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Strategies of biochemical adaptation for hibernation in a South American marsupial, *Dromiciops gliroides*: 3. Activation of pro-survival response pathways

Bryan E. Luu[a], Sanoji Wijenayake[a], Jing Zhang[a,1], Shannon N. Tessier[a,2], Julian F. Quintero-Galvis[b], Juan Diego Gaitán-Espitia[c], Roberto F. Nespolo[b], Kenneth B. Storey[a,*]

[a] Department of Biology and Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada
[b] Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile, Campus Isla Teja, Valdivia, Chile
[c] CSIRO Oceans & Atmosphere, GPO Box 1538, Hobart 7001, Tasmania, Australia

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ABSTRACT

The South American marsupial, monito del monte (*Dromiciops gliroides*) uses both daily torpor and multi-day hibernation to survive in its southern Chile native environment. The present study leverages multiplex technology to assess the contributions of key stress-inducible cell cycle regulators and heat shock proteins to hibernation in liver, heart, and brain of monito del monte in a comparison of control versus 4 day hibernating conditions. The data indicate that MDM2, a stress-responsive ubiquitin ligase, plays a crucial role in marsupial hibernation since all three tissues showed statistically significant increases in MDM2 levels during torpor (1.6–1.8 fold). MDM2 may have a cytoprotective action to deal with ischemia/reperfusion stress and is also involved in a nutrient sensing pathway where it could help regulate the metabolic switch to fatty acid oxidation during torpor. Elevated levels of stress-sensitive cell cycle regulators including ATR (2.32–3.91 fold), and the phosphorylated forms of *p*-Chk1 (Ser345) (1.92 fold), *p*-Chk2 (Thr68) (2.20 fold) and p21 (1.64 fold) were observed in heart and liver during hibernation suggesting that the cell cycle is likely suppressed to conserve energy while animals are in torpor. Upregulation of heat shock proteins also occurred as a cytoprotective strategy with increased levels of hsp27 (2.00 fold) and hsp60 (1.72–2.76 fold) during hibernation. The results suggest that cell cycle control and selective chaperone action are significant components of hibernation in *D. gliroides* and reveal common molecular responses to those seen in eutherian hibernators.

1. Introduction

All organisms must deal with changes in their environment and, when environmental stress becomes extreme, they employ adaptive mechanisms to help preserve viability for as long as possible while also working to attenuate cell death signals. Established means of dealing with unfavorable environmental conditions include the suppression of many energy-expensive anabolic and growth processes and the reprioritization of ATP use towards support for pro-survival actions (Storey and Storey, 2004). If such preservation measures fail, the balance can tip towards programmed cell death (apoptosis) when stress conditions persist (e.g. nutrient deprivation, hypoxia/ischemia, dehydration, disease, etc.). Although cell death is a natural way by which animals recycle old or damaged cells, many organisms show remarkable adaptive controls over these processes in order to endure taxing stresses without losing cell viability.

The South American marsupial, monito del monte (*Dromiciops gliroides*), has been called a "living fossil", a relic of a near-extinct marsupial lineage, the Order Microbiotheria, only distantly related to all other North and South American marsupials. It was known as the sole extant member of this group until recently when the single species was reclassified as three geographically-separate species of *Dromiciops*, all living in the temperate rainforests of southern Chile (D'Elía et al., 2016). This tiny nocturnal marsupial uses torpor to enhance survival under conditions of cold environmental temperatures or limited food availability. Both shallow daily torpor and prolonged multi-day

Abbreviations: ATR, ataxia telangiectasia and Rad3 related; Chk, checkpoint kinase; HSP, heat shock protein; H2A.X, histone 2A member X; MDM2, mouse double minute 2

* Corresponding author at: Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada.
E-mail address: kenneth.storey@carleton.ca (K.B. Storey).
[1] Schulich School of Medicine & Dentistry, Western University, London, ON, N6A 5C1, Canada.
[2] Center for Engineering in Medicine & BioMEMS Resource Center, Massachusetts General Hospital & Harvard Medical School, Boston, Massachusetts 02129, U.S.A.

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hibernation have been documented (Bozinovic et al., 2004). Understanding the biochemical adaptations that underlie hibernation in this ancient marsupial lineage will advance our overall understanding of mammalian torpor/hibernation and stress biology and of the conserved versus novel features utilized by marsupial versus placental mammals. For example, marsupials lack the brown adipose tissue that is the main thermogenic organ used to arouse placental (eutherian) mammals from torpor (Villarin et al., 2003).

In the previous two papers in this series on *D. gliroides* we have focused on signal transduction pathways (Wijenayake et al., 2018) that may be involved in inducing and mediating torpor and regulating protein synthesis (Luu et al., 2018), one of the most energy-expensive metabolic activities in cells (Storey and Storey, 2004). Regulated metabolic rate depression is a core feature of torpor/hibernation across the animal kingdom and, by strongly suppressing energy use by ATP-expensive functions (e.g. gene transcription, protein translation, the cell cycle), animals can prolong the time that a fixed reserve of body fuels can support survival. However, suppression of such ATP-expensive functions would predictably necessitate reduced turnover and greater stability of cell macromolecules and lead to a requirement for improved cytoprotective pro-survival measures (e.g. chaperone proteins, antioxidant defenses, anti-apoptosis mechanisms, etc.) during prolonged hibernation. Therefore, we predicted that cytoprotective mechanisms would be enhanced when *D. gliroides* transitioned into a hypometabolic state. The present paper examines this proposal by analyzing the responses to hibernation by selected cell cycle regulatory proteins and heat shock chaperone proteins in monito del monte tissues. Of further interest, regulatory proteins of the cell cycle (an energy-expensive process) and selected chaperone proteins (pro-survival) have been shown to be intimately co-regulated, in particular as responses to cold (Kühl and Rensing, 2000; Nakai and Ishikawa, 2001; Rice et al., 1986; Storey, 2004). As a result, we focused the present study on these two critical molecular hubs that play roles in balancing metabolism and survival.

The cell cycle is a complex biochemical process that requires significant coordination between multiple pathways to initiate or halt cell division. Cell cycle regulation is sensitive to many stresses (e.g. DNA damage, hypoxia, nutrient deficiency, etc.), allowing cell cycle arrest to be implemented as a versatile response to stress conditions. Factors that trigger cell cycle arrest often act through activation of the ataxia telangiectasia and Rad3 related (ATR) protein, a serine/threonine protein kinase that phosphorylates downstream targets such as the checkpoint kinases, Chk1 and Chk2 (Ding et al., 2013; Martin et al., 2012). Phosphorylation of Chk1 and Chk2 by ATR at Ser345 and Thr68, respectively, triggers a broader signaling cascade that spreads to encompass phosphorylation-mediated regulation of cell cycle arrest, DNA damage, and apoptosis responses (Ouchi and Ouchi, 2014; Wang et al., 2012). For example, in response to DNA damage, double-stranded breaks will trigger the immediate phosphorylation of the histone 2 variant (H2A.X) at Ser139 by ATR, and lead to the recruitment of DNA repair machinery (Rogakou et al., 1998; Singh et al., 2012). In addition to reversible protein phosphorylation, the cell cycle can also be controlled by proteins such as the mouse double minute 2 protein (MDM2) and p21, that directly interact with transcription factors and protein kinases including p53 and cyclin-dependent kinases (Moll and Petrenko, 2003; Xiong et al., 1993).

Heat shock proteins (HSPs) are a well-known family of chaperone proteins with actions that aid both folding of nascent proteins and refolding of misfolded or denatured proteins (Feder and Hofmann, 1999). Cells produce HSPs in response to numerous stresses including heat, cold, dehydration, changes in salinity, UV radiation and more, and HSPs have important roles in many diseases (Storey and Storey, 2011; Yu et al., 2015). Previous studies have determined that chaperone proteins are differentially regulated during torpor/hibernation as part of a cytoprotective response in eutherian mammals including lemurs, ground squirrels and bats (Lee et al., 2002; Mamady and Storey, 2006; Rouble et al., 2014; Wu et al., 2015). Enhanced levels of chaperones aid long-term viability during prolonged torpor since the scope for extensive repair/replacement of damaged proteins is reduced in the hypometabolic state.

The present study characterizes selected stress-responsive cell cycle regulators and HSPs to identify torpor-responsive cytoprotective pathways that aid *D. gliroides* hibernation. We used multiplex technology for analysis of three organs (liver, heart, brain), comparing aroused and hibernating (4 days of continuous torpor) conditions. The results indicate that cytoprotective mechanisms are employed during torpor in *D. gliroides*, and are differentially regulated in an organ-specific manner to manage cell cycle and chaperone pathways.

2. Materials and methods

2.1. Animals

Adult monito del monte, *D. gliroides*, was captured near Valdivia, Chile in January–February 2014. Expanded information on conditions of animal holding, acclimation and experimentation are described in Wijenayake et al. (2018). In brief, animals were acclimated at 20 ± 1 °C under a 12 h:12 h light:dark cycle with mealworms, fruits and water provided ad libitum. After two weeks, some were sampled as controls. Remaining animals were subjected to a decrease in ambient temperature over 2–3 days until 10 °C was reached; all had entered torpor by the time that temperature was lowered to ~15 °C. Experimental animals were sampled after 4 d of continuous torpor. Euthanasia followed protocols approved by the Committee on the Ethics of Animal Experiments of the Universidad Austral de Chile. Tissue samples were rapidly dissected, immediately frozen in liquid nitrogen, and air-freighted to Carleton University in a dry shipper. All animal capture, handling and maintenance followed the guidelines of the American Society of Mammalogists (Gannon and Sikes, 2007) and were authorized by the Chilean Agriculture and Livestock Bureau (SAG: Servicio Agrícola y Ganadero de Chile, permit resolution No. 1054/2014).

2.2. Protein extraction

Samples of frozen tissues (~50 mg each) were crushed under liquid nitrogen and homogenized 1:5 (w/v) using a Dounce homogenizer in pre-chilled lysis buffer (Milliplex MAP Assay Buffer 1; Cat. No. 43-010) with additions of 1 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate and 1% protease inhibitor cocktail (Cat. No. PIC001, BioShop). Samples were left to incubate on ice for 30 min with vortexing every 10 min, and were then centrifuged at 12,000 × *g* for 20 min at 4 °C. Supernatants were removed and soluble protein concentration was determined using the Bio-Rad protein assay (Cat. No. 500-0006). Samples were then standardized to 10 μg/μL with the addition of small volumes of lysis buffer and stored at − 80 °C until use.

2.3. Multiplex analysis

EMD Millipore magnetic bead kits were used to assay levels of six cell cycle markers (Milliplex DNA Damage/Genotoxicity Kit, Cat. No. 48-621MAG) and four heat shock proteins (Milliplex Heat Shock Protein Kit, Cat. No. 48-615MAG). Initial work tested dilutions of small aliquots of cell lysates to determine the limits of detection and ideal sample concentration for assays. Manufacturer-supplied negative and positive controls were run to assure functionality and performance of the assay. For cell cycle marker analysis, lambda phosphatase-treated HeLa cells were used as a negative control (Cat. No. 47-229), whereas Jurkat cells stimulated with 25 μM anisomycin for 4 h (Cat No. 47.207) and A549 cells stimulated with 5 μM camptothecin overnight (Cat. No. 47-218) were the positive controls. For heat shock protein analysis unstimulated HeLa cells were the negative control (Cat. No. 47-205) and HS/Ars-treated HeLa cells were the positive control (Cat. No. 47-

211).

To conduct assays, stock premixed magnetic beads were thoroughly sonicated and vortexed as specified by the manufacturer, and diluted to a 1 × working concentration with Assay Buffer 1. In a magnetic Milliplex 96-well plate, 25 µL of 1 × magnetic beads were combined with 25 µL of concentration-adjusted cell lysate resulting in the addition of 25 µg protein per well for cell cycle markers or 25 ng protein for heat shock proteins. Positive and negative controls as well as blanks were also prepared and loaded. Plates were sealed and incubated overnight at 4 °C on an orbital shaker protected from light. Using a magnetic plate, beads were held in place while liquid was decanted, and then beads were washed twice with Assay Buffer 1. An aliquot of 25 µL of biotin-labelled detection antibody cocktail was then added to each well and the plate was incubated at room temperature with orbital shaking for 1 h. Excess detection antibody was decanted and then 25 µL of 1 × streptavidin-phycoerythrin (25X SAPE, Cat. No. 45-001H) was added to each well and incubated for 15 min at room temperature with orbital shaking. Subsequently, 25 µL of Amplification Buffer (Cat. No. 43-024A) was added to each well, and orbital shaking was resumed for another 15 min. Excess SAPE/Amplification buffer solution was discarded and the beads were resuspended in Assay Buffer 1, shaken for 5 min, and then analyzed using a Luminex 200 system (Luminex, Austin, TX). Beads were analyzed using the following parameters: Events: 50 beads; Sample Size: 100 µL; Gate settings: 8000 to 15,000.

2.4. Data and statistics

Relative protein or protein phosphorylation levels were recorded as Median Fluorescence Intensity (MFI) values from control and torpor samples. Results are expressed as mean ± SEM for $n = 4$ independent biological replicates, and torpor values were expressed relative to their corresponding controls. Statistical analysis was done with the Student's *t*-test, with $p < 0.05$ accepted as a significant difference.

3. Results

Relative protein expression or site-specific protein phosphorylation was assessed with magnetic multiplex assays using Luminex instrumentation. A selection of cell cycle regulators and HSPs were characterized in the liver, heart, and brain of *D. gliroides* comparing control (aroused) and hibernating (4-day continuous torpor) conditions. Figs. 1–3 show the effects of torpor on cell cycle related proteins. Relative total protein levels of ATR, MDM2, and p21 were measured as well as the relative phosphorylation state of Chk1 (Ser345), Chk2 (Thr68), and H2A.X (Ser139). In the liver, ATR protein levels increased significantly by 2.32 ± 0.18 fold in torpid animals as compared with controls and MDM2 and p21 protein levels also increased by 1.76 ± 0.01 fold, and 1.64 ± 0.06 fold, respectively (all $p < 0.05$) (Fig. 1). Relative phosphorylation of checkpoint kinases also increased during torpor; *p*-Chk1 (Ser345) content increased by 1.93 ± 0.11 fold and *p*-Chk2 (Thr68) rose by 2.20 ± 0.24 fold (both $p < 0.05$). However, liver H2A.X (Ser139) did not change significantly. In the heart of torpid *D. gliroides*, ATR levels increased strongly by 3.91 ± 0.69 fold during torpor and MDM2 also increased by 1.60 ± 0.03 fold, as compared to control levels (both $p < 0.05$) (Fig. 2). However, the other four targets were unaffected. Brain of torpid *D. gliroides* showed a significant increase in MDM2 alone (1.57 ± 0.05 fold, $p < 0.05$), compared with controls whereas all other targets were unaffected (Fig. 3).

The effects of hibernation on the relative levels of HSP27, HSP60, HSP70 (HSP72), and HSP90α were also analyzed in liver, heart, and brain of *D. gliroides*. In response to torpor, relative levels of HSP27 and HSP60 increased significantly in liver by 2.00 ± 0.21 and 2.76 ± 0.32 fold ($p < 0.05$), respectively (Fig. 4). However, HSP70 (HSP72) and HSP90a levels did not change in liver. In heart, only mitochondrial HSP60 was torpor-responsive, showing a significant

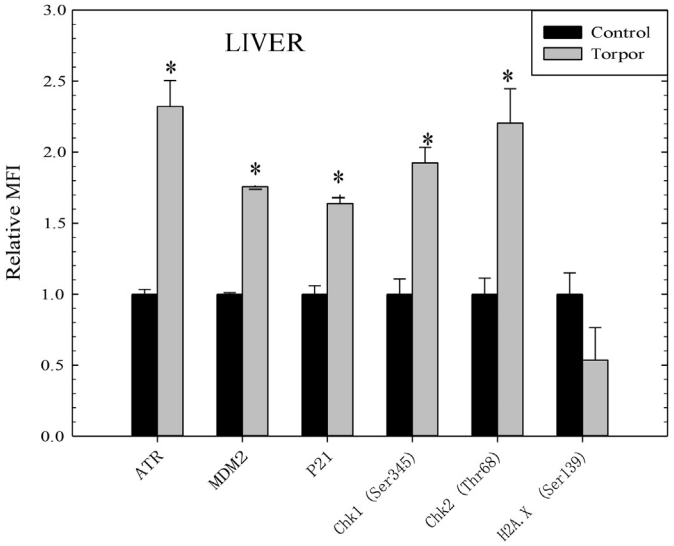

Fig. 1. Relative protein expression of cell cycle regulators in liver of control versus torpid *D. gliroides*. Torpid animals were sampled after 4 days of continuous torpor. Relative protein levels were determined for ATR, MDM2 and p21 along with the relative phosphorylation state of *p*-Chk1(Ser345), *p*-Chk2(Thr68), *p*-H2A.X(Ser139). Data were quantified as median fluorescence intensity (MFI) for each biological replicate and are shown as mean ± SEM, $n = 4$ independent samples from different animals. For each parameter, values for the hibernating condition were expressed relative to the mean control value that was standardized to 1.0. Data were analyzed using a two-tailed Student's *t*-test. * - Significantly different from the corresponding control value, $p < 0.05$.

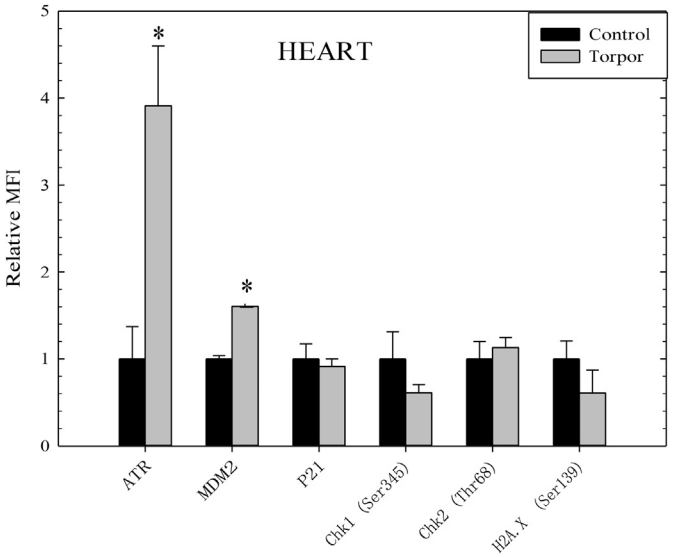

Fig. 2. Relative protein expression of selected cell cycle regulators in heart of control versus torpid *D. gliroides*. Other information as in Fig. 1.

1.73 ± 0.13 fold ($p < 0.05$) increase compared to euthermic controls (Fig. 5). Brain showed no statistically significant changes in any of the four HSPs during torpor (Fig. 6).

4. Discussion

Hibernation is a state of torpor and heterothermy involving biochemical and physiological adaptations that is typically used to allow various mammalian species to endure cold winter conditions. The results from the previous two papers in this series showed substantial changes in cell signaling pathways when the South American marsupial, *D. gliroides*, entered hibernation (Wijenayake et al., 2018; Luu

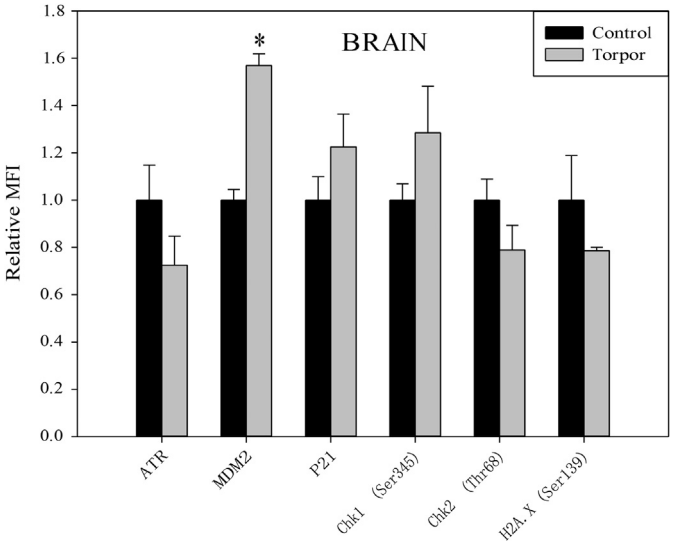

Fig. 3. Relative protein expression of selected cell cycle regulators the brain of control versus torpid *D. gliroides*. Other information as in Fig. 1.

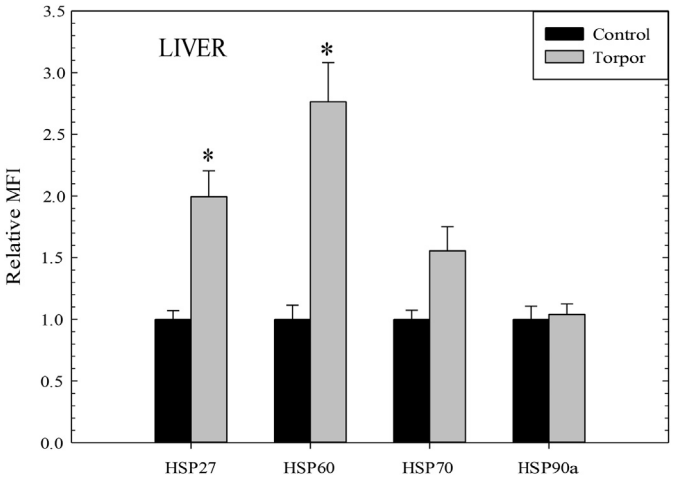

Fig. 4. Relative protein expression of four heat shock proteins (HSP27, HSP60, HSP70/HSP72, HSP90α) in liver of control versus torpid *D. gliroides*. Data are expressed as the relative mean ± SEM of median fluorescence intensity for each independent biological replicate; * - Significantly different from the corresponding control using a two-tailed Student's *t*-test ($p < 0.05$).

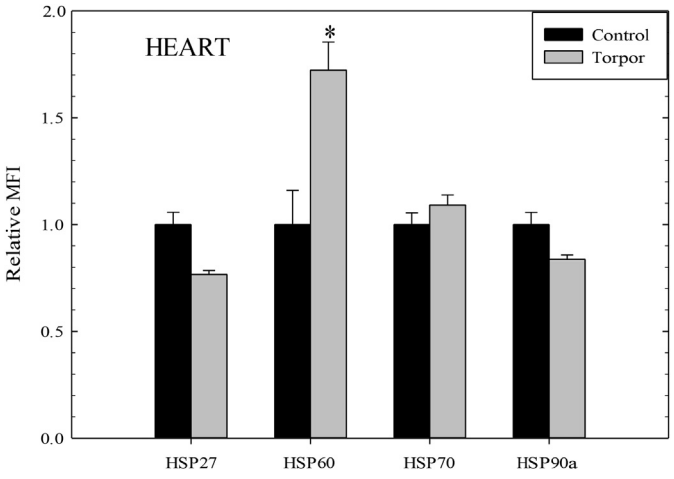

Fig. 5. Relative protein expression of four heat shock protein targets in heart of control versus torpid *D. gliroides*. Other information as in Fig. 4.

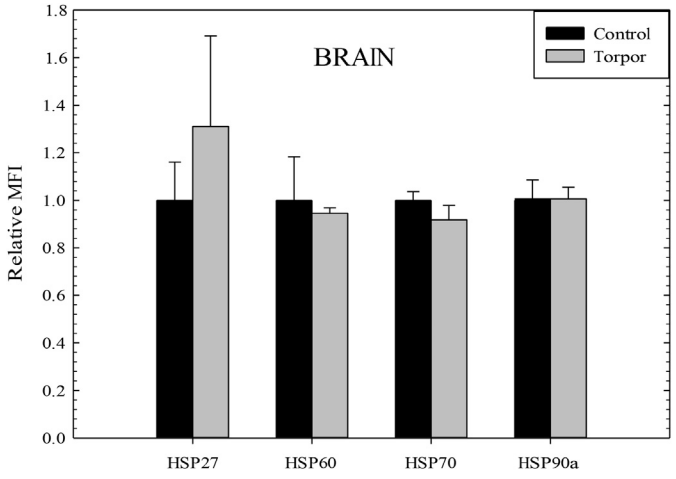

Fig. 6. Relative protein expression of four heat shock protein targets in brain of control versus torpid *D. gliroides*. Other information as in Fig. 4.

et al., 2018). Changes in signaling will clearly modulate multiple processes in cells. Hence, in the present study we chose to focus on two aspects of cellular metabolism that can be crucial for long term survival in a hypometabolic state and that are likely targets of modulated cell signaling during torpor. One is the role of pro-survival adaptations that are initiated in order to stabilize cells/organs for long-term survival in a hypometabolic state (in this case, selected heat-shock chaperone proteins) and cell cycle control. The latter is both a known energy-expensive process that needs to be regulated during torpor, and a process that is highly attuned/integrated to the metabolic state of animals to regulate energy and fuel metabolism. The results reveal a number of molecular adaptations that aid hibernation in *D. gliroides*.

Selected proteins involved in cell cycle regulation, such as ubiquitin ligases, are crucial to facilitating cytoprotective effects in other systems. The ubiquitin ligase MDM2 has an important role in the regulation of p53, a cell cycle regulating protein. MDM2 acts by tagging p53 for proteolysis, but many other organ-specific roles have also been identified for this ligase. For example, in ischemic brain, the ubiquitin-proteasome system is an important tightly-regulated system that is responsible for processing neuronal structures that become impaired from inflammatory responses or oxidative stress (Caldeira et al., 2014). Brain ischemia may also damage the ubiquitin-proteasome system, and ultimately result in unwanted protein deposits (Caldeira et al., 2014). The torpor-induced elevation of MDM2 (Fig. 3), which is known to participate in synapse elimination, may signify a neuroprotective response in brain of *D. gliroides* during torpor that involves the activation of the ubiquitin-proteasome system to mitigate against potential ischemia/reperfusion stress (Caldeira et al., 2014). In the heart, MDM2 expression is known to be cytoprotective in rodent cardiomyocytes; for example, upregulation of MDM2 is induced by H_2O_2 exposure and acts to suppress the pro-apoptotic effects of oxidative stress (Pikkarainen et al., 2009). Lastly, MDM2 is known to interact with both p53 and ribosomal proteins during periods of nutrient deprivation. A study on mice showed that MDM2 is involved in a signaling pathway that is sensitive to nutrient deprivation via its interaction with ribosomal proteins and p53 (Liu et al., 2014). The study found that mutations of MDM2 showed reduced binding to ribosomal proteins and this resulted in fat accumulation in liver under normal feeding and fatty liver disease under fasted conditions (Liu et al., 2014). The conclusion from this study was that the RP–Mdm2–p53 pathway appears to function normally as an endogenous sensor responsible for stimulating fatty acid oxidation in response to nutrient depletion (e.g. fasting, dietary restriction). Applied to a hibernator system, the data showing an elevation in MDM2 in liver of torpid *D. gliroides* suggests that this pathway could be involved in promoting fatty acid oxidation in liver as a fuel during torpor, leading

to an increase in lipid oxidative capacity. This could support both basal metabolism and also enhanced liver-based thermogenesis during torpor (Villarin et al., 2003). Indeed, torpor-induced MDM2 expression in *D. gliroides* could be a multi-tissue response that helps to regulate both cytoprotection and fuel metabolism. In the brain and heart, MDM2 may be primarily cardioprotective and neuroprotective during torpor, whereas MDM2 may be more involved in controlling fatty acid oxidation as a fuel in the liver (Figs. 1, 2 and 3). Interestingly, studies of skeletal muscle in a eutherian hibernator (the 13-lined ground squirrel, *Ictidomys tridecemlineatus*) showed that MDM2 protein levels were unchanged during torpor but rose during the arousal period – the time of lipid-fueled shivering thermogenesis by this organ (Hefler et al., 2015). To our knowledge, MDM2 has not been explored in other hibernating species but clearly should be a target for future study.

Cells have intricate molecular signaling pathways that detect and respond to stresses such as DNA damage and hypoxia. One of the responses to such stresses is cell cycle suppression to prevent replication of compromised genetic information, and to reduce oxygen and fuel demands required for cell proliferation. Whereas ATR has been shown to be activated by DNA damage, it can also be activated by hypoxia, a condition which involves changes in metabolic rate (Ding et al., 2013; Martin et al., 2012). In *D. gliroides* liver, phosphorylation levels of ATR, Chk1, and Chk2 significantly increased in response to torpor (Fig. 1). Previous studies confirmed that the ATR protein kinase directly phosphorylates Chk1 and Chk2 and thereby promotes cell cycle arrest (Ouchi and Ouchi, 2014; Wang et al., 2012). Thus, our results suggest that a torpor-induced increase in ATR may facilitate the increased phosphorylation of Chk1 and Chk2 observed in *D. gliroides* liver, leading to an energy-saving mechanism that regulates/suppresses cell cycle activity. This is comparable to results for a eutherian hibernator, the 13-lined ground squirrel, where *p*-Chk1 content increased by 2.5-fold in liver during entrance into torpor (Wu and Storey, 2012) and, along with evidence from changes in other cell cycle components, indicated cell cycle arrest during ground squirrel hibernation. Furthermore, in the livers of both monito del monte and thirteen-lined ground squirrels, an increase in p21 was observed during torpor (Fig. 1) (Wu and Storey, 2012). The p21 protein is a cyclin dependent kinase inhibitor, which contributes to cell cycle arrest by inhibiting the enzymes that facilitate cell cycle progression (Xiong et al., 1993). Whereas ATR activation and phosphorylation of Chk1 and Chk2 are characteristic responses to DNA damage, it should be noted that no changes in H2A.X phosphorylation were observed in any of the tissues in this study (Figs. 1, 2, and 3). Since DNA damage is known to induce H2A.X phosphorylation by ATR, the changes to the cell cycle regulators seen in this study may likely be induced as an accompaniment to natural torpor, rather than to DNA damage (Rogakou et al., 1998; Singh et al., 2012). Indeed, evidence of DNA damage is lacking in hibernating models. In the heart, a significant increase in ATR protein levels (by 3.91 fold) was also observed during torpor (Fig. 2). However, since the increase in ATR was not accompanied by changes in Chk1 and Chk2 phosphorylation, the results suggest that ATR may have organ-specific downstream targets in different organs of hibernating monito del monte (Fig. 2). It could be postulated that an elevation in cardiac ATR might be a response to shifts in oxygen levels in the torpid marsupial heart.

Lastly, we characterized the responses of four HSPs in *D. gliroides*. The proteins chosen were well-studied chaperones that are broadly known to respond robustly as markers of cell stress in many organisms and have been associated with cytoprotective roles in various forms of natural hypometabolism (Storey and Storey, 2011). HSP responses to hibernation in *D. gliroides* were moderate with significantly increased HSP60 levels found in liver and heart, as well as HSP27 elevation in liver (Figs. 4 and 5). However, HSP70/HSP72 and HSP90α did not change in any of the three tissues. In particular, the lack of an HSP70/HSP72 response may suggest that hibernation in monito is not a classic or generalized stress on the animal's metabolism but rather more of a coordinated reorganization of some facets of metabolic function, requiring only targeted changes in selected chaperones. This upregulation of selected HSPs during hibernation in *D. gliroides* parallels the selective responses by different HSPs also seen in organs of eutherian hibernators such as ground squirrels, lemurs, and bats (Lee et al., 2002b; Mamady and Storey, 2006; Rouble et al., 2014; Wu et al., 2015). However, as yet, there have been no clearly consistent responses by HSPs identified in mammalian hibernators, although that may be partly due to differences in the tissues analyzed, the hibernation time points chosen, and choice of protein vs mRNA for analysis in different studies (Storey and Storey, 2011). The role of HSP60 as a mitochondrial chaperone may reflect the importance of stabilizing proteins involved in aerobic ATP production in mitochondria under heterothermic conditions. Indeed, HSP60 was also elevated in the liver of torpid lemurs (Wu et al., 2015). Given that liver has a possible thermogenic role in marsupials, as reported by Villarin et al. (2003) and also consistent with various cell signaling responses seen in *D. gliroides* liver (Wijenayake et al., 2018; Luu et al., 2018), the strong increase in HSP60 in liver could be associated with a proliferation of liver mitochondria to support enhanced thermogenesis. HSP60 levels also increased in hearts of hibernating monito del monte (Fig. 5) and this chaperone has been previously shown to play an important role in the ischemia/reperfusion response of injured cardiomyocytes (Schett et al., 1999). Hence, HSP60 may also play a protective role in monito heart during hibernation or arousal. Lastly, the protein chaperone HSP27 has actions including inhibition of protein aggregation under stress conditions, protection of actin filaments, and activation of antioxidant defenses through HSP27-mediated upregulation of glutathione-related enzymes (Storey and Storey, 2011). An increase in HSP27 in monito del monte liver suggests that this protein may have similar roles during *D. gliroides* hibernation. Overall, the tissue-specific HSP protein expression responses in *D. gliroides* suggest that chaperone proteins are important members of the cytoprotective response that protects the cells of hibernating animals against stress conditions associated with prolonged torpor and/or that occur during entry into or arousal from the hypometabolic state.

5. Conclusions

The present study characterized selected targets in the canonical DNA damage and heat shock responses in liver, heart and brain of hibernating *D. gliroides*. The results indicate that these proteins can have organ-specific pro-survival and metabolic roles that facilitate animal survival during torpor. Enhanced levels of the ubiquitin ligase MDM2 may provide cardioprotective and neuroprotective responses during torpor, in addition to facilitating fatty acid oxidation in the liver. Modulation of cell cycle regulators in the liver suggest that torpor is accompanied by mechanisms for cell cycle arrest, whereas an elevation of cardiac ATR kinase may belong to a broader protein kinase response. Both cell cycle regulation and HSP modulation were most prominent in the liver and more modest in the heart. With the exception of MDM2, this study did not identify any differential regulation of cell cycle regulators or HSPs in the brain. Hence, similar to other hibernating animals, monito del monte employs a diverse assortment of pro-survival pathways during bouts of torpor.

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