Multistate proteomics analysis reveals novel strategies used by a hibernator to precondition the heart and conserve ATP for winter heterothermy

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Grabek KR, Karimpour-Fard A, Epperson LE, Hindle A, Hunter LE, Martin SL. Multistate proteomics analysis reveals novel strategies used by a hibernator to precondition the heart and conserve ATP for winter heterothermy. Physiol Genomics 43: 1263–1275, 2011. First published September 13, 2011; doi:10.1152/physiolgenomics.00125.2011.—The hibernator’s heart functions continuously and avoids damage across the wide temperature range of winter heterothermy. To define the molecular basis of this phenotype, we quantified proteomic changes in the 13-lined ground squirrel heart among eight distinct physiological states encompassing the hibernator’s year. Unsupervised clustering revealed a prominent seasonal separation between the summer homotherms and winter heterotherms, whereas within-season state separation was limited. Further, animals torpid in the fall were intermediate to summer and winter, consistent with the transitional nature of this phase. A seasonal analysis revealed that the relative abundances of protein spots were mainly winter-increased. The winter-elevated proteins were involved in fatty acid catabolism and protein folding, whereas the winter-depleted proteins included those that degrade branched-chain amino acids. To identify further state-dependent changes, protein spots were re-evaluated with respect to specific physiological state, confirming the predominance of seasonal differences. Additionally, chaperone and heat shock proteins increased in winter, including HSPA4, HSPB6, and HSP90AB1, which have known roles in protecting against ischemia-reperfusion injury and apoptosis. The most significant and greatest fold change observed was a disappearance of phospho-cofilin 2 at low body temperature, likely a strategy to preserve ATP. The robust summer-to-winter seasonal proteomic shift implies that a winter-protected state is orchestrated before prolonged torpor ensues. Additionally, the general preservation of the proteome during winter hibernation and an increase in stress response proteins, together with dephosphorylation of cofilin 2, highlight the importance of ATP-conserving mechanisms for winter cardioprotection.

2D DiGE; CFL2; hibernating; Ictidomys (spermophilus) tridecemlineatus; mass spectrometry

HIBERNATION IN MAMMALS is characterized by dramatic, but reversible, reductions in body temperature (Tb) and metabolic rate. During this torpid phase, respiration and heart rate also decline to 1–4% of their euthermic values (for review, see Ref. 11). Torpor bouts are punctuated by interbout arousals (54), when physiological rates are restored to or even surpass euthermic levels (11). For circannual hibernators such as 13-lined ground squirrels, Ictidomys tridecemlineatus, the ability to orchestrate reversible metabolic depression exists only in winter (reviewed in Ref. 38). This heterothermic winter phenotype contrasts markedly with summer, when animals remain homotherms. A “two-switch” mechanism in which animals first switch from summer homeothermy to a state that is permissive for winter heterothermy (switch 1), and then cycle between periods of torpor and arousal in winter (switch 2) appears to model the phenomenon of circannual hibernation (31, 58).

Heterothermy poses a significant challenge to a hibernator’s physiology. This may be particularly acute for the heart, because it maintains functionality across a wide Tb range. The acceleration of heart rate from 3–5 to ≥300 beats/min during arousal from torpor (44) is likely to be especially stressful. As metabolic rate peaks during this period, oxygen delivery fails to keep pace, creating transient hypoxic conditions (45) analogous to ischemia-reperfusion events. Furthermore, the rapidly increasing metabolic activity may generate a surge in oxidative stress (16, 52). Yet, remarkably, the hibernator’s heart continues to function throughout all of these dramatic transitions. Understanding this cardioprotected phenotype, which is capable of low-temperature function, offers untapped potential to benefit human medicine by identifying novel strategies to improve outcomes in recovery from heart attack, thoracic heart surgery, and the treatment of epilepsy-associated arrhythmia and ischemic heart disease (14, 69).

There is evidence supporting a cardioprotected winter phenotype in hibernators. In winter-torpid animals, the upregulation of some peroxiredoxins (49) and inducible heat shock proteins (18) suggests strategies for managing oxidative stress. Winter elevations of Ca$^{2+}$/ion transporter abundance and activity facilitate the maintenance of Ca$^{2+}$/ion homeostasis and cardiac contraction (2, 42, 73). Depolarizing signal conduction is also promoted at the low Tb of torpor by increased levels of connexins 43 and 45 (27). These findings provide discrete mechanistic explanations for continued cardiac function under conditions pathological to nonhibernators. For example, hibernator hearts maintain contractility during torpor (Tb 0–7°C), while those of nonhibernators become arrhythmic between 30 and 16°C (35). Hibernators are also markedly resistant to ventricular fibrillation (35) and to the cardiac conduction block that generally accompanies hypothermia in mammals (26). Moreover, the hibernator’s heart maintains Na$^{+}$/K$^{+}$/ion homeostasis during torpor, while nonhibernating mammals lose ion homeostasis over time (36).

While insightful, these studies provide a partial view of cardioprotection in hibernators, because they sampled few physiological states and focused on relatively few gene products. To maximize its potential to reveal novel medical treatment strategies, however, the cardiac phenotype of hibernators must be characterized in detail. Therefore, we applied an unbiased screening method to examine the heart proteome that
underlies the dynamic physiology of hibernation. To capture the protein components of the two switches in hibernation, we quantified differences in the 13-lined ground squirrel’s heart among eight states defined by season or physiology using two-dimensional difference gel electrophoresis (2D DiGE). The components of the winter-protected phenotype were identified by comparing states representing summer homeothermic and winter heterothermic physiology, whereas the components of the intrawinter cycle were identified by analysis of four winter groups from both torpid and aroused states. Finally, we hypothesized that fall represents a transition between homeothermy and heterothermy that could illuminate the process underlying the summer-winter switch. Therefore, for the first time, we included two fall sampling states. This comprehensive sampling strategy and unbiased experimental approach provides new insight into elements supporting the dynamic cardiovascular physiology of the hibernator heart.

EXPERIMENTAL PROCEDURES

Animals

Procurement and monitoring. Thirteen-lined ground squirrels were purchased from the University of Wisconsin, Oshkosh captive breeding program in July-August 2006–2009. Squirrels were individually housed under standard conditions (18–21°C, 14:10 light-dark cycle, fed cat chow supplemented with sunflower seeds and water ad libitum). All animals except those in the summer active group (SA, n = 6; Fig. 1) were surgically implanted in August or early September with both an intraperitoneal datalogger (iButton, Embedded Data Systems) and a radiotelemeter (VM-FH disks; Mini Mitter, Sunriver, OR) for remote Tb monitoring until tissue collection. In early October, squirrels were moved to the hibernaculum, which was kept in constant darkness. Ambient temperature was lowered step-wise from 18 to 4°C over a 2 wk period, and food and water were withdrawn as squirrels exhibited multiday torpor bouts, assessed by Tb arousing (IBA: Tb days after spontaneous termination of hibernation); summer active (SA: n = 2.5 days with Tb torpid/H11005 6; Fig. 1) were surgically implanted in August or early October, squirrels were moved to the hibernaculum, which was kept in constant darkness. Ambient temperature was lowered step-wise from 18 to 4°C over a 2 wk period, and food and water were withdrawn as squirrels exhibited multiday torpor bouts, assessed by Tb arousing (IBA: Tb days after spontaneous termination of hibernation); summer active (SA: n = 2.5 days with Tb torpid/H11005 6; Fig. 1) at least 10 days before euthanasia. All animal protocols were approved by the University of Colorado Institutional Animal Care and Use Committee.

Tissue collection. Hearts were collected from animals representing the eight different seasonal and physiological groups (n = 6 per group) defined in Fig. 1. Animals were euthanized by exsanguination under isoflurane anesthesia. They were perfused with ice-cold isotonic saline and decapitated before dissection.

2D DiGE

Protein sample preparation. Each heart was frozen in N2(l) immediately after dissection and stored at ~80°C. Protein extracts were prepared as described previously (20) in ice-cold homogenization buffer (0.5 M sucrose, 100 mM phosphate, 5 mM MgCl2, 1 mM PMSF, 10 μg/ml protease inhibitors). The homogenate was centrifuged and the nuclear-free supernatant was snap frozen in aliquots using N2(l). Aliquots were stored at ~80°C and each aliquot was used only once. Protein concentration was determined using a BCA assay (Pierce, Rockford, IL).

2D DiGE labeling. DiGE labeling was completed as previously described (46). A pooled reference standard containing equal amounts (μg) of n = 3 heart protein extracts from each sampling group (24 samples total) was stored in single-use aliquots. For labeling, 90 μg of each experimental sample and reference standard were denatured overnight at room temperature in lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, and 25 mM Tris, pH 8.8). Denatured protein samples were labeled with Cy2, Cy3, or Cy5 (CyDye DiGE Fluors; GE Healthcare, Piscataway, NJ). Cy2 always labeled the pooled reference sample. Cy3 and Cy5 labels were alternated among the experimental samples to control for bias in the dye labeling: in each group of six, three samples were labeled with Cy3 and the remaining three samples were labeled with Cy5.

2D DiGE and pick gels. 2D electrophoresis was completed as described previously (22). For each gel, two experimental samples (one Cy3 and the other Cy5-labeled) were mixed with a Cy2-labeled reference sample. The experimental gels were always prepared with two group combinations (e.g., FT-Cy3 and IBA-Cy5 or IBA-Cy3 and FT-Cy5) so that no two samples of the same group were used in a single gel. The labeled protein mixture for each gel was precipitated with methanol-chloroform and then resuspended as previously described (22). For first dimension protein separation, resuspended samples were absorbed overnight onto Immobiline DryStrips (pH 3–10 NL, 18 cm; GE Healthcare) and focused (Multiphor II isoelectric focusing apparatus, GE Healthcare). The strips were incubated in reducing (50 mM Tris pH 6.8, 2% SDS, 15% glycerol, 6 M urea, and 1% DTT) and alkylating (50 mM Tris pH 6.8, 2% SDS, 15% glycerol, 6 M urea, 1.25% iodoacetamide, and 0.05% bromphenol blue) buffers, and then proteins were size separated by SDS-PAGE using 9–16% polyacrylamide gels. Each gel was scanned with three lasers (Typhoon 9400, GE Healthcare) to collect the Cy2, Cy3, and Cy5 images within 4 h of completion of electrophoresis.

Gels used for spot picking were prepared as described above, except that an unlabeled reference sample was used, and the SDS-PAGE gels were poured onto a bind-silane (PlusOne, GE Healthcare)-treated plate. After electrophoresis, these “pick” gels were fixed in 10% methanol and 7.5% acetic acid, stained in SYPRO Ruby gel stain (Bio-Rad, Hercules, CA) overnight, and fully destained in 10% methanol-chloroform and then resuspended as previously described (22). For first dimension protein separation, resuspended samples were absorbed overnight onto Immobiline DryStrips (pH 3–10 NL, 18 cm; GE Healthcare) and focused (Multiphor II isoelectric focusing apparatus, GE Healthcare). The strips were incubated in reducing (50 mM Tris pH 6.8, 2% SDS, 15% glycerol, 6 M urea, and 1% DTT) and alkylating (50 mM Tris pH 6.8, 2% SDS, 15% glycerol, 6 M urea, 1.25% iodoacetamide, and 0.05% bromphenol blue) buffers, and then proteins were size separated by SDS-PAGE using 9–16% polyacrylamide gels. Each gel was scanned with three lasers (Typhoon 9400, GE Healthcare) to collect the Cy2, Cy3, and Cy5 images within 4 h of completion of electrophoresis.

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Quantitative analysis of 2D gels. A total of 24 analytical gels containing data from 48 individuals, plus 5 SYPRO Ruby stained “pick” gels were evaluated with DeCyder 2D 7.0 software (GE Healthcare). The Cy2 gel containing the most protein spots was designated the master gel, and spots in the remaining 23 Cy2 images and 5 pick gels were matched to it using DeCyder’s Biological Variation Analysis (BVA) Module. We first manually landmarked ~275 spots then applied the automated spot matching feature. Each Cy3 or Cy5 spot intensity value was normalized to its respective Cy2 internal reference spot value.
Random Forests (9), an unsupervised machine learning classification method, was applied to cluster individual samples based solely upon protein spot abundance variation and to identify the protein spots that best discriminated the clusters. The initial analyses considered all spots present in every sample, i.e., 6/6 samples per group. Subsequent Random Forests were run with the top 6–10 classifiers from the top 20 identified by the initial analyses. The Random Forests that used the fewest spots to give the greatest biological separation of individual samples into clusters were considered the best. Random Forests were generated in R (64), n = 50,000 classification trees.

To identify the proteins that changed significantly between the summer homeothermic (SpD and SA) and winter heterothermic [interbout arousal (IBA), entrance (Ent), late torpor (LT), and early summer homeothermic (SpD and SA)] groups, a student’s two-tailed t-test followed by a Benjamini-Hochberg false discovery rate (FDR) correction (6) was performed on spots present in 5/6 individuals per group across all groups. These same spots were evaluated by a one-way ANOVA analysis followed by FDR correction. Post hoc Tukey pairwise comparisons were performed for all spots significant by one-way ANOVA. All tests were performed in R (64) with a set to 0.05.

Spot picking and mass spectral analysis. Protein spots comprising the 20 most important classifiers from the Random Forests were identified by tandem mass spectrometry, as were those significant by either t-test or ANOVA. Spots were robotically picked (1.4 mm diameter, 1.5 mm deep head) and digested from three pick gels using generated in R (64), identified by the initial analyses. The Random Forests that used the top 6–10 classifiers from the top 20 were run with the collision gas. An in-house script was used to create deisotoped centroided peak lists from the raw spectra (.mgf format). The spectral data were then analyzed with Spectrum Mill as described above. All of the resulting peptide data were combined to account for multiple MS/MS runs and orthologous identifications using an in-house program, ExtracTags (23). Spots were considered unambiguously identified if they returned an overall score ≥30 and contained ≥2 peptides for only one protein. If multiple proteins were identified in a spot but one (with ≥2 peptides) had 4× greater spectral intensity than any other, this was also accepted as the unique ID, as previously described (46). The resulting peptide data are available in Supplemental Table S1. To eliminate ambiguity inherent with protein nomenclature, we generally refer to proteins by their unambiguous gene ID throughout the text and figures; both protein name and gene symbol appear in Supplemental Table S2.

Biological pathway classification. To identify meaningful biological components underlying the cycles of hibernator heart physiology, we used the functional annotation clustering tool in DAVID (Database for Annotation, Visualization and Integrated Discovery; 34). Official gene symbols of identified proteins were submitted to the DAVID database; functional annotation clusters (FACs) were generated using a Homo sapiens background and clustering stringency set to “high,” with default settings for all other parameters. The Entrez Gene database at NCBI was used to assign functions to individual genes.

2D Gel Analysis of Phosphoproteins

Phosphoprotein abundance in heart extracts from IBA, Ent, LT, and E-Ar samples (n = 3 per state) were evaluated on 2D gels as above, except that 90 μg (unlabeled) were separated on a single gel. After electrophoresis, the free-floating gels (12 total) were fixed overnight (50% methanol, 10% acetic acid), then stained in 1/3 diluted Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR) for 2 h, destained 4× (50 mM sodium acetate, pH 4.0, 20% acetonitrile) and scanned (1). The gels were then stained in SYPRO Ruby for >24 h, destained in 10% methanol and 7.5% acetic acid, and resanned for total protein.

The Pro-Q Diamond phosphoprotein and SYPRO Ruby total protein 2D gel images were uploaded into DeCyder’s BVA module for spot matching to the Cy2 experimental gel images. Because the phosphoprotein-stained gels did not reveal every protein spot seen in the SYPRO or Cy2 gels, each Pro-Q Diamond 2D gel and its corresponding SYPRO Ruby gel image were overlaid in Adobe Photoshop, which served as a visual guide during matching in DeCyder. Overlay images were generated in Adobe Photoshop (CS3 Extended; Adobe Systems, San Jose, CA).

Western Blotting

Heart protein samples (30 μg) from all eight groups (Fig. 1, n = 3 per group) were denatured in 1× Laemmli sample buffer and separated by electrophoresis through 15% polyacrylamide gels. Proteins were transferred to PVDF membranes (Immobilon-FL, Millipore, Bedford, MA), stained with Memcode protein blot stain (Pierce, Rockford, IL), and then scanned to quantify total protein loading. Blots

1 The online version of this article contains supplemental material.
were blocked for 1 h (Odyssey blocking buffer; LI-COR, Lincoln, NE), and incubated overnight at 4°C in both anti-cofilin 2 (CFL2, 1:5,000, goat polyclonal; Abcam, Cambridge, MA) and anti-phospho-cofilin 2 (P-CFL2, 1:500, rabbit polyclonal; Upstate, Lake Placid, NY) primary antibodies. This was followed by 50 min incubation in secondary antibodies: anti-rabbit IgG (1:20,000, IRDye 800CW conjugated; LI-COR) and anti-goat IgG (1:30,000, IRDye 680LT conjugated; LI-COR). Protein bands were visualized using the Odyssey near-infrared imaging system (LI-COR) and analyzed with ImageQuant TL software (GE Healthcare). To correct for inconsistencies in protein loading, band intensities were normalized to the lane containing the greatest protein pixel volume. Bands were further normalized by setting the most intense band for each antibody in each blot to 100. A one-way ANOVA followed by a Tukey post hoc test was used to assess both total CFL2 and P-CFL2 for significant abundance differences among the groups.

RESULTS

Protein Spot Separation and Matching

Proteomic changes in the heart of 13-lined ground squirrels from eight stages across their circannual hibernation cycle were assessed by 2D DiGE. A representative 2D pick gel showing the separation of heart protein spots is presented in Fig. 2. Of the analytical gels (24 total), the “master” gel contained 2,080 Cy2 spots and was used to match spots in all of the other Cy2 gel images. A total of 278 spots were matched in all 24 analytical gels and used for Random Forests. For t-tests and ANOVA statistical analyses, 432 spots were used that matched in at least 5/6 samples per group for all sampling groups.

**Top Discriminating Proteins for Unsupervised Group Classification**

A striking seasonal separation between the summer homeothermic (SpD and SA) and winter heterothermic (IBA, Ent, LT and E-Ar) groups was revealed by Random Forests classification (Fig. 3A). However, our a priori defined groups within each season (Fig. 1) were not clearly distinguished. Thus, these data define a seasonal heart proteome with distinct summer and winter components. When the two fall groups, fall torpor (FT) and fall active (FA), were added to the analysis, a separation between the summer and fall active (SpD, SA, FA) versus the winter groups (IBA, Ent, LT, E-Ar) was apparent (Fig. 3B). It is noteworthy, however, that 4/6 FT individuals clustered between summer and winter (Fig. 3B); these four were torpid with T_b at 4 – 6°C when the hearts were collected. The remaining two FT samples, taken from squirrels with T_b at 37 or 19°C, clustered adjacent to FA and closer to the homeothermic individuals, indicating that T_b as well as seasonal components define the fall cardiac proteome in 13-lined ground squirrels. The proteome of the four FT individuals that fell between the summer and winter clusters may be truly distinct or comprise proteins characteristic of both.

We examined the top 20 discriminating protein spots of the Random Forests classification and determined that using only the top six protein spots: 1) 1727, CFL2; 2) 161, heat shock 70 kDa protein 4 or HSPA4; 3) 750, dihydrolipoyl transacylase or DBT; 4) 1210, unidentified; 5) 1486, apolipoprotein A-I or APOA1; and 6) 1878, transthyretin or TTR (listed in order of
importance; Fig. 4, A–F;) best clustered the summer and winter seasonal groups in the absence of fall (Fig. 3C). Using this limited dataset, we could also visualize distinct clusters that contained the IBA and LT states in addition to the seasonal separation seen by the initial analysis. When the fall samples were included (Fig. 3B), the best clustering occurred using the top eight protein spots (Fig. 3D). In this analysis, 5/6 FT individuals clustered together; the lone outlier was the individual with Tb at 37°C, again highlighting the importance of Tb in addition to season. The FA samples were also better separated from SA and SpD, and E-Ar clustered apart from the remaining winter groups. Although differing slightly in their order of importance, six of the top eight discriminating proteins for the Random Forests analysis including fall states were the same as in the analysis without fall. Not surprisingly, the abundance variation for several of these proteins supports the seasonal summer-winter switch, which is the dominant feature of these data. The remaining two discriminating proteins underlie the improved clustering of the fall groups. These eight discriminating protein spots were, in order of importance: 1) 1727, CFL2; 2) 750, DBT; 3) 1210; 4) 1878, TTR; 5) 1486, APOA1; 6) 1314, 14-3-3 protein or YWHAQ; 7) 1904, heart fatty acid-binding protein or FABP3; and 8) 161, HSPA 4 (Fig. 4).

The top discriminating proteins for both of these analyses showed several different abundance level patterns among the groups, including seasonal changes in the proteins HSPA4, DBT, TTR, and FABP3 (Fig. 4, B, C, F, and H; mean fold changes are listed in Supplemental Table S2) as well as torpor-arousal cycle changes in CFL2, HSPA4, spot 1210, APOA1, and YWHAQ (Fig. 4, A, B, D, E, and G; Supplemental Table S3). The fall groups displayed intermediate patterns, such as protein spot abundance similar to SA and SpD but different from the winter groups (e.g., DBT; Fig. 4C) or similarity to winter groups but not to summer groups (e.g., FABP3; Fig. 4H). Finally, for CFL2, APOA1, and TTR, (Fig. 4, A, E, F) relative abundance levels were different between FA and FT. Such shifts in protein spot abundance among sampling states were therefore responsible for the separation and clustering of the two seasonal groups, the FT samples, and the limited intrawinter separation that was observed.

Seasonal Changes: Homeothermy vs. Heterothermy

Because the Random Forests prominently classified the data by season, a Student’s t-test was used to discern protein spots with an overall difference between the homeothermic (summer: SpD and SA) and heterothermic (winter: IBA, Ent, LT, and E-Ar) states. Significant seasonal changes occurred in 46 spots (q < 0.05, Fig. 5). Of these, the majority of spots (34 spots) were winter elevated (28 of these were identified with LC-MS/MS, 19 of these were unique proteins). Of the 12 summer-elevated protein spots, nine were identified as a single ID in the protein spot (Supplemental Table S2).
Seasonally elevated proteins were functionally clustered using DAVID. The summer-elevated proteins returned four functional annotation clusters (FACs): two were related to the mitochondrion, the other two terms were tricarboxylic acid cycle enzyme complex and lipoic acid binding. The valine, leucine, and isoleucine degradation pathway was also significantly summer-enriched ($P < 0.001$). This indicates that catabolism of essential branched-chain amino acids is downregulated during the hibernation season, a time when amino acid dietary intake ceases. FACs for the winter elevated proteins included the chaperonin-containing T-complex and fatty acid metabolism, indicating that pathways involved in protein folding and energy generation from fat oxidation are overrepresented in the winter cardiac phenotype.

Annual Changes and Fall Transition

We expanded our analysis to identify spots that were significantly affected by both seasonal and physiological state using all eight sampling groups, including FA and FT. A one-way ANOVA detected significant changes in 82 spots. Tukey’s post hoc tests revealed that the majority of statistically significant changes occurred between the non-hibernating (SpD, SA) and hibernating groups (IBA, Ent, LT, E-Ar), while relatively fewer changes occurred among these groups (Table 1; see Supplemental Table S4 for Tukey $P$ values of all pairwise comparisons). In agreement with the Random Forests analyses, FT differed from both groups and FA was more similar to the summer groups.

When picked for protein identification (Fig. 2), 70 of the 82 ANOVA significant ($q < 0.05$) spots were unambiguously identified, while 11 spots contained multiple proteins and one spot was not identified (Supplemental Table S1). The 70 positively identified spots (Supplemental Table S2) represented 50 unique genes.

These 50 unique proteins were clustered using DAVID to highlight functional pathways that alter across the circannual rhythm of a hibernator. DAVID returned 17 FACs with an enrichment score $> 1.3$ (Table 2). Many of the FACs included terms relating to metabolism such as mitochondrion, tricarboxylic acid cycle and lipid metabolism, demonstrating the adjustments made to the energy-generating pathways in the heart throughout the hibernator’s year. Additionally, the terms “chaperone” and “response to heat stress” point to stress response or protein folding pathways as important for phenotypic changes that characterize the hibernator’s heart. A stepwise analysis of significant Tukey pairwise protein differences that occur with each transition of the seasonal and torpor-arousal cycle (Fig. 6) not only provides further evidence that most proteins are regulated seasonally but also places these changes in the context of the full circannual rhythm of hibernation. For example, the abundances of heat shock proteins HSP90AB1 (heat shock protein 90-β), HSPA4, HSPB6 (heat shock protein β-6), and the protein chaperone CCT7 (T-complex protein 1 subunit η) all increased in the transition from summer (SA) to winter (IBA) and then decreased in the transition back to post-hibernation (IBA to SpD). Additionally, proteins involved in branched chain amino acid catabolism decreased in the transition to winter (SA to IBA; proteins DBT and DLD, dihydrolipoyl dehydrogenase) and increased in the transition back to summer (IBA to SpD; proteins DBT and BCKDHB, 2-oxoisovalerate dehydrogenase β). Protein levels altered in the fall as well; plasma transport proteins APOA1,
ALB (albumin), TTR, and TF (transferrin) increased from FA to FT but fell in the transition to winter heterothermy (FT to IBA; proteins ALB, TTR, and TF). Finally, the proteins CRAT (carnitine O-acetyltransferase) and ECI1 (dodecenoyl-CoA isomerase), both involved in fatty acid oxidation, increased from SA through FT to IBA, remained elevated throughout the winter, and then subsequently decreased in the transition back to summer homeothermy (IBA to SpD). This visualization of significant pairwise comparisons confirmed the results of our seasonal t-test and Random Forests analyses: expression of chaperone and fatty acid metabolic proteins increased during the hibernation season while proteins involved in branched chain amino acid catabolism were concurrently downregulated. Furthermore, the FT group displayed a unique proteome of which plasma transport proteins comprised a substantial component.

CFL2

CFL2 (spot 1727) was selected for further analysis because it was the top discriminating protein for the Random Forests and showed the greatest fold change of all protein spots (~7-fold; Supplemental Table S3). CFL2 also had the lowest q value of all spots examined (Supplemental Table S2). Phospho-staining indicated that spot 1727 (CFL2) was phosphorylated (Fig. 7A). We confirmed this result with Western blotting for both P-CFL2 and total CFL2. While total CFL2 was

Table 1. Counts of significant pairwise comparisons reveal that most protein adjustments occur between summer homeothermy and winter heterothermy

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All significant Tukey tests (P < 0.05) for protein spots (n = 82) significant by ANOVA (q < 0.05) were counted. SpD, spring dark; SA, summer active; FA, fall active; FT, fall torpor; IBA, interbout aroused; Ent, entrance; LT, late torpor.
similarly abundant in all states, P-CFL2 decreased to near nondetectable levels in the FT, LT, and E-Ar samples (Fig. 7B). A one-way ANOVA to compare normalized band intensities confirmed that P-CFL2 fluctuated significantly among sampled states \( (F = 8.06, P = 0.0003) \), while total CFL2 was unchanged \( (F = 0.73, P = 0.65; \text{Fig. 7C}) \). Furthermore, the pattern among the sample groups of relative P-CFL2 protein abundance detected by Western blotting mimicked the pattern obtained by 2D DiGE (Fig. 7C). Hence, we concluded that spot 1727 was the phosphorylated form of CFL2.

**DISCUSSION**

The present study quantified protein differences among eight distinct seasonal and physiological states in the heart of the 13-lined ground squirrel using 2D DiGE proteomics. We anticipated detection of proteins that contribute to the maintenance of cardiac function during winter heterothermy. Specifically, we hypothesized the identification of summer-winter seasonal proteomic changes that protect the heart during the low \( T_b \) of torpor and rapid rewarming of arousal and, by virtue of our unique winter season sampling strategy, recovery of at least some components of the torpor-arousal switch.

The dominant proteomic signature of the hibernator’s heart that emerged from this analysis is one of a seasonal shift between the homeothermic (nonhibernating) and heterothermic (hibernating) portions of the year with relatively few changes detected within the hibernation cycle. This finding is in line with two previous heart screening studies that compared at least two heterothermic states and a homeothermic state (70, 72). The relative constancy of the cardiac proteome throughout the hibernation cycle is also consistent with the fact that the heart must continually function throughout torpor-arousal cycles. Moreover, new protein synthesis is an inefficient way to regulate heart function during the hibernation cycle, especially during its dramatic increase in activity from torpor to arousal.

### Table 2. **DAVID FACs identify metabolism and energy generation as pathways having circannual regulation in 13-lined ground squirrels**

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Enrichment Score</th>
<th>Annotations, ( n )</th>
<th>Genes, ( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrion</td>
<td>20.24</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>Mitochondrial lumen</td>
<td>15.28</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>Organelle lumen</td>
<td>7.82</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>Tricarboxylic acid cycle</td>
<td>7.59</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Glucose catabolic process</td>
<td>4.87</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Lipoic acid binding</td>
<td>4.65</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Oxidoreductase activity</td>
<td>4.41</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Mitochondrial alpha-ketoglutarate dehydrogenase complex</td>
<td>3.78</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>3.05</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>Mitochondrial membrane</td>
<td>2.56</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>NAD or NADH binding</td>
<td>2.27</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>1.76</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Cytoplasmic vesicle</td>
<td>1.69</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Macromolecular complex assembly</td>
<td>1.53</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>ATP binding</td>
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<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Chaperone</td>
<td>1.44</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Response to heat</td>
<td>1.42</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Functional Annotation Clusters (FACs) with an enrichment score \( \geq 1.3 \) are listed for the 50 unique proteins found significant by ANOVA \( (q < 0.05) \) and identified by mass spectrometry. For each enriched FAC, the cluster term that provided the best biological meaning is presented.

Peak metabolic rate in the heart occurs mid-arousal and is estimated to be \( 10^x \) greater than in eutheria (32). As the animal is fully committed to rewarming during this period, heart rate is maximal in order to support whole body reperfusion (44). ATP generation and use may be completely directed at maintaining ion homeostasis and heart contractility during arousal instead of synthesizing and/or degrading proteins. Furthermore, initiation of protein translation is inhibited at \( T_b \) as low as \( 18^\circ C \) (67), implying that de novo synthesis of any protein required to regulate heart function must occur during the IBA and Ent states. Yet, our pairwise Tukey tests revealed few differences among any of the winter states. Enhanced protection from protein degradation may be at least partly responsible for the constancy of the proteome throughout winter heterothermy, and the observed adjustments to relative protein spot abundance may in fact largely result from posttranslational modifications, a rapidly reversible and less energy-consuming form of protein regulation (11). The phosphorylation state of the winter cycling spot, 1727, or P-CFL2, was indeed responsible for explaining its robust change by DiGE. Regardless of whether synthesis, degradation, or posttranslational modifications is responsible for the detected spot changes in the hibernation cycle, it is important to note that only 10/432 spots differed significantly within winter, while a total of 82/432 spots differed among all groups. Taken together, the paucity of within-winter changes and the relatively large number of interseasonal changes in the heart proteome are consistent with the hypothesis that seasonal reprogramming of gene expression enables enhanced heart function during heterothermy (17, 26–27, 73), whereas sympathetic and parasympathetic innervation (47), likely signaling via posttranslational modifications of proteins to modulate activity, and \( Q_10 \) effects (29) act concertedly to facilitate the winter cycle.

**Fig. 6. Seasonal changes dominate the hibernator heart proteome.** Schematic representation of the 2 cycles of a hibernator’s year; the heart protein changes in each transition are labeled. Only identified proteins (indicated by gene symbols) that change significantly between states of the cycle \( (P < 0.05, \text{Tukey}) \) are shown. Large colored arrows reflect the state-to-state transitions within both annual and hibernation cycles. Small arrows show increased (↑) or decreased (↓) abundance for each transition. IBA is placed between the 2 cycles because it is the winter state that is the most physiologically similar to homeothermy and eliminates the confounding variable of reduced \( T_b \).
Metabolic Pathways

The cardiac proteomic cycles are dominated by representatives of pathways involved in metabolism (Fig. 6, Table 2). Functional annotation revealed overrepresentation of proteins related to the TCA cycle as well as to glucose and lipid metabolism. Our data support the well-documented enhancement of lipid metabolism during hibernation (reviewed by Refs. 2, 11) by a winter abundance increase in ECI1, CRAT, FABP3, and ACADVL (acyl-Coenzyme A dehydrogenase, very long chain) proteins. Additionally, OXCT1 (3-oxoacid-CoA transferase 1), which is involved in ketone catabolism, increased in winter, consistent with the study of Russeth et al. (57). Thus, our results agree with previous studies that highlight the fundamental importance of lipid metabolism as the main energy-generating source during hibernation.

Proteins involved in branched chain amino acid catabolism decreased in winter. Specifically, the catalytic subunits BCKDH (E1 subunit), DBT (E2 subunit), and DLD (E3 subunit) that compose the branched chain α-ketoacid dehydrogenase complex (BCKDC) were all reduced compared with summer. BCKDC is involved in the breakdown of leucine, isoleucine, and valine by catalyzing a reaction that commits these essential amino acids to their degradation pathways (33). Branched chain amino acids are only available in the diet, and BCKDC downregulation does occur in response to long-term low-protein diet in rats (74). Because the 13-lined ground squirrel does not eat during the hibernation season, a downregulation of the BCKDC complex in response to the elimination of protein intake would be expected in order to spare essential amino acids. In fact, this response has been previously noted in the liver (22) and plasma (21) of this species. Furthermore, the mRNA of BCKDHB was also found to decrease in the heart of torpid versus summer active Arctic ground squirrels (72).

Chaperone and Heat Shock Proteins

A controlled stress response is a key aspect of the heart proteome in hibernating 13-lined ground squirrels. Functional
annotation analysis of the protein spots that varied significantly by ANOVA for all sampling states highlighted the enrichment of FACs related to chaperone and heat stress responses. When protein spots that specifically increased in winter were examined, an enrichment of the chaperonin containing T-complex (abbreviated CCT or Tc; 61) was detected. This apparent strong reliance on chaperones during winter heterothermy may be a mechanism by which protein damage is reduced or prevented in the hibernator’s heart, thereby minimizing the requirement for new protein synthesis to replace degraded proteins.

The CCT is barrel-shaped and composed of two rings, each with eight subunits (65). All three of the CCT subunits recovered in our dataset, CCT5 (T-complex protein 1 subunit β; spot 526), CCT7 (spot 581), and TCP1 (T-complex protein 1 subunit α; spot 609), increased in hibernation, indicating their importance for winter cardioprotection. CCT folds a subset of nascent cytosolic proteins, many of which are essential to the cell, including actin and tubulin (65); it specifically folds proteins with complex topologies that are prone to aggregation or have slow folding kinetics (71). The detected winter seasonal increase of CCT may regulate the replenishment of required proteins during IBAs by preventing nascent protein aggregation and ensuring that slowly folding proteins reach completion during this brief euthermic stage.

Heart proteins may be further preserved in hibernation by the chaperone and antiapoptotic activities of a number of heat shock proteins elevated in the transition from SA to IBA (Fig. 6). The small heat shock protein HSPB6 (spot 1709) is related in several ways to improved cardiac function; it is associated with enhanced myocardial contraction (13) as well as protection against ischemia-reperfusion injury (25). Furthermore, HSPB6 inhibits apoptosis by binding and stabilizing the actin cytoskeleton and by directly interacting with the proapoptotic protein BAX (24). HSPA4 (spot 161), a member of the HSP110 family, is induced under stressful conditions such as ischemia (41), radiation (37), and hepatocellular carcinoma (30). HSPA4 may depress apoptosis by inhibiting protein aggregation but is unable to fold proteins itself (30). Instead, it binds and holds substrates for refolding as well as serving as a nucleotide exchange factor for HSP70 (68). We also detected a winter increase, including a significant pairwise increase from SA to LT, in HSP90AB1 protein content (spot 285). HSP90AB1 stabilizes proteins, especially those involved in signal transduction and protein secretion (5). Furthermore, it plays an antiapoptotic role by complexing with AKT (protein kinase B), which leads to downstream activation of antiapoptotic NF-κB (4). Carey et al. (12) observed fluctuations in NF-κB levels of hibernating ground squirrel intestinal mucosa, with this transcription factor constitutively activated throughout the torpor-arousal cycle. The increase of HSP90AB1 protein during hibernation may represent a component of the antiapoptotic NF-κB regulatory pathway, which is in turn an important aspect of cardiac cellular defense strategies. Interestingly, HSP90AB1 mRNA was reported to be significantly underexpressed in the hearts of torpid versus summer active Arctic ground squirrels (72).

The discrepancy between the mRNA and protein levels of this heat shock protein may represent bona fide species-specific differences but more likely serves to underscore the likely importance of mechanisms in addition to differential gene expression to modulate protein content and function throughout the hibernation cycle. Alternative mechanisms include protection of protein from degradation or posttranslational modifications for the regulation of HSP90AB1 specifically, but also as a general, low-energy means to achieve cellular protection in the hibernating heart.

Resistance to ischemia-reperfusion injury and the apoptosis that typically follows (60) is a remarkable feature of the hibernating phenotype. Despite some evidence for apoptotic pathway adjustments in the small intestine (e.g., Ref. 28), the regulation of apoptotic signaling pathways during hibernation is still poorly characterized (66). All three heat shock proteins that increased in our dataset during winter heterothermy, HSPB6, HSP90AB1, and HSPA4, have antiapoptotic properties. Moreover, we detected a winter seasonal increase in the antiapoptotic YWHAQ protein (i.e., spot 1314; Fig. 4G), which also binds to and sequesters BAX (50). These proteins suggest a link between ischemia-reperfusion tolerance and apoptosis inhibition, and further study of their functional roles in relation to hibernation is thus warranted. Nonetheless, our finding of heat shock and chaperone protein winter elevation along with previous reports of upregulated heat shock protein 27 (18) and several antioxidant peroxiredoxins (49) in hibernating ground squirrel hearts suggests that enhanced cardioprotection (15, 39) during heterothermy is a fundamental component of the winter phenotype.

CFL2

CFL2 is a muscle-specific protein that belongs to the actin depolymerization factor/cofilin family; it regulates actin dynamics by promoting actin filament turnover (51, 53). CFL2 binds actin; phosphorylation prevents actin binding (7). In hibernating ground squirrels, P-CFL2 is not detected during FT, LT, and E-Ar and only faintly detected during Ent (Fig. 7B). The quantity of total CFL2 remains unchanged (Fig. 7B), suggesting that the pool of CFL2 is mostly in its active form during these states. A similarly robust dephosphorylation of the cofilin pool occurs during conditions of ATP depletion and oxidative stress in cultured cells (63), neurons (48), and vascular smooth muscle (40). Moreover, Bernstein et al. (8) proposed that hyperactivating cofilin by dephosphorylation is a protective mechanism to preserve ATP as well as inhibit apoptosis during ATP-limited conditions such as ischemia. When the entire cofilin pool is dephosphorylated, it is sequestered into actin-cofilin rods; because cofilin is not able to participate in the ATP hydrolyzing activity of actin turnover, this sequestration prevents further declines in available ATP. Additionally, cofilin sequestration prevents its localization to the mitochondria where it plays a role in initiating apoptosis. Although the observed decrease of P-CFL2 in the hibernator’s hearts (Fig. 7) could indicate that ATP availability is limited during the entrance, torpid, and early arousal periods, others have shown that tissue ATP levels (43, 58, 75) and/or energy charge (10, 19) are maintained throughout hibernation. Furthermore, the hibernator’s heart is able to maintain ion homeostasis and contractility in the cold, therefore it is unlikely that ATP is depleted in the heart during these states. Instead, the dephosphorylation of CFL2 observed here likely represents a preprogrammed mechanism to maintain the energy charge and conserve ATP for essential functions during the depressed metabolic states of Ent, FT, and LT and during the E-Ar.
period, which is transiently ischemic (45). It may instead or in addition be a mechanism for the heart to suppress apoptosis during these states. We speculate that without dephosphorylation and sequestration of CFL2, ATP would deplete in the heart and compromise the maintenance of homeostasis.

**Fall Transition**

Two fall sampling groups were included in our proteomics screen: this is the first proteomics study that has specifically examined the seasonal transition undertaken by hibernators between homeothermy and heterothermy. The two-switch model of hibernation predicts that torpor-arousal cycles are only possible once the switch to a heterothermy-permissive winter physiology has occurred (58). Because the Tb of all squirrels classified as FT had previously reached at least 10°C, and 4/6 were torpid at the time of tissue collection, we hypothesized the FT animals would have switched to the winter proteome. Instead, Random Forests clustering and ANOVA revealed FT to be an intermediate phase between summer and winter. The euthermic FT squirrel clustered near the FA and homeothermic groups, and the five FT animals with lower Tb moved toward but did not merge with the winter groups (Fig. 3D). This result indicates that FT is neither analogous to SpD, SA, or FA nor to any of the winter groups. Furthermore, Tukey pairwise comparisons revealed numerous spot abundance differences between FT and sampling states considered both homeothermic (SpD and SA) and heterothermic (IBA, Ent, and LT; Table 1), which supports the idea that FT is not biochemically identical to either season.

The intermediate nature of the two fall sampling states further implies that the transition from summer to winter requires a protracted and perhaps sequential reprogramming phase rather than a simple rapid switch. This phenotypic transition period is evidenced by specific spot abundance patterns. For example, levels of DBT (Fig. 4C) and YWHAQ (Fig. 4G) were comparable between the fall (FA, FT) and summer (SA, SpD) groups. Similar patterns were noted for OXCT1 (spots 691, 692, and 700), PDHX (pyruvate dehydrogenase protein X component, spot 748), PARK7 (parkinson-associated protein 7, spot 1660), PCCA (propionyl-CoA carboxylase alpha component, spot 748), MDH1 (cytosolic malate dehydrogenase, spot 1162), ECI1 (spot 1388), and CRAT (spot 524) (Supplemental Table S3). The involvement of CRAT and ECI1 in fatty acid catabolism and of OXCT1 in ketone body catabolism suggests that the seasonal switch to enhanced lipid metabolism is not yet complete in the heart when animals initiate their first torpor bout. In contrast, the proteins FABP3 (Fig. 4H) and PDP1 (pyruvate dehydrogenase phosphatase, spot 643; Supplemental Table S3) display comparable levels between fall (FA, FT) and winter (IBA, Ent, LT, and E-Ar) states. Because FABP3 promotes intracellular transport of long-chain fatty acids and other hydrophobic moieties (62), the heart is poised for increased delivery of fatty acid substrates to mitochondria or peroxisomes, although their subsequent catabolism is not fully optimized for winter. Increased FABP3 in the fall would also support transport of n-6 polyunsaturated fatty acids (PUFA) as they accumulate in heart membrane phospholipids by a diet-independent mechanism during this period (3). This appears to involve a seasonal redistribution of n-6 PUFA from white adipose tissue to heart; elevated n-6 PUFA are hypothesized to boost the activity of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase SERCA2a at low temperature (55), which in turn leads to enhanced reuptake of Ca\(^{2+}\) from the cytoplasm at low Tb (42). Protein differences between FA and FT were also observed, including P-CFL2 (Fig. 4A), APOA1 (Fig. 4E), and TTR (Fig. 4F). In particular, the plasma proteins APOA1 (spots 1449, 1476, and 1486), ALB (spots 423, 436, and 462), TF (spot 333), TTR (spot 1878), and A2M (α2-macroglobulin, spot 105) were uniformly more abundant in the cardiac proteome of FT versus FA animals. As some of these plasma proteins have transport roles, e.g., fatty acid transport by ALB, thyroid hormone and vitamin A by TTR, and cholesterol with APOA1, it is possible that augmenting the ability to manage and transport lipids precedes the switch to fatty acid catabolism characteristic of winter heterothermy. However, we cannot rule out the possibility that the detection of plasma proteins in these tissue samples may result from a perfusion artifact that is enhanced when animals are at low Tb. Investigations of heart metabolites would reveal if cholesterol and fatty acid levels are indeed elevated during this transition period.

Finally, the gradual nature of the fall transition is revealed by those proteins that demonstrated incremental adjustments in abundance from summer to winter via the two fall sampling periods. For example, HSPA4 (Fig. 4B) increased steadily from summer to winter, perhaps in response to mounting cellular stress encountered in the heart during the short fall torpor bouts (56). In contrast, BCKDHB (spot 1144) declines steadily through the fall transition, likely a result of eliminating dietary protein intake with the onset of fasting. As this is the rate-limiting subunit (E1) of the BCKDC complex (74), its abundance likely reflects the actual decline of BCKDC activity in the catabolism of branched-chain amino acids.

**Conclusions and Perspectives**

Our study revealed that the heart proteome underlying summer homeothermy differs markedly from that supporting winter heterothermy. The findings are consistent with a need to establish fully a winter protected phenotype before prolonged multiday torpor bouts ensue. Remarkably, despite dramatic oscillations in temperature and heart and metabolic rates over the torpor-arousal cycle, very few protein changes were detected among winter heterothermic states. Yet the maintenance of ATP homeostasis stands out in this dataset. Because the heart must function in both the metabolically depressed state of torpor and the oxygen-limited but metabolically intense rewarming during arousal, available ATP resources are likely prioritized for essential functions such as contractility and ion homeostasis. The general lack of protein abundance changes detected across the torpor-arousal cycle provides evidence that use of ATP for protein degradation and resynthesis is limited; the increased abundance of chaperone and heat shock proteins preserve existing protein integrity and protect against apoptosis. An additional example of an ATP conserving mechanism in the heart was the near complete dephosphorylation of CFL2 in all of the hibernation states except IBA. Dephosphorylated CFL2 conserves ATP by limiting actin turnover and illustrates a strategy for acute posttranslational protein regulation. Enhanced ATP conservation facilitates the ability of the hibernator’s heart to maintain function under metabolically depressed and/or oxygen-limited conditions. Moreover, the unique car-
dioprotected physiology of hibernators may be derived through a novel form of preconditioning, initiated by endogenous mechanisms in a constant environment (e.g., FA and FT animals, Fig. 4H). This preconditioning is modulated (Fig. 4A) and amplified (Fig. 4B) by short torpor bouts in fall (56) prior to achieving the deep extended torpor of hibernation. Therefore, the initial fall torpor bouts may be particularly stressful to the heart. Further study of this intermediate phase is imperative to characterize the mechanisms that permit survival of prolonged torpor in hibernators.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES