mortar and pestle in liquid N_2 and homogenized with a P10 homogenizer, 1:2 w/v in chilled homogenization buffer (20mM HEPES, 200mM NaCl, 0.1mM EDTA, 10mM NaF, 1mM Na_3VO_4, 10mM β-glycerophosphate, pH 7.4) with a

few crystals of PMSF and 1 µL/mL of protease inhibitor (Catalogue # PIC002; BioShop Canada Inc., Burlington, ON, Canada). The homogenates were then centrifuged at 10,000 x g for 15 min at 4°C and the supernatant containing the soluble proteins was collected as samples. Total protein concentration for each sample was determined using the BioRad protein assay (Catalogue # 5000002; BioRad Laboratories, Hercules, CA, USA) at 595 nm on an MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA, USA). Concentrations were then adjusted to 10 µg/µL using the same homogenization buffer as before. Aliquots of samples were taken at this stage to be used for HMT activity assays. The remaining sample volumes were used for western blotting. These samples were mixed 1:1 v/v with SDS buffer (100mM Tris-base, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercaptoethanol), ending with a final concentration of 5 µg/µL. All samples were then boiled in a water bath for 10 min to fully denature and linearize the proteins. Samples were stored at -40°C until use.

2.3 Western blotting

Equal amounts of control, 24 h frozen, and 8 h thaw (15-40 µg, depending on tissue, and protein to be probed) were loaded into 6-15% discontinuous SDS-polyacrylamide gels (percentage of acrylamide in resolving gel dependent on protein to be probed) with 4 µL of PiNK Plus pre-stained protein ladder (10.5-175 kDa; Catalogue # PM005-0500K; FroggaBio, Toronto, ON, Canada). The upper stacking gel (pH 6.8) was comprised of 5% acrylamide v/v in pH 6.8 Tris buffer (1M Tris) with 0.1% SDS (Sodium dodecyl sulfate), 0.1% APS (ammonium persulphate), and 0.1% TEMED (N,N,N',N'-Tetramethylethane-1,2-diamine) whereas resolving gels were 6-15%

acrylamide v/v in pH 8.8 Tris buffer (1.5M Tris), with 0.1% SDS, 0.1% APS, and 0.1% TEMED.

Loaded gels were run in the BioRad Mini Protean III system (BioRad Laboratories, Hercules, CA, USA) for 30-150 min at 180V in running buffer (25 mM Tris-base, 190 mM glycine, 0.1% w/v SDS, pH 7.6). Proteins from the gels were then transferred onto 0.45 μm pore PVDF membranes by electroblotting at room temperature for 60-180 min at 160mA in 1X transfer buffer (25 mM Tris-base, 192 mM glycine 10% v/v methanol, pH 8.5).

To prevent non-specific binding of primary and secondary antibodies, the membranes were incubated with skim milk (1-10%, on a rocker at room temperature for 30 min) in 1X TBST (20mM Tris-base, 140 mM NaCl, 0.05% Tween-20) or M_w 30,000 – 70,000 PVA (1 mg/mL, on a rocker at room temperature for 30-90 sec) in 1X TBST. Membranes were then probed with primary antibodies (1:1000 v/v dilution in 1X TBST) at 4°C for 18 hours (Please refer to ***Appendix A: Antibody information and suppliers for western blotting*** for a list of primary antibodies used). Blots were washed 3 x 5 min with 1X TBST and probed with HRP-conjugated goat anti-rabbit secondary antibodies (1:8000 v/v dilution in TBST; Catalogue # APA002P, BioShop Canada Inc., Burlington, ON, Canada) at room temperature for 30 min (Please refer to ***Appendix B: Western blotting conditions*** for a detailed list of optimized conditions for each target). Membranes were washed 3 x 5 minutes again in 1X TBST and were visualized by chemiluminescence (1:1 v/v H2O2, Luminol) using the ChemiGenius Bio Imaging System (Syngene, Frederick, MD, USA). Membranes were then stained with Coomassie blue (0.25% w/v Coomassie

brilliant blue, 7.5% v/v acetic acid, 50% methanol) to visualize all protein bands for loading standardization.

2.4 Histone methyltransferase activity assays

Relative levels of total histone methylation activity on individual histone residues were measured using the EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kits (Epigentek, H3K4-Catalogue # P-3002-96; H3K9-Catalogue # P-3003-96) following the manufacturer's instructions. Briefly, a dilution curve ranging from 20 to 100 µg of protein for each kit and tissue was tested using a pooled sample containing 10uL of total protein extracts from each sample point to determine the linear range of protein needed for the assay. Based on the dilution curve, 30-60 µg of total protein extracts from liver and skeletal muscle of control, 24 h frozen, and 8 h thaw wood frog tissue were incubated with the supplied adomet (Epigentek) and biotinylated substrate (Epigentek) in the provided 96-well microplate for 60 min at 37°C. Along with the protein samples, a blank well was prepared per run by replacing total protein extracts with the provided assay buffer, and a positive control was prepared by replacing total protein extracts with the provided control enzyme (300 µg/ml; Epigentek). After incubation, each well was washed 3 times with 150 µL of 1X wash buffer. 50 µL of capture antibody provided with the kit (100 µg/ml; Epigentek) diluted 1:100 in 1X wash buffer was added to each well and incubated for 60 min at room temperature on an orbital shaker. The wells were washed again 3 times with 150 µL of 1X wash buffer before adding 50 µL of supplied detection antibody (100 µg/ml; Epigentek) diluted 1:1000 in 1X wash buffer, which was incubated for 30 min at room temperature. After a final 5 washes with 150 µL of 1X

wash buffer, 100 µL of developing solution supplied with the kit was added to each well and incubated for 10 min at room temperature until the color of the positive control wells had reached a dark blue (indicating the presence of methylated histone H3K4). At this stage, 50 µL of the supplied stop solution was added to each well, and the sample wells were read using a microplate reader (Multiscan Spectrum, Thermo Labsystems) at 450 nm.

A standard curve was created for each kit by replacing total protein extracts with supplied HMT standard at different concentrations (0.1 – 5.0 ng/µL) as per the manufacturer's instructions. The protein concentrations used fit on the linear portion of the standard curve, and the slope of this curve was calculated and used to calculate HMT activity using the following equation:

$$Activity\left(\frac{\frac{ng}{h}}{mg}\right) = \frac{OD(sample - blank)}{[Protein\ amount\ (ug) * incubation\ time(h) * slope]} \times 1000$$

For information regarding mechanistic details of the activity assay see ***Appendix C****:* ***Commercial HMT activity assay mechanistic details***.

2.5 Quantification and statistics

Chemiluminescent protein bands on the immunoblot were quantified by densitometry using ChemiGenius Bio Imaging System GeneTools Software (Syngene, Frederick, MD, USA). Band densities were standardized against the combined intensity of multiple Coomassie blue stained bands in the same lane. For more information on the validation of this method see ***Appendix D****:* ***Loading control for western blotting***. Data

for each experimental condition are expressed as mean ± SEM with n = 4 samples from different animals. Statistical analysis was performed by one-way ANOVAs and Tukey's post-hoc test ($P < 0.05$) using SigmaPlot 12 statistical package software (Systat Software Inc., San Jose, CA, USA).

Chapter 3

Histone Methylation in Skeletal Muscle

3.1 Introduction

As mentioned in **Chapter 1**, freeze tolerant animals such as *Rana sylvatica,* use metabolic rate depression as a survival strategy during periods of freezing (Storey and Storey 1988; Storey and Storey 1992). To conserve energy resources until temperatures rise and the animal can thaw, a coordinated remodeling of the metabolic landscape takes place in every organ and tissue leading up to freezing. The entrance of a hypometabolic state allows animals like the wood frog to survive for an unknown length of time on a finite reserve of energy resources. Although the length of the winter season does not vary by a significant amount year-to-year, the duration of a given bout of freezing can. This is especially true at the beginning and end of the winter season when temperatures fluctuate between freezing and non-freezing temperatures frequently. The wood frog has no capability to predict how long it will be frozen for, and thus one strategy is to reduce its metabolic rate to a level allowing it to survive an extend period of time, even if temperatures rise and thawing occurs in a short time frame. Since each organ and tissue has unique functions before, during, and after freezing, it is no surprise they each also have unique adaptations for surviving freezing temperatures.

During periods of freezing, skeletal muscle loses 22-36% of its water content as pure intracellular water is drawn across the membrane and incorporated into the growing ice crystal lattice (Lee *et al.* 1992). To counteract the stresses induced by cellular dehydration and volume loss, glucose is transported from the liver and pumped into skeletal muscle cells by plasma membrane glucose transporters where its colligative properties limit the loss of intracellular water (Storey 1987a; Costanzo *et al.* 1993). Since the ice crystals form in the extracellular space surrounding skeletal muscle, muscle tissue

is completely obstructed from contracting during freezing (Layne 1992). Without the ability to perform one of its primary function (contraction), skeletal muscle necessarily exists in a dormant state while frozen. To reduce the unnecessary use of energy resources, the wood frogs employs biochemical changes specific to skeletal muscle. For example, during freezing calcium uptake by the sarcoplasmic reticulum is suppressed to just 8% of unfrozen levels (Hemmings and Storey 2001). Since muscle contractions will not occur when the wood frog is frozen, energy is conserved by limiting processes that would allow contraction to take place. It's also been shown that unlike in the liver, skeletal muscle glycogen levels do not change during freezing, meaning that energy requirements are low enough for an alternative source, such as phosphocreatine, to take over (Storey and Storey 1984; Storey 1984; Hemmings and Storey 2001; Dieni and Storey 2009). This hypometabolic state provides the conditions necessary for surviving the unknown length of time until temperatures rise and thawing occurs.

Biochemical and molecular changes in skeletal muscle during freezing are almost certainly modulated by transcriptional and translational regulation. As mentioned previously, the serial nature of gene expression means that through transcriptional regulation, there is indirect regulation of translation as well. Since transcription and translation are highly energy intensive, this top-down regulation of molecular and biochemical processes within the cell is one of the most efficient in terms of saving energy resources. Regulation at any further stage (e.g. mRNA or protein level) would necessarily use more energy since energy must be invested just to progress to that stage of gene expression. The wood frog is dependent on metabolic rate depression and the

conservation of energy to survive the winter, and thus regulation of transcription may play a key role in survival, especially in a dormant tissue such as skeletal muscle.

Histone methylation is one method for regulating gene expression at the transcription level (Zhang and Reinberg 2001; Berger 2002; Martin and Zhang 2005). The functional outcome of histone methylation depends on which lysine residue is methylated, and the degree to which it is methylated (mono-, di-, or trimethylated) (Lachner *et al.* 2001; Santos-Rosa *et al.* 2002; Sims and Reinberg 2006). Certain methyl-histone marks are associated with transcriptional activation (H3K4me1 and H3K27me1) (Schneider *et al.* 2004; Martin and Zhang 2005), while others are the opposite, silencing transcription and changing the conformation of chromatin (H3K9me3 and H3K36me2) (Peters *et al.* 2002; Keogh *et al.* 2005). These mechanisms for promoting or silencing gene transcription can act on any gene (Schubeler *et al.* 2004; Pokholok *et al.* 2005; Barski *et al.* 2007), and thus can be used genome wide and may be used in a coordinated fashion in animals that undergo MRD. If the wood frog uses histone methylation in skeletal muscle to aide its entrance into a hypometabolic state, a few outcomes may be seen with regard to methylated histone residues. Possibilities include: transcriptionally permissive methyl-histone marks (H3K4me1 and H3K27me1) may decrease, transcriptionally repressive methyl-histone marks (H3K9me3 and H3K36me2) may increase, or some combination of the two. Whichever of these outcomes is the case, the results will help us better understand how skeletal muscle in the wood frog can enter and survive in a dormant state throughout periods of freezing.

In this chapter, the protein levels of HMTs were measured in response to the freeze-thaw cycle in skeletal muscle of the wood frog. Protein levels of seven HMTs

(SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8) were measured in control, 24 h freezing, and 8 h thaw frogs. Additionally, the enzymatic activity of HMTs on H3K4 were measured, as well as the levels of methylated histone residues (H3K4me1, H3K9me3, H3K27me1, and H3K36me2). Finally, the non-histone HMT targets p53, and its methylated forms p53k370me2, and p53k373me1 were measured in the skeletal muscle to help elucidate the range of functions these proteins have during freeze tolerance in the wood frog. Overall, the results show that HMTs and their targets are differentially expressed and methylated during freezing and thaw, and suggests that histone methylation may be used to suppress transcription in skeletal muscle during periods of freezing.

3.2 Methods and materials

3.2.1 Animal experiments

Animal experiments were performed as described in **Chapter 2.1.**

3.2.2 Total soluble protein extraction

Total soluble protein extraction was performed on skeletal muscle as described in **Chapter 2.2.**

3.2.3 Western blotting

Western blotting was performed on skeletal muscle as described in **Chapter 2.3.** Protein levels of the seven studied HMTs (SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8), the histone residues they methylate (H3K4me1, H3K9me3, H3K27me1, and H3K36me2), and non-histone targets (Total p53, p53k370me2, and p53k372me1) were measured. See ***Appendix A**: **Antibody information and suppliers for western blotting*** for a full list of antibodies used, and ***Appendix B**: **Western blotting conditions*** for a detailed list of western blotting parameters for each target.

3.2.4 Histone methyltransferase activity assays

Histone methyltransferase activity on H3K4 was measured in skeletal muscle as described in **Chapter 2.4** using the Epigentek EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kit (Catalogue # P-3002-96).

3.2.5 Quantification and statistics

Quantification and statistics were performed as described in **Chapter 2.5.**

3.3 Results

3.3.1 HMT protein expression in skeletal muscle

Relative protein expression levels of seven HMTs (SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8) were measured by western blotting in skeletal muscle across the freeze-thaw cycle of the wood frog, *Rana sylvatica* (**Fig. 3.1**). The HMTs that methylate H3K4 (SETD7, RBBP5, ASH2L, and SMYD2) showed varying responses to 24 h freezing and 8 h thaw. ASH2L protein levels decreased to 50 ± 5% of control values during 24 h freezing and remained at that level during 8 h thaw. SETD7, RBBP5, and SMYD2 showed no significant change across experimental conditions. As for the HMTs that methylate H3K9, SUV39H1, increased 1.4 ± 0.13 fold during 8 h thaw, while EHMT2 had no significant change. SET8 decreased to 40 ± 6% of control during 24 h freezing and remained at that level for 8 h thaw.

3.3.2 HMT enzymatic activity in skeletal muscle

Total HMT activity to monomethylate H3K4 was measured in skeletal muscle (**Fig. 3.2**) across the freeze-thaw cycle using the Epigentek EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kit as in **Chapter 2.4**. Relative activity decreased to 50 ± 10% of control values after 24 h freezing, and further decreased to 26 ± 10% of control values after the 8 h thaw (Actual control value: 5.04 ± 0.42 ng/h/mg).

3.3.3 Methylated histone residues in skeletal muscle

The relative levels of methylated histone residues (H3K4me1, H3K9me3, H3K27me1, and H3K36me2) in skeletal muscle were measured by western blotting during control, 24 h freezing, and 8 h thaw (**Fig. 3.3**). H3K4me1 levels showed a significant decrease to 2 ± 1% and 4 ± 1% of control values during both 24 h freezing and 8 h thaw respectively. H3K9me3 did not change across the three conditions, while H3K27me1 decreased to 20 ± 4% of control values during 24 h freezing and maintained that level during 8 h thaw. H3K36me2 also showed no change during 24 h freezing and 8 h thaw.

3.3.4 Methylated p53 in skeletal muscle

Relative protein levels of p53 and two methylated p53 species (p53K370me2 and p53K372me1) were measured by western blotting in skeletal muscle (**Fig. 3.4**). Total p53 proteins levels remained constant across the experimental conditions, however changes in both methylated p53 species were observed. p53K370me2 increased 2.0 ± 0.13 fold in response to 24 h freezing, and increased further to 2.3 ± 0.34 during the 8 h thaw, whereas p53K372me1 decreased to 70 ± 5% of control values after 24 h freezing and further decreased to 40 ± 1% of control values in response to 8 h thaw.

3.4 Discussion

HMTs have been implicated in a wide range of cellular functions (Zhang *et al.* 2005; Huang *et al.* 2007; Shi *et al.* 2007; Rathert *et al.* 2008), however since their primary function is to modify histones, it comes as no surprise that most research has characterized their functional outcomes with respect to gene regulation. Unlike other families of histone modifying enzymes such as HATs and HDACs, HMTs can be both positive and negative regulators of transcription depending on which histone lysine residue they methylate, and to which degree (mono, di, or tri) it becomes methylated (Kouzarides 2002; Barski *et al.* 2007; Kouzarides 2007). Although transcriptional regulation and output has been characterized in the wood frog and other freeze tolerant animals (Hofmann and Hand 1994; Frerichs *et al.* 1998; Fraser *et al.* 2001; Larade and Storey 2002; Storey and Storey 2004a), the role of HMTs in freezing survival, and metabolic rate depression in general, has yet to be explored. Skeletal muscle provides an ideal starting point because of the striking changes this tissue undergoes when going through the freeze-thaw cycle. During freezing, skeletal muscle is completely restricted from contracting by physical interactions with the surrounding ice, but also metabolically restricted by the ischemic state the frog enters during freezing (Layne 1992; Irwin *et al.* 2003). Since skeletal muscle cannot produce ATP aerobically during freezing, and is limited to a finite supply of energy resources, it must enter a hypometabolic state and reduce all unnecessary cellular processes, including transcription. This study aims to provide the first characterization of HMTs and their downstream targets (histone and non-histone proteins) throughout the freeze-thaw cycle in skeletal muscle of the wood frog, and successfully demonstrate evidence to further this line of research.

The functional outcome of histone methylation relies on HMTs since they are the enzymes that catalyze the reaction. Each HMT is specific to a given target (Tachibana *et al.* 2001; Xiao *et al.* 2005; Fritsch *et al.* 2010),therefore, changes in methylation of an HMT target may be implied through the levels and activity of a specific HMT itself. Thus, the logical starting point for this study is to examine the relative protein expression levels of HMTs through the freeze-thaw cycle. The seven HMTs I measured can be grouped together by the histone lysine residues they target. By examining the measured HMTs in this way, we can see similarities between HMTs that methylate the same target (**Fig. 3.1**). For example, the HMTs that monomethylate H3K4 (SETD7, RBBP5, ASH2L, and SMYD2) were downregulated during freezing and thaw or did not change significantly compared to the control condition. Although there are other HMTs that monomethylate H3K4, this may suggest a decrease in net methyltransferase capacity on H3K4 during freezing and thaw. Since H3K4me1 is tightly associated with transcriptional activation (Schneider *et al.* 2004; Barski *et al.* 2007; Heintzman *et al.* 2007), a decrease in HMTs that monomethylate H3K4 could be an indication that transcription may be reduced in the skeletal muscle during periods of freezing in order to establish a hypometabolic state. The HMTs that trimethylate H3K9 (SUV39H1 and EHMT2) did not change significantly between control and frozen frogs, while SUV39H1 increased after thawing (**Fig. 3.1**). Interestingly, it may be expected that these HMT would increase during freezing to increase the amount of transcriptionally repressive H3K9me3. However, the same outcome (e.g. silencing transcription) may be accomplished by decreasing HMTs that methylate transcriptionally permissive lysine residues (H3K4me1 and H3K27me1) while maintaining those that silence (H3K9me3

and H3K36me2). This strategy for decreasing transcription by downregulating HMTs that methylate transcription activators is further exemplified in these results by SET8, which is heavily downregulated during freezing and thaw in skeletal muscle (**Fig. 3.1**). SET8 monomethylates H4K20 (Nishioka *et al.* 2002; Xiao *et al.* 2005), which is found in the promotor regions of highly transcribed genes (Barski *et al.* 2007; Wang *et al.* 2008), and thus the global downregulation of SET8 could decrease transcriptional activity across the genome. While the exact relationship between these HMTs and transcriptional activity cannot be conclusively determined by this study, these results indicate that these proteins may be involved in the response to freezing temperatures in skeletal muscle, and likely play a role in transcriptional silencing.

To determine the net effect freezing has on the HMTs that methylate H3K4, I measured the total HMT enzymatic activity that monomethylates H3K4 (**Fig. 3.2**). Activity levels of HMTs that monomethylate H3K4 significantly decrease during freezing and thaw. These result align with the protein levels of the measured HMTs that methylate H3K4 (**Fig. 3.1**), in that they were either downregulated or did not change during freezing and thaw. The cause of this decrease in activity is due to either a decrease in protein levels, or due to negatively regulating the activity of these HMTs. Although which of these possibilities is responsible cannot be conclusively determined, these results show a coordinated decrease in the methylation of this transcriptionally permissive residue in skeletal muscle.

The effect HMTs have on transcription is mediated through the methylated histone residue itself by its interaction with effector proteins such as methyl-binding proteins, or other epigenetic writers and erasers (Lachner *et al.* 2001; Fischle *et al.* 2005;

Flanagan *et al.* 2005; Adams-Cioaba and Min 2009). Methylated lysine residues can act as binding sites for transcription regulating proteins (Lachner *et al.* 2001; Fischle *et al.* 2005), or can enhance or repress the binding capability of nearby sites (Ben-Porath and Cedar 2001; Lee and Zhang 2008; Jobe *et al.* 2012). It is these interactions that determine whether transcription is activated or silenced in selected genes. Thus, in order to further characterize the role HMTs play in freeze tolerance in skeletal muscle, I measured the relative methylation levels of the lysine residues that the HMTs previously discussed methylate. Four methyl-lysine residues, H3K4me1, H3K9me3, H3K27me1, and H3K36me2, were measured in skeletal muscle of the wood frog through the freeze-thaw cycle (**Fig. 3.3**). These results show a tight correlation between residues that have the same end function. The two methyl-lysine residues that are associated with transcriptional activation, H3K4me1 and H3K27me1, are both significantly downregulated during freezing and thaw. This decrease in H3K4me1 aligns with the previous results on the proteins levels and activity of the HMTs that methylate H3K4. The wood frog is decreasing the activity of HMTs that methylate H3K4 (and likely with H3K27 as well), either by reducing protein levels or by negative regulation of protein activity, which is translating into a significant decrease in H3K4me1 and H3K27me1 during freezing. The other two methyl-lysine residues measured in this study, H3K9me3 and H3K36me2, are both associated with negatively regulating transcription. Both of these targets maintained the same level of methylation across the experimental conditions. These four methyl-lysine residues paint a picture of net transcriptional downregulation through the decrease of positively regulating methyl-lysine residues coupled with the maintenance of negatively regulating residues.

These results fit with what is already known about skeletal muscle in the wood frog during periods of freezing. Once ice nucleation begins, the metabolic landscape of the skeletal muscle changes rapidly (Storey and Storey 1984). With enough ice buildup the heart and lungs cease to function and the frog becomes ischemic (Storey and Storey 1984; Layne *et al.* 1989). This causes the skeletal muscle to switch energy production to an anaerobic method as cellular oxygen levels are quickly depleted. The result is a cellular environment that must survive an unknown length of time with a limited supply of energy. The best way to ensure survival is to ration these cellular resources, such as glycogen stores, and only use them for necessary processes. During freezing, one of the primary function of skeletal muscle, contraction is restricted, and thus proteins needed for this function are in lower demand. Skeletal muscle is also a non-proliferative tissue, meaning processes such as the cell-cycle can also be suppressed during freezing to conserve energy, which has been demonstrated previously (Zhang and Storey 2012). The most efficient method for capitalizing on energy saving is thus not to produce an abundance of proteins involved in these processes during freezing. With these savings, the wood frog can energetically afford to put in place adaptations needed for surviving freezing, such as antioxidants to deal with ischemia reperfusion injury when undergoing repeating freeze-thaw cycles (Joanisse and Storey 1996). It is at this stage that I propose histone methylation may play a role in freezing survival. Since histone methylation can result in both transcriptional activation and repression, there exists the possibility it is used to decrease transcription for the purpose of lowering the overall metabolic rate. My results lend evidence to this idea in that the methyl-histone residues associated with transcriptional activation, H3K4me1 and H3K27me1, were both significantly

downregulated during freezing, while the repressive methyl-histone residues, H3K9me3 and H3K36me2, were maintained. These results are reflected in the levels of HMTs that methylate these histone residues, and in the case of H3K4, methyltransferase activity. This shift in transcription regulatory equilibrium favors silencing, and therefore it may be a mechanism used in skeletal muscle to conserve energy. By establishing a new equilibrium between energy use and energy production through the conservation of energy, the wood frog extends the period of time it can live off of the finite supply of energy resources it has accumulated and can ultimately survive the period of freezing.

Histones are not the only type of proteins that HMTs can methylate. Although histones are the most studied substrate, in recent years the number of non-histone proteins that have been discovered to be regulated by methylation has grown substantially, and is expected to continue rising (Biggar and Li 2015). Methylation is similar to other post-translational modification, such as phosphorylation, in that it can regulate the function of the target protein, its subcellular localization, and inhibit proteasomal degradations (Hershko *et al.* 1991; Huang *et al.* 2007; Munro *et al.* 2010; Dhayalan *et al.* 2011; Calnan *et al.* 2012; Kassner *et al.* 2013; Biggar and Li 2015). The transcription factor p53 is regulated by a multitude of post-translational modifications (Tibbetts *et al.* 1999; Barlev *et al.* 2001; Tang *et al.* 2008; Lee and Gu 2009), including methylation, making it particularly interesting in the context of this study. p53 plays a part in many cellular processes such as the cell cycle (Agarwal *et al.* 1995), apoptosis (Lowe *et al.* 1993), and energy metabolism (Puzio-Kuter 2011), and thus could play a major role in the wood frog's survival during periods of freezing. In this study I look at two methyl-lysine residues on p53 (p53K370me2 and p53K372me1). When these lysine residues are

methylated, p53 is localized to the nucleus and its stability as a transcription factor increases. This results in more p53 binding to transcription factor binding sites, and increased transcription of the genes it regulates (Chuikov *et al.* 2004; Huang *et al.* 2007). My results show that although protein levels of total p53 do not change in skeletal muscle, there is differential regulation of the two methylated residues (**Fig. 3.4**). The most prominent change being a 2-fold increase in p53K370me2 during freezing and thaw. Since methylation of this residue is associated with p53 activation by increasing its association with p53BP1 (Chuikov *et al.* 2004), these results suggest an active role for p53 during and after periods of freezing in skeletal muscle. To conserve as much energy as possible, energetically expensive processes other than transcription, such as the cell cycle, should be reduced as well. The wood frog has previously been shown to initiate cell cycle suppression during periods of freezing (Zhang and Storey 2013), which was suggested as one method for conserving energy during a time in which energy resources are finite. Other species that undergo metabolic rate depression to survive harsh environmental conditions also show a similar trend of increasing p53 activity. The anoxia tolerant turtle, *Trachemys scripta elegans,* showed an increase in p53 protein levels (Zhang *et al.* 2013), whereas phosphorylation of p53 serine-15, which increases p53 DNA damage repair, was seen to increase in the liver of the hibernating gray mouse lemur, *Microcebus murinus* (Biggar *et al.* 2015). Since p53K370me2 increases p53 activity and the activity of p53BP1 (Chuikov *et al.* 2004), and p53BP1 is involved in initiating cell cycle checkpoints (Fernandez-Capetillo *et al.* 2002), p53 methylation may be contributing to cell cycle arrest in skeletal muscle during freezing. p53K370me2 may also be involved in the DNA damage response due to ischemia-reperfusion injury when

undergoing multiple freeze-thaw cycles (Joanisse and Storey 1996). Both p53K370me2 and subsequent p53BP1 activation have been implicated in DNA damage response pathways (Huang *et al.* 2007), and although the wood frog has well adapted antioxidant pathways, the methylation of p53 may serve as a further protection. These results fall into one of the main themes of freeze tolerance, halting resource intensive process to conserve energy until thawing occurs.

In summary, the present chapter provides evidence that methylation is involved in the metabolic rate depression of skeletal muscle in the wood frog during periods of freezing. Although more details will need to be elucidated, the data here suggests that the wood frog is reducing the amount of transcriptionally permissive methyl-histone residues (H3K4me1 and H3K27me1), while maintaining the residues that silence transcription (H3K9me3 and H3K36me2), and may be doing so by regulating the amount and activity of corresponding HMTs. There is now also evidence that methylation of p53 in skeletal muscle may be involved in metabolic rate depression in these animals as well, possibly by halting cell cycle progression. Continuing this line of research will help elucidate the complex mechanisms used by skeletal muscle in the wood frog to survive freezing, which could have implications for many other biological problems such as muscle preservation and stresses induced by cold or freezing temperatures.

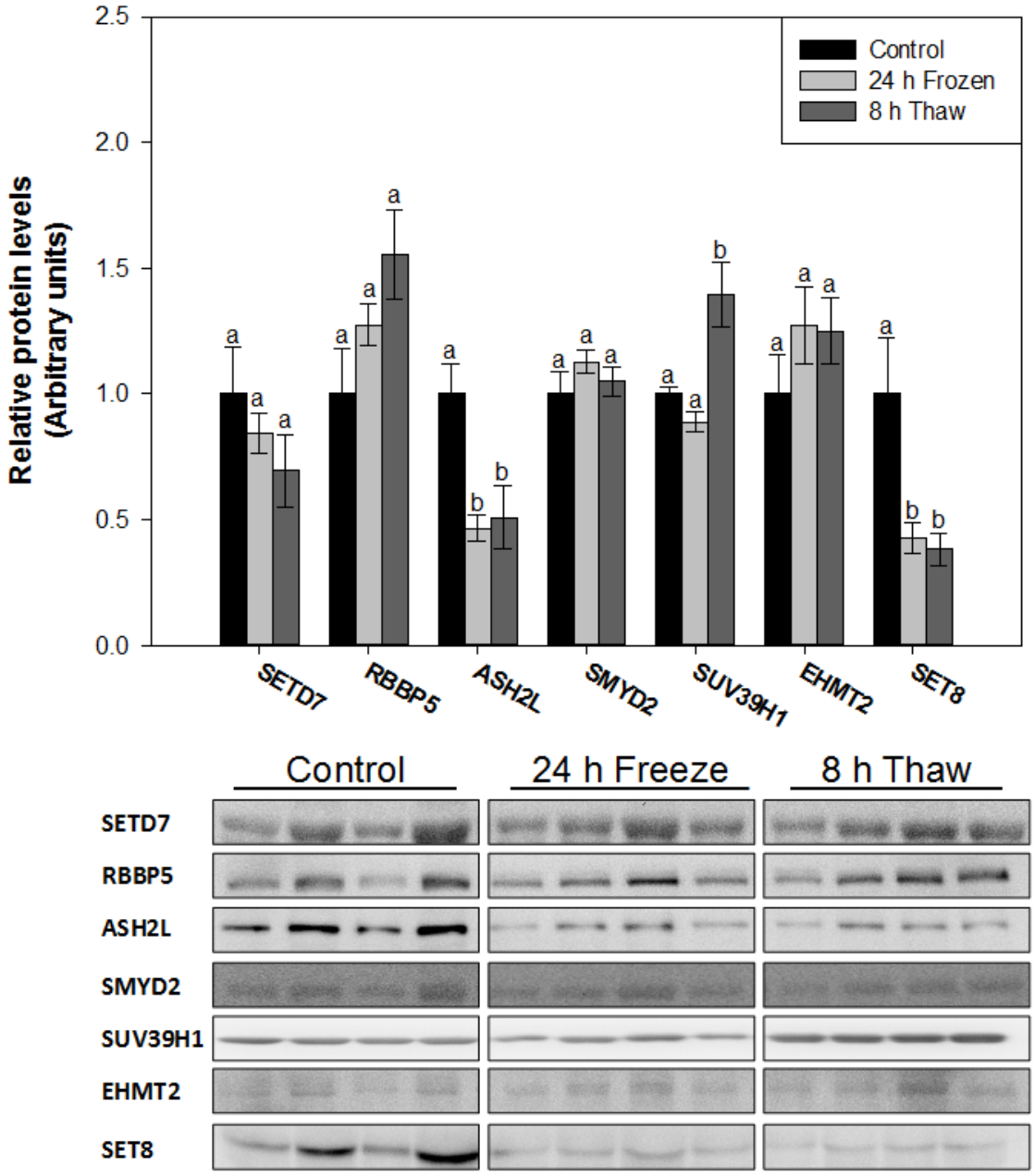

Figure 3.1: Effects of 24 h freezing and 8 h thaw on relative proteins levels of SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8 methyltransferases in *R. sylvatica* skeletal muscle as determined by Western immunoblotting. Data are mean ± SEM, n = 4 independent trials on samples from different animals. For each target measured, values that are not statistically different from each other ($P > 0.05$) share the same letter notation.

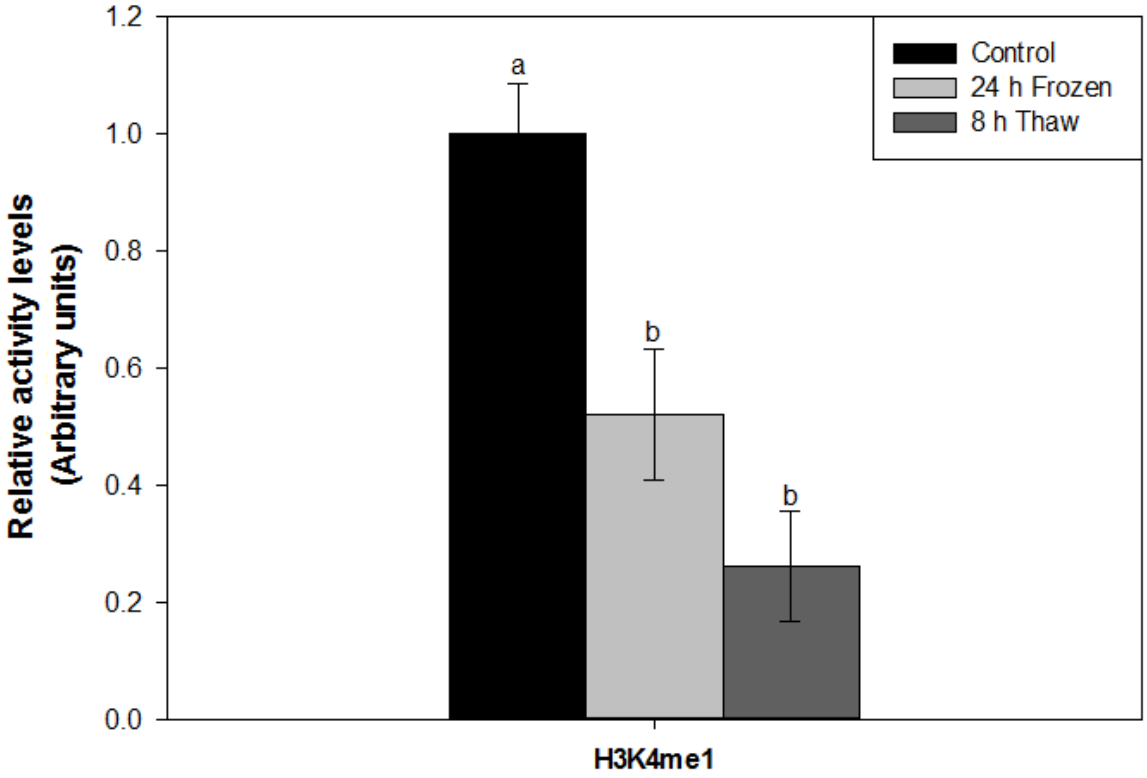

Figure 3.2: Relative HMT activity on H3K4 in skeletal muscle of *R. sylvatica* comparing the effects of 24 h freezing and 8 h thaw. Actual control value is 5.04 ± 0.42 ng/h/mg. Control, 24 h frozen, and 8 h thaw values are reported relative to this value. Data are mean ± SEM, n = 4 independent trials on samples from different animals. Values that are not statistically different from each other ($P > 0.05$) share the same letter notation.

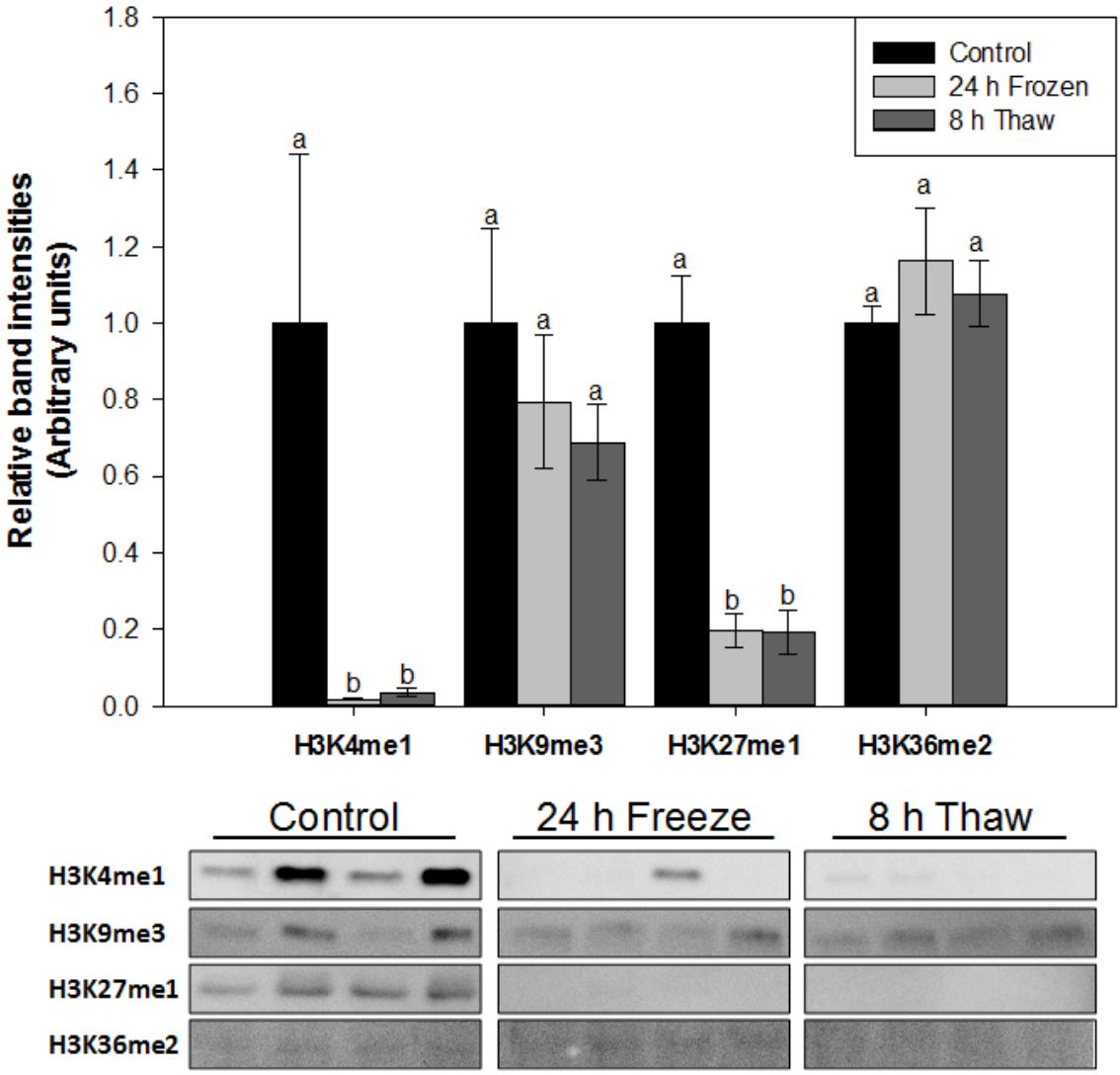

Figure 3.3: Effects of 24 h freezing and 8 h thaw on relative protein levels of H3K4me1, H3K9me3, H3K27me1, and H3K36me2 in *R. sylvatica* skeletal muscle. All other information as in Fig. 3.1.

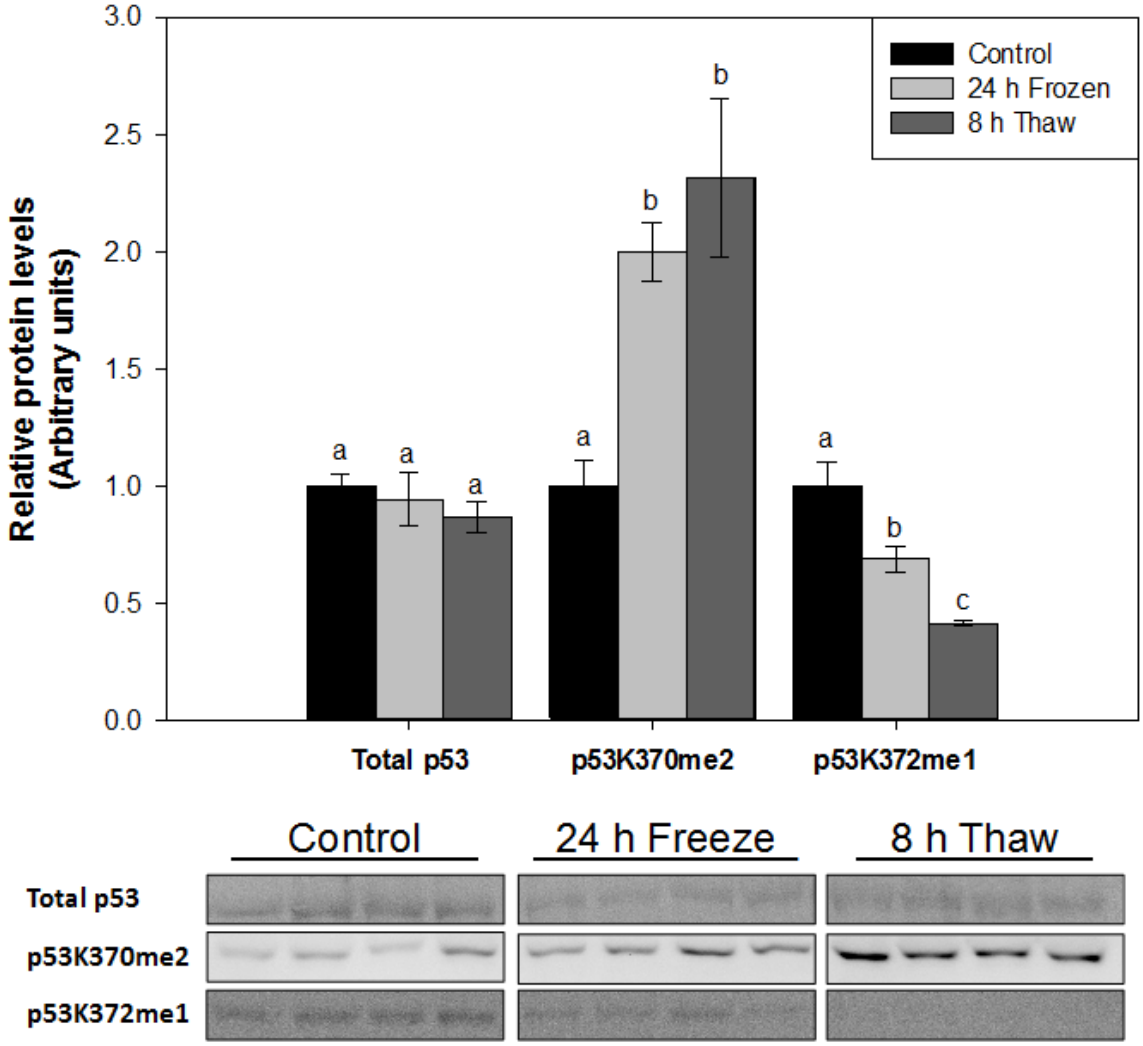

Figure 3.4: Effects of 24 h freezing and 8 h thaw on relative protein levels of total p53, p53K370me2, and p53K372me1 in *R. sylvatica* skeletal muscle. All other information as in Fig. 3.1

Chapter 4

Histone Methylation in Liver Tissue

4.1 Introduction

Wood frog liver is responsible for the production and distribution of cryoprotectant glucose to the entire body before and during freezing. Glucose is an ideal cryoprotectant in the wood frog for multiple reasons: (1) it is a small molecule with a low molecular weight (2) it has a high solubility (3) it does not interfere with the function of proteins (4) it is easier to produce than the polyhydric alcohols produced by other freeze tolerant organisms, and, (5) it is easily transported through the blood stream and across cell membranes (Storey and Storey 2004b). Glucose acts in a colligative fashion to prevent intracellular ice formation, and potential damage from ice formed in extracellular spaces. High glucose acts in a similar manner to other osmolytes in that it lowers the freezing point of fluid by disrupting the ordered organization of hydrogen bonds between water molecules that would otherwise form ice. When glucose is accumulated intracellularly, it also counteracts the osmotic gradient across the cell membrane when extracellular water is incorporated into the ice crystal lattice, leaving behind the osmolytes previously dissolved in that fluid. This means that less water is drawn out of the cell, thus preventing cell volume from dropping below the critical minimum at which membrane structure (and hence proper function) breaks down due to compression stress that forces the bilayer structure into an amorphous gel state. In preparation for winter, the wood frog accumulates large stores of liver glycogen in the late summer and early fall (Storey and Storey 1984). These glycogen stores are the principle source of cryoprotectants once freezing is initiated, since glycogen levels in other tissues, such as skeletal muscle, do not decrease during freezing (Storey 1984). Once an ice nucleation occurs event occurs, β-adrenergic signal cascades trigger the breakdown of glycogen to

free glucose via glycogenolysis, however, it has yet to be elucidated whether this is the main mechanism responsible for instigating other wood frog freeze-tolerance adaptations (Storey 1987a; Hemmings and Storey 1994; Storey and Storey 1996). The pathway from glycogen to free glucose is heavily regulated since its intermediates can also take part in other pathways (Cowan and Storey 2001), and thus regulation of the enzymes involved can direct the breakdown of glycogen into glucose. For example, the glycogenolytic product glucose 6-phosphate (G6P) can either be converted to glucose by glucose 6-phosphatase (G6Pase), catabolized via glycolysis, or enter the pentose phosphate pathway through the action of glucose 6-phosphate dehydrogenase (G6PDH). Due to the negative regulation of G6PDH and of glycolytic enzymes (mainly phosphofructokinase), G6P derived from glycogen follows the metabolic route to free glucose and glucose levels in the liver quickly rise within minutes of ice nucleation (Storey and Storey 1985a; Cowan and Storey 2001). Liver glycogen not only acts as a source of glucose for the liver, but also for the rest of the body. Glucose from the liver is shuttled out and results in extremely high blood glucose levels (Storey and Storey 1984; Storey 1984; Storey and Storey 1985a), and subsequent transport of glucose to all other tissues and organs (Storey 1984; Storey and Storey 1986). Not only does the production of glucose happen in a rapid fashion at the onset of ice nucleation, but glucose levels continue to increase for days after whole body freezing occurs (Storey and Storey 1984). This process exemplifies the critical role the liver plays during freezing and, that despite the frog being in a global hypometabolic state where most other tissues and organs are dormant, key liver processes that facilitate successful freezing survival remain active.

Although many processes in the liver are downregulated during freezing (Cowan and Storey 2001; Rider *et al.* 2006; Zhang and Storey 2012), some metabolic activity is retained for the production of the cryoprotectant glucose throughout the entire freezing period (Storey and Storey 1984; Storey and Storey 1986; Storey 1987b). Although the best studied upregulated process during freezing is glycogenolysis, select other processes and pathways are upregulated as well, including the expression of novel freeze-responsive proteins (Cai *et al.* 1997; Cai and Storey 1997b; Cai and Storey 1997a; McNally *et al.* 2002; McNally *et al.* 2003). Through differential cDNA library screening of the wood frog liver, it was found that fibrinogen, a protein involved in blood clot formation, was significantly upregulated during freezing (Cai and Storey 1997a). One of the main dangers of ice formation is the risk of physical damage to tissues by the ice crystals themselves. When temperatures rise and thawing occurs, any shearing or puncture damage to tissues or capillaries by ice crystals would be revealed as internal bleeding. Therefore, an anticipatory response that increased the supply of fibrinogen would aid in the formation of blood clots to repair the tissue quickly. Also identified as upregulated in the cDNA screening was mitochondrial ADP/ATP translocase (AAT) gene sand protein (Cai *et al.* 1997). The authors postulated that this increase was an adaptive response to the anoxia/ischemia that occurs during freezing to help preserve the intracellular adenylate pool size. This would both save energy, and allow for the quick rephosphorylation of adenine nucleotides when thawing occurs and oxidative phosphorylation resumes. Three novel freeze-induced genes, *fr10*, *li16*, and *fr47* were also found to be expressed in a freeze-responsive manner (Cai and Storey 1997b; McNally *et al.* 2002; McNally *et al.* 2003). Although the exact functions of the proteins

encoded by these genes have not been determined, evidence from these studies show they are most likely involved in adaptive responses to cellular volume changes, ischemia resistance, and the protein kinase C-mediated pathway, respectively. Fr10 in particular has been explored in further details, with evidence suggesting it can limit the recrystallization of small extracellular ice crystals into larger ones (Biggar *et al.* 2013). These findings demonstrate that novel freeze-specific genes exist and that their expression is in response to freezing itself, and thus must be facilitated in some way by active transcriptional regulation.

One mechanism that could potentially be involved in facilitating the selective gene activation required for freeze tolerance is the regulation of histone methylation. Histone methylation can result in both positive and negative regulation of gene transcription depending on the target residues and the degree of methylation (Kouzarides 2002; Kouzarides 2007). This dual regulation suggests that histone methylation may be involved in both downregulating some liver processes, while simultaneously increasing pro-survival processes during freezing. To date, it is unknown how the levels of individual HMTs and methyl-histone residues are regulated during periods of environmental stress, and elucidating these mechanisms will result in a better understanding of the role histone methylation plays in metabolic rate depression and freeze tolerance.

In the present chapter, responses of HMTs to the freeze-thaw cycle of the wood frog are characterized in the liver. Proteins levels of seven HMTs (SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8) were measured by western blotting in control, 24 h frozen, and 8 h thawed frogs. Furthermore, both the total methyltransferase

activity on specific histone residues (H3K4 and H3K9), and the levels of methylated histone residues (H3K4me1, H3K9me3, H3K27me1, and H3K36me2) were measured. Additionally, the protein levels of a non-histone target, the p53 tumor suppressor protein and its methylated forms (p53K370me2 and p53K372me1) were quantified by western blotting. These findings show levels of HMTs, their enzymatic activities, and the targets they methylate and suggest a role for HMTs in the regulation of freeze tolerance in wood frog liver.

4.2 Methods and materials

4.2.1 Animal experiments

Animal experiments were performed as described in **Chapter 2.1.**

4.2.2 Total soluble protein extraction

Total soluble protein extraction was performed on liver tissue as described in **Chapter 2.2.**

4.2.3 Western blotting

Western blotting was performed on liver tissue extracts as described in **Chapter 2.3.** Protein levels of the seven HMTs (SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8), the histone residues they methylate (H3K4me1, H3K9me3, H3K27me1, and H3K36me2), and non-histone targets (Total p53, p53k370me2, and p53k372me1) were measured. See ***Appendix A**: **Antibody information and suppliers for western blotting*** for a full list of antibodies used, and ***Appendix B**: **Western blotting conditions*** for a detailed list of western blotting parameters for each target.

4.2.4 Histone methyltransferase activity assays

Histone methyltransferase activity on H3K4 and H3K9 was measured in liver tissue extracts as described in **Chapter 2.4** using the Epigentek EpiQuik Histone

Methyltransferase Activity/Inhibition Assay Kit (H3K4-Catalogue # P-3002-96; H3K9-Catalogue # P-3003-96).

4.2.5 Quantification and statistics

Quantification and statistics were performed as described in **Chapter 2.5.**

4.3 Results

4.3.1 HMT protein expression in liver tissue

Relative protein levels of seven HMTs (SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8) were measured by western blotting in the liver across the wood frog freeze-thaw cycle (**Fig. 4.1**). The HMTs that methylate H3K4 (SETD7, RBBP5, ASH2L, and SMYD2) showed differential protein levels after 24 h freezing and 8 h thaw. SETD7 levels significantly increased 1.6 ± 0.08 fold during 24 h freezing, and remained elevated at 1.8 ± 0.16 fold during 8 h thaw, whereas RBBP5 did not change across the experimental conditions. SMYD2 and ASH2L significantly decreased to 30 ± 8% and 20 ± 2%, respectively, of the control value during 24 h freezing, and whereas ASH2L returned to above control levels (1.4 ± 0.13 fold), SMYD2 content remained suppressed after 8 h thaw (10 ± 2% of control values). The HMTs that methylate H3K9 (SUV39H1 and EHMT2) did not change across the experimental conditions, and SET8 also remained constant.

4.3.2 HMT enzymatic activity in liver tissue

Total methyltransferase activity on two histone lysine residues (H3K4 and H3K9) were measured in the wood frog liver across the freeze-thaw cycle (**Fig. 4.2**) using the Epigentek EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kit as in **Chapter 2.4.** Relative HMT activity to monomethylate H3K4 significantly decreased after 24 h freezing to 20 ± 7% and 30 ± 4% after 8 h thaw of control values (Actual control value: 21.1 ± 4.48 ng/h/mg). Likewise, the relative methyltransferase activity to

trimethylate H3K9 also significantly decreased after 24 h freezing to 40 ± 10% of control values and then increased to 80 ± 4% of control values (Actual control value: 3.28 ± 4.48 ng/h/mg).

4.3.3 Methylated histone residues in liver tissue

Relative methylation levels on four histone 3 residues (H3K4me1, H3K9me3, H3K27me1, and H3K36me2) were measured in wood frog liver across the freeze-thaw cycle using western blotting (**Fig. 4.3**). Levels of H3K4me1 were observed to significantly decrease after 24 h freezing to 24 ± 5% of the control value, and remained low at 30 ± 3% of the control values after 8 h thaw, whereas H3K9me3 did not change between the three conditions. H3K27me1 remained constant between control and 24 h freezing, relative methylation at this site increased 2.8 ± 0.62 fold during 8 h thaw. H3K36me2 content significantly increased by 1.3 ± 0.05 fold after 24 h freezing and then decreased to 60 ± 9% of the control after 8 h thaw.

4.3.4 Methylated p53 in liver tissue

The relative protein levels of p53 and the relative methylation status of two lysine residues on the protein, p53K370me2 and p53K372me1, were measured by western blotting in wood frog liver extracts across the freeze-thaw cycle (**Fig. 4.4**). Total p53 as well as p53K370me2 and p53K372me1 content all remained constant over the freeze-thaw cycle.

4.4 Discussion

Many studies have examined the role that post-translational modifications play in animals that undergo metabolic rate depression (Whitman and Storey 1990; Dieni and Storey 2011; Zhang *et al.* 2013; Childers and Storey 2016; Logan *et al.* 2016; Gerber *et al.* 2016) With that said, only a few have examined epigenetic modifications, i.e. those that were identified in association with DNA or histone control (Krivoruchko and Storey 2010; Rouble and Storey 2015; Storey 2015; Wijenayake and Storey 2016). Until now, none have examined the freeze-responsive epigenetic mechanisms of the wood frog, nor has histone methylation involvement in metabolic rate depression been examined in any of these animals. This chapter aims to build on the epigenetic findings described in wood frog skeletal muscle (**Chapter 3**) by examining the role that histone methylation plays in the liver, an organ crucial for freezing survival.

The liver of the wood frog is the primary source of cryoprotectant synthesis once ice nucleation occurs (Storey and Storey 1985a; Storey and Storey 1986; Storey 1987a). The glycogenolytic enzymes responsible for the conversion of glycogen to glucose remain active not only in the initial glucose loading phase shortly after ice nucleation begins, but also throughout the freezing period when the blood plasma is frozen (Storey and Storey 1984; Crerar *et al.* 1988). Since glycogenolytic activity continues for days while the frog is frozen (Storey and Storey 1984), this pathway is most likely supported through the transcription and translation of new enzymes, rather than solely relying on modulating the activity of the enzymes expressed prior to freezing. The upregulation of a select number of other genes has been discovered during freezing in the liver as well. Significant increases in fibrinogen are seen during freezing, which is most likely a

preemptive repair mechanism (Cai and Storey 1997a). Since fibrinogen is involved in forming blood clots, it may be produced and stored during freezing for use when thawing occurs, where any damage from ice crystals becomes evident. This would allow the wood frog to quickly clot areas of bleeding and increase chances of survival. Mitochondrial ADP/ATP translocase (AAT) is also known to be upregulated in response to freezing (Cai *et al.* 1997). While these process and the genes involved have been thoroughly characterized, the wood frog also expresses novel freeze-responsive genes not found in any other organisms to date. These freeze-responsive genes*, fr10, li16*, and *fr47*, unsurprisingly seem to be involved in adaptive responses to various stresses involved with freezing and thawing (Cai and Storey 1997b; McNally *et al.* 2002; McNally *et al.* 2003). Since these processes and genes are expressed and upregulated during freezing, there must be positive transcriptional regulation involved. Histone methylation is in direct control of transcription, both in a positive and negative regulatory manner, and therefore it stands to reason that HMTs may be involved in conferring freeze tolerance adaptations in the liver, such as cryoprotectant production.

The seven HMTs measured in liver showed differential protein expression which can be classified by the histone residues that each HMT methylates (**Fig. 4.1**). Similar to muscle (**Fig. 3.1**), the HMTs that monomethylate H3K4 showed differential regulation between control, 24 h freezing, and 8 h thaw. Both ASH2L and SMYD2 were significantly downregulated during freezing, whereas RBBP5 did not change, and SETD7 increased significantly during freezing and thawing (**Fig. 4.1**). Since H3K4me1 is a transcriptional activator, it may be expected that the HMTs that methylate H3K4 would decrease during freezing since many processes and pathways in the liver are

downregulated. Indeed, with the exception of SETD7, the HMTs measured herein portray a trend of decreased H3K4 methylation during freezing that was further confirmed by the significantly reduced total activity of HMTs that methylate H3K4 (**Fig. 4.2**). Whether the reduced total activity of HMTs observed during freezing is modulated through levels of expression, or through post-translational regulation, the net effect they have on methyl-histone residue levels can be measured by their global activity. An example of post-translational modifications controlling HMT activity is the phosphorylation of RBBP5. When RBBP5 is phosphorylated at serine-350, it promotes WRAD complex formation (a complex containing WRD5, RBBP5, ASH2L, and DPY30) which in turn increases H3K4 methyltransferase activity (Zhang *et al.* 2015). Dephosphorylation of RBBP5 may be one method for post-translationally regulating total H3K4 methyltransferase activity, leading to interesting lines of research to be explored more thoroughly. These results show that global methyltransferase activity on H3K4 decreases significantly during freeze/thaw, even with the increased expression of SETD7 (**Fig. 4.2**). The discordance between SETD7 protein abundance levels and the other three HMTs that methylate H3K4 can be explained by the other function SETD7 has in the liver. Other than histone H3, SETD7 also methylates the nuclear farnesoid X receptor (FXR), increasing the activity of FXR, and thus positively regulating the expression of FXR target genes in the liver (Balasubramaniyan *et al.* 2012). Although FXR is primarily known for its role in bile acid biosynthesis, more evidence is suggesting it is also involved in glucose metabolism, modulating the expression of gluconeogenic enzymes such as PEPCK, G6Pase, and fructose 1,6-bisphosphatase (FBPase) (Yamagata *et al.* 2004; Stayrook *et al.* 2005; Ma *et al.* 2006). FXR expression has been shown to be upregulated when glucose levels are

high (Duran-Sandoval *et al.* 2004), and when stimulated it results in the negative regulation of gluconeogenesis (Ma *et al.* 2006). SETD7 may be methylating, and thus activating, FXR during and after freezing to downregulate gluconeogenesis especially at this time when glucose is overly abundant. This idea aligns with previous research showing gluconeogenesis suppression in the wood frog during freezing (Storey 1987a; Pinder *et al.* 1992; Kiss *et al.* 2011). If this is the case during freezing, it may hold true during thawing of the wood frog. By contributing to negative feedback mechanisms already in place to block gluconeogenesis during thawing, glucose levels are able to efficiently return to basal levels. This connection between FXR methylation and gluconeogenesis in freeze tolerance of the wood frog will require further exploration. The HMTs that methylate H3K9 (SUV39H1 and EHMT2) exhibited constant protein levels across the experimental conditions in liver, much like in skeletal muscle, while SET8 also did not change (**Fig. 4.1**). Interestingly, the total methyltransferase activity acting on the transcriptionally repressive H3K9me3 residue, which is involved in the formation of heterochromatin, decreased during freezing and then returned to control levels (**Fig. 4.2**). This change may be due to post-translational regulation of SUV39H1, which is known to be phosphorylated (Park *et al.* 2014), and/or changes in other HMTs, such as ESET (Schultz *et al.* 2002), not measured in this study that methylate H3K9.

The end result of changes in methyltransferase activity is the altered levels of methyl-histone residues and, as such, this study also analyzed levels of two transcriptionally active histone marks, H3K4me1 and H3K27me1, and two that are repressive, H3K9me3 and H3K36me2 (**Fig. 4.3**). Levels of H3K4me1 decreased significantly during freezing, and remained at that level during thaw, which is in

accordance with the protein levels of HMTs that methylate H3K4 and their activity. As previously discussed, the liver reduces many of its functions during freezing, with the exception of a select number of pro-survival processes such as glycogenolysis, the upregulation of fibrinogen and ATT, and expression of novel freeze-induced genes to name a few. Any liver function requiring the perfusion of blood, such as transport of metabolites to liver for detoxification, is necessarily inactive when freezing occurs since the blood plasma freezes (Layne *et al.* 1989). Since freezing can last for days or weeks at a time (Storey and Storey 1984), the wood frog needs to conserve its internal energy reserves and one energy-saving method would be to not transcribe genes that are not essential for basic cellular survival, such as the genes involved in the non-crucial pathways highlighted above. H3K4me1 is associated with active transcription, and thus its abundance may lower to decrease liver pathways not needed for freezing survival. The results for H3K9me3 in liver fit this trend of a net decrease in liver activity, as levels of H3K9me3 remained constant throughout the freeze-thaw cycle. Similar results are seen in the methyl-histone residue most similar to H3K9me3, which is H3K36me2. This transcriptionally repressive residue increased slightly during freezing, then decreased below control levels during thaw (**Fig. 4.3**). The decrease in H3K4me1, maintenance of H3K9me3, and increase in H3K36me2 would all contribute to decreasing transcription, and thus these residues, either individually or in combination, may be a transcriptional regulatory mechanism for many of the downregulated processes in the liver during freezing. In skeletal muscle, H3K4me1 and H3K27me1 both decreased during freezing while H3K9me3 and H3K36m2 were maintained (**Fig. 3.3**). The liver showed a similar trend for these methyl-histone marks with the exception of H3K27me1, which did not

decrease during freezing, but rather increased strongly during thaw with respect to the control (**Fig. 4.3**). H3K27me1 is a histone modification associated with active gene transcription (Barski *et al.* 2007; Ferrari *et al.* 2014), and of the other histone modifications examined in this study, it is most similar to H3K4me1. Interestingly, these two transcriptional activators showed differential changes suggesting that they play different roles in the liver during freezing. Some pro-survival processes such as glycogenolysis increase strongly to support high rates of cryoprotectant glucose production (Storey and Storey 1984; Storey 1984; Storey and Storey 1986; Storey 1987a), whereas freeze-specific gene expression is also induced (Cai *et al.* 1997; Cai and Storey 1997b; Cai and Storey 1997a; McNally *et al.* 2002; McNally *et al.* 2003). If these processes are regulated at the transcriptional level, it stands to reason that a transcriptionally permissive histone modification may be involved. Thus H3K27me1 may be a candidate for regulating pro-survival genes, since it is the only methyl-histone residue associated with active transcription that is maintained during freezing. In the case of cryoprotectant production, once temperatures rise and the wood frog thaws, it must deal with the hyperglycemic state it has induced to survive freezing. Glucose in the liver must decrease to avoid organ damage, and liver glycogen levels must increase in preparation for the next sub-zero event. Within the first few hours of thawing, the liver enacts multiple methods for decreasing glucose levels. Glycogen production begins (Storey and Storey 1986), but is at a pace that takes multiple days to return to control levels, and therefore the liver secretes excess glucose into the blood, which is eventually excreted in the urine (Layne *et al.* 1996). Interestingly, the wood frog is unique in the sense that glucose transporters are present in the bladder to reabsorb glucose secreted into

the urine (Costanzo *et al.* 1997). The slow pace to return to euglycemia does have its advantages since the frog undergoes various freeze-thaw cycles over the course of the winter, and thus increasing glucose levels from an already elevated level requires less energy expenditure. Glycolysis also resumes during thawing, which is evident by an increase in fructose 2,6-bisphosphate (Storey 1987b) and subsequent utilization of glycolytic intermediates built up during the freezing period (Storey 1987a). These steps taken by the liver to return to euglycemic levels require coordinated regulation at the transcriptional level. If H3K27me1 regulates glucose production during freezing, it may also be a histone modification regulating glucose metabolism during thawing. If this is the case, the increase in H3K27me1 during thaw is explained by the need to quickly reduce glucose levels and thus transcribe genes involved in this process. These results suggest that H3K27me1 plays an interesting role in the liver with respect to freezing survival, the details of which should be explored further in a more focused and narrow fashion.

As reported in **Chapter 3**, two lysine residues on p53 were differentially methylated across the freeze-thaw cycle in skeletal muscle. Comparable changes were not seen in the liver; neither the amount of total p53, nor the levels of methyl-lysine residues changed over freeze-thaw (**Fig. 4.4**). A change in activity or function for p53 in the liver during freezing cannot be implied from these results, however it also cannot be ruled out since p53 is subject to many other PTMs (Barlev *et al.* 2001; Tang *et al.* 2008; Lee and Gu 2009; Zhang *et al.* 2013). The difference in the freeze-thaw methylation pattern on p53 between these two tissues may be explained by their phenotypic differences and their functional requirements during freezing. Since skeletal muscle is largely inactive, the

methylation of p53 in skeletal muscle may contribute to dealing with skeletal muscle specific challenges such as the prevention of muscle disuse atrophy, where control over apoptosis and stabilizing the proteome and subcellular structures by features including chaperones, antioxidants, and cryoprotectants is needed. In contrast, p53 may have a different function in the liver in freeze-tolerance, and/or modulation of its activity is controlled through other regulatory mechanisms, such as phosphorylation and acetylation during freezing, and thus the methylation status of p53 may not need to change.

In summary, this chapter provides the first investigation of histone and non-histone methylation in the liver over the freeze-thaw cycle. The liver plays a crucial role in surviving freezing temperatures. Differential regulation of histone methylation provides the base for downregulating genes and thereby the pathways in which they participate that are not vital while simultaneously maintaining the select few that are. The results herein show freeze-responsive changes in methyl-histone residues which are reflected in the activities and protein levels of the HMTs that methylate those residues. These results provide a “first look” into the inner workings and role of histone methylation in natural freeze tolerance and they build upon previous evidence that epigenetic mechanisms are involved in conferring adaptations for freeze tolerance and metabolic rate depression.

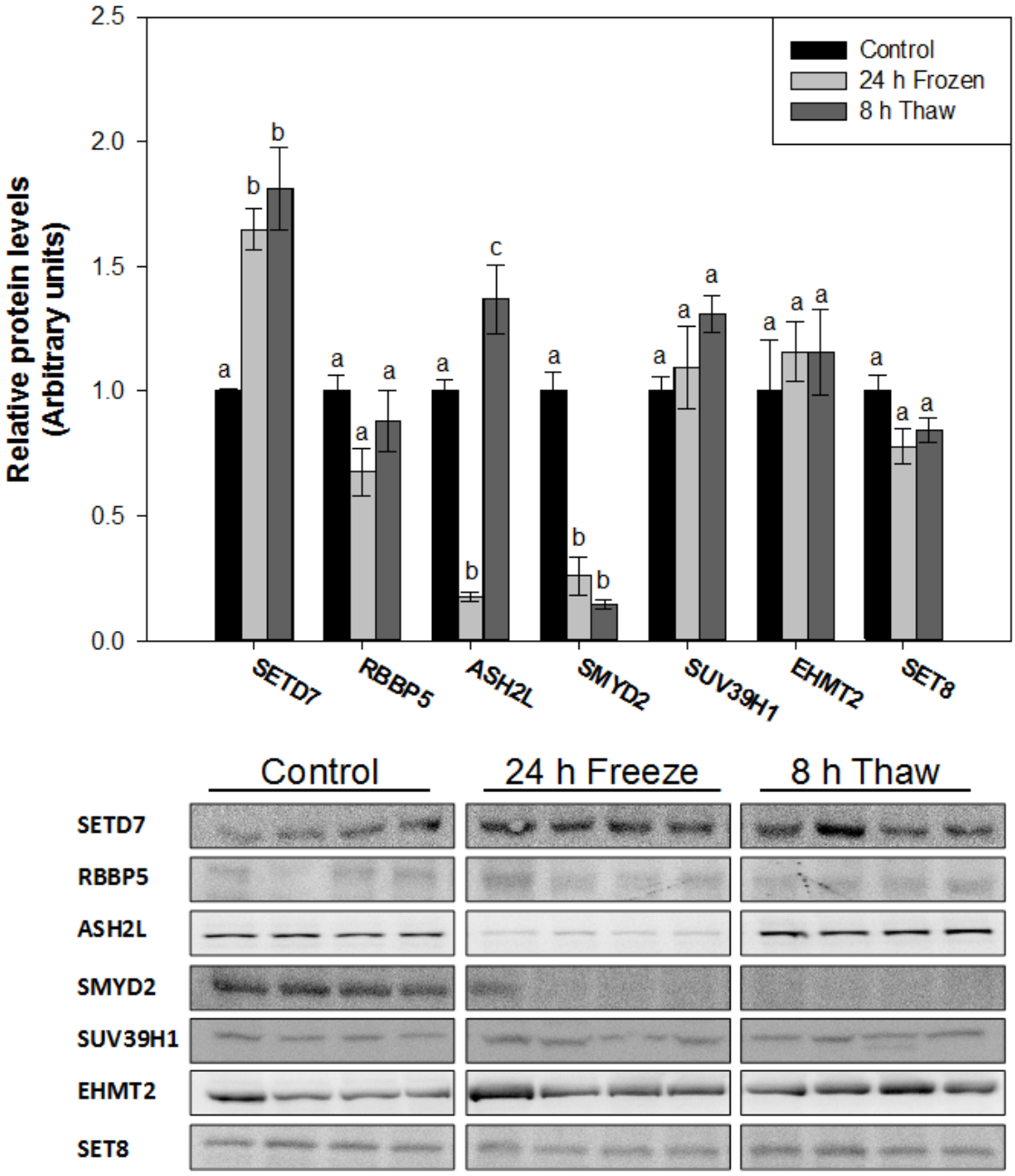

Figure 4.1: Effects of 24 h freezing and 8 h thaw on relative proteins levels of SETD7, RBBP5, ASH2L, SMYD2, SUV39H1, EHMT2, and SET8 methyltransferases in *R. sylvatica* liver tissue as determined by Western immunoblotting. Data are mean ± SEM, n = 4 independent trials on samples from different animals. For each target measured, values that are not statistically different from each other ($P > 0.05$) share the same letter notation.

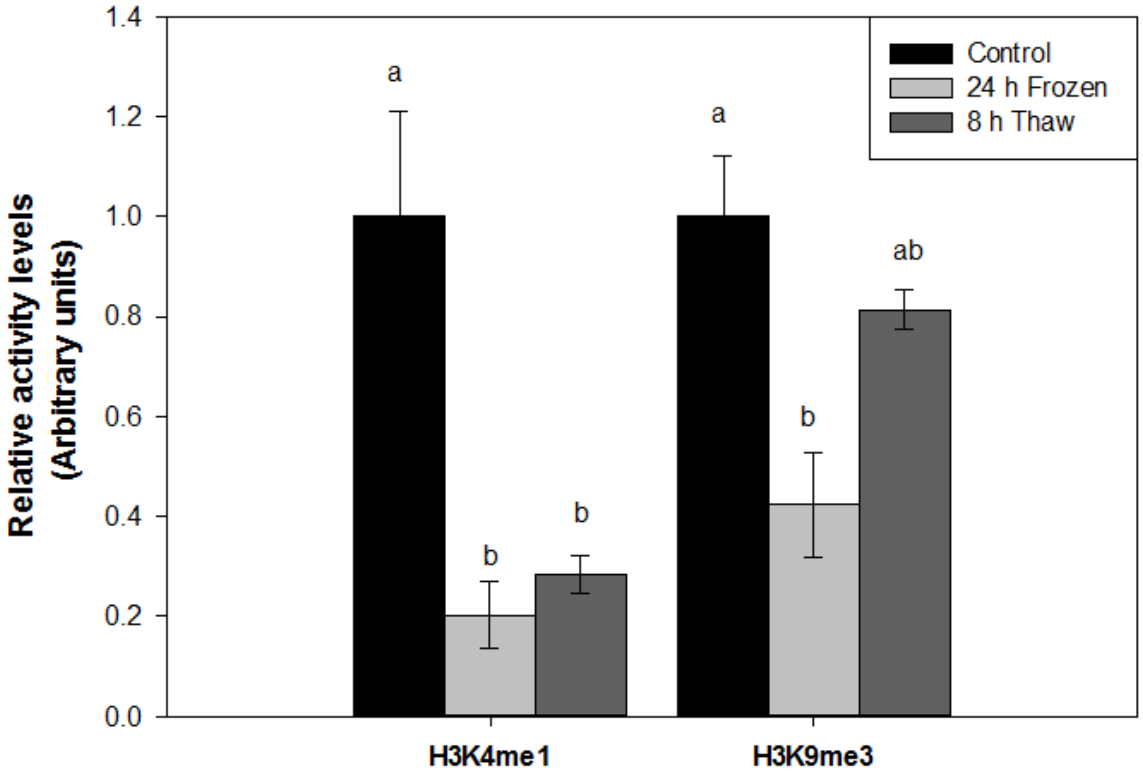

Figure 4.2: Relative HMT activity on H3K4 and H3K9 in liver tissue of *R. sylvatica* comparing the effects of 24 h freezing and 8 h thaw. Actual control values for H3K4me1 and H3K9me3 are 21.1 ± 4.48 ng/h/mg and 3.28 ± 4.48 ng/h/mg respectively. Control, 24 h frozen and 8 h thaw are reported relative to these values. Data are mean ± SEM, n = 4 independent trials on samples from different animals. Values that are not statistically different from each other ($P > 0.05$) share the same letter notation.

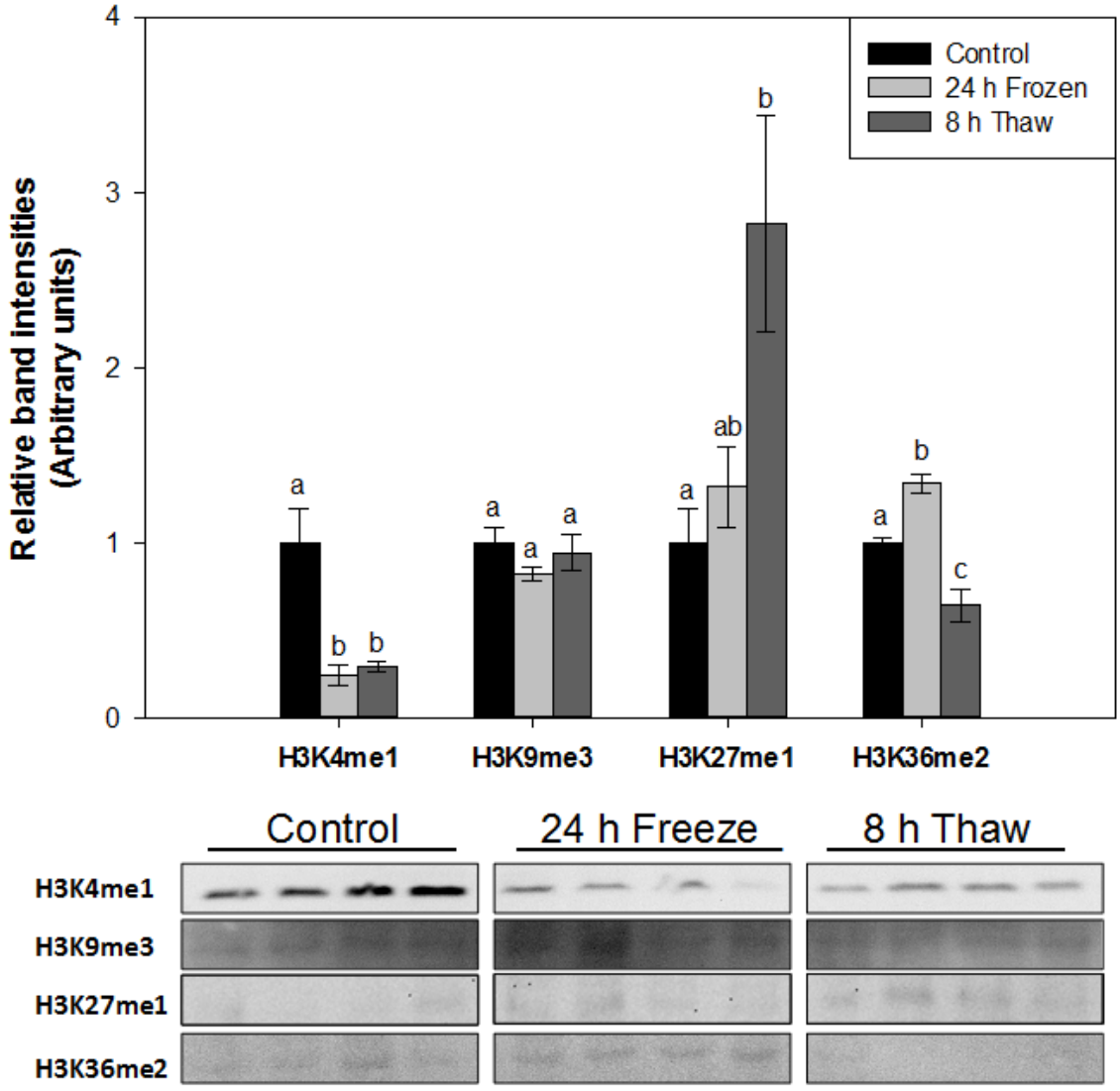

Figure 4.3: Effects of 24 h freezing and 8 h thaw on relative protein levels of H3K4me1, H3K9me3, H3K27me1, and H3K36me2 in *R. sylvatica* liver tissue. All other information as in Fig. 4.1.

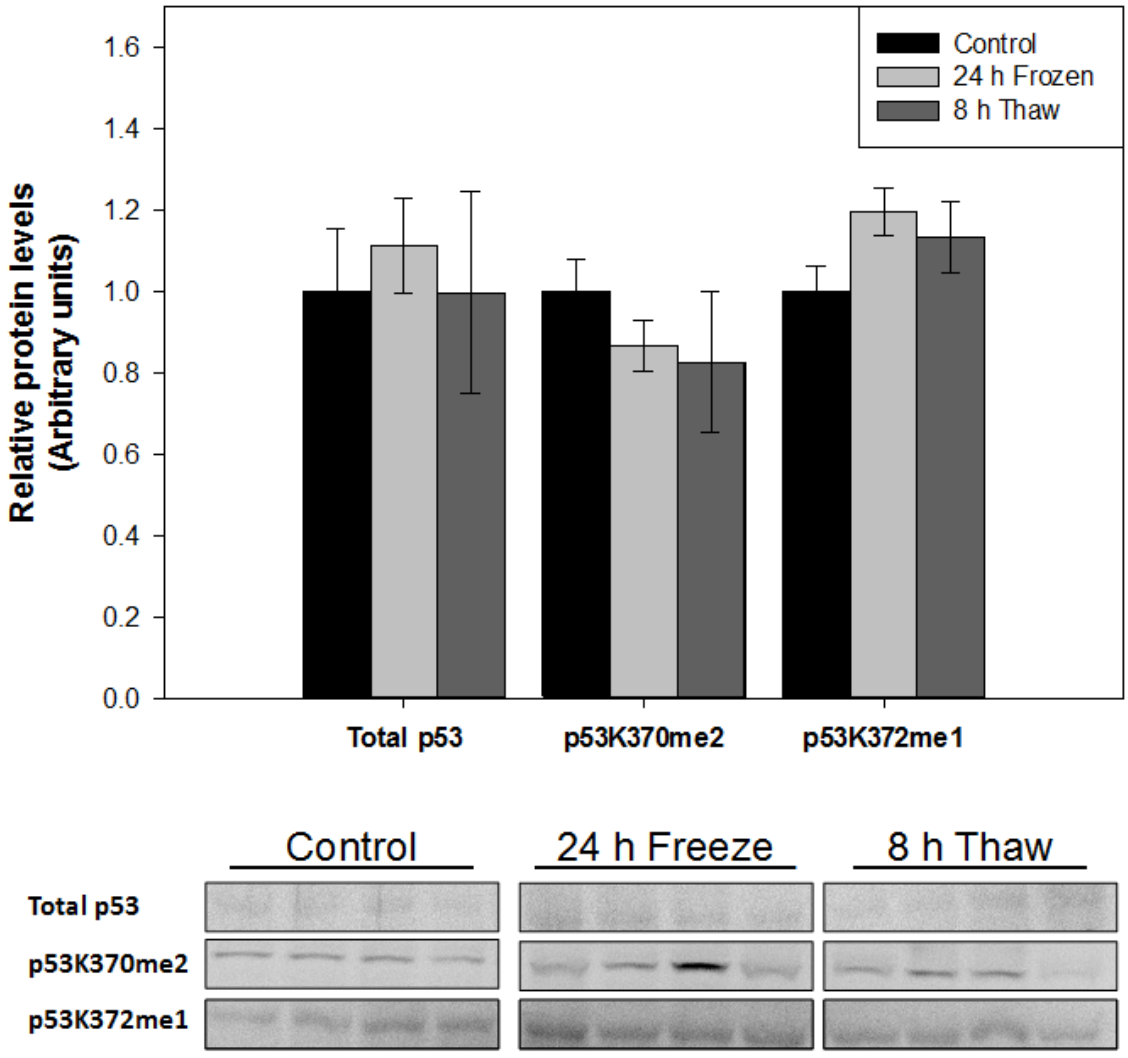

Figure 4.4: Effects of 24 h freezing and 8 h thaw on relative protein levels of total p53, p53K370me2, and p53K372me1 in *R. sylvatica* liver tissue. All other information as in Fig. 4.1

Chapter 5

General Discussion

5.1 Freeze tolerance and the wood frog

Seasonal shifts to sub-zero temperatures create harsh conditions for animals to survive and without the proper adaptations, cold- and freeze-induced stresses can be fatal. The damage induced during periods of freezing comes in many forms such as mechanical damage from ice crystal formation, physically blocking heart and lung function, increased osmotic pressure from freeze induced dehydration, and ischemic reperfusion injury, to name a few (Storey and Storey 1988). Animals that have adapted to live in climates that regularly experience these temperatures have survival strategies that generally involve avoiding sub-zero temperatures altogether by migration, or seeking thermally buffered microhabitats. A small subset of species has adapted to these freezing temperatures by either supercooling their body fluids and avoiding ice crystal formation (Lowe *et al.* 1971; Sømme 1982), or by surviving ice nucleation and living in a frozen state for days to weeks at a time. These freeze-tolerant animals must still deal with the potential stressors and damage as mentioned above, and thus have developed specialized survival strategies for each one (Storey and Storey 1992). The first of the hallmark strategies employed by freeze-tolerant animals is the production and distribution of small molecule cryoprotectants (Storey and Storey 1985a). Usually in the form of glycerol or glucose, these cryoprotectants are shuttled into cells of every tissue and organ in the body to counteract the osmotic pressure as pure water is drawn out of cells and incorporated into extracellular ice (Storey and Storey 1986). Cryoprotectants also lower the equilibrium freezing point of intracellular fluid, and therefore avoid the dangers of intracellular ice formation by relegating all ice to extracellular spaces. The second strategy that freeze-tolerant animals use when temperatures drop below zero is

modulation of energy utilization and metabolic rate (Storey and Storey 1990; Storey and Storey 2004a; Storey 2015). Once an ice nucleating event occurs, the propagation of ice quickly spreads to all available extracellular fluid (Rubinsky *et al.* 1994). Consequently, the heart and lungs cease to function due to physical constraints and limited energy imposed by the ice (Layne *et al.* 1989). An ischemic state is thus imposed on the animal, and cellular oxygen levels decline rapidly. Freeze-tolerant animals switch from aerobic respiration, to anaerobic ATP production, relying solely on the finite stores of energy accumulated prior to the freezing event. To maximize chances of surviving prolonged periods in a frozen state, these animals impart a new energy expenditure/production equilibrium. By reducing their metabolic rate, they lower their ATP expenditure needs and therefore lower their ATP production needs, which prolongs the length of time internal energy reserves can last, thus increasing the chances that temperatures will rise before they lose the capacity to produce ATP altogether (Storey and Storey 2004a). This metabolic rate depression is carried out in a selective manner, where all non-essential cellular processes are suppressed, while those needed for surviving the freezing stress are maintained.

The wood frog, *Rana sylvatica*, has a broad natural range across North America, making it the primary vertebrate model for studying freeze tolerance since it's discovery in 1982 (Schmid 1982). The wood frog has the highest capacity for natural vertebrate freeze tolerance, this is supported by the accumulation of vast stores of liver glycogen in the months leading up to the winter season. Once the temperature drops below zero and ice nucleation occurs, liver glycogen is converted to glucose through the glycogenolysis pathway (Cowan and Storey 2001). This glucose is the primary cryoprotectant deployed

by the wood frog and is rapidly transported throughout the body by the blood stream before ice propagation prevents the heart from pumping. Once the heart stops beating, oxygen can no longer be transported to tissues and organs around the body, causing the wood frog to switch from oxidative phosphorylation to anaerobic fermentative glycolysis, producing a buildup of lactate (Storey and Storey 1984; Storey 1987b). Like all other freeze-tolerant animals, this means the wood frog relies on internal stores to produce ATP, and cannot supplement these resources until temperatures rise and thawing occurs. The disequilibrium between euthermic energy utilization levels and the limited quantities of ATP producing resources when frozen provides the necessity to transition into a hypometabolic state. The wood frog accomplishes this through coordinated regulation at all levels of gene expression and protein activity. For example, enzymes such as G6PDH are post-translationally modified to block glycogenolytic intermediates from entering the pentose-phosphate pathway (Cowan and Storey 2001). At the mRNA level, gene transcripts are differentially regulated by mechanisms such as microRNA silencing, and possibly through mRNA storage in stress granules and p-bodies throughout the freeze-thaw cycle (Biggar *et al.* 2009; Biggar and Storey 2011; Zhang and Storey 2013). Like these previous examples, most regulatory studies using the wood frog have focused on regulation that occurs after transcription. As posited in **Chapter 1**, regulation of transcription may be more energy efficient than regulation of steps post gene expression. For example, regulation by microRNA saves on the energy needed to translate the mRNA transcript, and any energy used by the protein once it is translated, however the same energy savings are gained by transcriptional regulation, plus any energy used to transcribe the mRNA and microRNA transcripts. It is for this reason that transcriptional

regulation may play a critical role in the entrance of a hypometabolic state by the wood frog when energy resources become limited.

Histone methylation is an epigenetic mechanism that can be both a positive and negative regulator of transcription (Barski *et al.* 2007; Kouzarides 2007). The addition of a methyl-group to histone proteins creates binding sites or modifies the availability of existing sites on the nucleosome. This means that the functional outcome of histone methylation is dependent not necessarily on the methyl-group itself, but rather on the proteins that interact with that methyl-group. With that said, a given location along the histone tail, and the degree to which it is methylated are generally associated with either transcriptional activation or silencing, but not both. Histone methylation is thus a single type of chemical modification that can have a multitude of functions, however those functions can be deduced based on their placement and context. This dynamic mechanism for controlling transcription can be applied to any location on the genome, and thus has been implicated in countless cellular processes.

5.2 The role of HMTs in freeze-tolerance

Given that histone methylation is a general mechanism for controlling transcription, this thesis hypothesized that histone methylation is involved in the freeze tolerance response the wood frog employs when exposed to sub-zero temperatures. To test this hypothesis, this study aimed to create a preliminary characterization of select HMTs, their enzymatic activity, and the state of their downstream targets throughout the freeze-thaw cycle of the wood frog. **Chapter 3** explores HMTs and their targets in

skeletal muscle, a tissue that exemplifies the dormant state most tissues exist in while frozen. **Chapter 4** then builds on these results in the liver of the wood frog, an organ that plays a critical and active role when ice nucleation begins, but also retains some activity throughout the freezing period. The results from these studies provides evidence to suggest that histone methylation is a part of the freeze tolerance adaptations employed by the wood frog during periods of freezing.

Skeletal muscle and liver represent opposite sides of a spectrum in terms of activity and function in frozen wood frogs. While skeletal muscle loses its main locomotive function as the frog is frozen in place and thus myofibrils cannot contract, the liver acts as the entire body's supply of cryoprotective glucose, and thus it is expected that the transcriptional output from each of these tissues will differ. Since HMTs are one of the gatekeepers of transcription, changes in transcription levels are expected to be in part due to changes in HMTs and their histone targets, which was indeed found to be the case. Skeletal muscle was seen to have a consistent pattern of expression of methyl-histone residues (**Fig. 3.3**). The two residues that are associated with active transcription (H3K4me1 and H3K27me1) were both significantly downregulated during freezing and thaw, while the two residues associated with silencing transcription (H3K9me3 and H3K36me2) were maintained at control levels. Protein and activity levels of the corresponding HMTs also matched this pattern, providing supporting evidence (**Fig. 3.1; 3.2**). This trend suggests a net decrease in methyl-histone residues associated with active transcription, which aligns with previous results showing the establishment of a hypometabolic state in skeletal muscle during freezing in the wood frog (Hemmings and Storey 2001; Zhang 2013; Bansal *et al.* 2016). These epigenetic findings match what has

been shown in other animals that undergo metabolic rate depression. For example, the transcriptionally active acetyl-histone marks H3K9ac and H3K23ac both significantly decreased in the skeletal muscle of the red-eared slider turtle, *Trachemys scripta elegans*, when exposed to anoxic conditions (Krivoruchko and Storey 2010). Similar results have also been shown in the mammalian hibernator, *Spermophilus tridecemlineatus,* where H3K23ac decreased during torpor, resulting in a reduced transcriptional state in skeletal muscle (Morin and Storey 2006). Furthermore, this same mechanism for regulating transcription by histone methylation, is also used to modify the function and activity of non-histone proteins such as p53. p53 in skeletal muscle was shown to be differentially methylated across the freeze-thaw cycle (**Fig. 3.4**), suggesting the involvement of methylation in downstream processes such as cell cycle control, and apoptotic pathways. The multifunctional role of HMTs further adds to the energy savings that HMTs allow during metabolic rate depression. Not only can they be used to silence the expression of any gene at the transcriptional level, but they also act to modulate the activity of downstream proteins, such as p53, that are in control of metabolically expensive processes.

As previously mentioned, skeletal muscle and liver are examples of two tissues with vastly different activity levels leading up to, during, and after freezing in the wood frog. This dichotomy manifests itself in the methylation of histone and non-histone proteins observed in this system. While skeletal muscle presented results that match closely with what would be expected in a dormant tissue, the results from liver show differential methylation with respect to methyl-histone marks that have similar functions (**Fig. 4.3**). The most striking difference between skeletal muscle and liver are the levels

of the transcriptionally active methyl-histone residue H3K27me1. While in the dormant skeletal muscle H3K27me1 was downregulated, however, in liver H3K27me1 was maintained during freezing and increased during thaw (**Fig 3.3; Fig 4.3**). Since the metabolic activity within the liver largely switches to cryoprotectant production and other pro-survival mechanisms during freezing, and subsequent reincorporation into glycogen during thaw, it may be the case that H3K27me1 is responsible for maintaining and promoting these pathways. Unfortunately, no other freeze tolerant species have been examined within the context of histone methylation, and thus a methyl-histone mechanism for maintaining cryoprotectant production is speculative at this point. Another interesting observation was the lack of differential methylation of p53 throughout the freeze-thaw cycle in the liver. A more comprehensive look into the numerous other post-translational modifications of p53 will be needed to gain a better understanding of its role in freeze tolerance.

5.3 Summary

The ability for wood frogs to survive in a frozen state for an extended period of time is the result of a coordinated remodeling of the cellular, metabolic, and biochemical landscape, involving every tissue and organ and is highly regulated at all levels. Perhaps the most fundamental problem to overcome is the thermodynamic limitations of surviving an extended amount of time without adding additional energy to the system. At a euthermic rate of energy use the wood frog would quickly run out of resources and not survive until thawing. Thus, globally coordinated metabolic rate depression establishes a new equilibrium between energy use and production, which lasts a sufficient amount of

time until temperatures rise. Given that histone methylation controls the ATP-expensive processes of transcription and subsequent translation, it comes as no surprise that the results from this study suggest a role for histone methylation in freeze tolerance in the wood frog. Dormant skeletal muscle showed a pattern of methyl-histone residues consistent with a state of decreased transcriptional output, while the more active liver presented a more nuanced state of methyl-histone residues and their corresponding HMTs. These results open the door for more in depth studies on histone methylation and other histone modification in the context of freeze tolerance, with the goal of one day applying what we learn to other systems involving tissue preservation or damage due to freezing.

5.4 Limitations and future directions

This thesis is far from a comprehensive explanation of the role histone methylation plays in conferring freeze-tolerance in the wood frog. Much is still unknown about the mechanisms and functions that histone methylation has within the cell, and each day new discoveries are being made. With the tools currently available, the combinatorial complexity of histone methylation, which is a subset of histone modifications, which in turn is a subset of epigenetic mechanisms, makes it exceedingly difficult to fully elucidate these intricate networks in a system such as the wood frog. However, this does not mean we need a complete understanding of histone methylation to gain useful insights from measurements of HMTs or the methyl-histone residues themselves. Much progress has been made in the past two decades to elucidate the connection between HMTs, histone lysine residues, and their functional outcomes, and

this will continue to grow with the development of a methyl-proteome (Erce *et al.* 2012; Biggar and Li 2015), and the mapping of interactions between histone modifications, DNA modifications, and effector proteins (Ben-Porath and Cedar 2001; Lee and Zhang 2008; Cheng and Blumenthal 2010; Jobe *et al.* 2012; Brinkman *et al.* 2012).

To better describe the complexity of histone methylation, one can examine the number of additional experiments that could be performed by simply switching which targets were measured in this study. We can first look at the number of HMTs that have been discovered. This study measured a subset containing seven HMTs, however dozens more that methylate the same histone residues exist (Kouzarides 2002; Greer and Shi 2012). This study also measured a subset of methyl-histone residues and although these are the most well-studied methyl-residues, there are other residues such as H3K79 and H4K20 that also have distinct functions (Jones *et al.* 2008; Lu *et al.* 2008). To add to this point, as previously mentioned, each histone lysine residue can have up to three methyl groups added. This means that each of the residues not measured in this study have three possible functional states, and that the residues that were measured also have two additional methyl-states that could have been measured. While this thesis focused exclusively on lysine methylation, arginine residues can also be methylated on histone and non-histone proteins (Wysocka *et al.* 2006; Di Lorenzo and Bedford 2011). Similar to lysine methylation, arginine methylation can come in three forms, monomethylation, asymmetrical dimethylation, or symmetrical dimethylation (Bedford and Richard 2005). Arginine methylation can also occur on multiple different histone arginine residues such as H4R3, H3R17, and H3R26 (Di Lorenzo and Bedford 2011). The addition of methyl-groups to arginine residues is facilitated by protein arginine methyltransferases (PRMTs).

Like HMTs, a large number of PRMTs exist and each is specific to a given arginine residue. However, both HMTs and PRMTs are one side of the methylation equation. Analogous to reversible protein phosphorylation, where there are proteins that add the modification (kinases/phosphorylases), there are also proteins that remove the modification (phosphatases). For HMTs and PRMTs these proteins are histone demethylases (HDMs). The existence of HDMs was a contested issue (Bannister *et al.* 2002) until 2004 when the first HDM, LSD1, was discovered (Shi *et al.* 2004). It is now known that many HDMs exist, and act in concert with HMTs to determine the methyl-status of any given histone residue. As described, there are an untold number of possible future studies available in the wood frog using the same experimental design, all pertaining to methylation. This says nothing about the exact same paradigm, writers (HMTs/PRMTs) and erasers (HDMs), that exists for other histone modifications such as acetylation, phosphorylation, ubiquitination, SUMOylation, deamination, ribosylation, and glycosylation to name a few (Strahl and Allis 2000; Zentner and Henikoff 2013; Huang *et al.* 2014).

All the results from this thesis are from measuring the total levels of a given target in the sample. For example, this means that a global decrease in H3K4me1 is an average of the H3K4me1 on all histones, rather than a definitive account of the level of H3K4me1 on any single gene. Since histone methylation generally works on a gene-to-gene level, this thesis is limited to a broad scope of changes on the level of the entire genome. At this level, the results provide useful information on broad over-arching trends going on at the transcription level, especially in a model species that undergoes dramatic changes in transcriptional output. These types of preliminary results also help guide and focus future

specific studies. There are techniques available for measuring the levels of different histone modification at the single gene level, however these techniques require a larger time, skill, and monetary investment, which further establishes the importance of global preliminary studies such as the one reported herein. Generally, the techniques used to measure histone modification at the level of a single gene are based on, and built off of chromatin immunoprecipitation (ChIP). ChIP creates a cDNA library from all the locations in the genome where the target of interest is bound. By immunoprecipitating a specific histone modification, and by fragmenting the DNA, you are able to precipitate the nucleosome and DNA that is in close association with that histone modification. The more the target is bound to a region of DNA, the higher the number of DNA fragments containing that region will be in the cDNA library. The creation of this ChIP library can then be followed up with DNA microarray analysis, or PCR (ChIP-on-chip and ChIP-PCR) to measure where in the genome and to what extent the target of interest binds. These two techniques are limited to a relatively small number of genes, which in recent years has been overcome by advances in next-generation sequencing. ChIP-sequencing (ChIP-seq), which is ChIP followed by next-generation sequencing, allows for the same type of analysis as ChIP-on-chip and ChIP-PCR, but can measure where a target binds to every gene in the genome at once instead of a select few. The use of ChIP-seq has accelerated research involving histone modification in recent years (Barski *et al.* 2007; Park 2009; Pekowska *et al.* 2011), and would have the same effect if applied to histone methylation in the wood frog. By measuring the levels of a given methyl-histone residue on every gene in the wood frog genome we would gain a much more detailed understanding of the transcription landscape during periods of freezing and thaw,

especially when combined with all the previous freeze-related findings on the wood frog from the literature. Currently however, the full power of this technique is limited by the fact that the wood frog genome has yet to be sequenced and annotated. This limitation extends to many other techniques and methods of data analyses, such as bioinformatic comparisons, although with the ever decreasing per megabase cost of DNA sequencing this may not be a problem in the near future (Goodwin *et al.* 2016).

Histone methylation relies on external proteins to interact with the methyl-histone residue itself to confer its function. This property is unlike some other histone modification such as histone acetylation, in which the addition of a charged acetyl-group modifies the charge distribution of the histone, affecting the histone-DNA interaction directly, leading to changes in transcriptional availability. “Reader” proteins, within the “reader”, “writer”, “eraser” paradigm of many epigenetic mechanisms have yet to be examined within the context of metabolic rate depression, and thus will ultimately need to be explored in the wood frog to gain a complete understanding of the role histone methylation has. For example, the transcriptional silencing associated with H3K9me3 is at least in part mediated through the recruitment of HP1 to the H3K9me3 residue (Lachner *et al.* 2001; Fischle *et al.* 2005), which is a protein involved in heterochromatin formation (James and Elgin 1986). In a similar manner, the function of H3K4me1 is also mediated by third-party proteins, CHD1 recognizes methyl-H3K4 to confer transcriptional activation (Pray-Grant *et al.* 2005; Gaspar-Maia *et al.* 2009). It is these types of interactions that ultimately control transcription, and thus are a great starting point for future studies.

All of the above mentioned targets, techniques, and experiments are only some examples of how to push forward our understanding of histone methylation and epigenetic mechanisms in the incredible system that is the freeze-tolerant wood frog. While the number of possible targets, and cost of some of these techniques are out of the scope of this thesis, it will not be long before the advent of new technologies and decreasing costs will make it possible to fully examine the histone code to learn exactly how the wood frog is able to control transcription and enter a hypometabolic state.

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Appendices

Appendix A: Antibody information and suppliers for western blotting.

Antibody	Company	Catalogue #
ASH2L	Cell Signaling	#5019
EHMT2	Cell Signaling	#3306
H3K4me1	Abcam	#ab8895
H3K9me3	Abcam	#ab8898
H3K27me1	ActiveMotif	#39890
H3K36me2	ActiveMotif	#39256
p53	Abcam	#ab28
p53K370me2	Ameritech Biomedicines	#ATB-H0007
p53K372me1	Genetex	#GTX117515
RBBP5	Cell Signaling	#13171
SET8	Cell Signaling	#2996
SETD7	Cell Signaling	#2813
SMYD2	Cell Signaling	#9734
SUV39H1	Cell Signaling	#8729

Appendix B: Western blotting conditions.

Antibody	Tissue	Protein Amount (µg)	Gel (%)	Block	[1°]	Reactivity	[2°]
ASH2L	Muscle	25	8	1.5% milk	1:1000	Anti-rabbit	1:8000
	Liver	25	8	2.5% milk	1:1000	Anti-rabbit	1:8000
EHMT2	Muscle	40	6	0% milk	1:1000	Anti-rabbit	1:8000
	Liver	25	8	2.5% milk	1:1000	Anti-rabbit	1:8000
H3K4me1	Muscle	35	15	1% milk	1:1000	Anti-rabbit	1:8000
	Liver	40	15	2.5% milk	1:1000	Anti-rabbit	1:8000
H3K9me3	Muscle	40	15	4% milk	1:1000	Anti-rabbit	1:8000
	Liver	15	15	1.5 min PVA	1:1000	Anti-rabbit	1:8000
H3K27me1	Muscle	40	15	1% milk	1:1000	Anti-rabbit	1:8000
	Liver	25	15	0% milk	1:1000	Anti-rabbit	1:8000
H3K36me2	Muscle	30	15	1 min PVA	1:1000	Anti-rabbit	1:8000
	Liver	30	15	1 min PVA	1:1000	Anti-rabbit	1:8000
p53	Muscle	25	10	2.5% milk	1:1000	Anti-mouse	1:8000
	Liver	30	12	5% milk	1:1000	Anti-mouse	1:8000
p53k370me2	Muscle	30	10	2.5% milk	1:1000	Anti-rabbit	1:8000
	Liver	30	12	2.5% milk	1:1000	Anti-rabbit	1:8000
p53k372me1	Muscle	30	12	2.5% milk	1:1000	Anti-rabbit	1:8000
	Liver	25	10	5% milk	1:1000	Anti-rabbit	1:8000
RBBP5	Muscle	35	10	1.5% milk	1:1000	Anti-rabbit	1:8000
	Liver	25	10	2.5% milk	1:1000	Anti-rabbit	1:8000
SET8	Muscle	35	15	5% milk	1:1000	Anti-rabbit	1:8000
	Liver	25	15	2.5% milk	1:1000	Anti-rabbit	1:8000
SETD7	Muscle	25	10	5% milk	1:1000	Anti-rabbit	1:8000
	Liver	25	15	10% milk	1:1000	Anti-rabbit	1:8000
SMYD2	Muscle	25	10	3.5% milk	1:1000	Anti-rabbit	1:8000
	Liver	25	15	5% milk	1:1000	Anti-rabbit	1:8000
SUV39H1	Muscle	20	10	5% milk	1:1000	Anti-rabbit	1:8000
	Liver	20	10	5% milk	1:1000	Anti-rabbit	1:8000

Appendix C: Commercial HMT activity assay mechanistic details.

The commercial HMT activity assays used in this thesis, Epigentek EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kit (H3K4-Catalogue # P-3002-96; H3K9-Catalogue # P-3003-96), use an antibody-based detection mechanism for measuring the activity of HMTs on a given histone lysine residue. An outline of the mechanism is shown below (**Fig. C1**). A biotinylated histone H3 N-terminal tail peptide containing the histone lysine residue of interest is coated to the inside of the supplied streptavidin-coated 96-well microplate. The extracted protein samples containing active HMTs are then added to the wells and the HMTs methylate the available histone lysine residues over a given amount of time at 37°C. The now methylated histone residues are recognized specifically by the supplied primary antibody. A secondary antibody conjugated to horse-radish peroxidase (HRP) then recognizes the primary antibody. The chromogenic substrate TMB (3,3',5,5'- Tetramethylbenzidine) is added to the well and is oxidized by the HRP conjugate secondary antibody to produce a blue color. This reaction is then halted by the addition of the stop solution which changes the solution to a yellow color which is read at 450 nm on a spectrometer. The absorbance at this wavelength is proportional to the amount of the specific methylated histone residue, and thus proportional to the activity on that residue of the HMTs in the added sample.

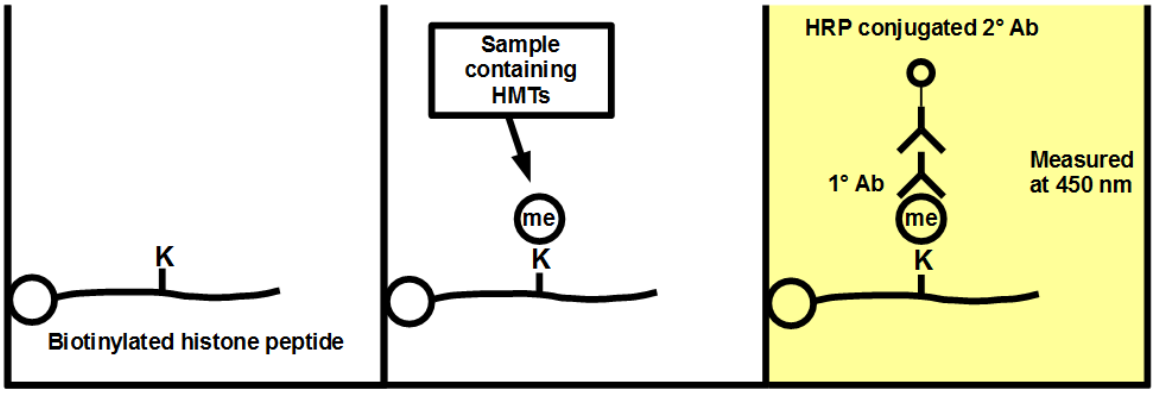

Figure C1: Mechanism of action of Epigentek EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kits (H3K4-Catalogue # P-3002-96; H3K9-Catalogue # P-3003-96). This mechanism is the same for both HMT activity kits (H3K4 and H3K9), with the exception of the primary antibody being specific for each methyl-histone lysine residue.

Appendix D: Loading control for western blotting.

The method for standardizing loading for western blots in this thesis relies not on single housekeeping genes, but rather a method previously published (Welinder and Ekblad 2011) that uses multiple protein bands from Coomassie-stained blots. The summed intensity of multiple bands from a single lane are used as a representative measurement for total protein, which is then used to standardize the antibody probed band within that lane. This method controls for the possibility of uneven loading, while avoiding the pitfalls that have been shown previously with the classic housekeeping gene method (Dittmer and Dittmer 2006). Shown below is a representative Coomassie-stained blot (**Fig. D1**) which was used to standardize protein loading for the corresponding SMYD2 immuno-reactive band in the liver of the wood frog across the freeze-thaw cycle from **Chapter 4 - Figure 4.1.** Also shown below (**Fig. D2**) are two examples (**adapted from Figure 4.1**) of representative ECL data with corresponding the Coomassie stained membranes used to standardize protein loading. Means and standard errors from the bar graphs are derived from dividing the ECL signal by the signal of the summed intensity of the Coomassie bands.

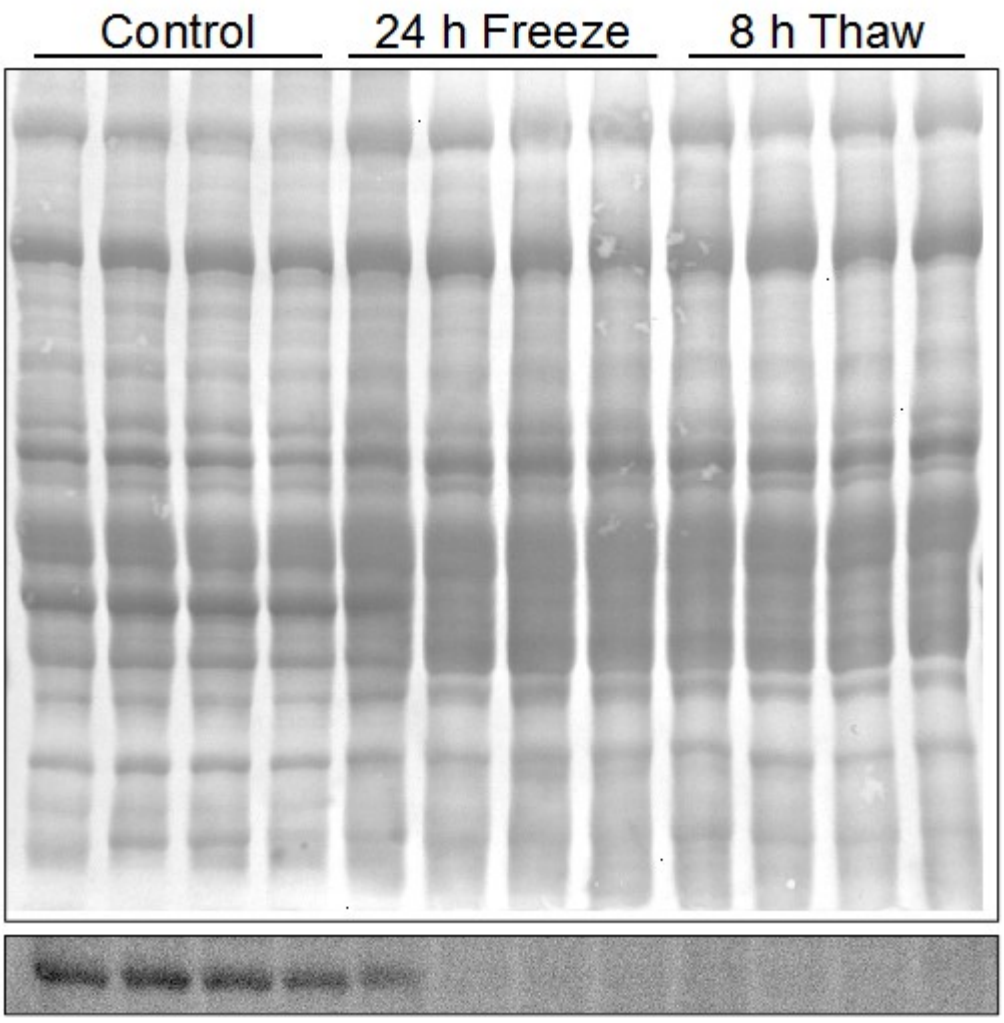

Figure D1: Representative Coomassie-stained blot (upper panel) and corresponding SMYD2 probed band (lower panel). Band of interest is standardized against the summed intensity of multiple Coomassie-stained bands from the same lane.

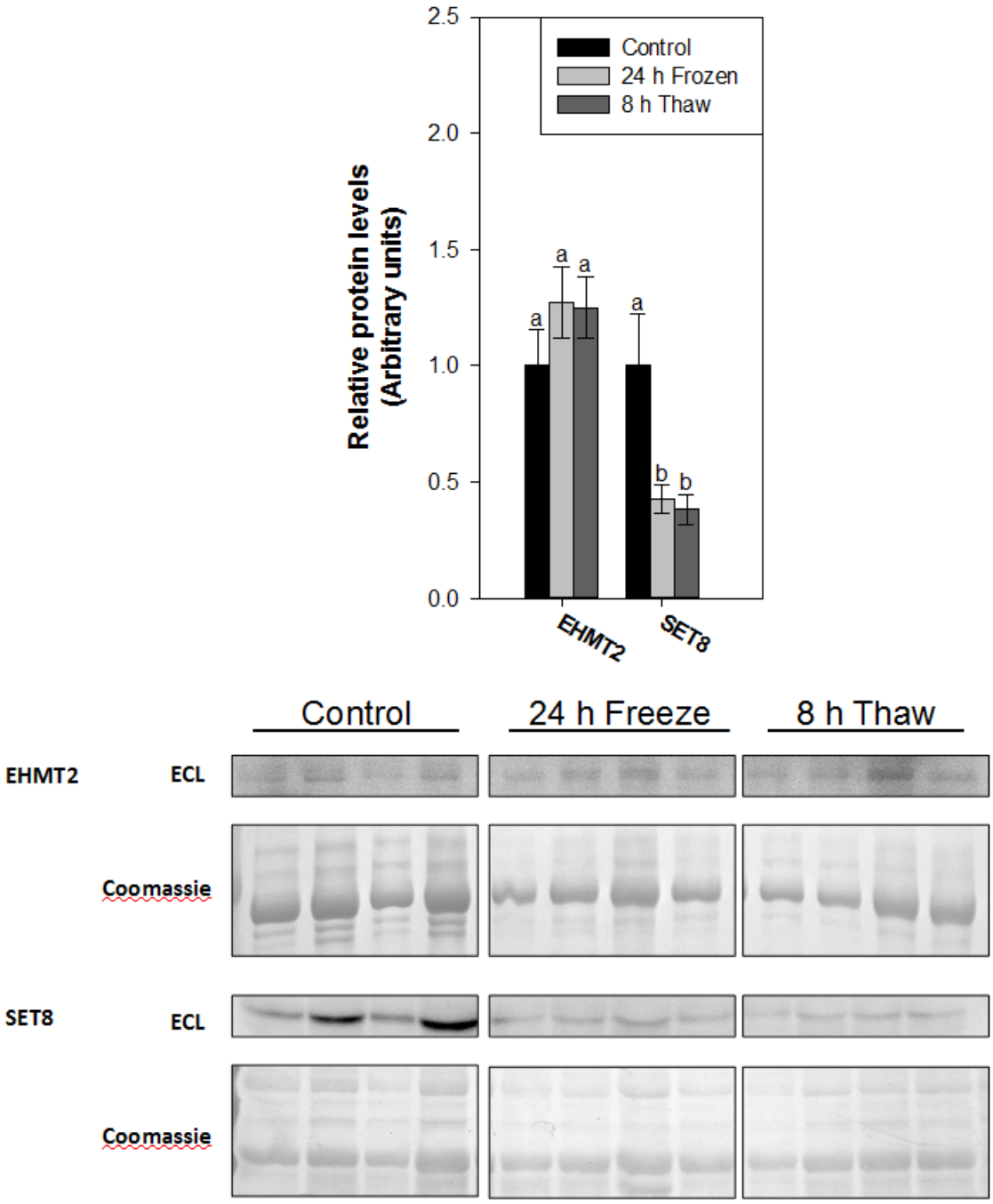

Figure D2: Examples of ECL bands with the corresponding Coomassie stained membranes images used to standardization and control for loading errors.

Appendix E: Ensuring antibody specificity for western blotting.

Several steps were taken to ensure antibodies cross-reacted at the expected molecular weight, and did not cross-react with multiple bands near that molecular weight when performing western blotting.

1. Blocking with milk or PVA before antibody probing was done to ensure non-specific background binding was minimized.
2. Blots were run (at least once) with a mammalian positive control homogenate (13-lined ground squirrel) to ensure that the antibody cross reacts at the correct molecular weight.
3. When possible, monoclonal antibodies that were tested on a wide range of species for cross-reactivity were purchased, thus increasing the chances of cross-reaction with the wood frog.
4. If the antibody company supplied the sequence of the epitope the antibody recognizes, that sequence was aligned using BLAST with multiple species (Human, rat, bovine, *Xenopus laevis*) to see whether the sequence is expected to be conserved in the wood frog. Antibodies recognizing epitopes with higher alignment scores were chosen over those with lower alignment scores.