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REVIEW

Proteomics approaches shed new light on hibernation physiology

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Abstract The broad phylogenetic distribution and rapid phenotypic transitions of mammalian hibernators imply that hibernation is accomplished by differential expression of common genes. Traditional candidate gene approaches have thus far explained little of the molecular mechanisms underlying hibernation, likely due to (1) incomplete and imprecise sampling of a complex phenotype, and (2) the forming of hypotheses about which genes might be important based on studies of model organisms incapable of such dynamic physiology. Unbiased screening approaches, such as proteomics, offer an alternative means to discover the cellular underpinnings that permit successful hibernation and may reveal previously overlooked, important pathways. Here, we review the findings that have emerged from proteomics studies of hibernation. One striking feature is the stability of the proteome, especially across the extreme physiological shifts of torpor–arousal cycles during hibernation. This has led to subsequent investigations of the role of post-translational protein modifications in altering protein activity without energetically wasteful removal and rebuilding of protein pools. Another unexpected finding is the paucity of universal proteomic adjustments across organ systems in response to the extreme metabolic fluctuations despite the universality of their physiological challenges; rather each organ appears to respond in a unique, tissue-specific manner. Additional research is needed to extend and synthesize these results before it will be possible to address the whole body physiology of hibernation.

Keywords Ground squirrel · Bear · Bat · Metabolic depression

The phenotype of hibernation

Hibernators exploit heterothermy and metabolic regulation to overcome environmental challenges. While this is, to some extent, a strategy used by all mammals, it is particularly extreme in hibernators, estivators and, to a lesser extent, daily torpidators (Heldmaier et al. 2004). Heterothermic mammals are broadly distributed among all three mammalian sub-classes, including the monotreme echidnas, the marsupial pygmy possums and fat-tailed dunnarts, and the eutherians, including lemurs, galagos, tenrecs, moles, hedgehogs, bats, shrews, ground squirrels, chipmunks, hamsters, mice, and bears (Dausmann et al. 2004; Geiser 2007; Kortner and Geiser 2009; Kortner et al. 2008; Lovegrove and Genin 2008; Tøien et al. 2011; Wilz and Heldmaier 2000). Due to their broad distribution, it is hypothesized that hibernation results from differential expression of genes common to all mammals rather than expression of novel genes found only in hibernators (Srere et al. 1992; van Breukelen and Martin 2002a).

Hibernation is characterized by a reduction in metabolic rate and body temperature set point to maximize energy savings during times of low food availability and unfavorable environmental conditions. Metabolic suppression and cell-level adjustments to manage limited food intake are

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common themes among species. Small-bodied mammalian hibernators in temperate climates or high latitudes actively suppress metabolic, respiratory and heart rates to 1–9 % of basal rates while lowering body temperature to enter a state called torpor (Ruf and Geiser 2014). Large-bodied bears also vary metabolism over nearly their entire metabolic scope, reaching 25 % of basal values (Tøien et al. 2011) and adopting mass-specific metabolic rates that approach those of torpor in small mammals (Geiser and Ruf 1995; Heldmaier et al. 2004).

Torpor is not maintained continuously, and temperate-zone hibernators must intermittently arouse back to euthermia, referred to as interbout arousals (IBA), or interbout euthermia (IBE). During rewarming, metabolic suppression is lifted, and physiological rates increase to levels that may actually exceed those of IBA (Carey et al. 2003; Hampton et al. 2010; Staples and Brown 2008). Metabolic re-activation precedes increasing body temperature (T_b), with rewarming achieved via endogenous heat production mechanisms. The arousal and euthermic period is generally <24 h in small mammals but is the most energetically costly phase of hibernation, consuming up to 86 % of the energy necessary to complete the torpor–arousal cycle in arctic ground squirrels (Karpovich et al. 2009) and cumulatively, as much as 70–80 % of the energy budget across the entire hibernation season (Heldmaier et al. 1993; Wang 1978). This large energy expenditure remains one of hibernation's biggest mysteries—why use energy to rewarm periodically while more could be saved by remaining torpid?

Body temperature depression is considerably less in bears (30–36 °C) compared to small mammals, and temperature oscillates over periods of several days (Tøien et al. 2011). Bears also exhibit a continued period of metabolic rate depression (47 %) on emergence from hibernation, despite euthermic body temperature. Bears, therefore, provide an example of body temperature and metabolic rate uncoupling (see also Ortmann and Heldmaier 2000), demonstrating how energetic benefits may derive both from active suppression and temperature-induced metabolic depression (Heldmaier et al. 2004).

Hibernation may persist for up to 9 months, with duration varying by climate and latitude (Zervanos et al. 2010). For many species, it is part of a circannual rhythm (Körtner and Geiser 2000). In the summer and fall, animals actively fatten, gaining as much as 50 % of body mass in fat deposits in advance of the hibernation season (Dark 2005; Martin 2008). Body weight fluctuation is an intrinsic rhythm entrained by light (Hiebert et al. 2000); although notably, it occurs in constant conditions, both in warm and light (Concannon et al. 2001; Hiebert et al. 2000), and in cold and dark (Kondo et al. 2006). Obligate hibernators such as ground squirrels also begin to use torpor even in the absence of environmental cues (Pengelley et al. 1976; Russell et al. 2010).

Based upon liver metabolite patterns and independent mathematical modeling, a two-switch model currently describes circannual hibernation (Hampton and Andrews 2007; Serkova et al. 2007). Conceptualized as a cycle within a cycle, a summer–winter or seasonal switch defines the components involved in the transition from a homeothermic to a heterothermic or hibernation-permissive state (Fig. 1). This model, therefore, predicts that prior to this seasonal transition, animals are incapable of entering torpor. A second switch, the torpor–arousal switch, controls the cycling between torpor and arousal in hibernation-competent individuals (Fig. 1).

Identification of differential gene expression in hibernation

Because of their innate capability to tolerate physiological extremes, hibernators are exceptional models for identifying natural solutions to improve the medical outcomes of a number of conditions, including hypothermic injury, organ transplantation, recovery from stroke, cardiac arrest and other ischemia/reperfusion insults, muscle and bone atrophy, and obesity and metabolic syndrome (Andrews 2007; Carey et al. 2012; Dave et al. 2012; Ivakine and Cohn 2014; McGee-Lawrence et al. 2008). To better understand how the dynamic hibernation phenotype is capable of tolerating extremes lethal to non-hibernators, many studies have focused on identifying differential gene expression across hibernation patterns, as this would ultimately manifest as phenotype via encoded proteins and their activity.

Because of the many unique aspects of the phenotype, attempts to characterize the molecular components underlying hibernation using candidate gene approaches based on knowledge gleaned from non-hibernating model organisms are likely to be inefficient and overlook crucial components. Additional limitations in the field have arisen from sampling imprecision or inconsistencies, e.g., comparison of just two groups of animals, most often those that have been torpid for an unknown time vs. a variably defined "euthermic control". Euthermic controls have ranged widely, and may include animals in the homeothermic season, heterothermic season animals housed in standard laboratory conditions and presumed to not be using torpor, or those in natural or artificially stimulated interbout arousal, or animals housed in the cold but not exhibiting torpor (e.g., McMullen and Hallenbeck 2010; Rouble et al. 2013). But the endogenous circannual rhythms and complexity of the hibernation phenotype (Drew et al. 2007; Russell et al. 2010) require deeper and more precise representation of their natural cycles. Fortunately, the phenotypic states within the hibernator's year can be precisely defined through continuous monitoring of, most easily, T_b (via
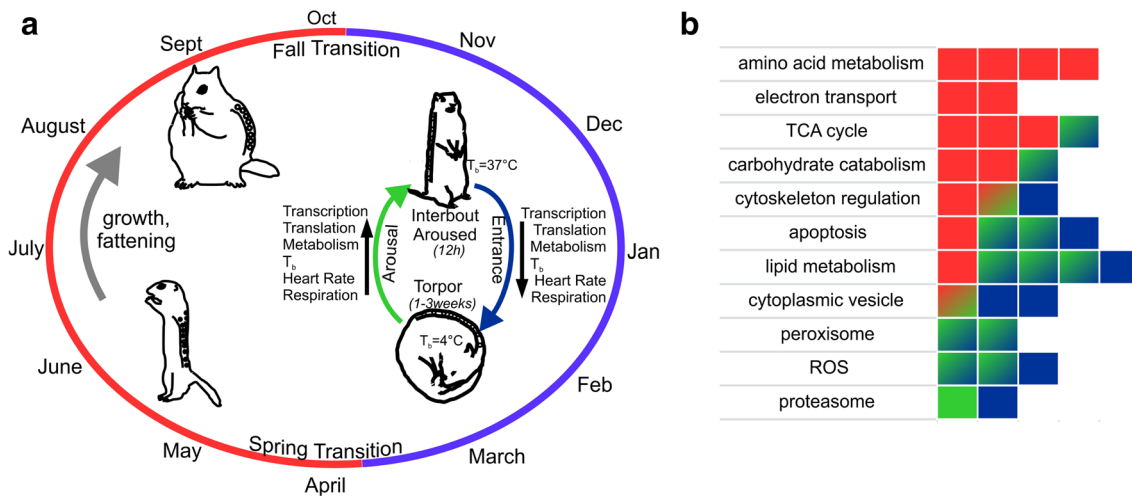
Fig. 1 Hibernation: a cycle within a cycle and linked proteomic adjustments. **a** Note the circannual rhythm that alternates between periods of homeothermy (occupied by reproduction, growth and fattening, *red* half of the year) and heterothermy (i.e., hibernation, *blue* half of the year). Ground squirrels emerge from hibernation lean, but immerge with large fat stores. Within and extending on either side of the winter months, heterothermy is caused by cycles between prolonged periods in torpor and short interbout arousals, with numerous physiological changes as indicated. **b** Each tissue or species sharing a functional enrichment category (Table 2) is represented by one square. The *color* of the *square* indicates the physiological state with the highest expression of that pathway's components: *red* the homeothermic phase of the annual cycle; *blue* the low T_b, and *green* the high T_b phases of winter heterothermy (**a**). Overall or non-specific (i.e., the original study considered just one group of heterothermic-phase animals) increases during winter heterothermy are denoted with a *green–blue transition square*. The transition of *red* to *green* represents shared elevation of that pathway in animals with warm T_b (homeothermic-phase animals and aroused hibernators). In some cases separate annotations were merged, these are: proteasome with ubiquitinylation; cytoplasmic vesicle with plasma proteins and endocytosis; lipid metabolism with fatty acid metabolism; ROS with redox homeostasis (color figure online)

surgical implantation of telemeters), or other physiological parameters such as heart and respiratory rates. Therefore, sample collection spanning endogenous rhythms of hibernation, rather than disrupting those patterns to induce specific states provides a unique opportunity to capture the underlying physiology (Fig. 2).

Discovery-based, "omics" approaches can be effective tools to characterize circannual changes in gene expression and identify shared and unique pathways underlying hibernation physiology. Compared to testing limited candidate genes, these methods enable the broad recovery of expression changes all at once, and are not predicated upon data from animals incapable of hibernation. Novel findings should then be used to develop testable hypotheses about the mechanisms underlying the hibernating phenotype. The most common approaches have included proteomic, transcriptomic and metabolomic screens. This review is focused on the discoveries made via proteomics screens (Table 1).

Proteomics methodologies The hibernating proteome has been interrogated mainly through 2D PAGE methodology, including the use of 2D DiGE (Table 1), where proteins are first fluorescently labeled and then visualized on 2D gels (Epperson and Martin 2011). The resulting protein "spots" are quantified and analyzed statistically for relative abundance changes before being chosen for identification by mass spectrometry. One advantage of this method is that protein isoforms with different molecular weights and isoelectric points (implying post-transcriptional and post-translational modifications) can be identified (Collier and Muddiman 2012). Another advantage to using this method specifically for hibernators, whose genomic resources were historically limited, is that multiple short peptides recovered from a single or small number of proteins facilitate identification of their parent protein via sequence identity with the orthologous, well-annotated protein sequence from other mammals (Epperson et al. 2004; Russeth et al. 2006). A high-coverage genome for the 13-lined ground squirrel was recently completed (spring 2012), and hence, a relatively comprehensive database of 13-lined ground squirrel protein sequences is now available. A disadvantage to using 2D gels, however, is that this method recovers only the most abundant, soluble proteins in the cell (i.e., ~3–5 % of the proteome) (Epperson et al. 2007). Newer proteomics methods that eliminate the need for 2D gels (Evans et al. 2012; Li et al. 2012; Nahnsen et al. 2013) and have been successfully applied to hibernation samples include: whole animal heavy isotope labeling (Rose et al. 2011), label free (Shao et al. 2010) and iTraq (Li et al. 2013). Since these methods provide more complete proteome coverage with
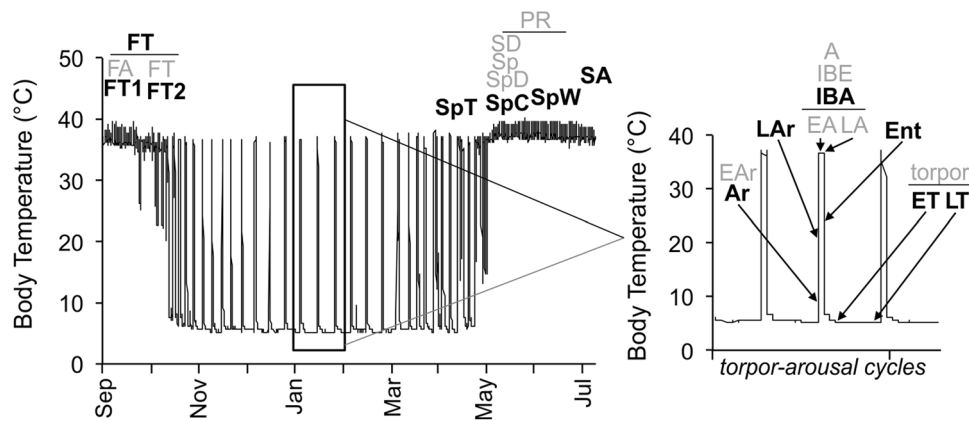
Fig. 2 Body temperature to define sampled states. The body temperature (T_b) of a laboratory-housed 13-lined ground squirrel (*Ictidomys tridecemlineatus*) over ~8 months denotes sampling timepoints in various proteomic studies of small-bodied hibernators, relating the variable nomenclature in the literature to a common physiological parameter, T_b. Abbreviations (*black labels*) are used in subsequent figures and tables. Spring (*Sp*) and summer (*SA*) samples represent the homeothermic portions of the annual cycle. Spring animals were given food and water but remained in the hibernaculum (L:D 0:24). Spring-cold (*SpC*, also called Spring-dark, *SD* or *SpD*) were maintained at low ambient temperature ($T_a = 4$ °C) and were killed 11–20 days after their last recorded torpor bout. Spring-warm (*SpW*) were collected 7–32 days after terminal arousal ($T_a = 14$–18 °C). Some studies examined the Spring post-reproduction period (*PR*), several weeks after re-establishing homeothermy. Heterothermic states are defined either generally (Pengelley and Fisher 1961) or specifically by T_b pattern, using time within that state or T_b telemetry. Some studies examined animals entering torpor (*Ent*) at 27 °C > T_b > 23 °C, and subsequently arousing from a torpor bout (early arousing, *EAr* or *Ar* at 7 °C < T_b < 12.8 °C and late arousing, *LAr*, at 18 °C < T_b < 25 °C). Samples from torpid animals may be from early torpor (*ET*) or late torpor (*LT*), collected at either 5–10 %, or (typically) 80–95 % of the previous bout duration, respectively. The euthermic period between torpor bouts is likewise defined by time elapsed once T_b reaches a certain threshold or activity resumes. This state is termed interbout arousal or euthermia (3–4 h after T_b stabilized; *IBA* or *IBE*, respectively) as well as aroused (1–15 h variable by study; *A*). The euthermic period is sometimes also divided into early and late (1–2 h; *EA*-Early Aroused, or > 10 h *LA*- Late Aroused). *FT* defines a highly variable Fall Transition period, where sampled animals may have undertaken previous torpor bouts, and were housed in warm or cold rooms. Spring Transition (*SpT*), animals maintained in a hibernaculum begin to display irregular or truncated torpor–arousal cycles in advance of spring emergence. Although some studies sampled hibernating versus non-hibernating animals (which could mean summer versus winter or torpid versus aroused), in most cases, sampling timepoints within homeo- or heterothermy were specified

robust quantification, they will likely become the methods of choice for future proteomics studies of hibernators.

Tissue-specific proteome characterization

Liver

To date, the liver is the most extensively studied organ in hibernation proteomics. It is the metabolic hub of the organism and thus both a crucial indicator and regulator of the highly dynamic physiology that characterizes the phenotype. An initial 2D gel analysis of golden-mantled ground squirrel liver samples (Table 1) from the homeothermic summer-active (SA) and heterothermic entrance (Ent) states (see Fig. 2 for state definitions) detected changes in 130/961 protein spots examined (13.5 %)—84 were reproducible across the 2D gels and 56 of which were uniquely identified by tandem mass spectrometry (Epperson et al. 2004). Several years later, comparing these same two states, a 2D-DiGE analysis of 13-lined ground squirrel liver (Epperson et al. 2010b) detected significant changes

in 269/2396 protein spots (11.2 %)—180 of which were reproducible across gels and a 115 of which were unambiguously identified containing unique proteins by tandem mass spectrometry. Lastly, using whole animal heavy isotopic (^{15}N) labeling of 13-lined ground squirrels and protein identification by MuDPIT, Rose et al. (2011) detected changes in 61/389 liver proteins (15.6 %) between these two groups. These studies reveal a seasonal shift that we now recognize as a global component of hibernation physiology, away from carbohydrate-based catabolism and lipid biosynthesis in summer homeothermy to lipid and ketone body catabolism in winter heterothermy, thereby allowing animals to survive using their fat stores during the fasting period of hibernation. In addition, the hibernation-increased proteins are involved in different aspects of protein turnover, including protein synthesis, stability, folding and RNA metabolism. In contrast, summer-increased proteins function in pathways related to amino acid and nitrogen catabolism and the urea cycle. Taken together, these results indicate that the hibernating ground squirrel switches from degradation to preservation of amino acids, supporting maintenance of homeostasis without additional

Table 1 Proteomic screens to identify hibernation state-specific protein differences

Species	Tissue	Physiological states	*State abbreviation	Method (quantification/protein identification)	Max. protein	Tested	Differential abundance	Unique	References
Rickett's big-footed bat	Brain	March artificially aroused vs. torpid bats	IBA, LT	2D PAGE/LC–MS/MS	2180	188	115	111	Zhang et al. (2014)
13-lined ground squirrel	Brain stem	2 groups; homeo- vs. heterothermic	SA (Aug), ET	2D DiGE/LC–MS/MS	2314	2196	286	119	Epperson et al. (2009)
13-lined ground squirrel	Brown adipose	10 groups; 3 homeothermic; 5 heterothermic; 2 transition	SpC, SpW, SA, IBA, Ent, LT, Ar, LAr, FT, SpT	2D DiGE/LC–MS/MS	2982		323	107	Hindle and Martin (2014)
13-lined ground squirrel	Forebrain	7 groups; 2 homeothermic; 4 heterothermic; 1 fall transition	SpC, SA, IBA, Ent, LT, Ar, FT	2D DiGE/LC–MS/MS	3471		84	34	Hindle and Martin (2013)
13-lined ground squirrel	Heart	2 homeothermic, 4 heterothermic and 2 fall transition states	SpC, SA, IBA, Ent, LT, Ar, FT1, FT2	2D DiGE/LC–MS/MS	2080	432	82	50	Grabek et al. (2011)
Woodchuck	Heart	Active homeotherms vs. torpid heterotherms	SA (June/July), LT	iTRAQ	3577	3577	162	3577	Li et al. (2013)
13-lined ground squirrel	Intestine	Active homeotherms vs. active heterotherms; both I/R and sham-treated	SA (Aug), IBA	2D DiGE/LC–MS/MS	2696	1012	153	51	Martin et al. (2008)
13-lined ground squirrel	Kidney	6 groups, 2 homeothermic; 4 heterothermic	SpC, SA, IBA, Ent, LT, Ar	2D DiGE/LC–MS/MS	2073	2073	564	100	Jani et al. (2012)
Golden-mantled ground squirrel	Liver	Late June/Early July homeotherms and Entrance heterotherms	SA (June), Ent	2D PAGE/LC–MS/MS		961	130	56	Epperson et al. (2004)
13-lined ground squirrel	Liver	August homeotherms vs. Entrance heterotherms	SA (Aug), Ent	2D DiGE/LC–MS/MS	2485	2396	269	74	Epperson et al. (2010a, b)
Arctic ground squirrel	Liver	May/June homeotherms; Interbout aroused and late torpor heterotherms	SA (May/June), IBA, LT	Label free/MuDPIT	3594	1209	517	3104	Shao et al. (2010)
13-lined ground squirrel	Liver	Active homeotherms vs. Entrance heterotherms	SA (Aug), Ent	Heavy isotope Labeling/MuDPIT	829	389	61	829	Rose et al. (2011)
Rickett's big-footed bat	Liver	Non-hibernating vs. torpid bats Non-hib., hib.		2D PAGE/LC–MS/MS	53	53	32	34	Pan et al. (2013)
13-lined ground squirrel	Liver	2 homeothermic; 4 heterothermic; 2 fall transition	SpC, SA, IBA, Ent, LT, Ar, FT1, FT2	2D DiGE/LC–MS/MS	2472	1317	525	176	Hindle et al. (2014)
13-lined ground squirrel	Liver mitochondria phosphoproteome	July homeotherms; Interbout aroused and torpid heterotherms	SA(July), IBA, LT	2D blue-native PAGE/MALDI-TOF/TOF MS			3	9	Chung et al. (2013)

Table 1 continued

Species	Tissue	Physiological states	*State abbreviation	Method (quantification/ protein identification)	Max. protein	Tested	Differential abundance	Unique	References
American black bear	Serum	Serial samples of Oct/Nov Pre-hibernators and Feb/Mar hibernators		2D DiGE	2230		70	15	Chow et al. (2013)
Greater tube-nosed bat (males)	Skeletal Muscle	Summer non-hibernating, winter torpid and aroused bats	SA, LT, IBA	2D PAGE/MALDI	>550	109	6	6	Lee et al. (2008)
13-lined ground squirrel	Skeletal Muscle	8 groups; 2 homeothermic; 6 heterothermic	SpC, SA, IBA, Ent, ET, LT, Ar, LAr	2D DiGE/LC–MS/MS	2426		216	138	Hindle et al. (2011)

Columns contain the: species, tissue, physiological states with specific abbreviations used (see Fig. 2); proteomics methods employed for both quantification and protein identification); maximum number of proteins (Max. protein) that were either seen or quantitatively compared (Tested), the number found to be significantly different (differential abundance), the number of unique proteins that differed (Unique, e.g., in 2D gel studies it is common to find more than one spot with the same protein identification due to isoform variation or post-translational modification, this becomes important because only unique IDs can be used for functional gene enrichment analyses); and the citation (references)

nutrient ingestion. Additional functional shifts related to the seasonal shift between feeding and fasting include hibernation-increased fatty acid transport and gluconeogenesis, and summer-increased detoxification pathways. Finally, the hibernation proteome exhibits increases of protective proteins involved in redox balance and anti-apoptosis, likely to survive the periods of low body temperature, blood flow and increased oxidative stress that are encountered within each torpor–arousal cycle.

Amazingly, although there was excellent concordance between the two 13-lined ground squirrel studies using different methods (Epperson et al. 2010b; Rose et al. 2011), only two proteins with synchronous changes were identified between 13-lined and golden-mantled ground squirrels (Epperson et al. 2004, 2010b): 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), important for ketone body formation, increased in Ent, and succinate-CoA ligase, a TCA cycle enzyme, increased in summer. Different methodologies and sampling times among these studies could explain the minimal overlap observed in individual protein responses. For example, two aldehyde dehydrogenases, ALDH1B1 and ALDH7A1, were summer-increased in the 13-lined ground squirrels, but hibernation-increased in the golden-mantled ground squirrels. The 13-lined ground squirrel studies sampled late summer animals (early August), compared to early summer sampling of golden-mantled ground squirrels (June). Thus, the discordance in ALDH1B1 and ALDH7A1 and the greater lack of overlap among these protein datasets in general may be partly explained by physiological differences within homeothermy, e.g., growth vs. lipogenic phases characteristic of early vs. late summer.

To detect both seasonal and torpor–arousal cycle changes, Shao et al. used shotgun proteomics to quantify changes in liver proteins from homeothermic post-reproductive Arctic ground squirrels (collected in May and June; referred to here as SA for ease of cross-study comparisons but originally abbreviated by authors as PR; see Fig. 2) and heterothermic Arctic ground squirrels in late torpor (LT) and early in interbout arousal (IBA; originally abbreviated EA) (Shao et al. 2010). Although 3104 unique proteins were identified, only 1209 were present in every sample in at least one of the three physiological states and, therefore, used in statistical testing: 517 proteins exhibited significant changes (42.8 %) in at least one pairwise comparison. Again, there were seasonal increases in fatty acid catabolic enzymes with concurrent decreases in glucose catabolic enzymes, as well as those involved with fatty acid synthesis, amino acid metabolism, urea cycle, and detoxification in LT and IBA hibernators, relative to the homeothermic SA group. HMGCS2 was most abundant in LT, together with several anticoagulant proteins, serpin peptidase inhibitor C1, histidine-rich glycoprotein and

alpha-2-macroglobulin (A2M). When comparing IBA to the LT state, pathways including oxidative phosphorylation and mRNA processing, protein translation and turnover were increased in IBA, supporting the suggestion that liver proteins are replenished in hibernation during the short returns to euthermia (Epperson et al. 2004). Interestingly, comparison of these protein abundances with their corresponding mRNAs measured in a previous study (Yan et al. 2008) indicate that only between and not within season differences are well correlated. This finding is consistent with the interpretation that a seasonal reprogramming of gene expression in the liver facilitates heterothermy, whereas post-transcriptional mechanisms are the principal means of controlling gene expression during heterothermy (i.e., during the torpor–arousal cycles of hibernation).

The most comprehensive temporal analysis of the hibernator's liver proteome to date characterized circannual and torpor–arousal protein changes in eight groups of 13-lined ground squirrels (Hindle et al. 2014). Among these eight groups were: animals representing two homeothermic states, spring (SpC, March–April) and summer active (SA, August); four heterothermic states, interbout aroused (IBA), entrance (Ent), late torpor (LT) and early arousal (Ar) hibernators; and two states sampled in the fall (September–October). Fall is a transitional period between summer homeothermy and winter heterothermy, which is often characterized by short ventures into torpor (Russell et al. 2010). The authors identified changes in 525/1317 (40 %) protein spots visualized on 2D DiGE gels among their six "base" states—i.e., the homeothermic and heterothermic groups excluding the fall transition, which will be discussed separately. Most protein changes occurred between the seasonal homeothermic and heterothermic groups, but, within winter heterothermy, the early arousal state was the most distinct (Fig. 3a). Consistent with the findings of the earlier liver proteomics screens that sampled fewer physiological states, proteins involved in hormone metabolism and the catabolism of glucose, amino acids and xenobiotics were highest in the homeotherms, whereas proteins involved in fatty acid catabolism, ion homeostasis and regulation of apoptosis were highest in the heterotherms. Because of the increased sample groups, these results confirm the indications of the work using fewer states that there is major seasonal switch supporting fatty acid-based metabolism and sparing of amino acids during the winter and link this switch to feeding and fasting behavior. Within the torpor–arousal cycle, the majority of changed proteins exhibited increased abundance during early arousal relative to the other states; these proteins were conspicuously enriched in non-metabolic pathways such as cytoplasmic vesicle and plasma proteins, protein localization, ubiquitin-dependent protein catabolism, redox homeostasis, response to oxidative stress and negative regulation of apoptosis

(Table 2). Increased abundance of these proteins during the metabolically intense and oxidatively stressful period of arousal likely facilitates transport of fatty acids for energy generation and serves to minimize apoptosis during this brief period of ischemia/reperfusion.

Because initiation of protein synthesis basically ceases at the temperature of torpor in hibernating ground squirrel liver (van Breukelen and Martin 2001), it is interesting to contemplate a mechanism to account for the observed increase of certain proteins during early arousal, despite continuously low body temperature for days to weeks. These protein increases could occur by a variety of mechanisms including: slow runoff translation of selected transcripts that initiated translation at higher temperature on the free ribosomes (van Breukelen and Martin 2001); elevated stability of a protein subset relative to the total protein pool (Sidell 1977), or increased post-translational modification despite no change in protein abundance.

Several post-translational modifications during hibernation (PTM) have been characterized in ground squirrel liver. Because PTMs such as phosphorylation and acetylation provide a rapidly reversible and relatively energy-efficient means to control protein activity, they are hypothesized to underlie the rapid, dramatic physiological oscillations in hibernation (Storey 1987). Post-translational modifications may activate or inactivate proteins by modifying secondary structure in a manner that affects binding of enzymatic substrates or subcellular localization. In Hindle et al., both phosphorylation and acetylation changes were characterized (Hindle et al. 2014). Although 56 significantly changed proteins were identified in at least two different spots on the DiGE gels, none of these changes were attributable to phosphorylation. There was also no clear seasonal shift in overall phosphorylation abundance; thus it appears that phosphorylation has a limited role in regulating protein activity in liver during hibernation cycles. However, there was one spot whose abundance change was attributed to phosphorylation: NSFL1 (p97) cofactor (p47). The phosphorylation state of this spot cycled within winter heterothermy, being highest in early arousal. One role for p47 is in the formation of autophagosomes; the authors hypothesized that phosphorylation of p47 in the torpor–arousal cycle could regulate its activity and thus generate substrates crucial for metabolic re-activation in early arousal.

The liver mitochondrial proteome of 13-lined ground squirrels was examined for changes in phosphorylation (Chung et al. 2013) that might explain the depression of state 3 respiration that accompanies metabolic depression in torpor–arousal cycles (Chung et al. 2011). This study also failed to find global shifts in phosphorylation among the homeothermic summer-active state and heterothermic interbout aroused and torpid states (Chung et al. 2013). While the authors did identify some phosphorylation
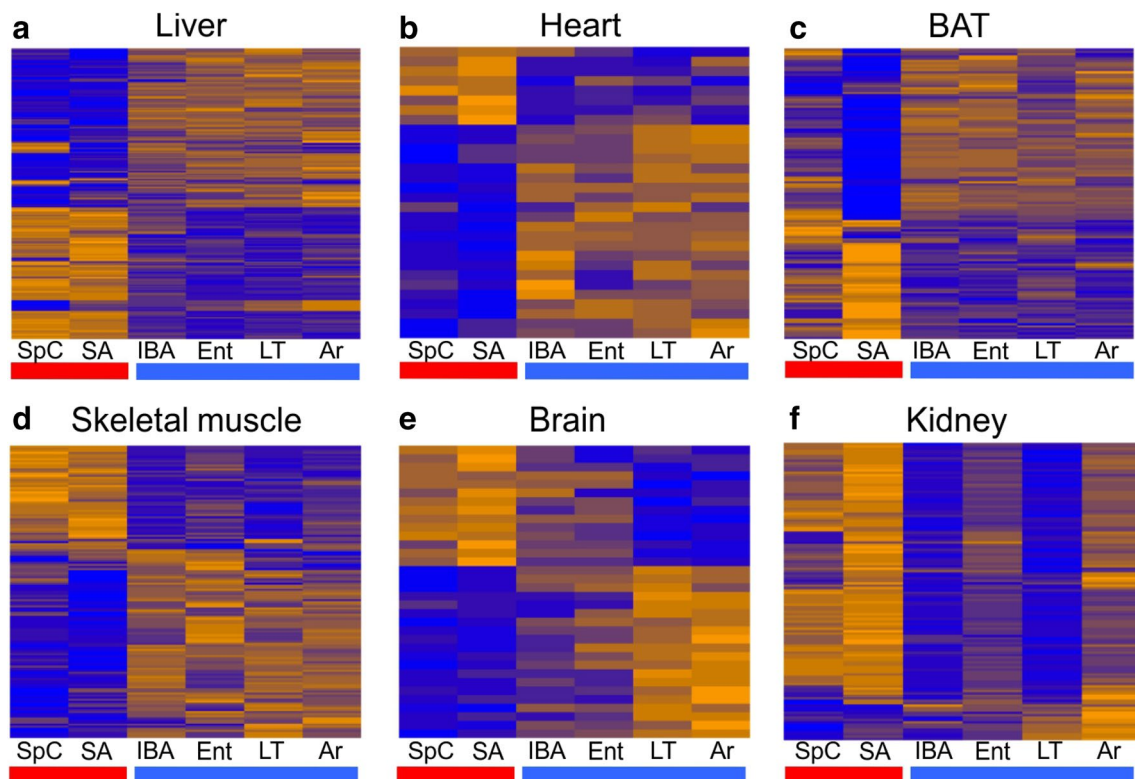
Fig. 3 *Heatmaps* illustrate the unique patterning of proteomic changes in hibernation cycles with tissue type. Each *row* displays the relative mean intensity (*deep blue* to *deep yellow* indicate hues represent least to most, respectively) among sample groups (*columns*) for one significantly changed protein spot. Homeotherms are indicated by the *red bar*, and heterotherms by the *blue bar*; groups are as labeled in Fig. 2. These data are re-plotted from the proteomic data published in **a** liver (Hindle et al. 2014), **b** heart (Grabek et al. 2011), **c** brown adipose tissue (Hindle and Martin 2014), **d** skeletal muscle (Hindle et al. 2011), **e** brain (forebrain lacking hypothalamus) (Hindle and Martin 2013) and **f** kidney (Jani et al. 2012). The deeper rows in brain and kidney compared to the other organs reflect the smaller numbers of protein spot changes; *rows* do not align or contain the same proteins across tissues (color figure online)

changes in specific proteins, these fluctuations were not associated with metabolic depression in torpor–arousal cycles. Rather, the detected phosphorylation changes occurred seasonally between the homeothermic and one or both heterothermic states.

Unlike the limited phosphoproteome changes, protein acetylation was globally increased in winter heterothermy relative to summer homeothermy, and, within the torpor–arousal cycle, acetylation levels were highest during torpor (Hindle et al. 2014). Because hyperacetylation is associated with a high-fat diet, fasting, caloric restriction and autophagy, the authors postulated that increased acetylation during hibernation further reflects adoption of a fasting metabolism, and more tantalizingly, exploitation of autophagy to maintain cellular energy levels. Several proteins that were previously identified as differentially expressed were also differentially acetylated, adding an extra layer for protein activity control in addition to relative abundance changes, especially within the torpor–arousal cycle. For instance, carbamoyl-phosphate synthase (CPS1), which catalyzes the first committed step of the urea cycle

and is downregulated during winter heterothermy, was also maximally acetylated during torpor. A seasonal reduction of CPS1 enhances amino acid sparing throughout hibernation, while additional activity inhibition by acetylation ensures toxic nitrogen removal occurs only during the short returns to euthermia within the torpor–arousal cycle itself.

Although the bulk of liver proteome studies have used the ground squirrel as a model hibernator, the liver proteome in bats has also been examined in at least one study (Pan et al. 2013). Liver proteins from torpid and artificially aroused Rickett's big-footed bats were visualized after fractionation on 2D gels. In contrast to ground squirrels, a number of enzymes involved in tyrosine and phenylalanine catabolic pathways were increased in torpor relative to the euthermic bats. Consistent with these enzyme changes, phenylalanine was 80 % reduced in torpid bat liver, while its ketone body catabolite, acetoacetate, was 60 % increased. CPS1 was also significantly increased in torpid bats. These findings suggest that hibernating bats catabolize at least some amino acids for gluconeogenesis and ketone body synthesis, unlike ground squirrels that appear

Table 2 Pathway functional enrichments associated with hibernation by species

Species	Tissue	Hibernation comparison	Enriched categories	References
Rickett's big-footed bat	Brain[a]	Torpor vs. aroused	Mitochondrial dysfunction, glycolysis/gluconeogenesis, TCA cycle, protein ubiquitination, aldosterone signaling, Huntington's disease signaling, NRF2-mediated oxidative stress response, 2-ketoglutarate dehydrogenase complex, Parkinson's signaling, tryptophan degradation, neuronal semaphorin signaling, arginine degradation, acetyl-coA biosynthesis	Zhang et al. (2014)
13-lined gs	Brain[b] (forebrain)	Elevated at high T_b	GTPase activity, neuron differentiation, endocytosis, synaptic transmission, neurotransmitter secretion, cytoskeleton regulation	Hindle and Martin (2013)
		Elevated at low T_b	Neurogenesis/differentiation, cytoskeleton regulation	
13-lined gs	Brown adipose tissue[b]	Decreased in SA (cluster 1)	TCA cycle, flavoprotein, fatty acid metabolism, glucose metabolism	Hindle and Martin (2013)
		Increased in winter heterothermy (cluster 2)	Fatty acid metabolism, TCA cycle	
		Increased in SA (cluster 3)	Mitochondrial transit peptide, aerobic respiration	
		Increased in SpC and SpW (cluster 4)	Oxidative metabolism, 14-3-3 pathway	
		Decreased in winter heterothermy (cluster 5)	Complex assembly, 14-3-3 pathway, apoptosis regulation, phospho-regulation	
		FT: preparative abundance change to winter level	Decreased from summer: Aerobic respiration, complex assembly, reactive O_2 species response, apoptosis regulation. Increased from summer: Fatty acid b-oxidation, TCA cycle, electron carrier activity, fatty acid binding, glucose catabolism	
		SpT: preparative abundance change to spring level	Decreased from winter: Coenzyme binding, fatty acid binding, fatty acid metabolism, endopeptidase activity regulation. Increased from winter: 14-3-3 pathway, complex assembly, phosphate metabolism regulation, apoptosis regulation	
13-lined gs	Heart[b]	Elevated in summer homeothermy	Tricarboxylic acid cycle enzyme complex, lipoic acid binding, valine, leucine and isoleucine degradation	Grabek et al. (2011)
		Elevated in winter	Chaperonin-containing T-complex, fatty acid metabolism	
Woodchuck	Heart[a]	Deep torpor vs summer homeothermy	Alpha-adrenergic signaling, actin cytoskeleton signaling, acute phase response signaling, CREB signaling, eNOS signaling, fatty acid metabolism, fatty acid elongation in mitochondria, glycolysis/gluconeogenesis, lysine degradation, mitochondrial dysfunction, nitric oxide signaling in the cardiovascular system, protein kinase A signaling, role of NFAT in cardiac hypertrophy	Li et al. (2013)
13-lined gs	Kidney[b]	Elevated at low T_b (cluster 1)	Cytoplasmic membrane-bound vesicle, plasma proteins	Jani et al. (2012)
		Decreased in heterothermy (cluster 2)	Mitochondria, generation of precursor metabolites and energy	
		Decreased in heterothermy (cluster 5)	Amino acid catabolism, TCA cycle, electron transport chain, oxidative phosphorylation	
13-lined gs	Liver[b]	Increased in Ent	Organelle lumen, fatty acid metabolism, endoplasmic reticulum, regulation of apoptosis, anti-apoptosis, cell redox homeostasis, fatty acid beta-oxidation, ribosome	Rose et al. (2011)
		Increased in SA	Mitochondrion, nitrogen compound catabolic process, aldehyde dehydrogenase, lysosome, ATP catabolic process, energy from oxidation of organic compounds	

Table 2 continued

Species	Tissue	Hibernation comparison	Enriched categories	References
13-lined gs	Liver[c]	Increased in SA vs. Ent	TCA cycle, glycolysis/gluconeogenesis; metabolism: pyruvate, propanoate, glutathione, butanoate, Ala, Asp, Gly, Ser, Thr, Glu, Trp, urea cycle, P-450 xenobiotic; and degradation: Lys, Val, Leu, Ile	Epperson et al. (2010a, b)
Arctic gs	Liver[b]	Increased in IBA vs. LT	Oxidoreductase, protein biosynthesis, proteasome, phosphoprotein, ribosome, ribonucleoprotein	Shao et al. (2010)
		Increased in LT vs. IBA	Steroid and lipid metabolic process	
		Increased in IBA vs. SA (May/June)	Translation, phosphoprotein	
		Increased in SA (May/June) vs. IBA	Oxidoreductase, electron transport, lipid metabolic process, response to chemical stimulus, amino acid metabolic process	
		Increased in SA (May/June) vs. LT	Electron transport, lipid and amine metabolic process, response to chemical stimulus	
13-lined gs	Liver[b]	Increased in homeothermy (cluster 1)	Mitochondria; metabolism: hexose, triglyceride and hormone; amino acid catabolism	Hindle et al. (2014)
		Increased in Ar (cluster 2)	Cytoplasmic vesicle, organelle lumen, cell redox homeostasis, regulation of apoptosis, ubiquitin-dependent protein catabolism, protein localization, plasma, response to oxidative stress	
		Increased in winter (cluster 3)	Peroxisome, mitochondrial membrane, regulation of apoptosis, fatty acid catabolism, cellular ion homeostasis	
Rickett's big-footed bat	Liver[a]	Non-hibernating vs. torpid	Network 1: Developmental disorder, gastrointestinal disease, hepatic system disease; Network 2: Amino acid metabolism, small molecule biochemistry, developmental disorder	Pan et al. (2013)
American black bear	Serum[d]	Increased in hibernation	Immune system process, adaptive and innate immune response, acute phase response, complement activation, response to wounding, digestion, platelet degranulation	Chow et al. (2013)
13-lined gs	Skeletal muscle[b] (hindlimb)	Elevated in summer	Carbohydrate catabolism, glycolysis, sarcomere and cytoskeleton regulation	Hindle et al. (2011)
		Elevated in winter	Aerobic respiration, monosaccharide metabolism, reactive O_2 species metabolism, regulation of cell death, peroxisome	

Columns report: the species (gs indicates 'ground squirrel'), tissue and hibernation states (abbreviations as in Fig. 2) investigated; and the functional gene enrichment categories found based on a quantitative analytical method, e.g., [a] Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, http://www.qiagen.com/ingenuity), [b] DAVID (Huang et al. 2009), [c] KEGG Spider (Antonov et al. 2008), [d] GoMiner (Zeeberg et al. 2003). The data were condensed to include only non-redundant terms describing biological processes

to broadly conserve amino acids (discussed above) and rely on triacylglycerols to meet their energetic needs.

Heart

The hibernator's heart must continually function under conditions that are pathological to non-hibernators, including near-freezing temperatures, hypoxia and oxidative stress. Several authors have highlighted the benefits of studying the hibernator's heart, including improved outcomes in the recovery from cardiac arrest, thoracic heart surgery and the treatment of ischemic heart disease, as well as resistance to arrhythmia that is common in epilepsy (Colugnati et al. 2008; Wang and Zhou 1999).

In an initial proof-of-concept study that primarily tested the performance of four different protein identification software programs, Russeth et al. (2006) detected significant upregulation of 3-oxoacid CoA transferase 1 (SCOT or OXCT1) in heterothermic torpid 13-lined ground squirrels vs. active homeotherms. This enzyme catalyzes the rate-limiting step in the metabolism of ketone bodies; the authors thus proposed a model in which ketone bodies are preferentially utilized by the heart in hibernation. A seasonal increase in OXCT1 was also detected by Grabek et al. (2011), who used 2D DiGE to characterize heart protein changes among the same eight distinct seasonal and physiological groups (Fig. 2) of 13-lined ground squirrels as described above for the liver.

In this comprehensive analysis of transitions across the hibernator's year, the authors detected significant protein changes in 82/432 (19 %) protein spots, most of which, again, were found to occur seasonally between the two homeothermic and four heterothermic groups (Fig. 3b). Proteins involved in fatty acid metabolism as well as heat shock and chaperone activity increased in winter heterothermy, while proteins involved in branched-chain amino acid catabolism characterized the homeothermic phase of the year. Although very few proteins changed within the torpor–arousal cycle (10/432 protein spots), the largest and most significant change occurred here, where the phosphorylation state of the actin-binding protein cofilin 2 cycled with changes in T_b. During the lowered T_b states of entrance, late torpor and early arousal, the pool of cofilin 2 became completely dephosphorylated, a phenomenon previously associated with ATP depletion (Suurna et al. 2006). The authors proposed that dephosphorylation of cofilin 2, in addition to regulating the actin cytoskeleton, serves as a mechanism to preserve ATP for essential functions during the metabolically depressed period of torpor. Coupled with the paucity of protein changes in the torpor–arousal cycle and a hibernation increase in stress response proteins, the seasonal shift in the winter period of heterothermy appears

geared towards increased cardioprotection and ATP preservation, in addition to lipid-based metabolism.

Increased cardioprotection during heterothermy also appears to be facilitated by changes in signaling pathways. Li et al. (2013) found that endothelial nitric oxide signaling was increased in torpid vs. homeothermic woodchucks using iTraq methodology; this signaling likely maintains myocardial blood flow during torpor via nitric oxide-induced vasodilation (Kudej and Vatner 2003). Of the 162 proteins identified as significantly changed between the two groups of woodchucks, many belonged to other pathways that are known to enhance cardioprotection and/or facilitate adaptive hypertrophy, including α-adrenergic and protein kinase A signaling pathways, as well as cAMP response element-binding protein and nuclear factor of activated T cell transcriptional regulation (Table 2). Not surprisingly, this study also detected a seasonal shift to lipid-based metabolism during winter heterothermy.

Thermogenic tissues: brown adipose and skeletal muscle

Body temperature rewarming relies on endogenous mechanisms of heat production. Brown adipose tissue (BAT) and skeletal muscle are the two most significant thermogenic tissues in hibernators. BAT is specialized for non-shivering thermogenesis (NST) and is highly elaborated in many small-bodied eutherians (Cannon and Nedergaard 2004). It generates heat by dispersing the proton motive force generated across the ETC via uncoupling protein 1. The relative contributions of shivering vs. non-shivering thermogenesis differ even among the clades of eutherians. Due to high thermal inertia, bears do not exhibit the precipitous T_b reductions and recovery that define torpor bouts during hibernation in small-bodied hibernators (Tøien et al. 2011). Bats can rely entirely on NST for rewarming, whereas rodent hibernators also employ thermogenesis by skeletal muscle shivering, particularly in the later stages of rewarming (Lyman et al. 1982). One early application of proteomics to the study of hibernation was performed by Russeth et al. (2006), which identified up to three proteins in 13-lined ground squirrel skeletal muscle as proof-of-concept, but did not compare hibernation states. A multi-state proteomics screen was subsequently conducted for both BAT (Hindle and Martin 2014) and hindlimb muscle (Hindle et al. 2011) in 13-lined ground squirrels, and a three-state (summer vs. torpid and aroused winter states) comparison in the pectoralis muscle of greater tube-nosed bats (Lee et al. 2008).

Both brown fat and skeletal muscle proteomes indicate the seasonal reliance on lipid fuel during hibernation observed in other ground squirrel tissues, with

carbohydrate metabolic signatures concordantly winter-reduced (Table 2). The hindlimb skeletal muscle proteome in 13-lined ground squirrels demonstrates that this substrate switch is coordinated in part through the seasonal abundance and activity of pyruvate dehydrogenase kinase 4, a key checkpoint enzyme in glucose metabolism; this regulatory role in substrate management during hibernation has been described (Buck et al. 2002). Fatty acid handling is also winter increased in both tissues. Fatty acid-binding protein 3 (heart-type) transports free fatty acids delivered by the circulation to cells—knockout mice are unable to acquire plasma fatty acids and lose cold tolerance (Vergnes et al. 2011; Yamashita et al. 2008). The elevation of both heart (type 3) and adipose (type 4) fatty acid-binding proteins underscores the importance of managing both plasma-delivered and local, intracellular lipid reserves during hibernation (Hindle and Martin 2014).

Skeletal muscle and brown fat both undergo annual and torpor–arousal cycles of metabolic activation. In hibernation, metabolic scope ranges from its minimum in torpor, with muscle essentially quiescent and BAT engaging at a low level to defend a species-specific body temperature setpoint for the brain and body (Cannon and Nedergaard 2004), to maximally active in rewarming arousals. Such a rapid activation of metabolic activity likewise requires rapid production of reducing equivalents and/or ATP. Perfusion, along with oxygenation and nutrient delivery, is restored to euthermic levels during interbout arousals (Lyman and Chatfield 1950), but may follow the development of peak metabolic rates (e.g., Hampton et al. 2010) in small eutherian hibernators. This creates hypoxemia (Ma et al. 2005) and the potential for oxidative stress in metabolically active tissues (Orr et al. 2009). Under these conditions, it would be advantageous for necessarily active tissues to favor anaerobic ATP production. Indeed, this appears to be the case in skeletal muscle (Hindle et al. 2011). The most striking torpor–arousal cycle protein abundance change in the muscle proteome of hibernating 13-lined ground squirrels was a peak in phosphorylated-PGM1 (phosphoglucomutase) during arousal, with immediate decline by IBA. As in heart, this dynamic indicates that the most dramatic torpor–arousal cycle protein changes can be due to post-translational modifications, consistent with regulatory PTMs being a key component of the torpor–arousal switch. Phosphoglucomutase-1 reversibly catalyzes the interconversion of glucose-1-P to glucose-6-P and is poised to regulate the mobilization of glycogen stores into glycolysis. When phosphorylated, PGM1 favors glucose-6-P production (Gururaj et al. 2004), suggesting that rewarming ground squirrels tap glycogen stores to meet cellular ATP requirements, possibly by anaerobic means.

Despite prolonged metabolic suppression during hibernation, many BAT and skeletal muscle proteins actually increase in winter (e.g., 56 % of differentially expressed, identified proteins in hindlimb muscle, Table 1; Fig. 3c, d). For 13-lined ground squirrels, this winter-enhancement of skeletal muscle and BAT proteomes includes mitochondrial proteins (Table 2) and likely supports transient but rapid energy production during periods of high metabolic activity. Rather than abundance changes in electron transporter proteins, structural mitochondrial proteins, such as mitofilin in skeletal muscle and components of membrane transporters in BAT, were winter increased. This striking seasonal component is consistent with a seasonal reprogramming of gene expression in both skeletal muscle and BAT. Once this new baseline is established, there are few changes across the torpor–arousal cycle—for identified proteins, there are only 0–6 (of 1623) significant pairwise differences among five states of hibernation in 13-lined ground squirrel BAT (Hindle and Martin 2014) and 0–14 (of 896) among six states in skeletal muscle (Hindle et al. 2011). Similarly, all six protein differences in bat pectoralis muscle were highest in hibernating vs. summer-active bats, and there were no differences between torpid and aroused winter animals (Lee et al. 2008).

Although the physiology of both skeletal muscle and BAT supports episodic activation and quiescence during hibernation, they display distinct seasonal attributes. BAT atrophies after animals emerge from hibernation but later proliferates and becomes highly recruited again as animals prepare for the next hibernation season; this is reflected by the relatively large proteome difference between the two homeothermic groups in BAT compared to the other organs (compare Fig. 3c to the other panels). In contrast, skeletal muscle contends with disuse during torpor and maximizes locomotory capability during the summer-active period. 13-lined ground squirrel axillary BAT mass peaked in mid-winter and was lowest in spring, with change in depot size occurring in conjunction with a strong enrichment of 14-3-3 proteins (Hindle and Martin 2014). Skeletal muscle generally mitigates winter atrophy (Ivakine and Cohn 2014), although some of this apparent mitigation appears to be due to early season loss and late season rebuilding (Hindle et al. 2015); proteomics results led Lee et al., to suggest that regulation of heat-shock protein chaperones protects against disuse remodeling of bat pectoralis muscle during torpor (Lee et al. 2008).

Brain

Despite its importance in regulating the process of hibernation, the brain also undergoes dramatically altered temperature, perfusion and metabolic rate (Drew et al. 2002, 2007; Frerichs et al. 1994). Proteomic changes in the small-bodied hibernators' brain must underlie documented neuroprotective features such as depressed metabolism to match

a 90 % reduction of cerebral perfusion in torpor (Frerichs et al. 1994), effective synapse re-establishment during brief euthermic periods (Dave et al. 2012) and cellular homeostasis at low T_b (van Breukelen and Martin 2002a).

The brain is expected to manifest winter fasting metabolism as a preference for metabolizing ketone bodies over glucose (Andrews et al. 2009; Schwartz et al. 2013). Metabolic signatures, albeit with different protein compositions, are featured findings from proteomic screens of both bat whole brain (Zhang et al. 2014) and 13-lined ground squirrel brainstem (Epperson et al. 2010a). Compared to winter arousal, torpid bats depress flux through the TCA cycle and demonstrate an enhanced capacity for anaerobic metabolism from glucose (Zhang et al. 2014). Glycolytic enzymes increased during torpor, including lactate dehydrogenase A, which catalyzes the final pyruvate breakdown step to lactate under anaerobic conditions. Bats also appear to increase their capacity to metabolize amino acids during torpor, a substrate pool that tends to be closely guarded in other hibernators (e.g., Nelson 1980; Riedesel and Steffen 1980). In contrast, the 13-lined ground squirrel brainstem increases its capacity for ATP production by streamlining the abundance of TCA cycle enzymes in early torpor compared to summer-active animals (Epperson et al. 2010a). Both species also show consistent modifications to electron handling in the ETC. In particular, subunits of complex I, III and ATP synthase, as well as voltage-dependent anion channel 2 in the mitochondrial outer membrane increase in torpid brains of both bats and ground squirrels (Epperson et al. 2010a; Zhang et al. 2014). That proteins associated with electron handling and ATP synthesis are enhanced at a time associated with dramatic metabolic rate reduction suggests that regulating ATP or possibly implementing mechanisms to maintain electron transport at low membrane temperatures is critical to successfully navigating torpor–arousal cycles.

As an additional contrast to the species specificity of metabolic adjustments during torpor, there also appear to be regional differences in the brain, because metabolic proteins are essentially unchanged in the 13-lined ground squirrel forebrain (Hindle and Martin 2013). In a multi-state comparison among summer-active, terminally aroused spring 13-lined ground squirrels and four hibernation states (IBA, Ent, LT and Ar) only four metabolic proteins differed (aconitase 2, lactate dehydrogenase B, transaldolase 1 and propionyl CoA carboxylase alpha subunit). The unique patterns observed in lactate dehydrogenase (LDH) among the three studies of brain proteome highlight the metabolic differences between brain regions (i.e., constitutively active brainstem vs. transiently active forebrain) and possibly between species of hibernator. While LDHA was elevated in torpid bat brains, this isoform was depressed in the

brainstems of torpid 13-lined ground squirrels and LDHB (the heart subunit, more active under aerobic conditions) was elevated.

The pattern of protein abundance changes in the multi-state evaluation of 13-lined ground squirrel forebrain also differs from other tissues. The forebrain lacks clear seasonal as well as torpor–arousal transitions. Rather, the predominant changes occur between warm (SA, SpC, IBA and Ent) and cold T_b (LT, Ar; Fig. 3e). PTMs are implicated to underlie differential protein expression across torpor–arousal in all existing proteomic screens of brain tissue (Epperson et al. 2010a; Hindle and Martin 2013; Zhang et al. 2014). In bats, PTMs were detected in multiple subunits of the pyruvate dehydrogenase complex, consistent with the strong metabolic signature in this proteomic dataset (Zhang et al. 2014). PTMs were also detected in the multi-state forebrain dataset; here, in contrast to all other tissues examined to date, abundance differences between cold and warm T_b states of the torpor–arousal cycle are predominant and there is little evidence of seasonal reprogramming (Hindle and Martin 2013). Cytoskeletal protein α-tubulin isoform 1A and microtubule assembly regulator dihydropyrimidinase-like protein 2 (CRMP2) were both confirmed to have phosphorylation changes between torpor and arousal.

Cytoskeleton regulation was the most consistent signal across available proteomic screens of hibernator brains. Indeed, the reduced T_b of torpor is associated with neural retraction and cytoskeletal depolymerization (Kirschner et al. 1974; von der Ohe et al. 2006). Resulting synaptic loss likely protects against excitotoxicity during torpor (Dave et al. 2012), but other proteomic changes could stabilize the cytoskeleton and promote rapid and reversible reorganization in each torpor–arousal cycle (Popov et al. 1992). Both two-state proteomic comparisons suggest a general reduction and stabilization of cytoskeletal polymerization in torpor, interpreted in the bat as reduced cytoskeletal plasticity (Zhang et al. 2014). As an example, all three studies report a difference in the abundance of actin-modulating cofilin-1, although the exact nature of its altered function in torpor is unclear as cofilin's phosphorylation state, unlike in heart (Grabek et al. 2011), was not determined in any study. The multi-state comparison in ground squirrel forebrain invokes a different interpretation—that suppressing cytoskeletal dynamics during torpor acts as a stabilization and preservation mechanism, which permits rapid re-establishment of microtubules and ultimately synapses during brief inter-bout arousals.

Brain tissue is composed of highly heterogeneous cell types, which confounds comparisons across hibernation states (Dave et al. 2012). In 13-lined ground squirrels, forebrain displays very few significant protein differences

(~2.5 % Hindle and Martin 2013). While brainstem of the same species shows considerably more differences between torpid and summer-active animals (13 % Epperson et al. 2010a), all differences were <twofold. These limited significant differences and suppressed fold changes may represent a dilution of larger cell type or nucleus-specific fold changes across the diversity of cells contained in a homogenized brain fraction (Epperson et al. 2010a).

Intestine

During hibernation the intestine shrinks, typical of a fasting animal. Despite this, mass-specific nutrient and electrolyte transport activities are not significantly different from the fed (summer) state when studied at 37 °C (reviewed in Carey et al. 2003). The intestine, like many other organs in the hibernator's body, is highly resistant to damage by ischemia followed by reperfusion (I/R injury); this resistance occurs during the heterothermic phase of the year in 13-lined ground squirrels. Specifically, using two models of I/R injury, Kurtz et al. (2006) demonstrated loss of villus epithelium and overall villus structure, infiltration of neutrophils and other immune cells, blood vessel congestion and increased intestinal apoptosis in summer ground squirrels compared to winter hibernators. Because the I/R challenge occurred in euthermic animals in both cases (IBA vs. SA) the protection is not attributable to low body temperature during torpor but rather appears to be a seasonal reprogramming, i.e., a manifestation of the winter-protected state. To gain insight into the protein components underlying the seasonal difference in response to I/R, Martin et al. used DiGE to compare the gut proteome among four groups, summer and IBA hibernators, both sham and I/R treated (Martin et al. 2008).

As with other organs, the intestinal proteome differs between summer and winter in the absence of I/R treatment. In fact a greater number of protein differences can be attributed to the seasonal reprogramming than to the response to I/R. Many of the seasonally changed proteins reflect the change in nutritional status, i.e., fed vs. fasting. For example, there is an increased capacity for ketogenesis (HMGCS2) and lipid binding and transport (albumin, apolipoprotein A1) in hibernators as seen in other tissues, and decreased enzymes for small molecule metabolism, e.g., arginase 2 and isovaleryl coenzyme A dehydrogenase. Perhaps the most striking feature of the proteins that differ between summer and hibernator sham groups, however, were those that distinguish immature from mature intestinal epithelium, i.e., changes that reflect the intestinal morphological differences between these two states. In the hibernator, proliferation and migration along the crypt–villus axis effectively ceases during torpor, although it activates again briefly during each IBA (Carey and Martin 1996).

This change in proliferative behavior apparently causes the enterocyte population to shift towards an increased abundance of mature, absorptive villus cells compared to the summer animals. The enhancement of this cell type explains many of the protein changes observed in the proteomic screen and retention of absorptive capacity during hibernation.

Summer and hibernator gut also differed in their response to I/R; the response of the intestinal proteome was greater in the summer animals than it was in the interbout-aroused winter hibernators. Eleven proteins differed between summer sham and summer I/R, most increasing in response to I/R. The increased proteins could reflect: (1) a damage response in summer animals that is repressed in the winter hibernators; or (2) the observed dramatic shift in the cell type distribution after I/R in the summer animals. Because of the substantial morphological change, specifically the dramatic loss of villus epithelium, which is observed in the intestines of just the summer I/R-treated animals, the second explanation is most likely. This interpretation is also consistent with increased abundance of the crypt cell marker, transferrin, after I/R treatment. In contrast, none of the six proteins that changed after I/R treatment of hibernator intestine were shared with the summer animals and all were decreased after I/R. Since most of these were also seasonally enhanced in the hibernators, their reduction may reflect some loss of the mature villus cells that characterize the winter intestinal epithelium, but not so much as to lead to the marked increase of crypt cell markers seen in the summer after I/R treatment (Martin et al. 2008). Taken together, these results highlight the sensitivity of proteomics results to cell type composition of a tissue under investigation. Given the complex dynamics of intestinal form and function under these extreme long-term fasting conditions, interpretation of the proteomic data necessarily must extend beyond simple changes in gene expression, or post-translational modification occurring in a constant cell type, to consider a redistribution of cell types.

Kidney

Hibernation challenges mammalian homeostasis in organs throughout the body. The lack of food and water intake for many months means kidneys must conserve water and electrolytes throughout winter. In addition, each torpor–arousal cycle sees renal function cease during torpor and recover during each interbout arousal. Because of its natural resilience to "cold storage" and warm reperfusion, the hibernator's kidney has become a model for discovery of novel approaches to improve kidney preservation during transplant (Jani et al. 2013). Just one kidney proteomics screen has been completed (Jani et al. 2012); this study captured

both seasonal and torpor–arousal cycle changes in 13-lined ground squirrels by sampling six states, two from the homeothermic season (Spring animals, cold exposed, SD) and SA (early August), and four from hibernation (IBA, Ent, LT and Ar). Among all states, there were 564/2,073 significantly changed spots, a relatively large proportion compared to other organs studied comparably. Additional observations based on protein spot differences that distinguish the kidney from the other organs are the relatively large number of proteins with increased abundance in Ar compared to the other hibernation states, and the fact that differences between summer homeotherms and winter hibernators are otherwise almost exclusively due to protein abundance decreases in winter (Fig. 3f).

Unique protein identifications of 150 of the protein spot differences were obtained after selecting those with at least 1.5-fold change in at least one pairwise comparison. The kidney proteome distinguished homeotherms from heterotherms, but also strongly separated arousing animals from the other hibernation states. Three distinct patterns of protein abundance changes among the six physiological states were apparent: (1) a small group of plasma and cytoplasmic vesicle proteins that increased with relatively large fold changes (4–13-fold) during each arousal; (2) a large group of proteins that were involved with energy capture and storage that were increased in summer; and (3) a selected subset of the latter that were also increased during each arousal, including enzymes for gluconeogenesis (fructose-1,6-bisphosphatase, pyruvate carboxylase, triosephosphate isomerase, phosphoenolpyruvate carboxykinase 1) and proteins that facilitate redox homeostasis (peroxiredoxins 3 and 6, glutathione reductase).

The plasma and endocytic pathway proteins that specifically increase during arousal from torpor provide unexpected insights into renal function. First, A2M, a winter-season upregulated protein that is secreted into the plasma by liver and too large for filtration (see liver section, above, and Epperson et al. 2007; Jani et al. 2012; Srere et al. 1992), is retained in the renal vasculature, indicating that the glomerular filtration barrier is not compromised during torpor. Its spotty distribution among glomeruli, however, indicates strong regional differences in blood flow during torpor bouts. Second, albumin, a protein that is partially filtered in glomeruli, clearly enters the urinary space within the renal corpuscle, and can be seen in parietal cells, podocytes and in adjacent proximal convoluted tubules. This accumulation is best explained by the cold temperature-sensitive block in receptor-mediated endocytosis, thus albumin accumulates along its normal pathway through the kidney until the animal rewarms during interbout arousal and the filtered albumin is rapidly either degraded or transported back into the bloodstream (Jani et al. 2012).

Serum

The rationale for studying the serum proteome within the hibernating phenotype is to identify altered immune components, as well as indicators of adaptive changes in other tissues, or possibly to identify the elusive "hibernation induction trigger" (Dawe et al. 1970). In one such study, serial samples of American black bear serum were taken from pre-hibernating and hibernating bears (Chow et al. 2013) and analyzed using 2D DiGE methodology. Indeed, proteins involved in immune-related processes, including adaptive and innate immunity, complement activation and acute phase response increased in the hibernating bears. Further, proteins involved in response to wounding were enriched in the hibernating bears, consistent with reports of maintenance of sub-cutaneous wound healing during hibernation (Iaizzo et al. 2012). This contrasts with small-bodied hibernators, where both wound healing and immune system components appear suppressed during torpor (Billingham and Silvers 1960; Bouma et al. 2010). Chow et al. (2013) hypothesized that this discord is due to differences in the degree of body temperature suppression, as well as unique strategies used by bears to cope with the extended metabolic depression that occurs independent of body temperature reduction. On the other hand, A2M is increased in both hibernating bears and ground squirrels (Srere et al. 1992); hence, hypocoagulation appears to be a theme shared across hibernators, likely in response to low cardiac output and increased blood viscosity during torpor. Finally, bears maintain bone mass despite months of inactivity; Chow et al. (2013) detected one protein involved in bone remodeling, alpha-2-HS-glycoprotein, which decreases during hibernation, suggesting that changes in this protein's expression, along with immune system-related changes, are important for balancing bone formation and resorption.

Proteomic changes during the fall transition

Transition periods are important in understanding the regulation of the hibernation phenotype. In general, summer animals behave as normal homeotherms, not capable of orchestrating reversible metabolic depression, but they do retain varying degrees of improved resistance to ischemia–reperfusion injury (Dave et al. 2006; Lindell et al. 2005). Interestingly, bears can extend reversible metabolic depression into the summer months (Tøien et al. 2011), consistent with the extension of the hibernation phenotype into the post-emergence period; however, metabolic depression has not been documented at the end of the summer season, pre-hibernation.

Cues for initiation of the transition to the hibernating phenotype include photoperiod and temperature, but there

is also an intrinsic clock component (Dark et al. 1990; Pengelley et al. 1976). In the fall, obligate hibernators such as 13-lined ground squirrels have been observed to spontaneously enter shallow torpor bouts, despite consistent laboratory housing (light/temperature) and available food (Russell et al. 2010). It has been suggested that these "test drops" are necessary to prepare for hibernation as they precondition critical tissues to hypothermia and ischemia/reperfusion associated with torpor (Hittel and Storey 2002). By comparing tissue-specific proteomes across the fall period, we can evaluate their proximity to the summer/winter condition, relative to details of housing condition and prior torpor use to determine: (1) the order in which tissues adopt a hibernating phenotype; (2) whether the transition to the winter proteome is preparatory or responsive to changes in environment; and (3) if initial "test drops" confer preconditioning to specific tissues.

Only a few studies thus far have included samples in the fall period for the purpose of examining the transition into hibernation. Protein abundance patterns reveal that most changes in brown adipose tissue are preparatory (Hindle and Martin 2014), while those in liver are responsive (Hindle et al. 2014, Fig. 4b). The heart proteome exhibits a mix of preparatory (Fig. 4d), responsive (Fig. 4e) and pre-conditioning patterns in the fall (Grabek et al. 2011, Fig. 4f). Despite the prevalence of a transition pattern within a given tissue, it is important to note that examples of all three patterns generally exist (e.g., BAT and liver pre-conditioning; Fig. 4a, c). The fact that most proteins in BAT achieve their winter phenotypic levels before any external stimulus likely reflects the need for recruitment in advance of cold exposure, although further enhancement has been documented in response to cold and torpor use (e.g., Fig. 4a; Burlington et al. 1969; Hoffman et al. 1965). The proteomic transition observed in liver, on the other hand, likely corresponds to the cessation of eating, which is expected to be hormonally regulated (Florant and Healy 2012).

Examining the abundance patterns of proteins that differ seasonally in more than one tissue provides insight into the role and function of specific tissues in coordinating the transition to hibernation. Unique tissue roles are highlighted by the observation that some proteins with concordant seasonal adjustments differ between tissues in their fall expression pattern. For instance, fatty acid-binding protein 3 (heart-type) is elevated in the heart in early fall (Fig. 4d), before the onset of torpor and exposure to cold. This differs from BAT, where the increase in fatty acid-binding protein 3 lags, having intermediate expression between summer and winter levels (Fig. 4a). Local, tissue-specific regulation likely accounts for this difference in timing, and suggests that the ability to handle plasma-delivered fatty acids must be maximized in the heart prior to the initial onset of torpor bouts.

There are also examples that suggest coordinate regulation of common proteins or distinct proteins in common pathways among tissues. For example, dihydrolipoamide-branched-chain transacylase (E2 subunit) involved in branched-chain amino acid catabolism, remains at summer-elevated levels throughout the fall in both liver and heart (Fig. 4b, e), suggesting that the transition to winter levels occurs in response to or as a final element of the complete adoption of a heterothermic phenotype. Two additional proteins involved in branched-chain amino acid catabolism, 3-hydroxyisobutyrate dehydrogenase in liver, and branched-chain keto acid dehydrogenase (subunit E1, beta chain) in heart, both exhibit gradual reduction across the fall transition to winter levels (Fig. 4c, f), the pattern expected for a pre-conditioning effect. Such pre-conditioning could derive from global physiological signals including the onset of fasting, circulating hormones, or short forays into torpor.

In sum, the fall transition involves a complex interplay of factors, including endogenous circannual timing, external environmental cues as well as whole body and tissue-specific signaling. Clear differences in hibernation preparation patterns among tissues drive home the necessity of a whole body approach to understanding the physiology of hibernation. Further, the distinct timing of the fall transition to hibernation among tissues, coupled with observations of a circannual phenotype that continues over the course of winter hibernation, emphasizes the importance of careful sampling in future hibernation experiments. Specific attention must be paid to the time of year of sample collection, as increased heterogeneity (especially evident in the fall) will lead to underpowered statistical analyses.

Transition from hibernation into the spring post-emergence phenotype is also likely complex, but has thus far only been evaluated in brown adipose tissue of 13-lined ground squirrels (Hindle and Martin 2014). Similar studies in additional tissues will provide information about the physiology at the termination of hibernation. Examination of this period will define species limits of hibernation duration and overall phenotypic plasticity, leading to a better understanding of additional physiological extremes, such as fasting tolerance, as well as to an understanding of how hibernating species might respond to changing climates (Lane et al. 2012).

Summary of changes across tissues and future directions

A surprising but striking feature revealed by the larger, multi-state screens is the relative stability of the proteome across the extreme physiological changes of hibernation. Most of the observed protein changes are seasonal,
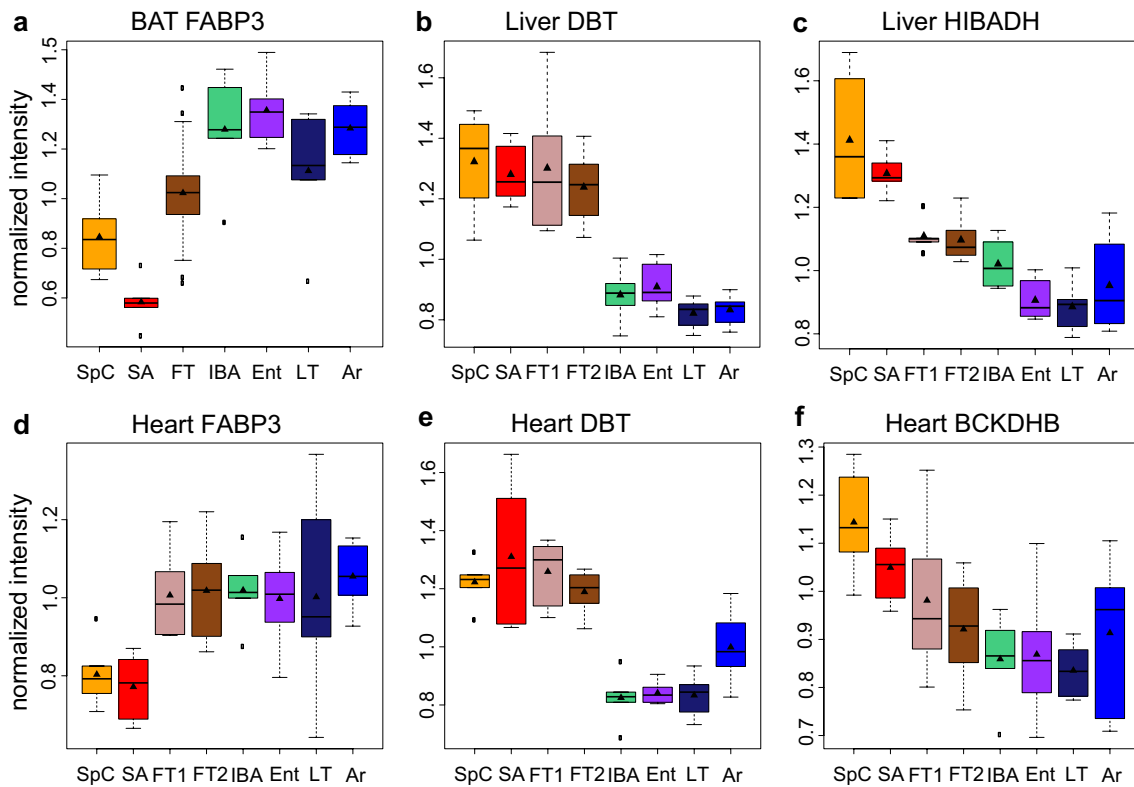
Fig. 4 *Fall box* plots illustrate different patterns of the fall transition into hibernation. Each *panel* plots the normalized intensity (*y*-axis) of one protein spot, labeled by respective tissue and gene symbol on top, among the sample groups (labeled as in Fig. 2); *triangles* mark the mean. Fall transition patterns include **a** brown adipose tissue pre-conditioning; liver **b** responsive and **c** pre-conditioning; heart **d** preparatory, **e** responsive and **f** pre-conditioning. Data are re-plotted from Hindle and Martin (2014), BAT; Hindle et al. (2014), liver; and Grabek et al. (2011), heart

i.e., between the homeothermic and heterothermic period, and maximum fold changes are generally low (<2). With the exception of the forebrain (lacking hypothalamus) proteome in 13-lined ground squirrels (Hindle and Martin 2013), few abundance changes are reported between cold and warm T_b states across the torpor–arousal cycle. Many of the limited protein changes are presumed to result from post-translational modifications, which may resolve as unique shifted spots on 2D gels or can be identified in shotgun methods by MS[3]. Attributing even some of the already limited torpor–arousal cycle differences in protein abundance to protein modifications implies an extremely stable total protein pool across the hibernation season, despite prolonged fasting and widely ranging metabolic levels. This stability is not completely unexpected, because the profound temperature-mediated suppression of both transcription and translation machinery drastically limits the ability to synthesize new gene products during torpor (van Breukelen and Martin 2001, 2002b), and protein degradation is also depressed (Velickovska et al. 2005). Thus, any observed protein abundance increase across the torpor bout is likely the consequence of: (1) post-translational

modification to convert an existing protein into a new isoform; (2) re-localization (e.g., receptor-bound plasma protein that accumulates because of the temperature sensitivity of endocytosis, as best exemplified by the kidney proteome, Jani et al. 2012); or (3) a relatively high intrinsic protein stability compared to the bulk of the protein pool.

Few themes of enrichment are common across systems (Table 2). These include the well-documented substrate switch from carbohydrate- to lipid-based metabolism that appears essential for the winter fast, with concordant amino acid and protein sparing adaptations, as well as increased management of cellular stress during winter heterothermy (Fig. 1b). A closer examination of enriched pathways indicates that cytoskeletal regulation may be an important, unifying theme across several tissues. Enriched cytoskeleton/cell-architecture signals among differentially abundant proteins across hibernation could reflect regulation of a variety of processes, including reducing ATP turnover (as highlighted above in the heart), altered cell type and morphology (as highlighted in the brain and intestine), or regulation of disuse atrophy (as highlighted in skeletal muscle and intestine). The specific, differentially abundant cytoskeletal

proteins that are enriched in the population of proteins that differ across the hibernation cycles are, however, distinct among tissues, again demonstrating tissue-specificity with this complex physiological strategy.

These existing proteomic studies of hibernation, while offering solid support for important discoveries that can be made, are limited by sample depth (e.g., abundant soluble proteins, tissues, time points) and the depth of the proteome evaluated. There is considerable variation among studies, within and between species, at least in part reflecting inconsistencies in methodology (e.g., number and timing of sampled groups, protein identification criteria) and statistics (e.g., fold change filters for inclusion of data, application of a false discovery rate correction). Nevertheless, we have unquestionably learned that changes in the proteome correlate with different hibernation states and that individual tissues and possibly individual species exploit discrete strategies to deal with the dynamics of the hibernation cycles. Given that the mammalian proteome is likely comprised of more than 20,000 gene products, current efforts have sampled only a small fraction of the complexity of the possible protein changes linked to hibernation (Table 1). Recent advances in proteomic methods can support a comprehensive analysis of the proteome in model organisms (Nagaraj et al. 2012), and nearly so in humans, provided a large number of tissues are examined (Kim et al. 2014). Future work needs to expand existing findings using these new methods on precisely collected samples from multiple tissues of hibernators. Once such a dataset is in hand, it can be integrated to identify testable, global hypotheses about the whole body physiology of hibernation.

Conflict of interest The authors declare that they have no conflict of interest.

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