Chapter 7
Epigenetics and the Extreme Stress Response

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Abstract Environmental conditions can be highly unfavorable for many organisms, imposing a variety of extreme stresses onto animal inhabitants. Winters are typically synonymous with shorter photoperiods, lack of food resources, and subzero temperatures. While some species migrate to avoid these conditions, many others have evolved defensive responses to combat these otherwise lethal situations. Such strategies can be classified into major categories including: freeze tolerance, freeze avoidance, anoxia tolerance, diapause, and hibernation. These types of strategies have been documented in a range of organisms including soil microfauna, intertidal

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marine invertebrates, insects, mammals, and various ectothermic vertebrates including some turtles, snakes, salamanders, and frogs. Extreme survival responses are possible thanks in part to metabolic rate depression (MRD), in which animals dramatically suppress energy expenditure and production to varying degrees. MRD necessitates holistic changes to the transcriptome of these specialized species. Unsurprisingly, recent research is showing that epigenetic mechanisms are invaluable contributors to stress adaptation, as is also true of gene silencing by noncoding RNAs. Epigenetic controls are a collection of regulatory mechanisms that alter gene expression without changing the DNA sequence itself, thereby making them ideal for implementing rapid, transient changes in phenotype as is characteristic of seasonal MRD. The current review will summarize the recent literature regarding epigenetic regulatory mechanisms, MRD, and adaptation to extreme environmental conditions. We also document where current research is directed, and what the most consequential and pressing inquiries are in the field.

Keywords Epigenetics · Freeze tolerance · Torpor · Hibernation · Hypoxia · Anoxia · Estivation · Dehydration

Abbreviations

5caC	5-carboxycytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
bp	Base pair
ChIP-seq	Chromatin immunoprecipitation sequencing
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EA	Early arousal
EN	Entrance (into torpor)
ET	Early torpor
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor 1 alpha
IA	Interbout arousal
KAT	Lysine acetyltransferase
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
KMT	Lysine methyltransferase
LT	Late torpor
miRNA	MicroRNA
MRD	Metabolic rate depression

mRNA	Messenger RNA
nt	Nucleotide
qPCR	Quantitative PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT-PCR	Real-time PCR
SAM	*S*-adenosyl methionine
snoRNA	Small nucleolar RNA
T_b	Body temperature
TET	Ten-eleven translocation
tRNA	Transfer RNA
WAT	White adipose tissue

7.1 Introduction

Many organisms live in environments where seasonal conditions can vary widely and can impose extreme stresses onto animal inhabitants, i.e., extreme cold or heat, oxygen limitation, and dehydration, among others. For example, winters are typically synonymous with shorter photoperiods, lack of food resources, and subzero temperatures. While some species can migrate to avoid seasonal extreme conditions, many others have evolved defensive responses to elude lethal situations, using behavioral, physiological or biochemical strategies including freeze tolerance, hibernation, estivation, and anaerobiosis (Storey and Storey 2010a, 2012a, 2017; Krivoruchko and Storey 2015). Such strategies have been documented in a range of organisms including soil microfauna, insects, intertidal marine invertebrates, mammals, and various ectothermic vertebrates including turtles and frogs (Ring 1982; Murphy 1983; Thomashow 1999; Costanzo et al. 2008; Holmstrup 2014; Storey and Storey 2017). Extreme survival responses are possible thanks in part to the phenomenon of metabolic rate depression (MRD), by which animals dramatically suppress energy expenditure and descend into a hypometabolic (torpid) state. MRD necessitates holistic changes to the transcriptome of these specialized species. Not surprisingly, recent research is showing that epigenetic mechanisms are valuable contributors to stress adaptation; these mechanisms include epigenetic transcriptional controls on DNA and the histone proteins that guard DNA, as well as translational silencing by noncoding microRNA. Epigenetic controls are a collection of regulatory mechanisms that alter gene expression without changing the DNA sequence itself, making them ideal for implementing rapid, transient changes in phenotype as are needed to achieve both global control of MRD and short-term adaptive adjustments to changing environmental conditions. The current review

summarizes recent literature regarding epigenetic regulatory mechanisms, MRD, and adaptation to extreme environmental conditions.

7.1.1   MicroRNA

While there is some contention about whether microRNA should be classified as a form of epigenetic regulation, these small molecules nonetheless constitute a highly conserved, vital method of post-transcriptional control of gene expression that has been documented across eukaryotic organisms and plays major roles in regulating the translation of gene transcripts. MicroRNAs (miRNA) are short, single-stranded, noncoding RNA of 21–24 nt in length that bind to mature mRNA transcripts to suppress their translation by mediating either the degradation or sequestration of mRNA transcripts (Bartel 2004). Since the discovery of the first miRNAs *let-7* and *lin-4* in 1993, hundreds of miRNAs have been identified across animal and plant species, all of which exhibit very high conservation between species. Several important features of miRNA:mRNA binding have been established: (a) miRNAs bind to gene transcripts through complementarity with the seed sequence, a stretch of 8 nt at the 5′ end of the miRNA sequence that corresponds with the 3' UTR of the mRNA sequence; and (b) perfect complementarity leads to cleavage and degradation of the mRNA transcript, whereas imperfect binding leads to translational suppression via isolation of the mRNA transcript into p-bodies or stress granules (Bartel 2004). MiRNAs are synthesized via the miRNA biogenesis pathway (Fig. 7.1). Given the high conservation of miRNAs observed among vertebrates, and the possession of unique features that make miRNA ideal for implementing reversible, transient phenotypes, miRNAs are a robust mode of post-transcriptional regulation that play a critical and dynamic role in allowing organisms to endure extreme environmental stresses. The miRNA studies which will be discussed in this chapter are laid out in Table 7.1.

7.1.2   DNA Methylation

DNA methylation is one of the three main mechanisms of conventional epigenetic control of DNA expression, the other two being histone acetylation and deacetylation, and histone methylation and demethylation. Table 7.2 displays the available DNA methylation studies on extreme environmental stress responses, while Table 7.3 highlights those on histone modifications. DNA methylation is the transfer of a methyl (-CH$_3$) group from *S*-adenosylmethionine, catalyzed by the DNA methyltransferase (DNMT) family of enzymes, to the 5′ carbon of a cytosine base to form 5-methylcytosine (5mC). Such 5mC methylation patterns often occur
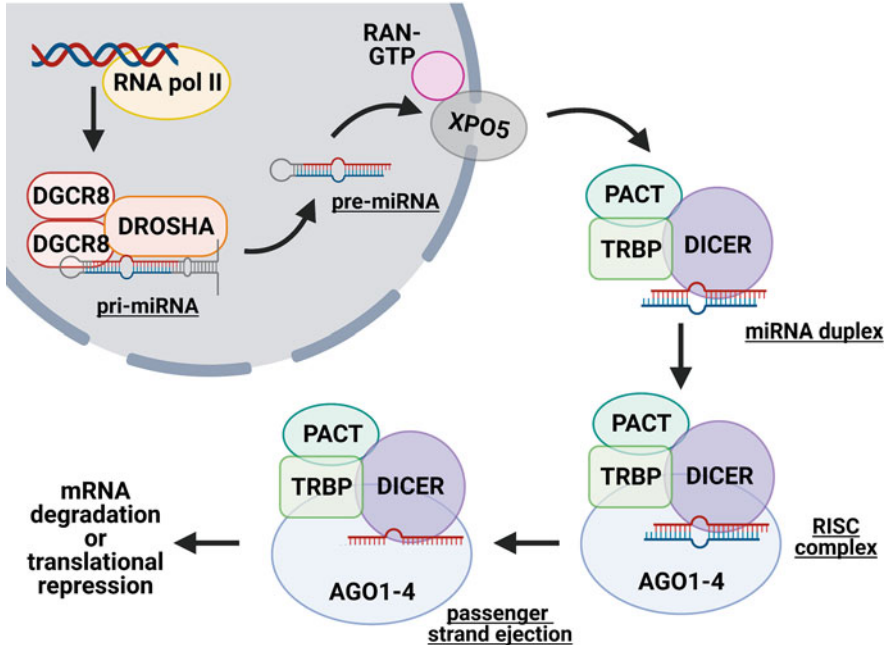
Fig. 7.1 The canonical miRNA biogenesis pathway. miRNAs are transcribed in the nucleus by RNA polymerase II to form the ~70 nt double-stranded primary miRNA (pri-miRNA), where the mature sequence is enclosed within a hairpin turn. Hairpin cleavage by the microprocessor complex (DROSHA/DGRC8) forms the double-stranded precursor-miRNA (pre-miRNA). With the help of RAN-GTP, the pre-miRNA is exported out of the nucleus via Exportin 5 (XPO5) and in the cytoplasm the RNase III enzyme DICER along with cofactors TAR RNA-binding protein (TRBP) and protein activator of PKR (PACT) process the pre-miRNA into the 21–24 nt long duplex miRNA. The 3′ end of the duplex miRNA has a two-nucleotide overhang which is used to load the duplex miRNA onto the Argonaute (AGO) proteins. The strand holding the mature miRNA sequence is kept whereas the other strand is discarded (named passenger strand ejection), and this structure constitutes the miRNA-induced silencing complex (RISC) which is fully prepared to target mRNAs. Figure created with BioRender.com and adapted from Ingelson-Filpula and Storey 2022

on CpG residues: a cytosine and a guanine nucleotide separated by a single phosphate group (Bird 1986). Regions of high CpG density are called CpG islands and these are commonly associated with the promoter region of genes. Hypermethylation of CpG islands correlates with transcriptional silencing of the downstream gene by (1) direct blockage of transcription factor binding, and/or (2) recruitment of repressive methyl-CpG-binding proteins which include MBD1, MBD2, and MeCP$_2$ (Bogdanović and Veenstra 2009; Moore et al. 2013). These three "reader" proteins bind methylated CpG regions, and then recruit chromatin remodeling complexes (like histone deacetylases) to block access of the transcriptional machinery to promoter elements (Nan et al. 1998).

Table 7.1 The collection of miRNA studies as discussed in this chapter

Stress	Animal	Tissue	Method	References
Freeze tolerance	*Rana sylvatica*	Brain	Western blot, RT-PCR, bioinformatics	Hadj-Moussa and Storey (2018)
		Heart	qPCR	Bansal et al. (2016)
		Skeletal muscle	qPCR	
	Dryophytes versicolor	Liver	Western blot, bioinformatics	Ingelson-Filpula and Storey (2022)
		Skeletal muscle	Western blot	
		Kidney	Western blot	
	Eurosta solidaginis	Whole larvae	RT-PCR	Lyons et al. (2016) Lyons et al. (2015)
Hibernation/ torpor	*Ictidomys tridecemlineatus*	Liver	RT-qPCR	Lang-Ouellette and Morin (2014)
		Skeletal muscle	RT-qPCR	
		Liver	RT-PCR	Wu et al. (2016)
		Heart	RT-PCR	
		Skeletal muscle	RT-PCR	
	Microcebus murinus	Liver	RT-PCR	Biggar et al. (2018)
		Skeletal muscle	RT-PCR	Hadj-Moussa et al. (2020)
	Ursus arctos	Skeletal muscle	RT-qPCR	Luu et al. (2020)
Hypoxia and anoxia	*Orconectes virilis*	Hepatopancreas	RT-qPCR, bioinformatics	English et al. (2018)
		Tail muscle	RT-qPCR, bioinformatics	
	Trachemys scripta elegans	Liver	RT-PCR	Biggar and Storey (2017)
		White muscle	RT-PCR	
		Spleen	RT-PCR	
		Kidney	RT-PCR	
Dehydration/ estivation	*Xenopus laevis*	Liver	RT-PCR	Wu et al. (2013)
		Skin	RT-PCR	
		Kidney	RT-PCR	
		Brain	RT-qPCR	Luu and Storey (2015)
		Heart	Bioinformatics, RT-qPCR	Hawkins and Storey (2020)
	Otala lactea	Foot muscle	qPCR	Hoyeck et al. (2019)

DNMT functions fall into two major categories: (1) maintenance methyltransferases, like DNMT1, that bind hemi-methylated DNA to copy methylation patterns onto a newly replicated DNA strand and (2) de novo

Table 7.2 The collection of DNA methylation studies discussed in this chapter

Stress	Animal	Tissue	Method	References
Freeze tolerance	*Rana sylvatica*	Liver	Western blot, activity assay, methylation kit	Zhang et al. (2019)
		Skeletal muscle	Western blot, activity assay, methylation kit	
		Brain	Western blot, activity assay	Bloskie (2021)
Hibernation/ torpor	*Ictidomys tridecemlineatus*	Liver	PCR, methylation kit	Alvarado et al. (2015)
		Skeletal muscle	PCR, methylation kit	
Hypoxia and anoxia	*Trachemys scripta elegans*	Liver	Western blots, activity assay, methylation kit	Wijenayake and Storey (2016)
		White muscle	Western blots, activity assay, methylation kit	
		Heart	Western blots, activity assay, methylation kit	

methyltransferases like DNMT3A and DNMT3B that place new methyl marks onto DNA (Lyko 2018). DNMT3L is a noncanonical DNMT given that it possesses no catalytic activity, but instead forms complexes with DNMT3A and DNMT3B, as well as other epigenetic enzymes, to regulate their activity (Chédin et al. 2002; Suetake et al. 2004).

Methyl marks are in turn removed through a two-step process via Ten-Eleven Translocation (TET) enzymes, which oxidize the 5mC to form 5-hydroxymethylcytosine (5hmC) before removing the methyl group altogether (Shi et al. 2017). Alternate pathways involve oxidation to 5-carboxycytosine (5caC) or 5-formylcytosine (5fC) before removal of the methyl group.

7.1.3 Histone Modification

Histones are small positively charged proteins that make up the functional unit of chromatin, the nucleosome. The nucleosome consists of ~200 bp of DNA wrapped around a histone octamer, made of pairs of histones H2A, H2B, H3, and H4 that enable intense condensation of genetic material within cell nuclei. Like other proteins, histones are subject to post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitylation, SUMOylation, citrullination, and serotonylation. Among these, the acetylation/methylation of lysine residues on N-terminal tails are the best studied, particularly due to their functional consequences on nearby gene transcription. Chromatin remodeling is a characteristic effect of histone modification, involving the dynamic interconversion between transcriptionally permissive euchromatin and repressive heterochromatin (Kouzarides 2007).

Table 7.3 The collection of histone modification studies as discussed in this chapter

Stress	Animal	Tissue	Method	References
Freeze tolerance	*Rana sylvatica*	Liver	Western blot, activity assay	Hawkins and Storey (2018)
		Skeletal muscle	Western blot, activity assay	
		Brain	Western blot, activity assay	Bloskie (2021)
Hibernation/ torpor	*Tamias asiaticus*	Liver	ChIP-seq	Tsukamoto et al. (2017; Tsukamoto et al. (2018)
	Ictidomys tridecemlineatus	Skeletal muscle	Western blot, activity assay	Morin and Storey (2006; Hawkins and Storey (2017; Rouble et al. (2018)
		White adipose tissue	Western blot, activity assay, acetylation kit	Rouble and Storey (2015)
		Brown adipose tissue	Western blot, activity assay, acetylation kit	Rouble et al. (2018)
		Liver	Western blot, activity assay, methylation kit	Watts and Storey (2019)
		Skeletal muscle	Western blot, activity assay, methylation kit	
Hypoxia and anoxia	*Trachemys scripta elegans*	Skeletal muscle	Western blot, activity assay, PCR	Krivoruchko and Storey (2010)
		Liver	Western blot, activity assay, PCR	
		Heart	Western blot, activity assay, PCR	
		Liver	Western blot, activity assay	Wijenayake et al. (2018 Wijenayake and Storey (2020)

The acetylation of lysine residues on histone tails is facilitated by lysine acetyltransferases (KATs), a name that reflects their ability to also act on a variety of nonhistone protein targets, whereas histones are deacetylated by histone lysine deacetylases (HDACs). KATs transfer acetyl groups from acetyl-CoA donors onto side chain ε-amino residues, and HDACs generate acetate as a by-product. KATs are subdivided into GNAT, MYST, and p300/CBP families (Berndsen and Denu 2008) whereas HDACs exist in four major classes: zinc-dependent class I, II, and IV, and NAD-dependent class III, which are also called sirtuins (SIRTs) (Haberland et al. 2009). Acetylation is tightly linked to gene activation, sometimes referred to as "permissive" to transcription, through (1) locally relaxing the tight electrostatic

interactions of positively charged histone proteins with negatively charged DNA, (2) providing binding sites for bromodomain "reader" proteins to recruit transcriptional machinery, and (3) preventing positions from being occupied by silencing modifications, since multiple modifications cannot co-exist at the same lysine residue.

Conversely, histone lysine methylation has much more variable outcomes depending on the particular methyl-lysine binding effector proteins that are recruited. Histone lysine methylation involves the addition of one, two or three methyl groups onto side chains of lysine residues. This epigenetic mechanism is reversible; marks are added by "writer" lysine methyltransferases (KMTs), removed by "eraser" lysine demethylases (KDMs), and interpreted by "reader" Chromo, Tudor, PWWP, PHD, WD or MBT domain proteins (Hyun et al. 2017). Through chromatin immunoprecipitation sequencing (ChIP-seq) analysis, most methyl-lysine marks have been shown to be strongly associated with actively or lowly transcribed genes (Barski et al. 2007; Mikkelsen et al. 2007). Methylation of H3K4 is linked to gene activation (H3K4me1 to primed enhancers, H3K4me3 to active promoters), whereas methylation of H3K9 and H3K27 is associated with repression that provides recruitment sites for heterochromatin protein 1 (HP1) (Bannister et al. 2001) and polycomb repressive complexes (PRCs), respectively. Mono-methylation of H4K20 is also generally deposited near start sites of actively transcribed genes (Evertts et al. 2013). Most KMTs contain the consensus Su(var) 3–9, Enhancer of zeste, and Trithorax (SET) domain as their key catalytic site and use methyl groups from the donor substrate, S-adenosyl methionine (SAM), to methylate the side chain amino group of lysine residues.

Epigenetic regulation including DNA methylation, histone lysine acetylation, and histone methylation coupled with post-transcriptional control via miRNAs are all potent tools for widespread, reversible methods of phenotypic variation. We have posited that animals, which transition into hypometabolic states as part of their survival strategy for enduring harsh environmental conditions, may use epigenetic modifications as an ideal mode of regulation to rework their metabolic needs without committing to lifelong changes in the genome. This introduction, as well as other chapters in this book, lay the groundwork for the multiple examples of environmental stress-mediated MRD that contributes to natural adaptive strategies including: freeze tolerance, freeze avoidance, hibernation/torpor, hypoxia/anoxia, and dehydration endurance.

7.2 Freeze Tolerance

Freeze tolerance is a survival strategy that has been documented for many species living in seasonally cold environments, including several species of vertebrates (frogs, salamanders, hatchling turtles) and many invertebrates (insects, molluscs) (Murphy 1983; Storey and Storey 1988; Storey and Storey 2017). Freeze tolerance is typified by the formation of ice in extracellular and extra-organ spaces and the

complete cessation of heartbeat, breathing, and movement. While this allows animals to mitigate harsh winter conditions including subzero temperatures, scarcity of food, and short photoperiods, freeze tolerance brings with it a collection of physiological dangers that require attention. Of primary importance is the prevention of intracellular ice crystal formation that poses mechanical threats of rupturing cell membranes and destroying subcellular architecture. Therefore, strategies are employed that prevent intracellular ice formation and restrict freezing to extracellular compartments only. Extracellular ice formation leads to exclusion of solutes from the growing ice lattice such that remaining extracellular fluid becomes hyperosmotic and draws water out of cells. Hence, cells risk both dehydration and extreme shrinkage. To counteract this, freeze-tolerant animals synthesize high levels of low molecular weight cryoprotectants that are packed into cells (Ring 1982; Storey and Storey 2017). The cryoprotectant used varies depending on the species and includes a variety of small molecules: polyhydric alcohols (e.g., glycerol, sorbitol), sugars (e.g., glucose, trehalose), and small nitrogenous compounds (e.g., urea).

Another consequence of ice formation in extracellular and extra-organ spaces (e.g., abdominal cavity, between skin and muscle) is ischemia caused by the interruption of blood flow with the consequence of hypoxia/anoxia as oxygen is depleted (Storey and Storey 2017). Gas exchange via the lungs is halted, kidneys do not remove waste, and skeletal muscle may atrophy from lack of use. To survive using only their own endogenous fuel reserves, cells/organs switch their metabolism and ATP generation from aerobic respiration to anaerobic fermentation, with the accumulation of end-products such as lactate and alanine (Storey and Storey 2017). On a holistic level, prevention of widespread damage severe enough to cause cell/organ death must be successfully managed by changes in antioxidant defenses, chaperone proteins, and antiapoptotic measures (Storey and Storey 2017). Recent studies of epigenetic regulatory mechanisms have illuminated the role of these controls in both downregulation of nonessential genes and processes as a part of MRD, and in facilitating pro-survival mechanisms in response to the threats posed by whole body freezing. The following section highlights studies of this nature, and a brief graphical overview of epigenetic influence during freeze tolerance is given in Fig. 7.2.

7.2.1 MiRNAs in Freeze Tolerance

Freeze tolerance among vertebrate species has been extensively studied in the main model for this process: the wood frog *Rana sylvatica*. During the winter months, wood frogs can endure the freezing of ~65% of total body water as extracellular ice when temperatures drop below about $-2\ ^{\circ}C$ (Costanzo and Lee 2013). MiRNA regulation of gene expression has been identified as a significant regulator of both entrance into and maintenance of freeze tolerance. In wood frog brain, miRNAs may serve a protective role by stabilizing existing, crucial neural networks, thereby acting as neuroprotectants (Hadj-Moussa and Storey 2018). They are synthesized via the
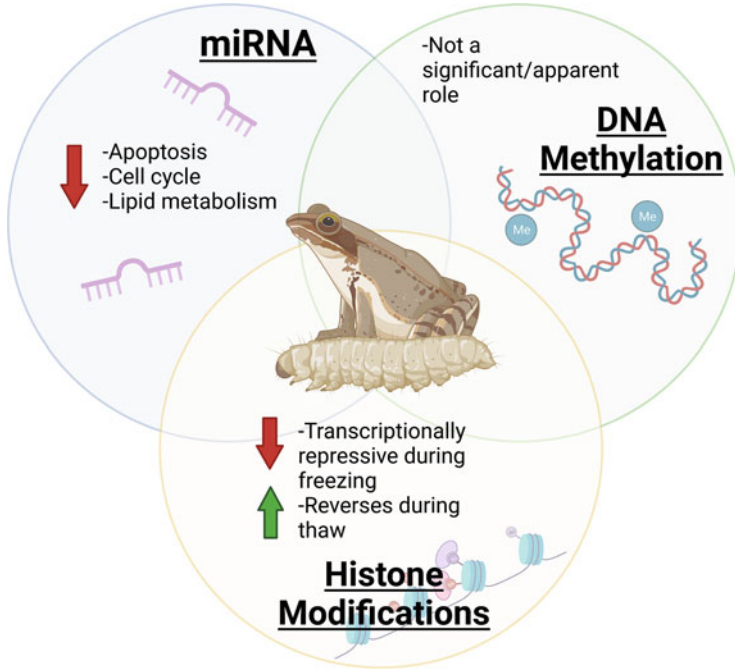
Fig. 7.2 An overview of epigenetic influences during freeze tolerance. Figure created using BioRender.com

miRNA biogenesis pathway (Fig. 7.1) and, in brain, protein levels of four members of the pathway decreased significantly during freezing. This indicated reduced synthesis of miRNAs in brain during freezing and may infer that widespread translational repression by miRNAs is not occurring (Hadj-Moussa and Storey 2018). However, it is possible miRNAs are acting in a cryoprotective manner by stabilizing existing, crucial neural networks. Furthermore, 113 miRNAs were quantified in wood frog brain via RT-PCR, with 24 of these exhibiting differential expression during freezing (Hadj-Moussa and Storey 2018). Nearly all of these miRNAs were downregulated save for one, miR-451-5p. Significantly, miR-451-5p has been previously characterized as a glucose-sensing switch which leads to downstream suppression of the PI3K/AKT pathway and activation of mTOR (Godlewski et al. 2010). The PI3K/AKT/mTOR network is a wide-ranging pathway with regulatory effects in actin cytoskeleton, apoptosis, autophagy, cell cycle progression, cell survival, DNA repair, epigenetic regulation, genetic stability, ion transport, metabolism, protein synthesis, regulation of gene expression, and ribosomal RNA synthesis (Ersahin et al. 2015). As mentioned, this pathway has many processes that we observe being differentially regulated during freeze tolerance, including cell cycle progression (an energy-expensive process that may be downregulated), cell survival processes including apoptosis and DNA repair, and epigenetic components. A study by Zhang and Storey hypothesized that AKT may

be playing an antiapoptotic role, demonstrating that AKT was inhibited in skeletal muscle, kidney, and heart after 24 h freezing exposure with a reversal after thawing (Zhang and Storey 2013). It is possible that miR-451-5p upregulation is downregulating energy- and metabolism-related processes of the AKT pathway, instead focusing on AMPK. AMPK is colloquially known as the energy sensor of the cell, and is responsible for fuel use switches by increasing glucose uptake and promotes fatty acid oxidation by phosphorylating ACC and decreasing malonyl-CoA production (Ke et al. 2018). Under conditions of glucose withdrawal, miR-451 downregulation is necessary for AMPK pathway activation, leading to suppressed proliferation rates and increased cell survival. Glucose is the cryoprotectant used by wood frogs with levels rising from ~5 μmol/g wet weight (gww) in unfrozen frogs to over 200 μmol/gww in frozen animals. It is possible that strong upregulation of miR-451-5p effectively targets the suppression of genes that would otherwise be upregulated by high glucose levels, including those that would funnel glucose into glycogen storage or use glucose as a metabolic fuel to support anabolic biosynthesis (Rider et al. 2006). Hence, the unexpected novel response to freezing by miR-451 may be a crucial factor in the ability of wood frogs to inhibit metabolizing glucose so it can be used as cryoprotectant. By contrast, many of the downregulated miRNAs affected genes/proteins involved in signal transduction and RNA processing and were linked with regulating intracellular signaling pathways, coupled with decreased miRNA biogenesis. This may infer that continued function of these signaling pathways is critical to survival during freeze tolerance, thereby highlighting a potential role of miRNAs in brain tissue to support freezing survival.

Studies on miRNA regulation over the freeze/thaw cycle in *R. sylvatica* have also been undertaken for heart and skeletal muscle, in which qPCR was used to quantify levels of 53 miRNAs in these tissues (Bansal et al. 2016). In heart, only one miRNA was upregulated whereas four were downregulated during freezing, although larger subsets of twenty were downregulated after 8 h thawing (Bansal et al. 2016). The widespread downregulation of miRNAs during thawing may signify that many cellular processes need to be reactivated after thawing to cope with any accumulated damage from freezing. Indeed, selected miRNAs from the group that was analyzed are known to play roles in heart function, such as miR-145 and miR-208 that are overexpressed in various heart diseases (Cooley et al. 2012). Skeletal muscle showed an alternate trend, with 16 miRNAs upregulated and one downregulated during freezing, as well as six remaining upregulated after thawing (Bansal et al. 2016). The miRNAs affected in skeletal muscle targeted genes in the cell cycle and apoptosis, thereby suggesting that these processes are suppressed during freezing and remain this way throughout the thaw. Bioinformatic prediction of pathways affected by these miRNAs yielded targets including actin cytoskeleton, PI3K-Akt, and MAPK signaling as being disproportionately affected by miRNAs (Bansal et al. 2016). The focus on intracellular signal transduction has been observed during freeze tolerance in wood frogs previously, suggesting a more global theme for the functions of miRNA in regulating signaling pathways during freezing.

Emerging data from our lab also show the miRNA responses by another freeze tolerant amphibian, the gray tree frog, *Dryophytes versicolor* (Ingelson-Filpula

2021). Members of the miRNA biogenesis pathway (Fig. 7.1) were differentially regulated across three tissues between control and frozen states, with liver showing upregulation of miRNA synthesis proteins and skeletal muscle/kidney exhibiting downregulation of some biogenesis proteins, thereby indicating suppression of miRNA synthesis. Noteworthy was the increased expression of ribonucleases DICER and DROSHA in hepatic tissues, and the strong reduction of RNA-binding argonaute proteins in frozen *Dryophytes* kidneys and muscle. Like wood frogs, *D. versicolor* produced copious amounts of cryoprotectant in liver, this organ being the most metabolically active tissue during freezing and the last organ to be affected by freezing. Skeletal muscle and kidney are less important with respect to freeze tolerance, and downregulation of miRNA biogenesis may be contributing to global MRD in these two tissues in order to conserve cellular energy. To further elucidate the functions of miRNA in liver tissue, unpublished data involved bioinformatic analysis of a small RNA dataset to filter out all non-miRNA reads (e.g., rRNA, tRNA, snoRNA, etc.), comparing control versus frozen states Ingelson-Filpula (2021). A subset of miRNAs were differentially regulated, both up and down, in response to freezing. Targets for these miRNAs appeared to center around downregulating intracellular signal transduction, apoptosis, and nuclear processes.

Many insects are also freeze-tolerant and, indeed, most tolerate temperatures far lower than frog species can, often to −40 °C or even lower (Denlinger and Lee 2010; Storey and Storey 2012b). Studies into insect freeze tolerance are currently limited to miRNAs, and these will be introduced herein. A major model for studies of the metabolic adaptations used for insect freeze tolerance is the goldenrod gall fly, *Eurosta solidaginis*, whose larvae overwinter inside galls on the stems of goldenrod. To survive, the larvae accumulate high levels of glycerol and sorbitol for cryoprotection, as much as 400 mM glycerol and 150 mM sorbitol in their tissues. MicroRNA also contributes to regulating freeze survival in this species. A study of freezing-associated miRNAs monitored responses over a time course of 3 weeks at 5 °C followed by 3 weeks at −5 °C, and finally by 3 weeks at −15 °C. A group of 24 miRNAs were differentially regulated at −15 °C, with four downregulated and 20 upregulated (Lyons et al. 2016). Lipid metabolism seems to be a focus of regulation by miRNAs in these insects, given miR-1-3p can regulate the expression of Liver X receptor alpha and modulate levels of lipogenic enzymes in humans (including fatty acid synthase and acetyl-CoA 1 carboxylase) (Zhong et al. 2013). In cold-hardy insects, selected lipid metabolism-related enzymes are known to be downregulated in *E. solidaginis* (Lyons et al. 2016). Although the larvae enter the winter season with huge lipid reserves, they are a poor fuel for winter metabolism given the need for oxygen to produce ATP from fatty acid catabolism (not an option when larvae are frozen). Instead, lipid catabolism is suppressed in winter and lipids are largely reserved for the spring pupation, emergence, mating, and egg laying by nonfeeding adults. MiR-14-3p was also bioinformatically predicted to be upregulated in this study and has been associated with stress responses and fat metabolism in *Drosophila melanogaster*. Other miRNA functions may include activation of the hypoxia-inducible transcription factor 1 alpha (HIF-1α), as evidenced by upregulation of miR-31a-3p under −15 °C conditions (where the

larvae are solidly frozen) and other work supporting HIF-1α activation during freezing in *E. solidaginis* (Morin and Storey 2005; Lyons et al. 2016). Further study by Lyons et al. revealed quantification of miR-8 and its relevance to freeze tolerance in *E. solidaginis*, given the miR-8/miR-200 family have been found to influence other models of hypometabolism (discussed elsewhere) by modulating expression of signaling pathways including Wnt, Toll, and PI3K (Lyons et al. 2015). An upregulation of miR-92b was also reported and suspected to play a role in regulating PTEN which is a regulator of cell growth (Lyons et al. 2015).

7.2.2 DNMT Enzymes in Freeze Tolerance

DNA methylation has been assessed in freeze-tolerant wood frogs to investigate whether hypermethylation of the genome is a potential contributor to inducing and maintaining a hypometabolic state during the winter. Because wood frogs utilize glucose as their primary cryoprotectant during freezing, this has the side effect of creating extreme hyperglycemia. Therefore, a study by Zhang et al. used glucose-loading as an experimental condition to investigate whether glucose cryoprotectant itself caused measurable changes in the expression/activity of the DNA methylation machinery and compared this with the effects of freezing alone (Zhang et al. 2019). A marked difference in the responses of two tissues, liver and skeletal muscle, to both freezing and glucose-loading was seen. In liver, DNA methylation appeared to be less important during freezing given that freezing did not affect DNMT protein levels, whereas total DNMT activity fell to ~25% of the control (saline) value after 24 h freezing and 5mC genome methylation decreased by about 25% (Zhang et al. 2019). The lone exception was upregulation of DNMT3L during thawing. DNMT3L possesses no catalytic activity and has various roles as a cofactor, both with DNMT enzymes as well as other epigenetic enzymes including HDAC1 (Deplus 2002). It is possible that DNMT3L was associating with other proteins that are not directly causal to DNA methylation, thus leading to the overall minimum trend in DNA methylation observed during freeze/thaw.

During glucose-loading of wood frogs (mimicking the hyperglycemia of the frozen state), the situation was somewhat different. In liver, DNMT1 and 3A were downregulated but DNMT3L once again was upregulated. Global 5mC and 5hmc levels were unchanged as was total DNMT activity. This fell in line with the trends observed during freezing: DNA methylation appeared to be downregulated in liver during glucose-loading with the lone exception of DNMT3L, which may be serving other regulatory purposes as a cofactor. It can be postulated that liver, being the most metabolically active organ and the last to freeze, needs to have its DNA accessible to transcribe genes that are crucial to survival and, hence, hypermethylation of the genome may disrupt pro-survival mechanisms.

Wood frog skeletal muscle showed somewhat different responses; both DNMT1 and DNMT3L were upregulated in response to 24 h freezing, whereas DNMT3A/3B remained unchanged along with DNMT activity (Zhang et al. 2019). Global 5mC

levels increased, perhaps reflecting DNMT1 upregulation, in contrast to liver (Zhang et al. 2019). After an 8 h thaw, DNMT1 and 5mC had returned to control levels whereas DNMT3L increased even further. Interestingly, total DNMT activity was strongly suppressed in muscle after 8 h thawed. With regard to the glucose-loading condition, DNMT1 was downregulated whereas both DNMT3A and 3B were upregulated in skeletal muscle. There were no changes in genomic 5mC levels but total DNMT activity decreased to less than 40% of the control (saline) value. DNMT1 is a maintenance methyltransferase, responsible for methylating hemi-methylated DNA following a round of DNA replication (Lyko 2018). As mentioned earlier, DNMT3L is not a canonical DNMT and does not possess any methylation capabilities, but its action as a cofactor allows greater affinity for DNA and therefore more efficient function. However, glucose-loaded frogs showed upregulation of the *de novo* methyltransferases DNMT3A or 3B suggesting that the global increase in 5mC may be due to increased DNA replication in muscle, necessitating DNMT1 function. This may be counterintuitive given that skeletal muscle is one of the first tissues to freeze and has a low metabolic activity during freezing, thus bringing the necessity of DNA replication and cell division into question. Further study will be needed to elucidate the complete function of DNA methylation in this tissue.

Investigation of DNA methylation during freeze tolerance in *R. sylvatica* has been extended to brain tissue (Bloskie 2021). Preliminary results suggest that during freezing, only levels of DNMT3B increase, while the other DNMTs remain unchanged. Thawing resulted in decreased expression levels of DNMT3A and DNMT3L. Total DNMT activity decreased during freezing and decreased further during thaw.

7.2.3 Histone Modifications Accompany Freeze Tolerance

Despite its energetically costly mechanism, transcriptional suppression is an important characteristic of hypometabolic states (Bocharova et al. 1992; Van Breukelen and Martin 2002). Like DNA methylation, a recent study has highlighted histone methyl-lysine patterns indicative of a repressed chromatin state in two tissues of wood frogs in response to freezing (Hawkins and Storey 2018). In both liver and skeletal muscle, hypomethylation of the H3K4 residue was identified, along with reduced levels of transcriptionally permissive H3K4me1. Analysis of the relevant KMT enzymes indicated that reduced expressions of SMYD2 and ASH2L were the contributing activities (Hawkins and Storey 2018). In liver, repressive H3K36me2 also appeared to be involved, but underlying mechanisms are currently unknown. H3K27me1, an intragenic-deposited permissive mark, was reduced in skeletal muscle during freezing but enriched during liver freeze-recovery. Similar to arousal from hibernation (as described in the next section), thawed recovery after freezing appears to facilitate transcriptional activation. This is evidenced in wood frog brains, where hypomethylation of H3K9 is observed during freeze-thawing (Bloskie 2021). Additionally H3K9me3, a chromatin mark highly associated to suppression of

nearby gene transcription, was significantly reduced in thaw recovery, which may be attributed to decreased expression of catalyzing enzymes SUV39H1 and ESET.

Unfortunately to date, the specific transcriptomic implications of these histone modifications have not yet been elucidated. However, several studies have highlighted the freeze-induced transcription of a number of genes (*li16, fr10, α/γ fibrinogen, glut2, ADP/ATP translocase, pyruvate kinase, ribosomal phosphoprotein P_0*) (Cai and Storey 1997a; Cai et al. 1997; Cai and Storey 1997b; Wu and Storey 2005; Sullivan and Storey 2012; Rosendale et al. 2014; Al-attar et al. 2020), which are expected to be, at least partly, due to epigenetic controls. Hepatic transcriptomic analyses in the related freeze-tolerant Cope's gray treefrog, *Dryophytes chrysoscelis*, show several DNA damage repair and heat shock response genes to be activated in cold-acclimated and frozen frogs, whereas those involved in cellular responses to oxidative stress and oxygen limitation were either downregulated or unchanged (Do Amaral et al. 2020).

Overall, freeze tolerance is a highly complex phenomenon, involving the implementation of multiple adaptations that address a variety of factors: (a) metabolic rate depression to halt or minimize metabolic processes that are not needed in a regulated manner, (b) tolerance of anoxia/ischemia to deal with lack of breathing and blood circulation while frozen, (c) a tolerance of cell and tissue dehydration due to water loss into extracellular and extra-organ ice formation, and (d) accumulation and tolerance of extreme concentrations of low molecular mass metabolites that provide colligative protection of cell volume and of macromolecular structures (e.g., glucose in wood frogs). All of the aforementioned phenomena are alien to the human condition but occur as one or more survival strategies in diverse organisms. For example, metabolic rate depression underlies hibernation, estivation, and anaerobiosis. Estivation also requires mechanisms to minimize cell and tissue dehydration (particularly in animals with highly permeable skins such as amphibians) by elevating the levels of compatible solutes like urea. Anoxia/hypoxia tolerance requires not just MRD but also pathways of ATP generation that are not oxygen-dependent. All of these survival strategies come together in freeze tolerance but they are also utilized by many other species and are regulated, at least in part, by conserved epigenetic controls on gene expression. The following sections analyze the roles of epigenetic mechanisms in some of these strategies.

7.3 Torpor/Hibernation

For mammals, homeothermy is a "double-edged sword" providing key advantages (e.g., regulated warm body temperature, ability to remain active in cold environments, fast locomotion, etc.) and disadvantages (a need for high food intake to fuel a high metabolic rate). The latter is a particular problem for small mammals, where a consistently high metabolic rate demands a huge daily food intake. As a result, many species implement energy-conserving strategies such as: (a) daily torpor—a reduction in metabolic rate during the inactive nonforaging hours, or (b) hibernation—

seasonal entry into prolonged multiday torpor to survive the winter (Jansky et al. 1986; Körtner and Geiser 2000). In both strategies, body temperature can fall to near ambient, although regulation is re-initiated if the body cools to near 0 °C (Ruf and Geiser 2015). Metabolic energy expenditure during hibernation can be reduced to as low as 1–5% of euthermic rates (Carey 2003). Prolonged torpor bouts in hibernating species are interspersed with short periods of arousal where T_b rises back to euthermic levels (near 37 °C) for several hours during which restorative actions occur before animals sink into another bout of torpor (Carey 2003). Regulation of torpor/arousal involves global controls that are used to reorganize an animal's metabolic needs including actions at physiological, biochemical, and molecular levels that downregulate nonessential processes during torpor. Such regulation, and its reversal during rewarming, includes controls at transcriptional (Srere et al. 1992; Morin and Storey 2006), translational (Wu and Storey 2012), and post-translational levels (Morin and Storey 2006; Abnous et al. 2012) including activation of selected transcription factors that have pro-survival roles (Tessier and Storey 2010; Tessier and Storey 2012). Given that all these molecular and physiological changes are transient in nature and need to be reversed during arousal back to euthermia, it is reasonable to assume that epigenetic regulation of gene expression plays a key role in facilitating cellular adaptations for daily torpor and hibernation.

Both daily torpor and seasonal hibernation have physiologically discrete phases that require distinct metabolic actions (Carey 2003). Additionally, the physiological characteristics of torpor/arousal can vary greatly from one species to the next. Henceforth, our discussion of 13-lined ground squirrel hibernation will involve these phases: EC designates euthermic control animals in the 5 °C cold room that have a stable body temperature (T_b, ~37 °C) and could enter torpor, but had not done so for at least three days. When triggered to enter a torpor bout, body temperature falls over time during the entrance (EN) phase ($T_b = 18$–31 °C) before T_b stabilizing at 5–8 °C; animals sampled after 1 day at this T_b are termed early torpor (ET). Late torpor (LT) is defined as $T_b = 5$–8 °C for >5 days into the torpor bout. Squirrels can remain in torpor for many days but, ultimately, arouse back to euthermia for short periods of time. The early arousal (EA) period is characterized by a rising T_b with an increase to 9–12 °C being indicative of a full arousal to come. Interbout arousal (IA) typically lasts ~18–24 h during which T_b stabilizes at euthermic values. Since EA and IA periods are characterized by high metabolic rates, re-establishing euthermic values during IA before decreasing again into another torpor bout, this presents the unique challenge for the animals that need to implement and reverse MRD multiple times over the hibernation season, adding another level of intricacy onto metabolic reorganization and regulatory control. Figure 7.3 highlights the major ways that epigenetics underlies torpor and hibernation.
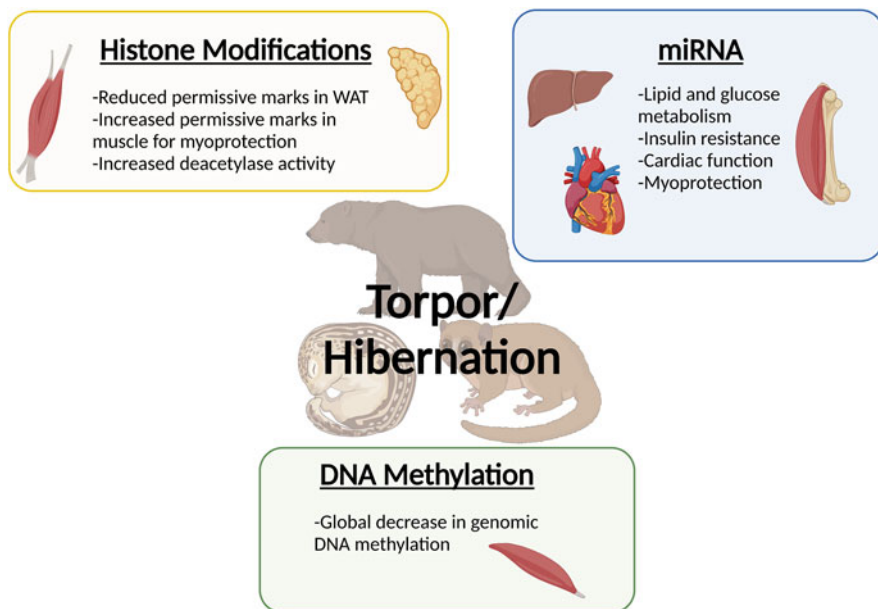
Fig. 7.3 A graphical depiction of the main biological processes affected by epigenetics during torpor and hibernation. Figure created using BioRender.com

7.3.1 MiRNA Involvement in Torpor and Hibernation

A first foray into analyzing the role of microRNAs in mammalian hibernation used RT-qPCR to evaluate the responses by microRNAs in liver and skeletal muscle of 13-lined ground squirrels, *Ictidomys tridecemlineatus* (Lang-Ouellette and Morin 2014). Increased levels of miR-29a were observed in liver of hibernating animals and were linked to functions including reduced glucose production via G6Pase and PGC-1α. Additionally, the fatty acid synthesis pathway appeared to be highly regulated given the reduced expression of fatty acid synthase in liver coupled with the overexpression of miR-195, a regulator of the fatty acid synthesis pathway. Other miRNAs have been reported to target the fatty acid synthesis pathway, confirming that lipid metabolism plays a key role during torpor (Lang-Ouellette and Morin 2014). Tangentially related were FOXO1 and SR-BI, targets of miR-223, that were also elevated in liver; these have ties to oxidative stress and glucose metabolism and high-density lipoprotein cholesterol (Greer and Brunet 2005; Wang et al. 2013).

The study of miRNA involvement during hibernation of *I. tridecemlineatus* was greatly expanded by Wu et al. with an analysis of 117 miRNAs assessed in liver, heart, and skeletal muscle across four stages of torpor/arousal in this squirrel species (Wu et al. 2016). In heart and skeletal muscle, enriched miRNAs targeted pathways related to cell growth, microtubule cytoskeleton organization, and active transport. Liver showed a similar trend, with miRNAs linked to downregulation of

energy-intensive processes including endosome transport, growth factor receptor signaling, mitosis and nuclear division, and glycolysis regulation. With regard to specific miRNAs, miR-208b was strongly upregulated in heart during LT and IA, and its action is known to be directly linked to regulating cardiac arrhythmias. Hence, miR-208b was hypothesized to play a role in facilitating the major decrease in heart rate from ~300 bpm in euthermia to ~10 bpm in torpor. In skeletal muscle, however, miR-208b was downregulated. Other known functions of this miRNA involve muscle remodeling, leading to the proposal that suppression of miR-208b, as a negative regulator of gene expression, may facilitate some needed changes in muscle contractile proteins at cold temperatures such as may also contribute to the shivering thermogenesis that aids rewarming of the squirrel body during arousal from torpor. Finally, insulin resistance appeared to be regulated via miR-181a overexpression specifically in liver during ET.

MiRNAs involved in insulin sensitivity were also overexpressed in brown adipose tissue in hibernating *I. tridecemlineatus* (Logan and Storey 2021). These miRNAs targeted nearly all major genes in the glycolysis pathway, thus downregulating them, while KEGG pathway analysis predicted enrichment of gluconeogenesis (Logan and Storey 2021). This inhibition was continued for major enzymes in the electron transport chain and possible anaerobic metabolism via L-lactate dehydrogenase.

Several novel miRNAs were predicted from small RNA-sequencing data, screened against database miRDeep, and experimentally validated to be significantly altered during hibernation in liver, skeletal muscle, and heart of *I. tridecemlineatus* and revealed roles for miRNAs in metabolism and signal transduction cascades (Luu et al. 2016). The metabolism-focused miRNAs in liver reinforced the switch to lipid oxidation from glucose consumption, which strengthens the results of Lang-Ouellette and Morin (Lang-Ouellette and Morin 2014). Downregulation of miRNAs in skeletal muscle and heart also corroborated the findings of Wu et al. (Wu et al. 2016) since the observed downregulation of miRNAs may facilitate myoprotective roles and skeletal muscle remodeling in response to decreased mobility (Luu et al. 2016).

Studies of the gray mouse lemur, *Microcebus murinus*, native to Madagascar, provide further insights into the roles of microRNA in hypometabolism using an animal that commonly undergoes daily torpor with a relatively small decrease in T_b but can also exhibit multiday hibernation in the cool, dry winter season (Schmid and Kappeler 1998). As a primate, this species has the closest evolutionary link to humans of any hibernator and this makes studies of its torpor capacity more relevant for potential discovery of metabolic mechanisms that can be employed to induce metabolic rate depression in humans. A study by Biggar et al. (Biggar et al. 2018) measured novel and conserved miRNA in *M. murinus*, and found 122 conserved miRNAs along with 44 novel miRNAs in liver. Of these, 16 conserved miRNAs were upregulated in liver during torpor, whereas 30 were downregulated. Similarly ten novel miRNAs were upregulated during torpor while only one displayed significant downregulation (Biggar et al. 2018). Interestingly, miR-222 (downregulated in *M. murinus*) has been found in white adipose tissue of hibernating ground squirrels

and may allow for metabolic adaptation in insulin-sensitive tissues, such as liver and adipose (Wu et al. 2014). Pathways under increased translational repression via miRNAs involve cell differentiation and growth, whereas pathways "enhanced" by reduced levels of miRNA during torpor include immune processes and G-protein-coupled signaling.

In skeletal muscle of *M. murinus*, 20 miRNAs amid a group of 234 conserved miRNAs were significantly altered during torpor (Hadj-Moussa et al. 2020). Eleven were significantly upregulated, and nine were significantly downregulated. Key members of the myo-miR family were among those downregulated; suppression of this group may act to limit muscle growth and differentiation which are very metabolically expensive and not congruent with MRD during torpor (McCarthy 2011). Moreover, two myomiRs (miR-1 and miR-133) directly target other potentially crucial processes including apoptosis, where reduced levels of miR-1 and/or miR-133 have been shown to favor survival (Xu et al. 2007). Of the upregulated miRNAs, many were related to cell growth including miR-2478 and miR-889 that target TGFβ1, a receptor primarily linked to cell proliferation and differentiation; these, energy-expensive processes are typically suppressed during torpor/hibernation (Li et al. 2017).

The small marsupial, *Dromiciops gliroides*, is unique in that is the only hibernating marsupial in South America and the last living relative of the Order Microbiotheria (Bozinovic et al. 2004). *D. gliroides* undergoes daily torpor in response to environmental stress and is also capable of prolonged hibernation in the winter. Hibernation-responsive miRNAs have been studied in liver and skeletal muscle of this animal, which continued to draw parallels to torpor-sensitive processes with a heavy emphasis on signaling-related pathways (Hadj-Moussa et al. 2016). In liver, signaling including MAPK, mTOR, and PI3K/Akt protein kinases was enriched due to downregulation of relevant miRNAs, whereas skeletal muscle appeared to overexpress miRNAs that regulate the ErbB and mTOR signaling pathways. The tissue-differentiated response of miRNAs between liver and skeletal muscle has been robustly demonstrated in primates by Biggar et al. (Biggar et al. 2018) and Hadj-Moussa et al. (Hadj-Moussa et al. 2020). Recall that miRNAs are generally downregulated in skeletal muscle of *I. tridecemlineatus* to contribute to myoprotective roles, so the upregulation observed in skeletal muscle of *M. murinus* and *D. gliroides* may signal a unique, species-specific role for miRNAs in primates and marsupials as compared with rodents, or a potential molecular difference between low-T_b versus high-T_b hibernation.

Another "warm hibernator", that is, a hibernator that maintains T_b at or near euthermic levels during hibernation, is the brown bear *Ursus arctos*. Muscle atrophy, or the lack thereof during hibernation, is a primary area of study and may be due in part to the MEF2A (myocyte enhancer factor 2A) signaling pathway, that is responsible for skeletal muscle development, maintenance, and regulation (Taylor and Hughes 2017). An investigation by Luu et al. used RT-qPCR to analyze 36 miRNAs linked to MEF2A in muscle samples from hibernating versus summer-active bears (Luu et al. 2020). Three miRNAs under MEF2A regulation were increased during hibernation and their corresponding mRNA target transcript levels decreased

in turn (Luu et al. 2020). Another 18 miRNAs involved in skeletal muscle regulation were also quantified, six of which were upregulated in hibernating bears. Finally, 11 members of the myomiR family which play roles in skeletal muscle atrophy and regeneration were studied, and three were upregulated (miR-23a-5p, miR-221–3p, and miR-31-5p) whereas two were downregulated (miR-199a-5p and miR-223-5p) (Luu et al. 2020). Taken together, these results implicate miRNAs in facilitating MRD and skeletal muscle maintenance during hibernation, at least partially through upregulation of MEF2A. Other functions of miRNAs involved decreased glucose utilization and uptake along with decreased fatty acid oxidation/lipid metabolism (Luu et al. 2020).

7.3.2 DNMT Enzymes in Torpor

The role of DNA methylation was assessed in the model hibernator, the 13-lined ground squirrel, *I. tridecemlineatus*. Global methylation levels, mRNA transcript levels of DNMT1 and DNMT3B enzymes, and mRNA transcript levels of "reader" proteins MBD1–3 and MeCP$_2$ were measured in liver and skeletal muscle (Alvarado et al. 2015). Significant changes in DNA methylation patterns in the liver were seen only during the IA phase of torpor, and while there was altered expression of *dnmt* transcript levels during various stages of hibernation, they were not correlated with changes in genomic DNA methylation at the corresponding timepoints. In muscle, genomic DNA methylation decreased strongly during LT, EA, and IA stages of hibernation (Alvarado et al. 2015). This decrease in genomic methylation may represent implementation of global MRD in muscle, supported by the overall transcriptional activity observed in the skeletal muscle of hibernating mammals (Bocharova et al. 1992; Storey and Storey 2004; Morin and Storey 2006). Furthermore, no significant changes in *dnmt* expression were observed across the torpor-arousal cycle of hibernation in skeletal muscle despite decreases in global DNA methylation. As mentioned above, other trans-acting mechanisms known to regulate methylation include post-translational (Kang et al. 2001) and post-transcriptional events that may affect DNMT enzymes to alter the final methylation state of the genome; this may explain the contradictory results observed in skeletal muscle.

7.3.3 Histone Modifications during Torpor

Research on the role of epigenetic mechanisms in mammalian hibernation has largely focused on two models, the Siberian chipmunk (*Tamias asiaticus*) and 13-lined ground squirrel (*I. tridecemlineatus*). Histone modifications have been shown to be integral to the torpor-mediated knockdown of hibernating proteins HP20, HP25, and HP27 in the liver of Siberian chipmunks (Tsukamoto et al. 2017; Tsukamoto et al. 2018). HP20/25/27 are highly homologous, belonging to

the C1q and tumor necrosis factor (C1q/TNF) superfamily (Kondo and Kondo 1992; Kishore et al. 2004). They form a 140 kDa complex in circulating blood. Using chromatin immunoprecipitation analysis, researchers demonstrated that permissive histone modifications, H3K9ac, H3K14ac, and H3K4me3, were all reduced in the HP25 promoter during torpor. The data suggested that this is due, in part, to decreased DNA binding capacity from putative "writer" enzymes KAT3A, NCoA-1, KAT2B, and SETD1A that are triggered by disabled binding of the coactivator, hepatocyte necrosis factor 4 (HNF4), to the HP25 promoter by the small heterodimer partner (SHP) (Tsukamoto et al. 2017). Subsequent work showed similar repression at HP27 and HP20 promoters via decreased binding of the coactivators, USF2 and/or HNF1 (Tsukamoto et al. 2018).

Histone modifications are also implicated on a global scale during the torpor-arousal cycle of ground squirrels. Deacetylation was initially suggested as a contributor to transcriptional suppression in torpor where a reduction of transcriptionally permissive H3K23ac, along with increased activity and expression involving HDAC1 and HDAC3 were noted in the skeletal muscle of hibernating animals (Morin and Storey 2006). These results were later supported by data showing increased total class I/II HDAC activity in the skeletal muscle of torpid ground squirrels (Hawkins and Storey 2017). Torpor-mediated suppression of skeletal muscle KAT3A and hepatic KAT2A also occurred (Rouble et al. 2018). The complexity of acetyl-histone mechanisms in mammalian hibernation has become increasingly evident with continuing research, often with tissue-specific but sometimes unclear results. Tissue differences were particularly apparent when acetyl-histone profiles of brown (BAT) and white adipose tissue (WAT) were compared. In BAT, increased levels of H3K9ac, likely a result of increased KAT2A expression and total histone acetyltransferase activity, provided evidence of a more transcriptionally permissive state during ET and LT (Rouble et al. 2018). Analysis of WAT suggested a different pattern, since H3K9ac was reduced during ET, along with decreased global HAT activity and KAT1 expression in late torpor, suggesting the reverse. Another study showed increased SIRT2 levels during LT in WAT (Rouble and Storey 2015). The contrast between these results is likely explained, in part, by differences in function required of BAT and WAT during hibernation. Metabolically active BAT must oxidize lipid stores to support nonshivering thermogenesis to prevent body temperature from falling below 0 °C and to reheat the body during arousal, whereas a less active WAT provides fatty acids' fuels to other tissues during the winter. Hence, the markedly different functions of the two adipose tissues undoubtedly lead to differing requirements for gene transcription. Acetylated histone residues, that support active transcriptional states, were also linked to early arousal states (Tessier et al. 2017; Rouble et al. 2018). In skeletal muscle during EA, global H3K14ac and H3K18ac were elevated (Tessier et al. 2017). KAT2A and KAT2B protein levels were also upregulated in the liver during EA (Rouble et al. 2018), although KAT2A expression remained high across all torpor stages. This preliminary evidence suggests that induced transcription during early arousal might facilitate essential pro-survival mechanisms across the torpor-arousal transition.

Analysis of histone lysine methylation also showed marked trends during ground squirrel hibernation (Watts and Storey 2019). In both liver and muscle, permissive H3K4me1 marks peaked during entrance into and arousal from torpor and this was suggested to result from increased KMT2 complex enzyme (ASH2L, RBBP5) expression leading to myoprotective roles. G9a methyltransferase, a mediator of repressive H3K9me2/3, was also induced during these transitions and SMYD2 targeted H3K4 and H3K36 to allow transcriptional activation during these states in both tissues. A heightened need for myoprotective factors at times of higher metabolic activity (i.e., EA and IA) can be postulated to explain this, although gene-specific methyl-lysine dynamics are still being investigated.

7.4 Hypoxia and Anoxia

Hypoxia is defined as low (suboptimal) availability of oxygen, whereas anoxia is a complete lack of oxygen. Both of these conditions arise when the cellular need for oxygen is greater than the accessible supply. Many animals experience hypoxia/anoxia as a result of their environmental conditions, particularly among various aquatic species such as those that undergo breath-hold diving (e.g., turtles such as the red-eared slider turtle *Trachemys scripta elegans*) or gill-breathing species that experience seasonal depletion of oxygen in water (e.g., crayfish *Orconectes virilis*, or fish such as Crucian carp and goldfish) or are deprived of oxygen during each low tide (Nilsson and Renshaw 2004; Storey 2007). Hypoxia/anoxia stress is also one component of freeze tolerance due to the lack of blood flow and gas exchange in frozen animals. Most species that experience routine hypoxia/anoxia show regulated metabolic rate depression to lower their energy needs when oxygen is depleted and, coupled with cold water during the winter season, most can survive for many weeks using anaerobic pathways of metabolism alone. For example, red-eared sliders can survive for 12–18 weeks in cold water without breathing oxygen (Jackson 2002). Metabolism switches from aerobic to anaerobic, high glycogen stores are slowly consumed by tissues, antioxidant defenses are upregulated to combat reactive oxygen species (ROS) formation and damage, and end products of anaerobic glycolysis are excreted or buffered (Storey 2007). For example, turtles store lactate in their shells using Ca^{2+} and Mg^{2+} bicarbonate ions released from the shell to buffer acidosis.

All the hallmarks of MRD and hypometabolism hold true for animals that survive hypoxia/anoxia. Metabolic rate is often lowered to ~10% or less of the aerobic rate, and nonessential/energy-expensive processes are downregulated. Energy usage is reprioritized to survival mechanisms including antioxidant defenses, antiapoptotic mechanisms, and anaerobic glycolysis. Epigenetic and post-transcriptional controls on gene expression can assist with the implementation of these survival strategies, and this section highlights some relevant studies performed on anoxia-tolerant animals. For a visual depiction of the primary hypoxia- and anoxia-responsive epigenetic effects, see Fig. 7.4.
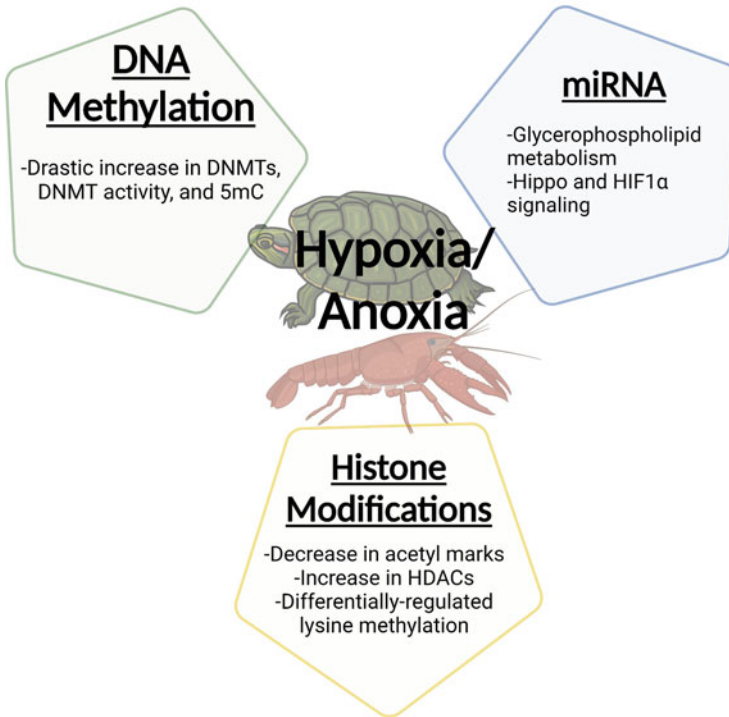
Fig. 7.4 A layout of the primary epigenetic roles during hypoxia and anoxia. Figure created using BioRender.com

7.4.1 MiRNAs in Anoxia

The northern crayfish *O. virilis* must contend frequently with hypoxic and anoxic water arising from high heat in shallow water in summer or ice-locked waters in winter. Whereas the molecular mechanisms by which *O. virilis* endures these conditions have not been well-studied, it is hypothesized that crayfish can enter a hypometabolic state similar to other anoxia-tolerant animals. Differential microRNA expression appears to contribute to their survival. An analysis of 76 miRNAs using RT-qPCR compared crayfish responses under acute (2 h) or chronic (20 h) anoxia exposures in two tissues, hepatopancreas and tail muscle (English et al. 2018). Interestingly, hepatopancreas metabolism appeared to be strongly regulated by miRNA action with 21 mRNA species downregulated under acute anoxia, whereas tail muscle showed significantly altered levels of only two miRNAs (one up- and one down-regulated) as well as two significantly upregulated in chronic anoxia (English et al. 2018). Bioinformatic analysis of the miRNAs altered in hepatopancreas suggested that the Hippo, JAK-STAT, and MAPK signaling pathways were particular targets under anoxia along with glycerophospholipid metabolism and mucin type O-glycan biosynthesis. The Hippo pathway has strong links to the hypoxia

stress response given that decreased signaling by this pathway promotes hypoxia-responsive genes indirectly via HIF-1α (Morin et al. 2005). Moreover, cell growth and proliferation are suppressed by Hippo under hypoxia stress, strengthening the importance of miRNA regulation of this pathway during anoxia. Many of the specific miRNAs predicted through bioinformatic analysis proved to be either direct or indirect regulators of HIF-1 itself, shedding more light on the importance of this transcription factor during anoxia in crayfish.

The importance of miRNAs in anoxia tolerance of turtles (*T.s. elegans*) began with RT-PCR quantification of a select group of miRNAs in liver, white muscle, spleen, and kidney in response to both acute (5 h) and chronic (20 h) anoxia exposure (Biggar and Storey 2017). Tissues showed variable expression of the miRNAs chosen for assessment. In liver, five miRNAs were upregulated under anoxic conditions, whereas three were downregulated. White muscle showed six upregulated and only one downregulated, kidney had six upregulated and three downregulated, and spleen showed five upregulated and one downregulated (Biggar and Storey 2017). Only one miRNA, miR-20a, showed similar anoxia-responsive upregulation across all four tissues and its gene targets center around cell division and proliferation. This signifies that increased expression of miR-20a helps to suppress the energy-expensive cell cycle during periods of oxygen deprivation (Biggar and Storey 2017). Another miRNA that was similarly expressed in muscle, kidney, and spleen was miR-21, which may mediate an antiapoptotic role in these tissues.

7.4.2 DNMT Enzymes under Anoxia

Altered DNA methylation also contributes to anoxia tolerance in *T. s. elegans*, as reported by Wijenayake and Storey (Wijenayake and Storey 2016). Enzymes responsible for reading, writing, and erasing DNA methyl marks were differentially regulated in both a tissue-specific manner and over time under anoxia stress. For example, in liver, DNMT1 and DNMT2 protein levels were strongly upregulated by 4- and 2- fold, respectively, in response to 5 h submergence in nitrogen-gassed water, before declining again to near control levels after 20 h anoxia. Liver MBD1 and MBD2 proteins also increased by ~three-fold after 5 h anoxic submergence but were partially reduced again after 20 h. Total DNMT activity and global 5mC levels also increased significantly in liver after both 5 h and 20 h of anoxia exposure. In white muscle, the primary responses were by DNMT3a and 3b whose protein levels increased by ~three-fold after 5 h anoxic submergence and DNMT3b remained high after 20 h anoxia whereas DNMT3a fell to below control levels. MBD1 protein showed no change over both anoxia conditions and DNMT3B levels also rose during 5 h anoxia, and increased even further after 20 h anoxia. Total DNMT activity was increased in both 5 h and 20 h anoxia, and global 5mC methylation rose in the 5 h anoxia condition but declined somewhat after 20 h anoxia but remained higher than control values.

In heart, DNMT1 protein levels did not change across anoxia but DNMT3A rose after 5 h anoxia and DNMT3B increased strongly after 20 h anoxia. Total DNMT activity in heart was essentially unchanged as were global 5mC levels.

It is noteworthy that DNMT activity was upregulated across all three tissues in response to 20 h anoxia exposure, with two tissues (liver and white muscle) also showing this elevated activity after 5 h anoxia. The protein expression levels of various DNMT enzymes were more varied: whereas some were upregulated after 5 h anoxia, others remained unchanged or were even downregulated after 20 h anoxia exposure. This would suggest that the cell's primary method of regulating DNMTs is not strictly from increased or decreased protein synthesis, which would strain available cellular resources, but another mechanism which affects DNMT activity and supports more efficient modulation of methyltransferase activity. Global 5mC levels reflected this increase in activity in both liver and white muscle, whereas 5mC levels in heart remained unchanged after 5 h anoxia (corresponding to the unchanged DNMT activity in this condition) and remained consistent during 20 h anoxia even though DNMT activity was increased at this timepoint.

7.4.3   Histone Modifications during Anoxia

Both histone lysine methylation and acetylation have been examined as parts of the anoxia tolerance response of red-eared sliders (*T. s. elegans*) (Krivoruchko and Storey 2010; Wijenayake et al. 2018; Wijenayake and Storey 2020). In terms of methylation, permissive H3K4me1 and repressive H3K9me3 levels were both elevated during prolonged (20 h) anoxia. The expression of corresponding methyltransferases ASH2L and G9a changed in agreement with their respective histone target residues, implying contributing roles. Global KMT activities at H3K4 and H3K9 were also increased under anoxia exposure (Wijenayake et al. 2018). Overall, this study suggested that lysine methylation plays a complex role in the gene regulation of anoxia survival, likely promoting transcription of anoxia-responsive genes, while actively suppressing nonessential pathways. Liver transcriptomic studies found mRNA significantly increased in pathways related to DNA damage repair and metabolic reprogramming (Biggar et al. 2019). Their results suggest that heightened succinate metabolism may be utilized during turtle anoxia to combat lactate accumulation, which is characteristic of other established models.

Equally intricate mechanisms were found upon investigation into histone acetylation. In a related study, H3K14ac, a hallmark of active promoters, was found to be consistently reduced across both short- and long-term anoxia stress conditions in turtle liver (Wijenayake and Storey 2020). KAT3A protein levels, an enzyme involved in H3K14ac catalysis, as well as global nuclear lysine acetyltransferase activity were similarly depressed, suggesting their involvement. KAT1 expression was also reduced during prolonged anoxia. This study built on previous work on deacetylases, which highlighted attenuated H3K9ac and H3K23ac levels in both liver and muscle tissues of turtles (Krivoruchko and Storey 2010). That study also

showed that a variety of HDACs were upregulated at both transcript and protein levels in response to anoxia. These results implied that transcriptional suppression may be mediated by deacetylation of key histone lysine residues, made possible by hypoactive acetyltransferase and hyperactive deacetylase activities that contribute to metabolic depression under anoxic conditions.

7.5 Estivation/Dehydration

Dehydration stress has been discussed above as a subcomponent in freeze tolerance, where cells lose liquid water that exits to join extracellular and extra-organ ice masses. However, more commonly, dehydration is a response to extreme heat and/or drying of the environment. Species with a poor capacity to resist water loss across their body surface are particularly vulnerable and often seek shelter underground including various species of frogs, toads, and lungfish. Some can minimize dehydration stress (at least initially) by constructing mucus or shed skin cocoons around their bodies or, for many amphibians, by slowly resorbing water from a very full bladder (a skill not found in other vertebrates) as well as retaining nitrogenous waste (urea) to elevate the osmolality of body fluids (Storey and Storey 2010b). Also key to survival in hot dry environments is metabolic rate depression, termed estivation that occurs widely among both vertebrates and invertebrates; for example, the milk snail, *Otala lactea*, is a well-studied model. These snails also limit water loss by constructing a mucus membrane across the aperture of the shell to minimize water evaporation, accumulating high concentrations of urea to provide colligative resistance against tissue water loss, and suppressing metabolic rate to only about 30% of their nonestivating rate (Bell et al. 2012). Estivating species can typically maintain aerobic metabolism for a long time but ultimately, as water loss progresses, blood plasma volume decreases (concentrating blood cells) and the workload on the heart increases to maintain circulation. Decreasing oxygen transport triggers an increased dependence on glycolysis for ATP generation resulting in lactate accumulation. To cope with this hypoxic and water-restricted state over a long period of time with no nutrient consumption, dehydration-tolerant animals then resort to metabolic rate depression to minimize substrate and ATP consumption. Figure 7.5 highlights the main functions of miRNA control during estivation and dehydration.

7.5.1 MiRNAs in Dehydration

As for other systems of MRD described above, a suppression of nonessential processes during estivation is key to survival and microRNA can play a significant role in this. Using the African clawed frog, *X. laevis*, as the model organism, a study of dehydration-induced changes in miRNA patterns in liver, skin, and kidney showed significant changes consistent with hypometabolism (Wu et al. 2013). An
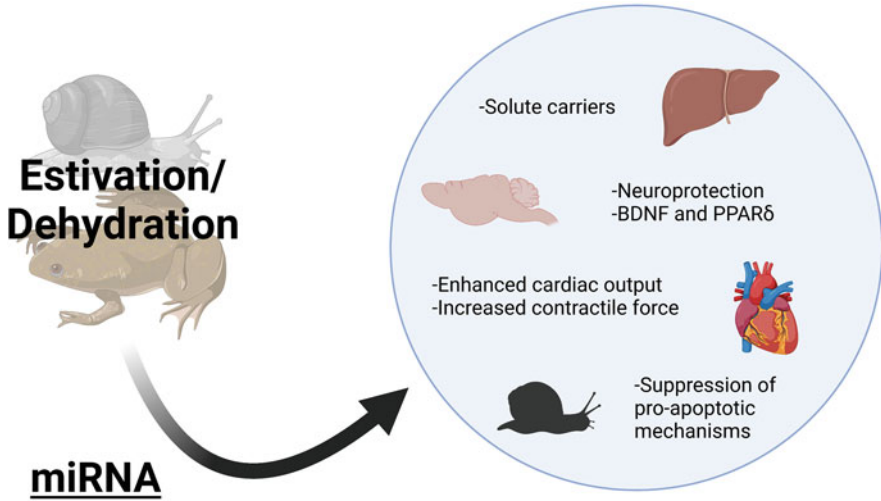
Fig. 7.5 The critical miRNA influences during estivation and dehydration between *X. laevis* and *O. virilis*. Figure created using BioRender.com

analysis of ten miRNAs revealed that three were downregulated and three upregulated in liver in response to the loss of >30% of total body water. Kidney showed three different miRNAs upregulated and skin showed only two upregulated whereas other miRNAs analyzed remained unchanged (Wu et al. 2013). MiR-203 was upregulated in both kidney and skin but unchanged in liver, whereas miR-34a was upregulated in skin and liver but not kidney. The downregulated miRNAs in liver were those that target genes for solute carriers. Various solute carriers have been observed to be upregulated during anoxia and hibernation, suggesting their general importance for MRD (Wu et al. 2013). There were other links to hibernation, e.g., upregulation of miR-29b which targets p85α, the catalytic subunit of the Akt pathway, which is reduced during hibernation (Abnous et al. 2008).

A second study that evaluated the responses of 43 miRNAs from brain of *X. laevis* via RT-qPCR revealed 12 that were downregulated during dehydration and none that showed upregulation (Luu and Storey 2015). Predicted functions for the downregulated miRNAs involved genes associated with axon guidance and long-term potentiation, which could be enhanced as a result and therefore worth investigating as coping mechanisms for dehydration tolerance. Other neuroprotective pathways may be activated given that downregulated miRNAs of interest in this study have also been shown to suppress neuroprotective factors such as BDNF and PPARδ in other species (Yin et al. 2010; Gao et al. 2015).

A further study of miRNA regulation during dehydration in *X. laevis* evaluated heart with a bioinformatics-centered study that identified 24 miRNAs that were differentially regulated in response to dehydration stress (Hawkins and Storey 2020). Of these, 21 were significantly downregulated whereas the remaining three were upregulated. The large number of downregulated miRNAs suggested a

facilitated upregulation of genes and processes with pro-survival actions. These could include actions that increase the contractile force of the heart to sustain circulation in the face of an increased thickening of blood due to water loss from the plasma as evaporative dehydration progresses (Hillman 1978). MiR-99b-5p showed the greatest change during dehydration with reduction to 15% of control values, and it was hypothesized that downregulation of this miRNA is linked to enhanced cardiac output. Functions of the collective group of differentially expressed miRNAs also related to RNA/DNA/transcription factor binding, with particular emphasis on proteins involved in other facets of post-transcriptional regulation (Hawkins and Storey 2020). The KEGG pathway Cardiac Muscle Contraction was the most significantly enriched, reflecting knowledge that heart function must increase to cope with the reduction in blood volume and increase in viscosity caused by water loss during dehydration, as mentioned earlier. Ion transporters were also targeted by specific miRNAs predicted to be downregulated in the study, and the Glycolysis/Gluconeogenesis pathway was also enriched. This is in line with known features of hypometabolic states, including increased reliance on anaerobic metabolism for ATP production.

The estivating snail *O. lactea* also showed differential miRNA expression as a facet of its stress tolerance (Hoyeck et al. 2019). A selection of 75 miRNAs were detected in foot muscle, of which 26 were upregulated during estivation and none were downregulated. The significantly upregulated miRNAs were implicated in regulating cell survival mechanisms revolving around antiapoptosis, tumor suppression, and muscle maintenance responses. The miR-2 family were among those upregulated and these suppress pro-apoptotic mechanisms, which would be crucial during estivation (Gennarino et al. 2012). Other antiapoptotic miRNAs upregulated in foot muscle included miR-153 and miR-124, further highlighting the importance of antiapoptotic measures in this animal (Hoyeck et al. 2019).

7.6 Conclusions

In this chapter, we provided a comprehensive look into modes of epigenetic regulation and how they interplay with extreme environmental stress conditions including freeze tolerance, torpor, hypoxia/anoxia, and estivation/dehydration. The main themes are outlined as follows:

During freeze tolerance, miRNA regulation seems to be greater in liver which is highly metabolically active, and therefore requires stricter control over essential processes. However, miRNA biogenesis seems to be downregulated in less critical tissues to aid in the theme of global MRD and hypometabolism and save on energy resources. The emphasis on signaling pathways (namely PI3K) in both invertebrate and vertebrate models of freeze tolerance may insinuate that miRNA is especially vital in maintaining intracellular transduction during this stress. Other functions may vary according to tissue-specific need, given the observed variation in expression in liver versus skeletal muscle and kidney, whereas closer analysis of miRNAs in brain

revealed neuroprotective roles, and heart showed cardioprotective roles. The rise of bioinformatic prediction tools and large-scale sequencing efforts may help shed more light on the functions of miRNAs across species in response to freezing stress and help elucidate their exact roles in enabling survival of this extreme environmental stress.

DNA methylation also appears to be less important in liver during freeze tolerance, so it is possible that miRNA is predominant in regulating cellular processes in this tissue in response to freezing stress. DNA methylation exhibited tissue-specific variations during freeze tolerance. Strong upregulation of DNMT3L was observed for three freeze-tolerant species, potentially signifying its importance as a cofactor to interact with many different forms of epigenetic control. The upregulation of DNMT1 also merits note, although downregulation in liver and upregulation in muscle (*R. sylvatica*) were opposite to the upregulation in liver and downregulation in muscle (*D. versicolor*). DNMT enzymes are also subject to a variety of post-transcriptional and post-translational modifications, all of which affect downstream activity and final methylation patterns of the genome. More research will uncover specific mechanisms and functions by which DNA methylation interplays with MRD and hypometabolism as a whole.

In torpor, miRNAs appear to play cross-species roles in muscle maintenance. Insulin resistance and lipid metabolism are also themes, with miRNAs targeting glycolysis and insulin-related pathways appearing in both *I. tridecemlineatus* and *M. murinus*, species with diverse profiles of torpor/hibernation use. DNA methylation during hibernation is understudied and with current preliminary data, it is hard to draw hypotheses regarding its function in any species. However, future research may elucidate a specific role, if any, for DNA methylation during hibernation.

Studies in hypoxia/anoxia strongly suggest a role for miRNAs and DNA methylation to suppress the cell cycle, as shown in *T.s. elegans*. Northern crayfish *O. virilis* appeared to utilize miRNAs in antioxidant defense, as evidenced by interactions with HIF1, which was echoed by antioxidant defense and protection against ROS observed in *T.s. elegans*. Overall, signaling pathways and antiapoptotic mechanisms have important roles across both anoxia-tolerant species, highlighting an area for future research in all aspects of epigenetic control.

Of all environmental stresses discussed, estivation/dehydration has received the least research. The model organisms explored so far have been *X. laevis* and *O. lactea,* each living in very different habitats that limits our ability for pattern-establishment between these models. The importance of miRNAs in regulating the expression of solute carriers appeared in *X. laevis,* as well as the need for increased cardiac contractility to cope with reduced blood volume. Analysis of *O. lactea* highlighted the importance of miRNA action in regulating antiapoptotic mechanisms.

The metabolic reorganization needed to both facilitate and maintain MRD is extensive and requires tight regulatory oversight through many factors, including by epigenetic and other modes of post-transcriptional control. The diversity of animals which use hypometabolism as a survival strategy for dealing with severe environmental conditions ranging from extreme cold to extreme heat is expansive, making it

all the more impressive that epigenetic and post-transcriptional mechanisms are crucial players in all the studies discussed in this chapter. The following years will see an increase in understanding of all these molecular mechanisms, and bring us closer to learning how animals ranging from molluscs to primates have evolved to survive and thrive in environmental conditions humans have yet to tolerate.

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