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REVIEW

Regulation of hypometabolism: insights into epigenetic controls

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ABSTRACT

For many animals, survival of severe environmental stress (e.g. to extremes of heat or cold, drought, oxygen limitation, food deprivation) is aided by entry into a hypometabolic state. Strong depression of metabolic rate, often to only 1–20% of normal resting rate, is a core survival strategy of multiple forms of hypometabolism across the animal kingdom, including hibernation, anaerobiosis, aestivation and freeze tolerance. Global biochemical controls are needed to suppress and reprioritize energy use; one such well-studied control is reversible protein phosphorylation. Recently, we turned our attention to the idea that mechanisms previously associated mainly with epigenetic regulation can also contribute to reversible suppression of gene expression in hypometabolic states. Indeed, situations as diverse as mammalian hibernation and turtle anoxia tolerance show coordinated changes in histone post-translational modifications (acetylation, phosphorylation) and activities of histone deacetylases, consistent with their use as mechanisms for suppressing gene expression during hypometabolism. Other potential mechanisms of gene silencing in hypometabolic states include altered expression of miRNAs that can provide post-transcriptional suppression of mRNA translation and the formation of ribonuclear protein bodies in the nucleus and cytoplasm to allow storage of mRNA transcripts until animals rouse themselves again. Furthermore, mechanisms first identified in epigenetic regulation (e.g. protein acetylation) are now proving to apply to many central metabolic enzymes (e.g. lactate dehydrogenase), suggesting a new layer of regulatory control that can contribute to coordinating the depression of metabolic rate.

KEY WORDS: Metabolic rate depression, Hibernation, Anoxia tolerance, Histone control, miRNA, RNA-binding proteins, Post-translational modification

Introduction

All organisms are susceptible to stresses that can put their survival in danger, whether they be environmental abiotic factors (e.g. too hot, too cold, restricted availability of oxygen, water or food, altered salinity, pollution, radiation, etc.) or physiological conditions (e.g. reproductive demands, exercise extremes, injury, disease, infection, etc.). Abiotic factors are particularly challenging, especially when they are long term or seasonal. Hence, many animal species respond to severe challenges by transitioning into hypometabolic states where they minimize their metabolic rate and thereby maximize the time that they can survive, hopefully maintaining viability until environmental conditions are once again conducive for active life and the resumption of growth, development and reproduction. Hypometabolism is the main underlying principle of phenomena with many names, such as torpor, hibernation, aestivation, diapause, dauer, anaerobiosis, freeze tolerance and anhydrobiosis. The biochemical mechanisms and adaptations that support hypometabolism have formed the core of research in my laboratory for many years (Storey and Storey, 1990; Storey and Storey, 2004; Storey and Storey, 2007; Storey and Storey, 2010; Storey and Storey, 2012a; Storey and Storey, 2012b; Storey and Storey, 2013). Central requirements for long-term viability in a hypometabolic state include (a) regulated suppression of net ATP turnover, (b) reprioritization of cell functions to conserve fuel/energy use, and (c) preservation and stabilization of cellular macromolecules to deal with both continuing effects of abiotic stress during the hypometabolic episode and to minimize the energy costs that would otherwise be needed to degrade and resynthesize damaged macromolecules. Much research has gone into defining the biochemical mechanisms of hypometabolism. One of these is the use of reversible protein phosphorylation, catalyzed by protein kinases and protein phosphatases, to regulate multiple ATP-expensive cellular processes when organisms transition into hypometabolism (Storey and Storey, 1990; Storey and Storey, 2004; Storey and Storey, 2007). This mechanism is known to be involved in regulating processes including fuel consumption, membrane transport, biosynthesis of many kinds (e.g. lipogenesis, protein synthesis), transcription factor activity and the cell cycle.

Depending on the tissue type, it is estimated that about 1–10% of cellular energy is used to support gene transcription (Rolfe and Brown, 1997). This is a significant metabolic cost and so it is not surprising that multiple studies have reported global suppression of transcription during hypometabolism, including during anaerobiosis in brine shrimp or intertidal snails (van Breukelen et al., 2000; Larade and Storey, 2002) and hibernation in mammals (Bocharova et al., 1992; van Breukelen and Martin, 2002; Osborne et al., 2004). However, despite the global suppression of gene transcription and protein translation (a much more ATP-expensive process), in most cases, selected proteins continue to be produced at low levels over the course of a hypometabolic excursion, including entry, torpid and arousal phases. Furthermore, the rapid arousal back to active life can be facilitated by the quick translation of selected mRNA transcripts that have been stored throughout the hypometabolic period.

The focus of the current article is on the mechanisms of transcriptional control that are used to coordinate global suppression of gene expression when animals transition into stress-induced hypometabolic states. In particular, several mechanisms that fall under the umbrella of epigenetic control are reviewed: (a) histone modification and DNA methylation, (b) RNA binding proteins that facilitate storage of mRNA transcripts during hypometabolism and (c) miRNA inhibition of mRNA translation. In addition, the use of mechanisms of post-translational modification that have long been known as epigenetic controls on gene expression – acetylation and methylation – are also examined in a new broader context of the control of metabolic enzymes.

Epigenetics and global suppression of gene transcription

The term epigenetics was first used in studies of embryonic development to describe the differentiation of cells from their original totipotent state (Waddington, 1942). Now, the term is more

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generally applied to describe functionally relevant changes to the genome (and the resulting phenotype) that are independent of changes in the primary DNA sequence of an organism (Wolffe and Matzke, 1999; Burggren, 2014). Hence, the epigenome sits at the interface between the dynamic environment and the static genome, responding to multiple environmental inputs and producing intragenerational modifications of the genomic expression of an individual and/or transgenerational heritable changes to genomic expression when epigenetic modifications to gamete cells have occurred (Burggren, 2014). The core mechanisms of epigenetic inheritance that were first identified are methylation of cytosine bases (on carbon 5) in DNA and histone regulation via post-translational modifications including serine phosphorylation, lysine acetylation, and methylation on lysine or arginine residues. Recently, controls by non-coding RNA have also been recognized as a part of epigenetics and, adding complexity, cross-talk among multiple epigenetic modifications creates a variety of outcomes (Molina-Serrano et al., 2013). For example, studies have shown that parental experience of abiotic stress, including hypoxia and cold exposure, can lead to increased tolerances of these stresses by offspring (Ho and Burggren, 2012; Zhou et al., 2013).

Given that stable epigenetic marks occur in response to external stresses experienced during an organism's lifetime and can be passed on to daughter cells or inherited by the next generation, it seems plausible that epigenetic mechanisms could also contribute to an organized (but reversible) genomic response to transient environmental stress. For example, these mechanisms might support global genome silencing and/or reprioritizing gene expression when organisms enter hypometabolic states. Epigenetic mechanisms could contribute to the depression of metabolic rate that occurs both as a response to predictable seasonal signals – such as those that trigger hibernation, cold hardening or diapause – or exposure to unpredictable episodic stresses – such as hypoxia/anoxia or desiccation. An initial exploration of this idea evaluated the potential involvement of a epigenetic mechanism, histone modification via acetylation and phosphorylation, in 13-lined ground squirrels *Ictidomys* (*Spermophilus*) *tridecemlineatus* and found reductions in both types of post-translational modification to histones during torpor in a manner that suggested that a more compact state of chromatin existed in torpid animals, which could lead to transcriptional suppression (Morin and Storey, 2006).

Post-translational control of histones is well known, extensively studied and demonstrated to be crucial to controlling chromatin structure and regulating gene expression by the 'histone code' (Jenuwein and Allis, 2001). The fundamental unit of chromatin is the nucleosome, which consists of a 147 bp DNA segment wound ~1.6 turns around a histone octamer of one $(H3\text{-}H4)_2$ heterotetramer and two H2A-H2B heterodimers (Li et al., 2012). The globular C-terminal domains of the histones make up the nucleosome scaffold, whereas the flexible N-terminal tails protrude outward and are the site for post-translational modifications that act to modify chromatin packing. In general, acetylation on lysine residues and/or phosphorylation on serine/threonine residues of histones act to open up chromatin structure (euchromatin) to allow the binding of the transcriptional apparatus. Particularly important to regulatory control are reversible post-translational modifications of histone H3, such as acetylation at Lys9 and Lys23 or phosphorylation at Ser10. By contrast, methylation on lysine or arginine residues is associated with compacting chromatin (heterochromatin) and contributes to transcriptional silencing.

A first analysis of histone H3 responses during mammalian hibernation found that the relative content of acetylated H3 (Lys23) and phosphorylated H3 (Ser10) were reduced by 25% and 40%, respectively, in skeletal muscle from hibernating animals compared with euthermic controls (Morin and Storey, 2006). This was accompanied by an increased amount of histone deacetylase (HDAC) protein (detected by immunoblotting) and a strong increase (82%) in HDAC enzymatic activity in the muscle of torpid squirrels, indicating that changes in lysine acetylation of histones are probably due to active changes in HDAC capacity when squirrels entered torpor. These data are suggestive of regulated transcriptional suppression during torpor as a result of increased chromatin packing. A new study of brown adipose tissue from ground squirrels adds further support for this. Non-shivering thermogenesis by brown adipose is crucial to the successful use of daily torpor or hibernation by mammals (Cannon and Nedergaard, 2004) and is responsible for the massive increase in heat production that is needed to rewarm animals from the low core body temperature (T_b) of the torpid state (often as low as 0–5°C during hibernation) back to euthermia. An analysis brown adipose tissue sampled from *I. tridecemlineatus* over a six-point time course of torpor and arousal showed that Lys23 acetylation on histone H3 decreased when squirrels entered torpor and stabilized at about ~50% of the euthermic value during both short (T_b of 5–8°C for ~24 h) and long (T_b of ~5–8°C for >5 days) periods of torpor (Biggar and Storey, 2014). This was correlated with changes in HDAC1 and HDAC4 protein levels that increased strongly during entry into torpor and reached peaks of 1.5- and 6-fold higher than control values, respectively, during long-term torpor. Two co-repressors of transcription, methyl-CpG-binding domain protein 1 (MBD1) and heterochromatin protein 1 (HP1), also rose by 1.9- and 1.5-fold, respectively, during torpor. MBD1, HP1 and HDACs all returned to near control values during the interbout period, suggesting a flexible reversal of their inhibitory actions on chromatin when animals roused themselves. The response of MBD-1 to torpor and arousal was particularly interesting. The primary accepted mechanism of MBD1-dependent transcriptional inhibition is via the recruitment of HDACs to regions of methylated DNA and the subsequent deacetylation of adjacent histones, resulting in chromatin condensation (Wade, 2001). A direct analysis of DNA modification during torpor showed a strong 1.7-fold increase in the content of methylated genomic DNA in brown adipose sampled from squirrels in long-term torpor compared with euthermic controls (T_b of ~36–37°C) (Biggar and Storey, 2014). Hence, enhanced DNA methylation and elevated MBD1 and HDAC protein levels would all go hand-in-hand to promote transcriptional repression during torpor.

Differential methylation of DNA in response to environmental stress was also reported recently as a response to hypoxia by primary cultures of mouse hippocampal neuronal cells (Hartley et al., 2013). Hypoxia applied on day 3 (1% O_2, 5% CO_2 for 24 h) had sustained effects on gene expression and DNA methylation that were still apparent on day 7 of cell culture. Hypoxia exposure resulted in differential expression of 369 genes, 225 being upregulated and 144 downregulated. Although both hyper- and hypo-methylation were detected on different genes as a response to hypoxia, many of the upregulated genes showed sustained hypomethylation of CpG islands or promoter regions, consistent with elevated expression. This indicates that short-term, sub-lethal hypoxia can have long-lasting effects on genome-wide DNA methylation and transcriptional activity of a variety of genes. It will be interesting to explore the responses of a hypoxia-tolerant species in the same manner, which might show a much greater downregulation of gene expression than the hypoxia-sensitive mammalian system.

Histone modification by reversible post-translational modification is now becoming well known as a mechanism of transcriptional

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suppression under anoxia. For example, anoxia exposure of *Caenorhabditis elegans* embryos led to dephosphorylation of histone H3 and selected cell cycle proteins (Padilla et al., 2002; Padilla and Ladage, 2012). Histone modification is also a feature of hypoxia tolerance in the red devil squid *Dosidicus gigas*. These oceanic squid undergo daily vertical migrations through the oxygen minimum zone and show a 25–50% reduction in the post-translational modification of histone H3 in several tissues under hypoxia compared with aerobic conditions (Seibel et al., 2014). The antibody used in these squid studies detected the dual modification of histone H3 (both acetylation on Lys9 and phosphorylation on Ser10), thereby indicating that coordinated suppression of both of these epigenetic modifications occurred in the hypoxic state. Squid tissues also showed a reduction in the phosphorylation of 4EBP, a binding protein that negatively regulates the ribosomal initiation factor eIF4E. Reduced phosphorylation of 4EBP increases its ability to sequester eIF4E and prevents it from doing its job of recognizing and binding the 7-methylguanosine (m7G) cap that is present on most mRNAs. As a result, this contributes to the suppression of cap-dependent translation, although other modes of translation initiation, such as via an internal ribosome entry site (IRES), may continue.

Epigenetic mechanisms also appear to be involved in suppressing gene transcription during anoxic submergence of turtles (*Trachemys scripta elegans*), a well-studied model of vertebrate anoxia tolerance (Storey, 2007; Krivoruchko and Storey, 2010a). This species (and some other freshwater turtles) undergoes frequent hypoxia/anoxia excursions as a consequence of diving, but most impressively can endure prolonged submergence in cold water without breathing for many weeks during the winter (Jackson and Ultsch, 2010). Turtles submerged in 5–10°C water have a metabolic rate that is only ~10% of the corresponding aerial value at the same temperature (Herbert and Jackson, 1985) as a consequence of strong anoxia-induced metabolic rate depression. Anoxic submergence in nitrogen-gas-bubbled water led to a significant reduction in acetylated histone H3 content (both Lys9 and Lys23) in white skeletal muscle; values fell to 40–60% of aerobic control values within 5 h and remained suppressed with prolonged 20 h submergence (Fig. 1D) (Krivoruchko and Storey, 2010b). This correlated with upregulation of five HDACs (isozymes 1–5) in muscle; transcript levels of *hdac1–hdac5* increased 4.6-, 2.9-, 2.5-, 1.3- and 1.7-fold, respectively, after 20 h anoxic submergence and protein levels rose in parallel 2-, 1.7-, 2.3-, 1.9- and 3.5-fold for HDAC1–HDAC5 (Fig. 1A,B). As a result, total HDAC enzymatic activity in muscle increased 1.5-fold after 20 h of anoxia (Fig. 1C). These results suggest that HDACs and deacetylation of histones are involved in coordinating chromatin condensation in response to anoxia in turtle muscle. Turtle liver responded similarly; anoxic submergence led to reduced acetylation of both Lys9 and Lys23 on histone H3 correlated with upregulation of *hdac1* and *hdac4* transcripts and HDAC1, HDAC4 and HDAC5 protein levels after 5 h of anoxia. However, turtle heart showed no change in histone H3 acetylation and a selective upregulation of only *hdac5* mRNA and HDAC5 protein, both peaking after 20 h of anoxic submergence (Krivoruchko and Storey, 2010b). Overall, these data show that

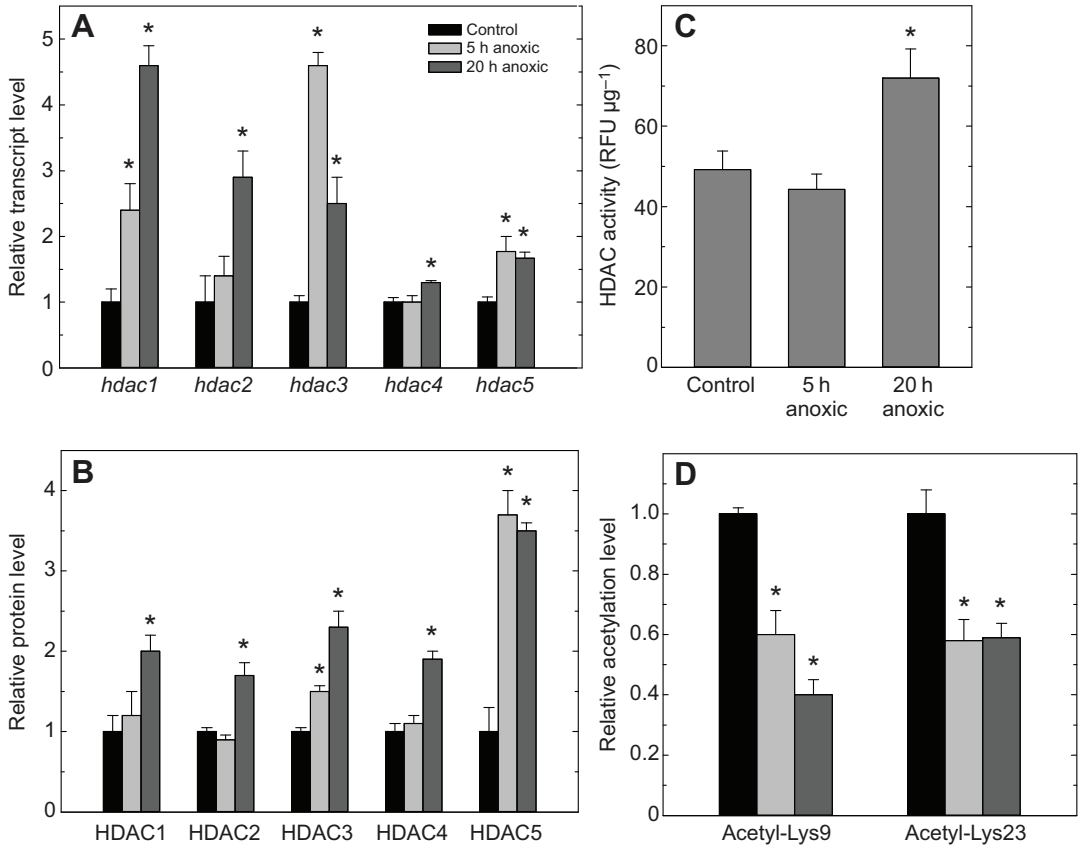

Fig. 1. Responses to anoxia by histone deacetylases and histone H3 acetylation in white skeletal muscle of turtles (*Trachemys scripta elegans*). Data compare aerobic control turtles with animals given 5 h or 20 h of anoxic submergence. (A) Relative *hdac1-hdac5* transcript levels. (B) Relative HDAC1-HDAC5 protein levels. (C) Total HDAC activity, expressed in relative fluorescence units per microgram protein. (D) Relative acetylation of histone H3 on Lys9 and Lys23. Data are means ± s.e.m., *n*=3–4; **P*<0.05 significantly different to the corresponding control. Figure redrawn from Krivoruchko and Storey (Krivoruchko and Storey, 2010b).

histone deacetylases respond robustly to low oxygen challenge in an anoxia-tolerant vertebrate. The response was also organ specific, which may reflect the metabolic demands on different organs under anoxia and/or a different time course of metabolic suppression in each organ.

Other approaches have also implicated epigenetic regulatory mechanisms in another form of hypometabolism – aestivation. The green-striped burrowing frog, *Cyclorana alboguttata*, spends 9–10 months of the year in aestivation with its whole-animal metabolic rate reduced by >80% (Kayes et al., 2009). Multiple metabolic adjustments support aestivation, including those aimed at transcriptional suppression. An analysis of mRNA transcript abundance of seven genes whose proteins play roles in gene silencing showed that two of these – DNA cytosine-5-methyltransferase 1 (DNMT1) and transcriptional co-repressor SIN3A – were upregulated in cruralis muscle during aestivation (Hudson et al., 2008). Both of these could have significant consequences for aestivation. DNMTs catalyze the methylation of DNA on the fifth position of cytosine (5mC) producing a chemically stable, reversible, post-replicative modification that inhibits transcription. Methylation contributes to transcriptional silencing by direct interference with transcription factor binding and/or through recruitment of repressive methyl-binding proteins such as MBD1, MBD2 and MeCP2 (Bogdanović and Veenstra, 2009). SIN3A is a co-repressor of transcription that associates with several transcription factors (including STAT3) to promote their deacetylation, thereby reducing their ability to upregulate genes under their control (Icardi et al., 2012). It was reported that the SIN3–deacetylase complex specifically repressed mitochondrial proteins that are encoded on either the nuclear or mitochondrial genome in *Drosophila* cells (Pile et al., 2003), raising the possibility that the action of SIN3 is linked to suppression of mitochondrial respiratory function during hypometabolism.

New concepts in post-translational modification for the control of gene expression

Post-translational modifications of histones by phosphorylation, acetylation and methylation are all well established as regulatory mechanisms in epigenetics but they are no longer the only ones that need to be considered. At least two others deserve a mention as well as further study as potential mechanisms of transcriptional regulation in hypometabolism. Our interest in these stems from the observation that the activity of RNA polymerase II (Pol II) decreased in skeletal muscle extracts of hibernating ground squirrels to a value that was just 42% of that in euthermic animals (Morin and Storey, 2006). This suggests that controls directed specifically at Pol II might also contribute to transcriptional suppression during hypometabolism. Studies with *C. elegans* support this idea. Larvae that hatch to find no food available quickly arrest their development (L1 arrest) and show enhanced stress resistance (both characteristics of stress-induced hypometabolism). During L1 arrest, Pol II continued transcribing a subgroup of starvation-response genes, but the enzyme was stalled on the promoters of growth and development genes (Baugh et al., 2009). However, in response to feeding, this was rapidly reversed; promoter accumulation of Pol II decreased, transcript elongation and mRNA levels increased and development restarted. New studies indicate that the link between nutrient availability and epigenetic control of gene expression is a post-translational modification called O-GlcNAcylation, which can target key transcriptional and epigenetic components, including Pol II, histones, histone deacetylase complexes, numerous transcription factors and many other proteins (Hanover et al., 2012; Lewis, 2013; Storey and Wu, 2013). This nutrient-sensitive sugar modification adds or removes O-linked β-D-*N*-acetylglucosamine (O-GlcNAc) moieties onto proteins catalyzed by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). O-GlcNAcylation of proteins seems to be nearly as prevalent as phosphorylation and frequently targets the same amino acid residues, creating an inverse relationship between these two protein modifications (Lewis, 2013). Given the huge range of metabolic functions (not just transcription related) affected by O-GlcNAcylation and the fact that hypometabolic states are virtually all nutrient limited, the scope for O-GlcNAcylation as a major regulatory mechanism, not just of epigenetic control, but also of metabolism during hypometabolism in general, is huge. This will be a fascinating area for future research in comparative biochemistry.

The second post-translational modification with potentially global influence on hypometabolism is SUMOylation. Proteins in the SUMO (small ubiquitin-related modifier) family are around 100 amino acids in length (~12 kDa) and their conjugation onto other proteins inhibits their action. Of particular relevance to epigenetic controls, SUMOylation is known to affect numerous transcription factors and alter multiple aspects of transcription factor behaviour, including DNA-binding activity, subcellular localization, interactions with co-regulators and chromatin structure. SUMO-conjugated protein levels rise dramatically in the brain, liver and kidney of ground squirrels during torpor, with an opposite reduction in free SUMO (Lee et al., 2007). This was rapidly reversed when animals roused themselves. Immunohistochemistry determined that SUMO-1 protein was distributed throughout neuronal cell bodies in euthermic squirrels but was highly concentrated in the nucleus of torpid animals, again arguing for the importance of SUMOylation in inhibiting transcription factors action during torpor. Subsequent research linked SUMOylation with improved ischemia tolerance of rat, mouse and human cells and to hypoxia/ischemia, oxidative, osmotic and genotoxic stresses in other systems (Tempé et al., 2008), suggesting that this modification is a universal contributor to suppressing the nuclear activity of transcription factors under stress and during hypometabolism (Lee et al., 2009).

Hypometabolism and post-transcriptional sequestering of mRNA

The processing and storage of mRNA transcripts is intimately associated with epigenetic mechanisms of transcriptional control during hypometabolism. Although transcription and translation are suppressed during entry into hypometabolic states, large pools of mRNA remain that are: (a) a metabolic resource that should not be wasted, and (b) needed to support metabolic recovery and renewed protein synthesis when animals rouse themselves out of hypometabolism. Indeed, there is evidence from hibernating mammals that transcripts of some genes (e.g. organic cation transporter) are upregulated during entry into torpor but not translated at this time; instead they associate with monosome and/or ribonuclear protein subcellular fractions (Hittel and Storey, 2002). Hence, they appear to be stored in anticipation of their need to support arousal and the restoration of the active state.

From the instant that mRNA molecules are synthesized in the nucleus until their destruction following translation in the cytoplasm, eukaryotic mRNAs are closely processed, shepherded, regulated and sometimes stored in either nuclear or cytoplasmic loci (Moore, 2005). These functions are carried out by RNA-binding proteins that act as mRNA chaperones and guide transcripts to a variety of fates. Proteins such as TIA-1 (T-cell intracellular antigen 1), TIAR (TIA-1-related) and PABP-1 (poly A-binding protein) influence transcription, mRNA splicing and the post-transcriptional

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localization and stability of mRNA, as well as association of transcripts with the translation machinery (Suswam et al., 2005; Kedersha and Anderson, 2007). With so many functions, these proteins are ideal candidates for regulatory roles in hypometabolism and a new study supports this with an analysis of RNA-binding proteins in liver of hibernating 13-lined ground squirrels (Tessier et al., 2014). Liver cryosections from euthermic squirrels versus squirrels that were in deep torpor for at least 5 days (T_b of 5–8°C) were compared using immunohistochemistry and fluorescence microscopy. This revealed the presence of large (~48 µm) subnuclear bodies within the nuclei of torpid ground squirrels (Fig. 2A). Targeting of TIA-1/TIAR and PABP-1 to subnuclear foci was seen in 98% and 90%, respectively, of nuclei from torpid squirrels but in <3% of nuclei from euthermic animals (Fig. 2B). TIA-1 splice variants proved to be major components of subnuclear structures with a 7-fold increase in relative TIA-1a protein levels in nuclear extracts of liver from torpid squirrels and a 3.7-fold increase in TIA-1b (Fig. 2C). In addition, TIARa and TIARb splice variants both decreased significantly in the cytoplasm during torpor to levels that were just 46% and 59%, respectively, of euthermic control values. The formation of subnuclear bodies with the prominent inclusion of RNA-binding proteins suggests that these can provide a framework for storing and preserving RNA transcripts when ground squirrels enter torpor, thereby contributing to the global suppression of translation but also providing the means for a immediate re-start of translation during the arousal phase. Although not yet widely explored, evidence from *C. elegans* showed an upregulation of *pabp* gene expression when larvae entered the dauer state (Cherkasova et al., 2000), suggesting that increased production of PABP may prove to be a widespread method to sequester gene transcripts when organisms enter hypometabolism.

The action of PABP in protecting mRNA transcripts during hibernation was nicely illustrated in studies with Arctic ground squirrels. Typically, poly(A) tail lengths shorten over time and when they get too short, the mRNA is enzymatically degraded. However, this did not happen in ground squirrel liver during torpor; mean lengths of poly(A) tails did not change over prolonged torpor and were not different from those in summer euthermic animals (Knight et al., 2000). As torpor duration increased, peaks representing overabundant poly(A) tail lengths appeared with a size distribution that indicated that protection was applied every 27 nt. This was consistent with the binding of molecules of PABP along the mRNA tails to halt their degradation. This pattern disintegrates when animals rouse themselves from torpor and was not found in mRNA from summer ground squirrels.

Structural rearrangements in cell nuclei have also been reported during torpor/hibernation in liver and brown adipose of two species of dormice. Electron microscopy revealed condensed coiled bodies and amorphous bodies in nucleoplasm during torpor as well as changes in the shape of nucleoli (Malatesta et al., 1994; Malatesta et al., 1999); these disassembled during arousal (Malatesta et al., 2001). Tissue-specific differences in pre-mRNA handling were also apparent; hepatocytes showed a preferential accumulation of pre-mRNAs at the splicing stage whereas brown adipocytes stored these at the cleavage stage (Malatesta et al., 2008). They suggested that storage at the cleavage stage could allow for immediate renewal of translation which fits with the need for the rapid activation of brown adipose metabolism to support thermogenesis that must begin instantly when arousal starts. Immunocytochemistry also revealed changes in the molecular composition of the nucleolus during hibernation (Malatesta et al., 2011). Ultrastructure analysis showed the appearance of nucleolus-associated domains during hibernation

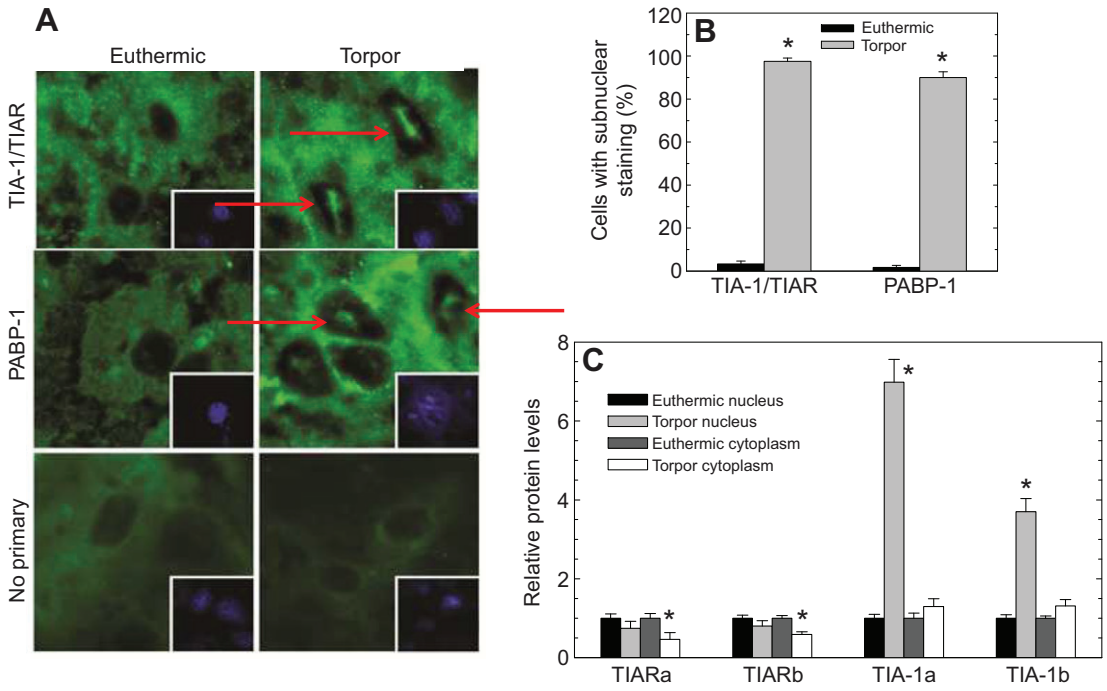

Fig. 2. Localization and subcellular distribution of RNA-binding proteins in liver of the 13-lined ground squirrel (*Ictidomys tridecemlineatus*) comparing euthermia and torpor (T_b of ~5–8°C for >5 days) conditions. (A) Immunostaining of cryosections for TIA-1/TIAR and PABP-1 showing strong fluorescence in distinct subnuclear foci during torpor (red arrows). Shown are representative cryosections immunostained with TIA-1/TIAR or PABP-1 as well as without primary antibody; inset in the lower right of each image confirms the position of nuclei via staining with 4′,6-diamidino-2-phenylindole, which binds to DNA. (B) Percentage of cells possessing subnuclear foci out of 120 cells assessed per condition. (C) Relative expression of TIARa/TIARb and TIA-1a/TIA-1b (± s.e.m., *n*=4) in cytoplasmic and nuclear extracts of liver from euthermic and torpid squirrels as determined by immunoblotting. **P*<0.05 compared with euthermic condition. Figure modified from Tessier et al. (Tessier et al., 2014).

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(which disappeared upon arousal) and immunocytochemistry showed that these contained several protein factors involved in transcription and processing of pre-rRNA or pre-mRNA as well as m3-G-capped snRNAs, snRNPs and hnRNPs. However, they lacked various typical nucleolar factors such as polymerase I, fibrillarin, nucleolin, and ribosomal phosphoproteins P0, P1 and P2. Therefore, it was proposed that the nucleolus-associated domains are a storage site for molecules involved in pre-mRNA splicing, keeping them ready for the rapid resumption of molecular trafficking through the nucleolus when animals rouse themselves.

Structural reorganization also occurs in the cytoplasm during hibernation. Electron microscopy has shown that liver structures associated with protein synthesis (rough endoplasmic reticulum) and post-translational processing (Golgi complex) also degrade during hibernation in dormice but reform again upon arousal (Malatesta et al., 2002). The authors proposed that this may be a fast and energetically efficient way to respond to a huge reduction in the demand for protein synthesis while still retaining the capacity to reactivate this ATP-expensive cell function immediately upon arousal. Multiple studies have documented polysome dissociation during mammalian hibernation (Whitten et al., 1970; Frerichs et al., 1998; Knight et al., 2000; Hittel and Storey, 2002) and other forms of hypometabolism (e.g. hypoxia, anoxia), with an opposite increase in monosomes and a sequestering of most mRNA transcripts into monosome and/or ribonuclear protein fractions (reviewed in Storey and Storey, 2004). However, some transcripts remain in the depleted polysome fraction in the hypometabolic state; for example, in hibernator kidney, the mRNA encoding fatty acid binding protein, which is strongly upregulated and translated during torpor to enhance subcellular trafficking of lipid fuels, remained in the polysome fraction whereas mRNAs encoding constitutively expressed genes (e.g. cytochrome c oxidase) were sequestered into monosome or ribonucleoprotein fractions (Hittel and Storey, 2002).

Hypometabolism and miRNA

Recent years have seen explosive growth in the exploration of the roles of non-coding RNAs in the regulation of gene expression. Of particular interest is miRNA (18–23 nt in length), which plays a regulatory role in controlling the post-transcriptional expression, storage or degradation of mRNA transcripts (Qureshi and Mehler, 2012; Suzuki et al., 2013). Hundreds of miRNAs are encoded in each animal genome, accounting for ~1–2% of all genes and at least 60% of protein-coding genes are computationally predicted to be targets for miRNA binding (Ebert and Sharp, 2012). Each miRNA species can regulate the mRNA transcripts of multiple genes and multiple miRNA species can control any given gene transcript making for an immense combinatory array of regulatory possibilities.

miRNA genes are transcribed by RNA polymerase II as transcripts of about 70 nt in length that fold into imperfect hairpin structures. These are then processed by the endonucleases Drosha in the nucleus and Dicer in the cytoplasm to form mature miRNAs that are incorporated into the miRNA-induced silencing complex (miRISC) (Fig. 3A). In these complexes, miRNAs guide the associated RISC proteins to the targeted mRNA strand, annealing to its 3′ untranslated region and promoting either degradation by the argonaute endonuclease within the complex or reversible translational repression of the mRNA (Bartel, 2004). This latter includes storage of mRNAs into cytoplasmic stress granules that are known to be stress-responsive in multiple systems; e.g. forming in response to heat shock, osmotic stress or anoxia in *C. elegans* (Jud et al., 2008; Kulkarni et al., 2010). Note that formation of these

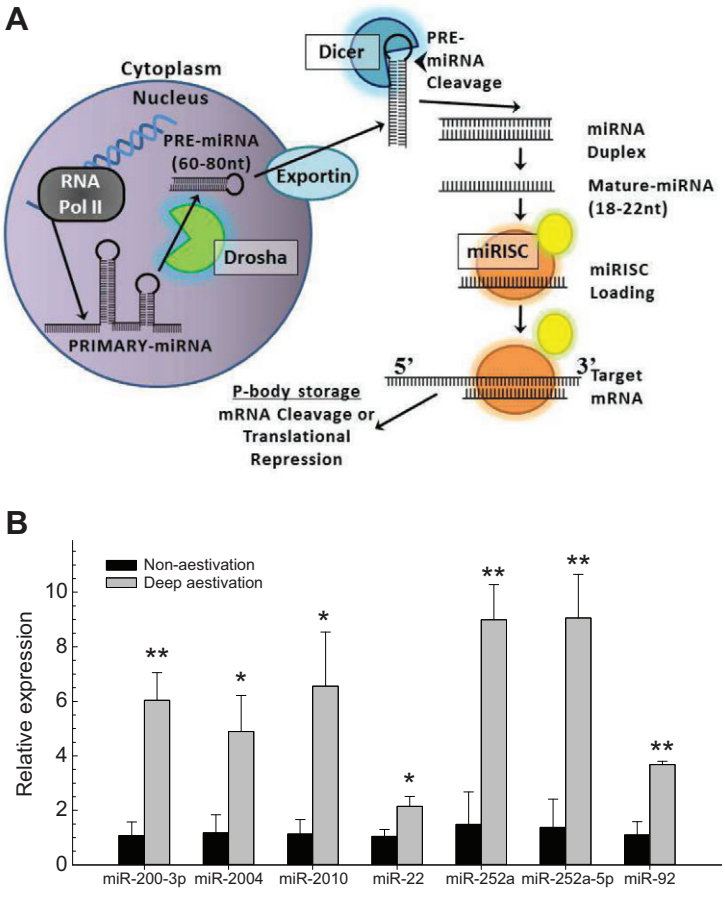

Fig. 3. Pathway of miRNA production and effect of aestivation on selected miRNA species in intestine of the aestivating sea cucumber, *Apostichopus japonicus*. (A) miRNA genes are transcribed by RNA polymerase II and then processed into single-stranded mature miRNAs by two riboendonucleases, Drosha in the nucleus and Dicer in the cytoplasm. Mature miRNAs join a miRNA-induced silencing complex (miRISC) and bind to the 3′-untranslated region of target mRNAs, leading to mRNA transcriptional repression and storage in cytoplasmic P-bodies or mRNA cleavage by the argonaute endonuclease in miRISC. Figure reproduced from Biggar and Storey (Biggar and Storey, 2009). (B) Differential expression of miRNAs in intestine of non-aestivating versus aestivating sea cucumbers as determined by real-time PCR. These data are supported by comparable results from both Solexa sequencing and microarray screening. Data are means ± s.d., n=3; *P<0.05; **P<0.01 compared with non-aestivation condition. Figure reproduced from Chen et al. (Chen et al., 2013).

cytoplasmic structures would correlate inversely with polysome degradation during hypometabolism.

miRNAs are emerging as important players in epigenetics in multiple ways (Molina-Serrano et al., 2013). First, single nucleotide polymorphisms in selected miRNAs have been linked with heritable susceptibilities to disease; e.g. mutations in miR-146a and miR-196a-2 increase susceptibility to human hepatocellular carcinoma (Xu et al., 2013). Second, epigenetic alterations to miRNA genes (including aberrant DNA methylation and histone modifications) alter miRNA expression in cancer and other diseases leading to the dysregulation of the expression of the mRNA targets of miRNAs (Suzuki et al., 2013). Third, because they can greatly influence the fate of gene transcripts (translation versus storage versus degradation), the action of miRNAs can change the ultimate functional phenotype resulting from gene expression.

The rapid and reversible actions of miRNA in determining the post-transcriptional fate of mRNA transcripts fit well with the need for the mechanisms of metabolic rate depression to be rapidly implemented in response to stress and readily reversed when the stress is removed (Biggar and Storey, 2011). Indeed, several recent studies have shown that differential miRNA expression (mainly increased amounts) is a component of hypometabolism in both vertebrate and invertebrate systems, indicative of a conserved principle of environmental stress-responsive gene regulation across the animal kingdom (Biggar and Storey, 2011; Storey and Wu, 2013). Altered miRNA expression is a demonstrated component of hibernation in ground squirrels and bats (Morin et al., 2008; Liu et al., 2010; Kornfeld et al., 2012; Maistrovski et al., 2012), anoxia tolerance in turtles and marine snails (Biggar and Storey, 2012; Biggar et al., 2012; Zhang et al., 2013), freeze tolerance by wood frogs and marine snails (Biggar et al., 2009; Biggar et al., 2012; Zhang and Storey, 2013), the response to dehydration in African clawed frogs (Wu et al., 2013) and aestivation in sea cucumbers (Chen et al., 2013; Chen and Storey, 2014).

miRNA links to specific events in hypometabolism have been made in some cases. For example, two studies show involvement of miRNA in anoxia-induced cell cycle suppression in turtles. The high energy costs and biosynthetic requirements of cell division make suppression of this process a crucial target for energy savings during long-term anaerobiosis. The p53 transcription factor is involved in stress responses to hypoxia, including cell cycle arrest, protecting against DNA damage and altering mitochondrial metabolism. Activation of p53 was evident as a response to anoxic submergence in turtle (*T. s. elegans*) tissues as shown by elevated p53 protein levels, altered post-translational modifications of p53, and increased p53 nuclear localization, as well as upregulation of downstream genes under p53 control including miR-34a (Zhang et al., 2013). miR-34a levels rose 2- to 3-fold in turtle liver and muscle under anoxia. In other systems, miR-34a overexpression leads to cell cycle suppression, including pronounced downregulation of cell cycle components including CDK4/6, cyclin E2 and cyclin E2F5 (Bommer et al., 2007; He et al., 2007). Upregulation of miR-16-1 and miR-15a also occurred during anoxia in turtle tissues. These two miRNAs are known to control cyclin D1, which is responsible for initiating the cell cycle by stimulating entrance into the G1 phase. Inhibition of cyclin D1 expression is a consistent feature of cell cycle arrest. Transcript levels of *cyclin d1* did not change under anoxia in turtle organs but cyclin D1 protein levels decreased by 30–40% (Biggar and Storey, 2012). The dichotomy between transcript and protein levels was linked to strong 2.5- to 3-fold increases in miR-16-1 and miR-15 levels to promote translational silencing (and storage) of *cyclin d1* transcripts under oxygen-limited conditions.

Recent studies have also applied large-scale sequencing technologies to identify and evaluate the involvement of miRNA in two diverse systems of hypometabolism – winter hibernation in the Arctic ground squirrel *Urocitellus* (*Spermophilus*) *parryii* (Liu et al., 2010) and summer aestivation in the marine sea cucumber *Apostichopus japonicus* (Chen and Storey, 2014; Chen et al., 2013). Expression of miRNAs in ground squirrels was analyzed using Illumina/Solexa deep-sequencing, an Agilent miRNA microarray and real-time PCR under three conditions: torpor (>8 days continuous), arousal (5 h out of torpor) and post-reproductive (1–2 months after hibernation ended). The authors identified about 200 miRNAs, 18 of which were specific to ground squirrels. Two main patterns were found: (a) those that were underexpressed in torpor/arousal compared with post-reproductive animals (miR-184, miR-200a, miR-211, miR-320, miR-378) and are known to promote cell survival, tumor growth and angiogenesis, and (b) those that were significantly overexpressed in torpor compared with arousal or post-reproductive states (miR-142, miR-144, miR-451, miR-486) and known to inhibit tumor growth. Hence, the responses of the miRNAs in both groups during torpor are consistent with suppression of the cell cycle and inhibition of tumorigenesis, suggesting a coordinated action to minimize cell proliferation in the hypometabolic state.

A similar conclusion was reached during analysis of miRNA involvement in sea cucumber aestivation. As a result of a large commercial fishery, as well as extensive aquaculture, there is much current interest in the biochemistry of the sea cucumber *A. japonicus*. This species undergoes an annual period of aestivation when sea water temperature rises above 25°C; dormancy can last for 4 months or more before a return to active life when water temperature cools below 18°C (Yang et al., 2006). During aestivation, animals cease eating and moving, rates of oxygen consumption and ammonia excretion fall to low levels and the intestine degenerates into a thin filament. Using Solexa sequencing, 308 sea cucumber miRNAs were identified, including 42 that were differentially expressed in the intestine during deep aestivation (at least 15 days of continuous torpor) compared with non-aestivating animals (Chen et al., 2013). A miRNA microarray made with probes for *A. japonicus* miRNAs and ~700 known miRNAs from three other marine invertebrates was also produced and screened to show upregulation of multiple miRNAs and then real-time PCR was used to validate the results for selected specific *A. japonicus* miRNAs. PCR analysis showed strong upregulation of seven of these target miRNAs by 2- to 9-fold during aestivation (Fig. 3B). A comparable study identified 30 differentially expressed aestivation-responsive miRNAs in respiratory tree tissue of *A. japonicus* and PCR confirmed 3- to 11-fold increases in miR-124, miR-124-3p, miR-79, miR-9 and miR-2010 during aestivation in this tissue (Chen and Storey, 2014). Roles for some of the miRNAs that were upregulated in intestine during aestivation have been proposed. miR-22 works in a regulatory loop with the AKT kinase and PTEN phosphatase (Bar and Dikstein, 2010) to modulate PTEN–AKT signaling pathway dynamics in response to extracellular signals. The AKT pathway is a major regulator of anabolism in cells, including positive control over protein synthesis and the cell cycle. AKT suppression occurs commonly during hypometabolism (Zhang et al., 2011). PTEN mediates suppression of Akt signaling by dephosphorylating members of the pathway and is well known as a tumor suppressor in many systems. Hence, altered miR-22 levels in the intestine during aestivation suggest a role in dampening AKT signaling during hypometabolism. MiR-92 has anti-apoptotic effects in cancer (Tsuchida et al., 2011) and may have a similar role in sea

cucumber aestivation to help to protect intestinal cells under low energy conditions that might otherwise trigger cell death.

Expanded roles for 'epigenetic' post-translational modifications in metabolic control

As discussed earlier, acetylation versus methylation of histones plays a major role in regulating chromatin accessibility, acetylation favouring transcriptional activity and methylation promoting transcriptional silencing (Jenuwein and Allis, 2001). However, with recent improvements to the detection of acetylation coupled with modern proteomic tools, it is now known that more than 2000 proteins in the cell can be acetylated, many of them being cytoplasmic or mitochondrial (Guan and Xiong, 2011). These include many enzymes whose catabolic properties and/or protein stability can be altered by acetylation. Furthermore, acetylation can effectively 'lock' lysine residues against other potential lysine post-translational modifications, not just methylation, but also ubiquitylation, SUMOylation, neddylation and biotinylation (Yang, 2005). Hence, multiple interacting regulatory opportunities can be envisioned; for example, if acetylation blocks ubiquitin tagging of proteins, then the rate of protein degradation by the proteasome should be reduced (Sadoul et al., 2008). This might be one way to extend protein lifespan during hypometabolism because net rates of both protein synthesis and protein degradation are known to be suppressed as part of metabolic rate depression, as occurs in anoxia tolerance in turtles (Hochachka et al., 1996; Storey and Storey, 2007). New work has also linked lysine acetylation with the control of autophagy, a process that is triggered by periods of nutritional deficit during which cells initiate recycling programmes to break down expendable intracellular structures and catabolize them for energy (Webster et al., 2014). Note that autophagy is a known component of dauer formation in *C. elegans*, a hypometabolic state induced by nutrient restriction (Meléndez et al., 2003). A new proteomics analysis of ground squirrel liver also indicated enhanced expression of phosphorylated p47 protein that is associated with autophagy during the hibernating heterothermic season (when animals are fasting) compared with the active homeothermic season (when animals are feeding) (Hindle et al., 2014). Interestingly, this study also showed a substantial increase in the overall acetylation state of liver proteins both during winter (nonfeeding) compared with summer (feeding) seasons and also within a torpor bout being greatest during torpor compared with both entrance and arousal periods. Selected specific proteins also showed differential acetylation (e.g. PEP carboxykinase, dimethylglycine dehydrogenase) over the seasons. In opposition to the general increase in protein acetylation in the winter, the amount of SIRT3 deacetylase decreased during the hibernating season (Hindle et al., 2014).

Biochemists have spent several decades investigating the roles of reversible protein phosphorylation in metabolic regulation, biochemical adaptation and hypometabolism, but it is now becoming obvious that much more remains to be done to determine how other forms of post-translational modification, including 'epigenetic mechanisms' of protein acetylation and methylation, contribute to metabolic control. That they are clearly involved in the regulation of some of the best-known enzymes and in the regulation of organismal response to environmental stress is now clear from recent research. As always, this work has been aided by advances in technology that have included development of site-specific and pan-specific antibodies to detect different post-translational modifications, development of inhibitors that can stabilize these modifications to allow their detection, and better prediction software for finding putative motifs in protein sequences that represent potential sites of post-translational modification.

Indeed, almost every enzyme in glycolysis, gluconeogenesis, the TCA cycle, the urea cycle, fatty acid metabolism and glycogen metabolism is now known to be acetylated (Zhao et al., 2010), including such well-known metabolic enzymes as glycogen phosphorylase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH) and phosphoenolpyruvate carboxykinase (PEPCK) (Ventura et al., 2010; Xiong and Guan, 2012; Zhang et al., 2012). For example, glycogen phosphorylase is acetylated on Lys470 and this promotes interaction with the protein phosphatase 1 targeting subunit to favour GP dephosphorylation, a process that is stimulated by glucose or insulin and inhibited by glucagon (Zhang et al., 2012). Acetylation of GAPDH at three lysine loci stimulates nuclear translocation of the enzyme in response to apoptotic stimuli (Ventura et al., 2010). Exposure of human liver cells to high concentrations of glucose enhanced the acetylation of the TCA cycle enzyme, malate dehydrogenase (MDH) and the urea cycle enzyme arginosuccinate lyase (ASL), but whereas acetylation activated MDH, it inhibited ASL (Zhao et al., 2010). However, exposure of cells to high levels of amino acids suppressed acetylation and increased the activity of ASL. These results, together with acetylation controls on PEPCK, suggest an integrated mechanism for fuels (glucose, amino acids) to regulate interlocking pathways (TCA cycle, urea cycle, gluconeogenesis) in response to changing fuel availability.

Recent studies by my group have been identifying post-translational modifications of LDH and their potential actions in modulating LDH kinetic properties. LDH has historically been viewed as a nonregulatory equilibrium enzyme that responds simply to changes in substrate availability (pyruvate, lactate, NADH, NAD^+). However, studies of purified turtle (*T. s. elegans*) liver LDH have shown that the enzyme is subject to both acetylation and phosphorylation in response to anoxia, the relative level of each modification increasing significantly by ~70% and 20%, respectively, under anoxia, compared with aerobic controls (Xiong and Storey, 2012). One or both modifications may be responsible for the observed anoxia-induced changes in LDH properties – a reduction in substrate affinity for pyruvate and an increase in enzyme sensitivity to high pyruvate as an inhibitor. Purified LDH from turtle skeletal muscle also showed anoxia-responsive changes in acetylation and phosphorylation, as well as two other post-translational modifications, ubiquitylation and SUMOylation. However, in all cases, the relative level of each modification decreased in anoxic muscle such that levels in anoxia were just 22% for serine phosphorylation, 42% for threonine phosphorylation, and 63%, 27% and 49% less for acetylated, ubiquitylated and SUMOylated LDH, respectively, compared with LDH from aerobic muscle (Dawson et al., 2013). Anoxia exposure increased the affinity of muscle LDH for lactate (K_m decreased by one-half) and strongly increased the maximum velocity of the enzyme in both directions. Hence, these modifications could have a physiological consequence in promoting LDH activity in anoxic muscle, thereby supporting glycolytic ATP production under oxygen-limited conditions. Skeletal muscle LDH purified from the freeze-tolerant wood frog (*Rana sylvatica*) also showed the presence of multiple post-translational modifications. These did not change significantly in a comparison of 5°C control frogs with −3°C frozen frogs but LDH acetylation levels decreased strongly to about 30% of control values in frogs that underwent strong dehydration (40% of total body water lost) (Abboud and Storey, 2013). Cellular dehydration is a major component of freezing stress (anoxia is another) and

multiple metabolic responses to freezing have previously been shown to be triggered by the dehydration component of freezing (Storey and Storey, 2013). LDH from skeletal muscle of dehydrated wood frogs showed significantly reduced K_m values for lactate, pyruvate and NAD^+, as well as greater sensitivity to inhibition by urea (Abboud and Storey, 2013). Hence, as also shown for turtle LDH, the stress-responsive change in post-translational modification of LDH (in this case by acetylation) could be the underlying mechanism.

Overall, these new studies of metabolic enzymes that are affected by acetylation and other post-translational modifications are providing tremendous possibilities for a wider understanding of how metabolism is regulated in response to abiotic stress and for coordinating gene expression in the nucleus with events in other cellular compartments. Molecular mechanisms that have long been thought of as epigenetic controls are now proving to be universal in their effects on cellular metabolism and an understanding of the multiple layers and interactions of these controls as they apply to biochemical adaptation and hypometabolism will keep comparative biochemists at work for many years to come.

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