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Characterization of adipocyte stress response pathways during hibernation in thirteen-lined ground squirrels

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Abstract To avoid the harsh conditions of winter climates, hibernating mammals undergo a systematic depression of physiological function by reducing their metabolic rate. During this process, hibernators are exposed to significant stresses (e.g., low body temperature, ischemia–reperfusion) that must be dealt with appropriately to avoid irreversible tissue damage. Consequently, we investigated the contribution of stress-responsive antioxidant enzymes, heat shock proteins, signal transduction pathways (e.g., mitogen-activated protein kinases, MAPK), and transcription factors for their role in conferring tolerance to stress in the hibernating thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*). Using a combination of multiplex protein panels and traditional immunoblotting procedures, we have focused on these stress factors in brown adipose tissue (BAT) and white adipose tissue (WAT) over cycles of torpor-arousal since they provide the means for heat production as a result of non-shivering thermogenesis and the mobilization of critical energy reserves, respectively. We show the differential and tissue-specific regulation of stress factors including a unified upregulation of the antioxidant enzyme Thioredoxin 1 in both tissues, an upregulation of superoxide dismutase (SOD1 and SOD2) in WAT, and an increase in heat shock proteins during the transitory periods of the torpor-arousal cycle (HSP90α in BAT and HSP60 in WAT). Additionally, an upregulation of the active form of ERK1/2 and p38 in BAT and select transcription factors (e.g., CREB-1 and ELK-1) in both tissues were identified. These data provide us with greater insight into the molecular mechanisms responsible for this animal's natural stress tolerance and outline molecular signatures which define stress resistance.

Keywords Brown adipose tissue · White adipose tissue · Hibernation · Mitogen-activated protein kinases · Antioxidant enzymes · Heat shock proteins

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Introduction

Hibernating mammals are a truly remarkable example of nature's ability to adapt to the many challenges of the physical world. In the face of the extreme environmental stresses of a winter climate (i.e., low temperatures, limited access to food sources), hibernators have evolved a strategy of avoidance that is characterized not by a geographical change, but by an escape from the normal energy demands of a euthermic lifestyle. Hibernation is a cyclic process characterized by periods of prolonged torpor (a metabolically reduced state, lasting days to weeks) interspersed with brief (<24 h) intervals of metabolic arousal. By depressing their metabolic rate during torpor (to as little as 2–4 % of euthermic values [1]), these animals avoid having to obtain the large amounts of resources (i.e., food) that would otherwise be necessary to maintain normal euthermic physiological functions during the winter months. This depression is facilitated by a drastic reduction in body temperature to near-ambient levels (sometimes <4 °C), decreases in breathing (2.5 % of normal) and heart rates (from 350 to 400 beats/min during euthermia to 5–10 beats/min during hibernation), lowered organ perfusion (<10 % of normal), and a suspension of high-energy processes at the cellular level (transcription, translation, ATP-pumps, etc.) [1–3]. To sustain this state, hibernators

rely upon energy stores in the form of internal lipid deposits in white adipose tissue (WAT), accumulated during the summer months in preparation for the hibernation season [3, 4]. The energy economy of the animal thus undergoes a drastic shift away from glycolytic pathways toward those of lipid catabolism, relying upon the sustained function of relevant lipid-processing enzymes and fatty acid-binding proteins [5, 6]. This also remains true during the brief periods of arousal, when the rapid reversal of these physiological changes is facilitated by a massive increase in non-shivering thermogenesis that is fueled by enormous levels of oxygen consumption and fatty acid oxidation in the specialized mitochondria of brown adipose tissue (BAT). Ultimately, these processes are controlled by tightly regulated molecular mechanisms involving the intricate adjustment of gene expression and protein function of selected targets [6].

Perhaps, the most remarkable aspect of the process of hibernation is the fact that these animals endure little to no damage to their tissues in the face of the extreme stresses associated with the torpor-arousal cycle [6, 7]. For example, despite low body temperatures and a limited oxygen supply (although tissue is not hypoxic [8]) during deep torpor, hibernators perform an intricate array of molecular adjustments which maintain harmony between ATP demand and supply. Additionally, hibernators withstand the stresses associated with rapid rewarming during arousal—a process fueled by a massive influx of oxygen to tissues causing the production of damaging reactive oxygen species (ROS). Indeed, the activation of cytoprotective pathways has been documented in many different species of mammalian hibernators during torpor and arousal, and appears to be a fundamental characteristic of most forms of metabolic rate depression [9]. Examples of such responses in hibernator tissues include the active regulation of antioxidant defenses (e.g., the increased expression of ROS-scavenging enzymes) [6, 10–12], heat shock proteins (HSPs—involved in the maintenance of protein stability/folding and the prevention of protein aggregation in response to various forms of stress) [13], and pro-survival signals (e.g., anti-apoptotic protein expression) [14], each with functions that ultimately serve to maintain cellular integrity during the stressful transitions to and from the hibernation state. Additionally, signal transduction cascades and the control they exert over transcription factors have been shown to contribute to the hibernating phenotype by providing a mechanism to sense the external environment and alter gene expression programs accordingly [3]. Key to this response are the mitogen-activated protein kinases (MAPK; extracellular-signal-regulated kinases, ERK; c-Jun N-terminal kinases, JNK; p38) which are widely studied protein kinases involved in the transduction of stress signals from various extracellular stimuli (e.g., osmotic stress, heat shock, and proinflammatory cytokines) through phosphorylation cascades that regulate a vast array of downstream targets including transcription factors [15–17]. Hence, hibernating mammals have become an important platform to study natural mechanisms of cytoprotection that may be applied to the treatment/prevention of diseases involving tissue degeneration and pathological stress responses, including conditions characterized by muscle wasting (muscular dystrophy and muscle disuse atrophy), neural degradation (Alzheimer's and Parkinson's disease), cardiomyopathy (in various cardiovascular diseases, especially in response to ischemia/reperfusion), and metabolic disregulation (diabetes and cancer) [3, 6]. The unique tissue-preservation tactics of hibernators may also prove to be useful in the development of strategies to extend the shelf-life of organs for transplantation purposes [6].

The maintenance of cellular integrity is not only essential in preventing the catastrophic deterioration of the hibernator's overall health, but it is also integral to ensuring the proper function of those tissues which are most responsible for facilitating the reversible metabolic decline. Two such tissues are particularly important to the maintenance, and reversal, of torpor: the brown and white adipose tissues, yet the mechanisms of stress tolerance which support torpor-arousal are largely uncharacterized in these tissues. Without BAT, the hibernator would be unable to maintain core body temperature above the lowest possible threshold (<4 °C in some instances), nor could it achieve the rapid thermogenesis required for the reawakening of arousal. Likewise, WAT provides the fundamental energy supply necessary to sustain these processes; without sufficient lipid stores (and the reliable mobilization thereof), the hibernator is incapable of surviving over the winter months [4]. Thus, the ability of these tissues to effectively respond to the stresses of hibernation (i.e., oxidative stress, rapid and extreme temperature fluctuations) is ultimately integral to the success of the phenotype.

The many protective strategies employed by hibernators represent an intricate program of stress tolerance that is governed by diverse mechanisms at the molecular level. When it comes to the hypothetical human-application of such strategies, a relevant question presents itself: what is the underlying molecular signature (or "fingerprint") that forms the basis of this stress-response program? To attempt to elucidate such an underlying molecular pattern, this study assessed the expression of several pathways/factors that are well-defined players in non-hibernating models of animal stress responses. We have measured the relative protein expression of downstream effectors of such responses, as well as that of possible signal transducers which may be responsible for relaying the appropriate molecular messages toward the promotion of stress-

tolerance, over the course of the torpor-arousal cycle in the BAT and WAT of a rodent model of mammalian hibernation, *Ictidomys tridecemlineatus* (the thirteen-lined ground squirrel). These targets include well-characterized ROS-scavengers/mediators of oxidative stress responses (antioxidant enzymes), HSPs, members of the mitogen-activated protein kinase (MAPK) signaling pathways, and transcription factors. By assessing these factors, we hoped to gain insight into both the specific mechanisms that ultimately confer cytoprotection upon these tissues, as well as the means by which such downstream responses are controlled and instigated. Our attempt to better define this "stress fingerprint" in BAT and WAT is described herein.

Methods

Animal experiments

Animal experiments were performed as described previously [14], and were carried out at the NIH facility in the laboratory of Dr. J. M. Hallenbeck. Animals were sacrificed at various points throughout the torpor-arousal cycle, such that tissues were frozen in liquid nitrogen upon immediate excision from squirrels under the following conditions: (1) Euthermic in the cold room (EC)—these euthermic control animals had not entered torpor for at least 72 h and had maintained a stable T_b of 36–37 °C during this time; (2) Entrance to torpor (EN)—these animals had begun to enter torpor and were identified by a T_b of 18–31 °C (at least two successive temperature readings showed a decreasing T_b); (3) Late torpor (LT)—these animals had remained in the deep torpor phase of the hibernation bout for at least 120 h (5 days), indicated by a stable T_b of 5–8 °C during this period; (4) Early arousal (EA)—these animals were characterized by an increased respiratory rate of more than 60 breaths/min accompanied by a persistent low T_b of 9–12 °C; (5) Interbout arousal (IA)—these animals were naturally aroused from torpor for approximately 18 h with a T_b of approximately 37 °C (reached the respiratory rate, metabolic rate, and T_b of euthermic controls). Tissues were stored in a −80 °C freezer upon delivery to Carleton University on dry ice.

Luminex® assays

Luminex® assay panels were purchased from EMD Millipore and were used according to the manufacturer's instructions. The assays employed in this study were used to measure oxidative stress response factors (5-Plex Oxidative Stress Magnetic Bead Panel, Cat#H0XSTMAG-18K), heat shock proteins (5-Plex Heat Shock Protein Magnetic Bead Panel, Cat#48-615MAG), and MAP Kinase/SAP Kinase phosphoproteins (10-Plex MAPK/SAPK Signaling Panel—Phosphoprotein, Cat#48-660) in both BAT and WAT of *I. tridecemlineatus* during each of the five sampled time points of the torpor-arousal cycle (as above). The multiplex Oxidative Stress Panel was used to determine the relative protein levels of key antioxidant proteins involved in universal responses to oxidative stress: catalase, superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), thioredoxin (TRX1), and peroxiredoxin 2 (PRX2). The Heat Shock Protein Panel measured relative protein levels of specific heat shock proteins: HSP27, phosphorylated HSP27 (pS78/pS82), HSP60, HSP70 (HSP72), and HSP90α. Finally, the 10-Plex MAPK/SAPK Signaling Panel was used to determine phosphorylated protein levels of specific MAPK/SAPK signaling factors. Targets reported include MEK1 (Ser221), ERK/MAP Kinase 1/2 (Thr185/Tyr187), p38 (Thr180/Tyr182), MSK1 (Ser212), JNK (Thr183/Tyr185), ATF2 (Thr69/71), c-Jun (Ser73), p53 (Ser15), and HSP27 (Ser78).

Protein extraction for Luminex® assays

Protein extracts were prepared from frozen tissue samples according to the manufacturer's instructions. Briefly, approximately 50 mg of frozen tissue from animals from each of the sampled time-points was weighed and immediately homogenized 1:4 w/v with chilled lysis buffer (EMD Millipore; Cat#43-045 or Cat#43-040, depending on the specifications of each kit) including added phosphatase (1 mM Na_3VO_4, 10 mM β-glycerophosphate) and protease (BioShop Cat# PIC001) inhibitors, using a dounce homogenizer. After incubation on ice for 30 min with occasional vortexing, samples were centrifuged at 12,000×*g* for 20 min (4 °C), and the supernatants were collected as total soluble protein lysates. The protein concentration of each sample was determined using the Bradford assay (Bio-Rad; Cat#500-0005) and standardized to 5 μg/μl using lysis buffer. Extracts were stored at −80 °C until further use. Before use in the assay, aliquots of each sample were combined with assay buffer (Milliplex MAP Assay Buffer 1 or Milliplex MAP Assay Buffer 2, depending on the specifications of each kit; EMD Millipore; Cat#43-010 or Cat#43-041, respectively), in at least a 1:1 ratio, such that the following amounts of total protein were added to each well for the respective kit/tissue: Oxidative Stress Panel (suggested working range: 5–20 μg/well), BAT = 17.5 μg, WAT = 16.25 μg; Heat Shock Protein Panel (suggested working range: 15–150 ng/well), BAT = 130 ng, WAT = 130 ng; 10-Plex MAPK/SAPK Signaling Panel (suggested working range: 1–25 μg/well), BAT = 17.5 μg, WAT = 15.0 μg.

Positive and negative controls provided by the manufacturer were used in each assay, and were prepared

according to the manufacturer's instructions. For the Oxidative Stress Panel, unstimulated HepG2 cell lysate (Cat#47-231) was used as a negative control. For the Heat Shock Protein Panel, unstimulated HeLa cell lysate (Cat#47-205) was used as a negative control, while HeLa cell lysate treated with arsenite and heat shocked (Cat#47-211) was used as a positive control. For the MAPK/SAPK Signaling Panel, unstimulated HeLa cell lysate (Cat#47-205) was used as a negative control, while HeLa cell lysate treated with arsenite and heat shocked (Cat#47-211); A431 cell lysate stimulated with epidermal growth factor, EGF (Cat#47-210), and NIH/3T3 cell lysate treated with anisomycin (Cat#47-219) were used as positive controls. Cell lysates were provided as lyophilized stocks, and were reconstituted in 100-μL ultrapure water (2 mg/mL total protein), vortexed, and incubated at room temperature for 5 min. The reconstituted lysates were prepared for use by the addition of an appropriate amount of assay buffer, as determined by the manufacturer's instructions for each respective kit.

Multiplex analysis

The protocol for multiplex analysis followed manufacturer's direction. Briefly, premixed antibody capture beads for each respective kit (EMD Millipore; Cat#H0XST PMX5-MAG, Cat#42-615K, Cat#42-660) were sonicated, vortexed, and diluted as required. After incubation with assay buffer, sample or control (positive or negative controls, as described above) lysates were individually combined with the premixed beads in a 96-well microplate. The assay wells were then incubated overnight at 4 °C on a plate shaker (600–800 rpm) while protected from light. The assay mixture was subsequently removed using a Handheld Magnetic Separator Block (Cat#40-285, leaving the beads in the well), and the wells were washed three times each with assay buffer. Biotin-labeled detection antibodies for each kit (EMD Millipore; Cat#H0XST-1018, Cat#44-615K, Cat#44-660) were vortexed and diluted as required, and were added to wells containing magnetic capture beads. After incubation for 1 h at room temperature on a plate shaker protected from light, the wells were decanted, washed, and incubated with appropriately prepared Streptavidin–Phycoerythrin (EMD Millipore; Cat#MC-SAPE6, Cat#45-001D) for 15–30 min at room temperature on a plate shaker protected from light. After this incubation, the wells were either decanted, washed, and the beads resuspended in Sheath Fluid (Oxidative Stress Panel) or amplification buffer (EMD Millipore; Cat#43-024A) was added to each well (15 min incubation at room temperature on a plate shaker) before decanting and resuspension in Sheath Fluid (Heat Shock Protein Panel and MAPK/SAPK Signaling Panel). Measurements were taken immediately after the assay was finished using either a Luminex 100® (MAPK/SAPK Panel) or a MAGPIX® (Oxidative Stress Panel and Heat Shock Protein Panel) instrument with xPonent software (Luminex® Corporation).

Total protein extraction and immunoblotting

Total protein extraction and the subsequent immunoblotting of the samples were performed as described previously [14]. Membranes were blocked using 2.5 % skimmed milk in TBST for 20 min, and were probed with specific primary antibodies for mammalian CREB-1 phosphorylated at serine 133 (Santa Cruz Biotechnology Inc., Cat#7978-R) and Elk-1 phosphorylated at serine 383 (Cell Signaling Technology, Cat#9181) (1:1,000 v/v dilution in TBST) at 4 °C overnight. Membranes were then probed with secondary anti-rabbit IgG HRP-linked antibody (1:4,000 v/v dilution in TBST) for 20 min at room temperature, and developed using enhanced chemiluminescence.

Quantification and statistical analysis

Bead-based assays used the net median fluorescence intensity (MFI) of a population of measurements (with a minimum bead count of 50 and subtracted from background wells) in order to determine the relative protein levels. Immunoblot protein bands were visualized using the ChemiGenius Bio Imaging System (Syngene), and quantified by densitometric analysis using the associated GeneTools Software (Syngene). Immunoblot band density was standardized against the summed intensity of a group of Coomassie-stained protein bands from the same sample lane, the latter of which were chosen based on their lack of variation between experimental conditions and because they were not located close to the protein band of interest. This method of standardization is employed due to the lack of change in the expression of the majority of proteins found in hibernator tissues throughout the torpor-arousal cycle. Data are expressed as mean ± SEM ($N = 3$–5 independent samples from different animals). All data were analyzed using a one-way ANOVA and a post hoc Tukey test (SigmaPlot 12 software). Differences between means were considered significant at $p < 0.05$.

Results

Antioxidant enzyme expression in BAT and WAT over the torpor-arousal cycle

Of the antioxidant factors measured in this investigation, TRX1 was the only target observed to fluctuate

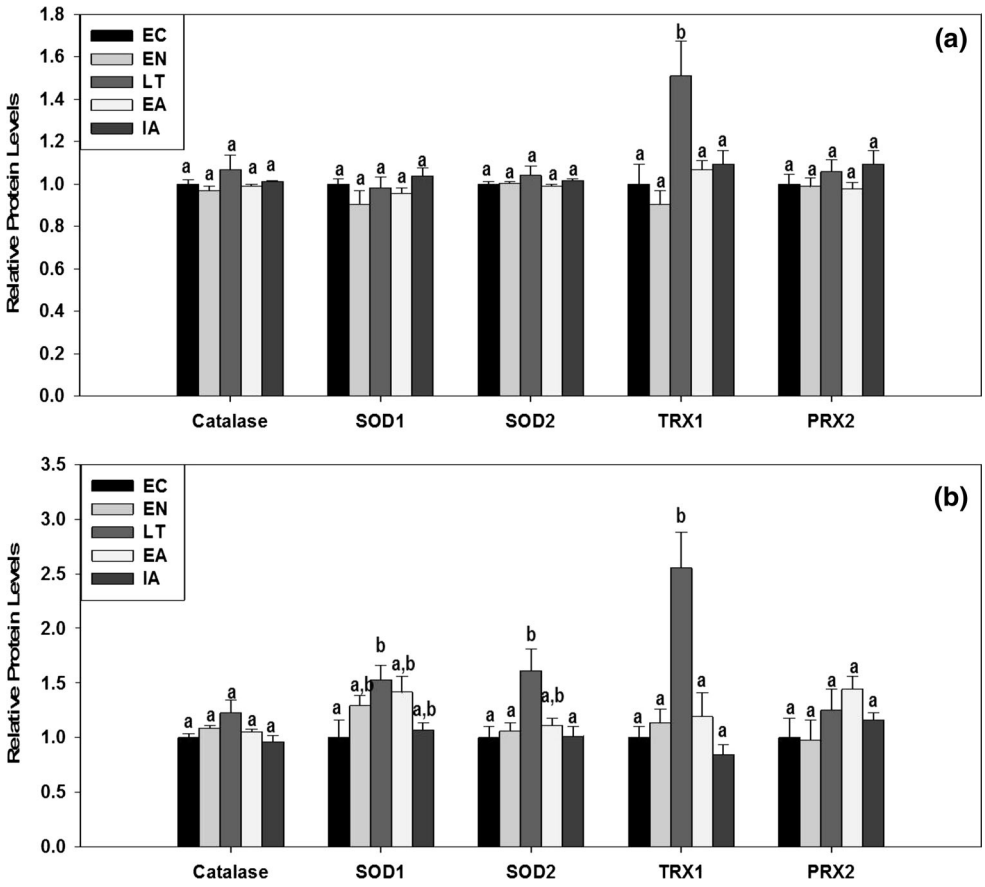

Fig. 1 Relative protein expression of the antioxidant factors Catalase, SOD1, SOD2, TRX1, and PRX2 in **a** brown adipose tissue (BAT) and **b** white adipose tissue (WAT) of *I. tridecemlineatus* over the torpor-arousal cycle. Sampling points were *EC* euthermic control, *EN* entrance to torpor, *LT* late torpor, *EA* early arousal, and *IA* interbout arousal. See the "Methods" section for more extensive definitions. Histograms showing relative protein levels for bead-based assays were determined using the net median fluorescent intensity (MFI) as measured by Luminex®. Data are mean ± SEM ($N = 3$–5 independent protein isolations from different animals). Data were analyzed using analysis of variance with a post hoc Tukey test ($p < 0.05$); values that share the *same letter notation* are not significantly different from one another

significantly, while Catalase, SOD1, SOD2, and PRX2 did not change significantly in BAT during hibernation (Fig. 1a). During the late torpor time point (LT), protein levels of TRX1 were significantly elevated (~1.5 fold) as compared to euthermic controls (EC) and all other experimental conditions. In WAT, protein levels of SOD1, SOD2, and TRX1 were found to be significantly elevated during LT as compared to euthermic controls (~1.5, 1.6, and 2.6 fold, respectively), with SOD2 levels also being significantly elevated as compared to EN and IA, and TRX1 levels being significantly elevated as compared to all other experimental conditions (Fig. 1b). Similar to the pattern observed in BAT, protein levels of Catalase and PRX2 did not change significantly in WAT throughout the hibernation cycle.

Expression of heat shock proteins in BAT and WAT over the torpor-arousal cycle

In BAT, protein levels of HSP90α were observed to increase significantly (~1.8 fold) during IA as compared to EC (Fig. 2a). However, protein levels of HSP27, HSP27(pS78/pS82), HSP27(pS78), HSP60, and HSP70(HSP72) did not change significantly during any of the experimental conditions tested. In WAT, levels of HSP60 were significantly elevated (~1.6 fold) during EN when compared to all other experimental conditions, while levels of HSP27, HSP27(pS78/pS82), HSP27(pS78), HSP70(HSP72), and HSP90α remained unchanged (Fig. 2b).

Activation of MAPK signaling cascades in BAT and WAT over the torpor-arousal cycle

Phosphorylated protein levels of several members of the MAPK-signaling pathway were observed to fluctuate significantly in both BAT and WAT over the course of hibernation (Fig. 3). In BAT, levels of p-ERK1/2 were found to be significantly elevated during EA as compared to EC (~3.8 fold) and all other experimental conditions while the levels of p-MEK1 and p-MSK1 did not change significantly (Fig. 3a). Levels of p-p38 were also significantly higher during EA (~2.8 fold) when compared to EC and LT experimental conditions. In contrast, levels of p-JNK were found to be significantly decreased during LT (~0.5 fold) when compared to EC and, when compared to EA, p-JNK levels were significantly lower during EN, LT, and IA. In WAT, a pattern of decreased phosphorylated protein levels was observed throughout the torpor-arousal

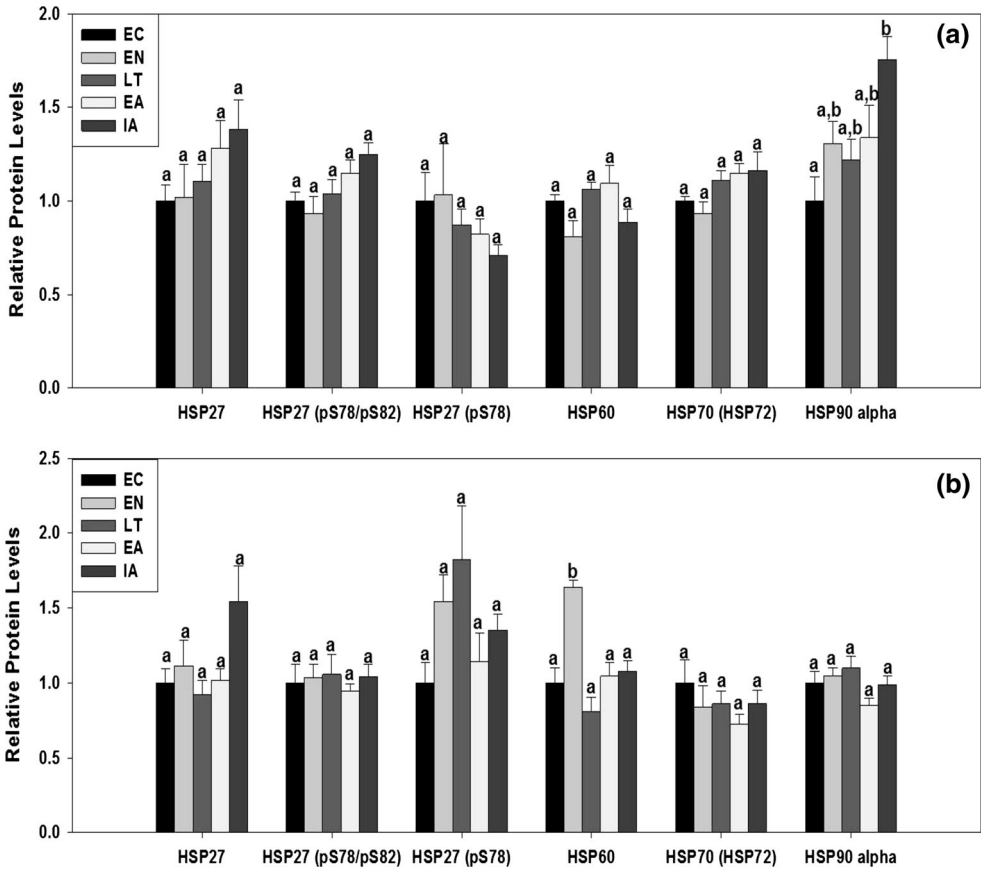

Fig. 2 Relative protein expression of the heat shock proteins HSP27, HSP27(pS78/pS82), HSP27 (pS78), HSP60, HSP70(HSP72), and HSP90α in **a** BAT and **b** WAT of *I. tridecemlineatus* over the torpor-arousal cycle. Histograms showing relative protein levels for bead-based assays were determined using the net MFI as measured by Luminex®. Data are mean ± SEM ($N = 3$–4 independent protein isolations from different animals). All other information as in Fig. 1

cycle (Fig. 3b). Protein levels of p-ERK1/2 decreased significantly during EN (~0.3 fold), LT (~0.1 fold), and EA (~0.2 fold) as compared to EC, before returning to euthermic levels during IA. Levels of p-p38 were also significantly lower during LT (~0.4 fold), EA (~0.2 fold), and IA (~0.2 fold) as compared to EC, as were levels of p-JNK during EA (~0.2 fold) as compared to EC. Levels of p-MEK1 also decreased significantly between the EN and IA time points (although no changes occurred relative to EC), and no changes were detected for p-MSK1 across all experimental conditions.

Activation of transcription factors in BAT and WAT over the torpor-arousal cycle

The transcriptional activity of many transcription factors is regulated by posttranslational modifications, such as protein phosphorylation, at distinct sites on the protein. As such, phosphorylated protein levels of several stress-activated transcription factors were measured in both BAT and WAT over the course of hibernation (Fig. 3). In BAT, western blotting revealed a significant increase in levels of p-CREB-1(S133) during LT (~5.0 fold) and IA (~6.0 fold) when compared to EC, EN, and EA, as well as a significant elevation of p-Elk-1(S383) levels during LT (~2.0 fold) when compared to all other experimental conditions (Fig. 4). In WAT, western blotting indicated a significant increase in p-Elk-1(S383) levels during LT (~4.0 fold) when compared to EC, EN, and IA, and during EA (~3.3 fold) as compared to EC and EN, while no changes were observed for levels of p-CREB-1(S133) (Fig. 5). In contrast, all other transcription factors measured over the torpor-arousal cycle did not change in BAT and WAT (Figs. 4, 5).

Discussion

The identification of general patterns of molecular regulation that characterize the remarkable adaptations of hibernating mammals is incredibly valuable to our understanding of how such animals are able to endure the extreme metabolic changes and associated stresses of torpor-arousal cycles. Hence, in the present study, we aimed to gain important insight into some of the possible mechanisms of molecular control that may be associated with the success of BAT and WAT in maintaining, and eventually reversing, the hibernation state. Since both of these tissues are absolutely integral to facilitating hibernation (by sustaining the animal's energy requirements via the

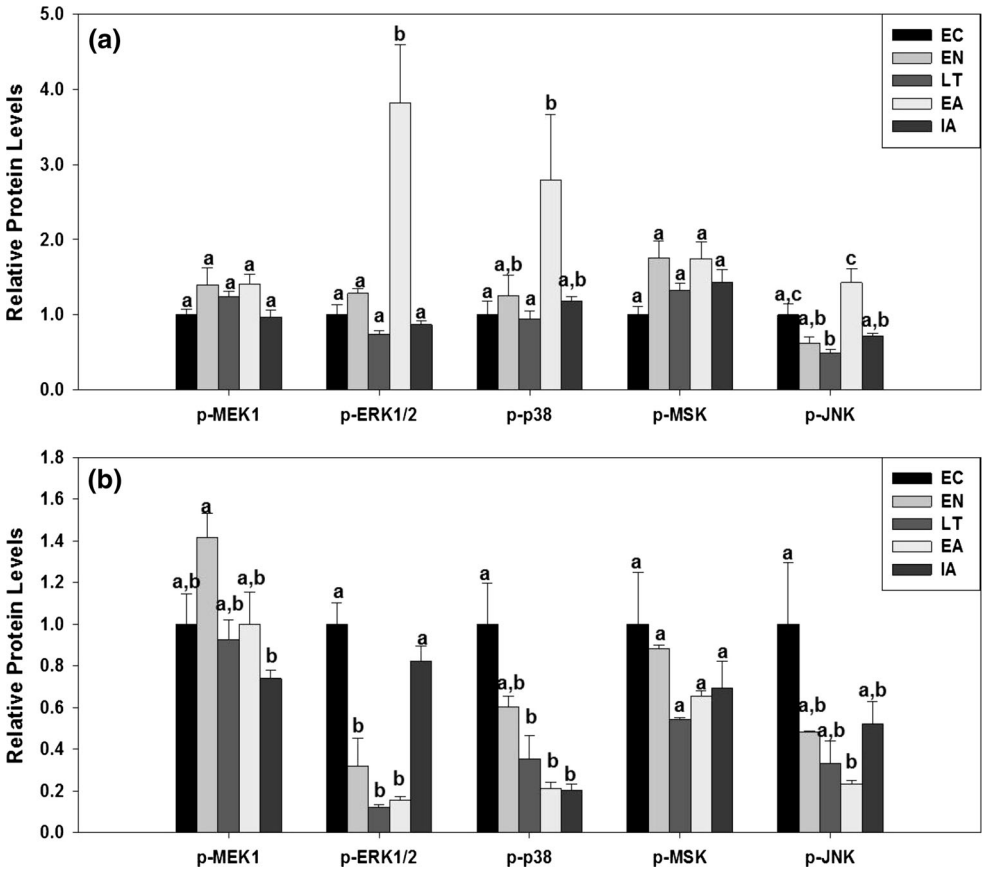

Fig. 3 Relative phosphorylation status of the MAPK-signaling pathway kinases MEK1, ERK1/2, p38, MSK, and JNK in **a** BAT and **b** WAT of *I. tridecemlineatus* over the torpor-arousal cycle. Histograms showing relative protein levels for bead-based assays were determined using the net MFI as measured by Luminex®. Data are mean ± SEM ($N = 3$–4 independent protein isolations from different animals). All other information as in Fig. 1

maintenance of the lipid-economy and fueling the massive thermogenesis of arousal), it is essential that the animal is able to prevent cellular damage and regulate the extreme stresses that could otherwise compromise tissue function. The pathways presently studied offer specific examples of how these challenges may be dealt with, providing plausible (and in many cases, previously established [3, 12, 13]) solutions for the detoxification of harmful ROS (i.e., increased antioxidant expression), the maintenance of protein integrity (i.e., increased heat shock protein expression), the transduction of the associated stress signals (i.e., differential MAPK signaling), and the response of transcription factors that underlie the proper functioning of BAT and WAT during hibernation. Indeed, the characterization of these processes will help to determine the essential patterns of molecular regulation that are integral to the cytoprotective abilities of these animals, and which may be most useful to future medical applications.

A protective role for antioxidant pathways in the context of hibernation and other models of metabolic rate depression has previously been established [3, 10–12], but the expression of several important antioxidant factors had yet to be characterized with respect to brown and white adipose tissue of *I. tridecemlineatus*. Our analysis revealed that the relative abundance of several of the measured antioxidant proteins changed throughout the course of hibernation (Fig. 1). A significant increase in TRX1 was observed in both tissues during late torpor, supporting previous studies that have identified increased antioxidant responses in BAT and WAT of other hibernator species [12]. TRX1 has multiple established functions in the regulation of oxidative stress and is known to promote cell survival in response to various challenges, including ischemia–reperfusion and hypoxia [18]. Thus, an increased expression of TRX1 in brown and white adipocytes may serve to prevent damage to these tissues at a time when ground squirrels experience low organ perfusion rates, decreased cellular function, and low T_b. Similarly, protein levels of SOD1 and SOD2 were also significantly elevated during late torpor in WAT (Fig. 1b), which supports prior work that identified a similar trend in WAT in another hibernator species [12]. The enhanced expression of these two highly conserved antioxidant enzymes points to an increased requirement of WAT for mechanisms of oxidative stress resistance during late torpor, and reflects a possible strategy by which the hibernator preemptively protects its tissues from elevated ROS production during the rapid period of rewarming that follows in arousal (as previously speculated [10, 12]). These findings, when taken together with previous studies, help to define a general

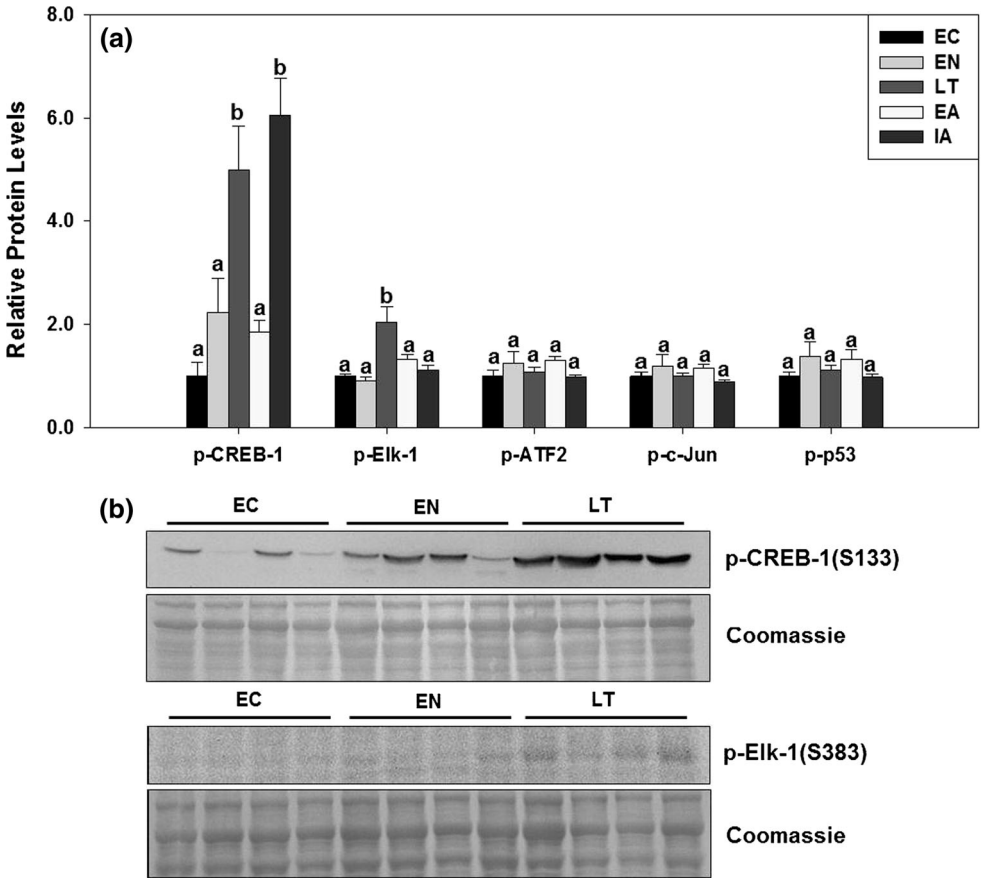

Fig. 4 Relative phosphorylation status of the transcription factors CREB-1, Elk-1, ATF2, c-Jun, and p53 in BAT of *I. tridecemlineatus* over the torpor-arousal cycle. **a** Histograms showing relative protein levels were determined using net MFI for bead-based assays (ATF2, c-Jun, p53) or band densities for immunoblotting (CREB-1, Elk-1). Data are mean ± SEM ($N = 3$–4 independent protein isolations from different animals). **b** Representative immunoblots and Coomassie loading controls are shown for p-CREB-1(S133) and p-Elk-1(S383) for selected experimental conditions that are labeled along the top of the blots. All other information as in Fig. 1

pattern of oxidative stress resistance in the hibernating mammal, and reinforce the notion of an underlying universal framework for the regulation of metabolic rate depression. Further investigation of regulatory mechanisms other than fluctuations in protein abundances (e.g., reversible post-translational activation of antioxidant factors via modifications such as differential phosphorylation and lysine acetylation [19, 20]) may also uncover other layers of control that remain unidentified by the current study, thereby adding to the present picture of the oxidative stress response in hibernator tissues.

Much like how antioxidant enzymes are well-characterized and highly conserved regulators of oxidative stress, heat shock proteins are established players in the universal response of organisms to a broad range of cellular stresses, functioning to prevent protein aggregation of unfolded and native polypeptides and facilitating the re-folding of denatured or malfolded proteins [21, 22]. For this reason, HSPs have previously been implicated as regulators of the stresses experienced in the context of several forms of metabolic rate depression, including hibernation [13]. In hibernators, HSPs have been suggested to serve cytoprotective roles in multiple tissues over the course of the torpor-arousal cycle, with the expression patterns of specific HSPs varying considerably between species and the tissues therein [13]. Notably, HSP expression is also highly variable in the traditional stress responses of non-hibernating organisms, suggesting that inter-species differences between hibernators are likely to be expected [22]. Our analysis of BAT and WAT from *I. tridecemlineatus* appears to reflect these previously established roles and variability (Fig. 2). Of the HSPs measured in both tissues, the relative abundance of two (HSP90α and HSP60) were found to change significantly, with each tissue exhibiting a different pattern of expression for each factor. In BAT, protein levels of HSP90α were elevated only during interbout arousal (Fig. 2a), while levels of HSP60 increased significantly in WAT during the entrance to hibernation (Fig. 2b). These two observations share some similarity to previous studies, which have identified fluctuations in these factors in different tissues of ground squirrels at specific points during the hibernation cycle [13]. Interestingly, such studies have also shown changes in HSP abundance during the transition periods of hibernation (i.e., entrance and arousal) [13], as seen in the current study. The observed increase of HSP90α in BAT during interbout arousal may indicate a role for this protein during the metabolic reactivation that is characteristic of this period, possibly via its

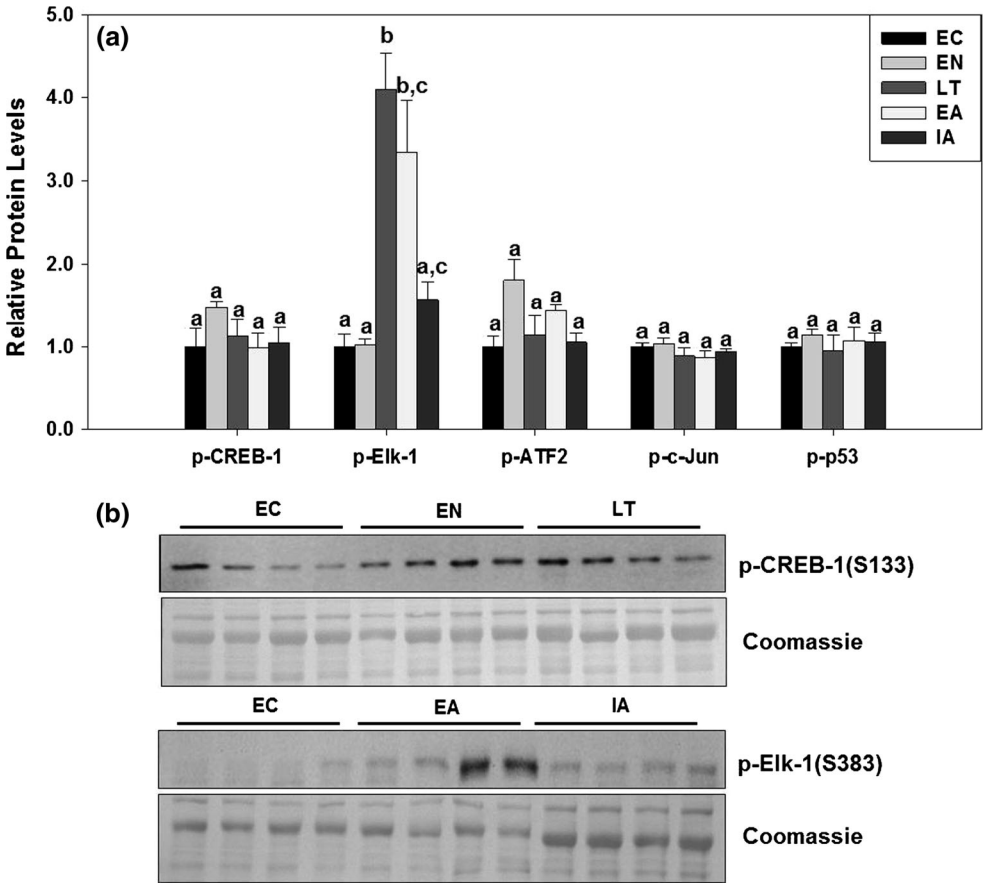

Fig. 5 Relative phosphorylation status of the transcription factors CREB-1, Elk-1, ATF2, c-Jun, and p53 in WAT of *I. tridecemlineatus* over the torpor-arousal cycle. **a** Histograms showing relative protein levels were determined using net MFI for bead-based assays (ATF2, c-Jun, p53) or band densities for immunoblotting (CREB-1, Elk-1). Data are mean ± SEM ($N = 3$–5 protein isolations from different animals). **b** Representative immunoblots and Coomassie loading controls are shown for p-CREB-1(S133) and p-Elk-1(S383) for selected experimental conditions that are labeled along the top of the blots. All other information as in Fig. 1

stabilization of cellular proteins and prevention of protein aggregation [21, 23] in the face of the rapid changes in ROS production and body temperature that occur during this time. Likewise, HSP60 may serve similar functions in WAT during the opposite transition, possibly ensuring the maintenance of protein integrity in the face of the rapid temperature reduction and other stresses associated with the metabolic decline. Indeed, HSP60, a mitochondrial HSP, has been implicated in the maintenance of proper mitochondrial function and the associated stabilization of cellular levels of ROS, whereby decreased HSP60 expression is linked to increases in ROS generation [24]. Of further possible significance is that HSP60 also appears to be crucial for the proper folding of the antioxidant protein SOD2 [25], which was observed in our study to be upregulated in WAT during late torpor (Fig. 1b). This may suggest a link between HSP expression and antioxidant responses in the hibernator. HSP60 is also required for the folding of proteins involved in fatty acid metabolism [26], a process which is absolutely fundamental to the success of the hibernation phenotype, and which is largely governed by lipid stores in WAT. By promoting the proper folding of such factors, the increased expression of HSP60 during entrance to torpor may support the drastic shift toward lipid mobilization, and the increased demand on fatty acid metabolic proteins. While further investigation of these proposed functions is required, the present results provide support for a general hibernation stress-response that includes a role for HSPs, and which may also link the regulation of associated antioxidant factors and metabolic processes to the heat shock response.

While antioxidant enzymes and heat shock proteins represent essential downstream effectors of stress-response pathways, our study also aimed to better understand the signaling mechanisms by which stress stimuli ultimately regulate these factors in the mammalian hibernator. MAP Kinase signaling networks are among the most widely studied of cellular signal transduction pathways and are known to serve remarkably diverse roles in stress responses, metabolism, apoptosis, proliferation, and differentiation [15,

17, 27]. MAPK pathways are characterized by three-tiered protein kinase cascades in which an external stimulus ultimately serves to activate a MAPK kinase kinase (MAP3K), which activates a MAPK kinase (MAP2K, e.g., MEK-1), which then activates a terminal MAPK (e.g., ERK1/2, JNK, and p38) [17], whereby phosphorylation serves as a molecular marker of activity. MAPKs then proceed to phosphorylate downstream targets such as transcription factors, either directly or via the activation of intermediate kinases (such as MSK1) [17]. Because of the broad range of functions they serve, it is unsurprising that MAPK pathway regulation varies incredibly between tissues, stimuli, and stress conditions, whereby numerous and astoundingly specific downstream functions are intricately achieved by the differential modulation of a core set of intermediate factors [28]. For instance, activation of the central MAP kinases ERK1/2, p38, and JNK have been identified as mediators of opposing cellular responses (e.g., both apoptosis and anti-apoptosis), depending on the nature and strength of the original stimuli, and the tissue/cell type by which the stimuli was received [29–32]. As such, our analysis identified clear differences in the phosphorylation/activation status of these central MAPKs (ERK1/2, p38, and JNK) and several of their downstream targets between BAT and WAT of our hibernator model (Fig. 3). Interestingly, we observed a somewhat opposite trend in MAPK phosphorylation between BAT and WAT whereby the former demonstrated increased phosphorylation of MAPKs during the transition between torpor and arousal (Fig. 3a) and the latter showed a general decrease in MAPK phosphorylation during late torpor and arousal (Fig. 3b). The trend in BAT toward MAPK activation during EA points to possible roles for these factors in regulating the processes involved in the transition between torpor and arousal, such as the dramatic increases in non-shivering thermogenesis and metabolic rate, and possible responses to the associated increase in ROS generation. Indeed, both ERK1/2 and JNK are known to promote lipolysis in adipocytes in response to certain stimuli [15], and p38 activation has been established to increase the expression of the brown adipocyte gene *ucp1* that is involved in uncoupled respiration/non-shivering thermogenesis in BAT [33, 34]. Additionally, ERK1/2 phosphorylation is known to promote cell survival specifically in BAT in response to cold-exposure [35], while both ERK1/2 and p38 have been shown to be activated in response to oxidative stress, with pro-survival functions [30, 32, 36, 37]. In contrast to BAT, phosphorylation of ERK1/2, p38, and JNK was generally reduced during torpor and arousal in WAT (Fig. 3b), suggesting deactivation of the MAPKs during these times. Since ERK1/2 signaling is generally involved in promoting cell proliferation and differentiation (including that of adipocytes) [15, 38], the inhibition of ERK1/2 signaling could potentially contribute to suspended adipocyte growth for the purposes of energy savings and the regulation of the lipid-based economy of hibernation, thereby serving a distinct but related function to that proposed in BAT. Indeed, suppression of cellular proliferation appears to be characteristic of the hibernation state [39], and likely occurs to reduce energy expenditure during metabolic depression. Additionally, p38 and JNK are known to mediate both pro-survival and pro-death signals in response to stress, depending on the nature of the stressor and the signaling context [29–31]. In WAT, the downregulation of p38 and JNK activity could therefore potentially promote survival during these periods of stress by inhibiting pro-death signals (rather than promoting pro-survival signals as proposed in BAT). While further studies are required in order to fully elucidate the cellular effects of MAPK activation/inhibition in this system, the current results implicate the differential regulation of these central MAPKs as a characteristic of hibernator adipose tissue.

One function of the signal-transducing MAPKs, alongside a suite of diverse signaling kinases, is the phosphorylation/regulation of transcription factors that mediate the gene responses to the initial signal stimulus [17]. Indeed, differential phosphorylation by kinases (MAPK or otherwise) allows transcription factors to respond to diverse signals in a tissue-specific manner. Perhaps unsurprisingly then, this study has revealed a possible role for the differential expression of phosphorylated transcription factors in BAT and WAT of our hibernator model. CREB-1 and Elk-1 are two well-characterized transcription factors whose regulatory functions in the nucleus ultimately serve to transduce cellular signals from various stimuli into changes in the expression of relevant response genes [40, 41]. Phosphorylation of CREB-1 at Ser-133 and Elk-1 at Ser-383 strongly increases their transcriptional activities, leading to the expression of genes whose functions include the mediation of stress responses [17, 40, 41]. In BAT of the hibernator, the phosphorylated forms of CREB-1 and Elk-1 were both differentially expressed over the course of the torpor-arousal cycle (Fig. 4), while the same was also evident for Elk-1 in WAT (Fig. 5). The observed increases in phosphorylated CREB-1 during late torpor and interbout arousal in BAT imply that the transcriptional activity of this factor is involved in the regulation of processes that are emphasized during these periods. The same is also true for Elk-1, whose phosphorylated form exhibited increased expression in BAT during LT and in WAT during LT and EA. Unsurprisingly, CREB-1 is a known regulator of several pathways that are important to the functions of BAT, including lipid catabolism and oxidative stress responses [42, 43], so it is plausible that its activation may serve similar roles in the hibernator system. Likewise, Elk-1 is known to control the expression of genes that may be relevant to adipose tissue during torpor and arousal, such as those that promote cell survival [44]. Elk-1 is also a

regulator of immediate early genes, which are characterized by rapid activation (minutes or hours) in response to cellular stimuli, and which function in biological processes ranging from cell differentiation and survival to the mediation of inflammation [41, 45]. Owing to its potential speed of response, the activation of Elk-1 during EA may therefore represent a mechanism by which select genes are rapidly induced in hibernator WAT during arousal from torpor. While the validity of such hypotheses will have to be assessed in subsequent studies, the observed fluctuations of the phosphorylated forms of CREB-1 and Elk-1 generally suggest a role for the activation of these factors at different points during hibernation. Notably, however, these observations did not appear to directly correlate with the apparent activation of the studied MAPKs, suggesting that the current CREB-1 and Elk-1 phosphorylation may be regulated by alternate means in the hibernator system. Indeed, CREB-1(S133) phosphorylation is known to be mediated by multiple other kinases, including protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinases [40]. Interestingly, PKA-mediated CREB-1 activation has been shown to be enhanced in response to the presence of the antioxidant factor TRX-1 [46], the expression of which we observed to increase in BAT during late torpor, along with phosphorylated CREB-1. Another disparity worth noting is the observed lack of differential phosphorylation of the other MAPK downstream targets measured in this study in BAT (Fig. 4), which is somewhat curious considering the previously discussed patterns of apparent ERK1/2, p38, and JNK activation. Of course, our measurements were not exhaustive in terms of the possible MAPK targets regulated in these tissues during hibernation, leaving open the possibility that other factors are responsible for the mediation of any relevant ERK1/2, p38, or JNK downstream functions. Further, since our measurements represent only an instantaneous moment during the given sampling period (i.e., a "snapshot" during each time point), it is possible that the downstream effects of the differential MAPK phosphorylation were simply not detected due to temporal factors.

In summary, we have identified what appears to be the general activation/differential regulation of known stress-response pathways (antioxidants, HSPs, MAPKs, and transcription factors) in BAT and WAT of the hibernator *I. tridecemlineatus*, which adds to previous research in other models of hibernation that describes similar responses [3, 12, 13]. However, while these general characteristics seem to be apparent, the specific molecular mechanisms regulating such processes certainly vary both within a particular organism (i.e., tissue-specific responses) and between species [3, 12, 13]. This study therefore reaffirms the notion of an overall trend toward the differential activation of general stress-response pathways in the tissues that regulate the process of hibernation, while highlighting specific molecular intricacies that may vary within, and between, models of this response. By increasing our understanding of such intricacies, we come closer to fully uncovering the secrets of hibernation that may be applied to the treatment of diseases that are characterized by pathological stress-responses or metabolic disregulation.

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References

1. Carey HV, Andrews MT, Martin SL (2003) Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol Rev 83(4):1153–1181
2. Geiser F (2004) Metabolic rate and body temperature reduction during hibernation and daily torpor. Annu Rev Physiol 66(1):239–274
3. Storey KB (2010) Out cold: biochemical regulation of mammalian hibernation—a mini-review. Gerontology 56(2):220–230
4. Dark J (2005) Annual lipid cycles in hibernators: integration of physiology and behavior. Annu Rev Nutr 25(1):469–497
5. Eddy SF, Storey KB (2004) Up-regulation of fatty acid-binding proteins during hibernation in the little brown bat, *Myotis lucifugus*. Biochim Biophys Acta 1676(1):63–70
6. Storey KB, Storey JM (2010) Metabolic rate depression: the biochemistry of mammalian hibernation. Adv Clin Chem 52:77–108
7. Von der Ohe CG, Darian-Smith C, Garner CC, Heller HC (2006) Ubiquitous and temperature-dependent neural plasticity in hibernators. J Neurosci 26(41):10590–10598
8. Storey KB (2003) Mammalian hibernation: transcriptional and translational controls. In: Roach RC, Wagner PD, Hackett PH (eds) Hypoxia: through the lifecycle. Advances in experimental medicine and biology, vol 543. Kluwer/Plenum Academic, New York, pp 21–38
9. Storey KB, Storey JM (2007) Tribute to P. L. Lutz: putting life on 'pause'—molecular regulation of hypometabolism. J Exp Biol 210(Pt 10):1700–1714
10. Allan ME, Storey KB (2012) Expression of NF-κB and downstream antioxidant genes in skeletal muscle of hibernating ground squirrels, *Spermophilus tridecemlineatus*. Cell Biochem Funct 30(2):166–174
11. Morin P, Storey KB (2007) Antioxidant defense in hibernation: cloning and expression of peroxiredoxins from hibernating ground squirrels, *Spermophilus tridecemlineatus*. Arch Biochem Biophys 461(1):59–65
12. Vucetic M, Stancic A, Otasevic V et al (2013) The impact of cold acclimation and hibernation on antioxidant defenses in the ground squirrel (*Spermophilus citellus*): an update. Free Radic Biol Med 65C:916–924
13. Storey K, Storey JM (2011) Heat shock proteins and hypometabolism: adaptive strategy for proteome preservation. RRB 2:57–68

14. Rouble AN, Hefler J, Mamady H, Storey KB, Tessier SN (2013) Anti-apoptotic signaling as a cytoprotective mechanism in mammalian hibernation. PeerJ 1:e29
15. Gehart H, Kumpf S, Ittner A, Ricci R (2010) MAPK signalling in cellular metabolism: stress or wellness? EMBO Rep 11(11): 834–840
16. Lawrence MC, Jivan A, Shao C et al (2008) The roles of MAPKs in disease. Cell Res 18(4):436–442
17. Plotnikov A, Zehorai E, Procaccia S, Seger R (2011) The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. Biochim Biophys Acta 1813(9):1619–1633
18. Arnér ES, Holmgren A (2000) Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem 267(20): 6102–6109
19. Chen Y, Zhang J, Lin Y et al (2011) Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. EMBO Rep 12(6):534–541
20. Rothgiesser KM, Erener S, Waibel S, Lüscher B, Hottiger MO (2010) SIRT2 regulates NF-κB dependent gene expression through deacetylation of p65 Lys310. J Cell Sci 123(Pt 24):4251–4258
21. Richter K, Haslbeck M, Buchner J (2010) The heat shock response: life on the verge of death. Mol Cell 40(2):253–266
22. Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282
23. Wegele H, Müller L, Buchner J (2004) Hsp70 and Hsp90—a relay team for protein folding. Rev Physiol Biochem Pharmacol 151:1–44
24. Kleinridders A, Lauritzen HP, Ussar S et al (2013) Leptin regulation of Hsp60 impacts hypothalamic insulin signaling. J Clin Invest 123(11):4667–4680
25. Magnoni R, Palmfeldt J, Hansen J, Christensen JH, Corydon TJ, Bross P (2013) The Hsp60 folding machinery is crucial for manganese superoxide dismutase folding and function. Free Radic Res 48(2):168–179
26. Corydon TJ, Hansen J, Bross P, Jensen TG (2005) Down-regulation of Hsp60 expression by RNAi impairs folding of medium-chain acyl-CoA dehydrogenase wild-type and disease-associated proteins. Mol Genet Metab 85(4):260–270
27. Kim EK, Choi EJ (2010) Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 1802(4): 396–405
28. Bardwell L (2006) Mechanisms of MAPK signalling specificity. Biochem Soc Trans 34(Pt 5):837–841
29. Liu J, Lin A (2005) Role of JNK activation in apoptosis: a double-edged sword. Cell Res 15(1):36–42
30. Gutiérrez-uzquiza Á, Arechederra M, Bragado P, Aguirre-Ghiso JA, Porras A (2012) p38α mediates cell survival in response to oxidative stress via induction of antioxidant genes: effect on the p70S6K pathway. J Biol Chem 287(4):2632–2642
31. Kuwabara M, Asanuma T, Niwa K, Inanami O (2008) Regulation of cell survival and death signals induced by oxidative stress. J Clin Biochem Nutr 43(2):51–57
32. Lu Z, Xu S (2006) ERK1/2 MAP kinases in cell survival and apoptosis. IUBMB Life 58(11):621–631
33. Cao W, Daniel KW, Robidoux J et al (2004) p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. Mol Cell Biol 24(7):3057–3067
34. Collins S, Bordicchia M (2013) Heart hormones fueling a fire in fat. Adipocyte 2(2):104–108
35. Lindquist JM, Rehnmark S (1998) Ambient temperature regulation of apoptosis in brown adipose tissue. Erk1/2 promotes norepinephrine-dependent cell survival. J Biol Chem 273(46):30147–30156
36. Adderley SR, Fitzgerald DJ (1999) Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. J Biol Chem 274(8):5038–5046
37. Matsuzawa A, Ichijo H (2008) Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. Biochim Biophys Acta 1780(11):1325–1336
38. Mebratu Y, Tesfaigzi Y (2009) How ERK1/2 activation controls cell proliferation and cell death: is subcellular localization the answer? Cell Cycle 8(8):1168–1175
39. Wu CW, Storey KB (2012) Pattern of cellular quiescence over the hibernation cycle in liver of thirteen-lined ground squirrels. Cell Cycle 11(9):1714–1726
40. Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821–861
41. Besnard A, Galan-Rodriguez B, Vanhoutte P, Caboche J (2011) Elk-1 a transcription factor with multiple facets in the brain. Front Neurosci 5:35
42. Herzig S, Hedrick S, Morantte I, Koo SH, Galimi F, Montminy M (2003) CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma. Nature 426(6963):190–193
43. Lee B, Cao R, Choi YS et al (2009) The CREB/CRE transcriptional pathway: protection against oxidative stress-mediated neuronal cell death. J Neurochem 108(5):1251–1265
44. Demir O, Aysit N, Onder Z et al (2011) ETS-domain transcription factor Elk-1 mediates neuronal survival: SMN as a potential target. Biochim Biophys Acta 1812(6):652–662
45. O'Donnell A, Odrowaz Z, Sharrocks AD (2012) Immediate-early gene activation by the MAPK pathways: what do and don't we know? Biochem Soc Trans 40(1):58–66
46. Hirota K, Matsui M, Murata M et al (2000) Nucleoredoxin, glutaredoxin, and thioredoxin differentially regulate NF-kappaB, AP-1, and CREB activation in HEK293 cells. Biochem Biophys Res Commun 274(1):177–182