Transcriptomic Analysis of Brown Adipose Tissue across the Physiological Extremes of Natural Hibernation

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Abstract

We used RNAseq to generate a comprehensive transcriptome of Brown Adipose Tissue (BAT) over the course of a year in the naturally hibernating thirteen-lined ground squirrel, Ictidomys tridecemlineatus. During hibernation ground squirrels do not feed and use fat stored in White Adipose Tissue (WAT) as their primary source of fuel. Stored lipid is consumed at high rates by BAT to generate heat at specific points during the hibernation season. The highest rate of BAT activity occurs during periodic arousals from hypothermic torpor bouts, referred to as Interbout Arousals (IBAs). IBAs are characterized by whole body re-warming (from 5 to 37 °C) in 2-3 hours, and provide a unique opportunity to determine the genes responsible for the highly efficient lipid oxidation and heat generation that drives the arousal process. Illumina HighSeq sequencing identified 14,573 distinct BAT mRNAs and quantified their levels at four points: active ground squirrels in April and October, and hibernating animals during both torpor and IBA. Based on significant changes in mRNA levels across the four collection points, 2,083 genes were shown to be differentially expressed. In addition to providing detail on the expression of nuclear genes encoding mitochondrial proteins, and genes involved in beta-adrenergic and lipolytic pathways, we identified differentially expressed genes encoding various transcription factors and other regulatory proteins which may play critical roles in high efficiency fat catabolism, non-shivering thermogenesis, and transitions into and out of the torpid state.

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Introduction

Almost all mammals possess Brown Adipose Tissue (BAT) as infants to help maintain their body temperature through nonshivering thermogenesis. Unlike White Adipose Tissue (WAT), which serves as the main fat storage depot in children and adults, BAT has evolved to generate heat in newborn and hibernating mammals (reviewed in [1]). Mammals that hibernate retain BAT as adults in order to efficiently re-warm themselves when arousing from hypothermic torpor. The discovery of BAT in adult humans [2-5] has generated excitement in the biomedical community because of its potential for accelerating weight loss in obese individuals [6-8]. However very little is known about the genes required for optimal BAT function.

In this study we examined BAT gene expression in a naturally hibernating mammal, the thirteen-lined ground squirrel (Ictidomys tridecemlineatus). During 5 months of hibernation these animals do not eat and use fat stores as their primary source of fuel. Stored lipid is consumed at high rates by BAT to generate heat at specific points during the hibernation season. The highest rate of BAT activity occurs during multiple arousals from hypothermic torpor bouts referred to as Interbout Arousals (IBAs). IBAs occur every 1-2 weeks and are characterized by rapid whole body re-warming and a resumption of normothermic metabolic activity for 12-24 hours (Figure 1). IBAs also provide a unique opportunity to determine the molecular factors involved in BAT that are responsible for the highly efficient fat burning activity that drives the arousal process. During the 2-3 hour transition out of torpor, O₂ consumption increases 50-fold, heart rate explodes from less than 10 beats per minute (bpm) to over 400 bpm, and body temperature increases from 5 to 37°C [9].

Lipid catabolism by BAT in natural hibernators is likely the highest capacity oxidation of fat (per mass of tissue) that occurs in mammals. With multiple mitochondria that uncouple the electron transport chain from ATP synthesis, and a high density of capillaries to deliver O₂, BAT has evolved to maximize the combustion of fat to generate heat in a short amount of time [1]. Despite the inherent advantages of knowing
how this fat-burning machine works, there is little information on the genes that control this process. In this study we identified genes that are expressed in BAT by purifying BAT RNA at four distinct phases of the hibernation season followed by Illumina sequencing of the corresponding cDNAs to profile gene expression at each phase.

**Results**

Total RNA was prepared from BAT during various activity states at four different times of the year: April active, October active, January torpid, and interbout arousal (Figure 1). Illumina HighSeq 2000 sequencing of cDNAs derived from 24 animals resulted in 179,979,787 high quality reads of 100 bases each. For comparison purposes we also obtained a total of 110,399,607 high quality reads of 76 bases each from WAT resulting in a far greater percentage than seen in other ground squirrel tissues (Table 1).

The twenty most highly expressed BAT transcripts based on the mean of the upper-quartile normalized counts of reads from the four collection points are shown in Figure 2 along with their related counts in WAT. Most of these are mitochondrial-targeted proteins or involved in beta-adrenergic and lipolytic pathways. Figure 3 shows the seasonal expression of four genes that are highly enriched in BAT versus WAT including the massively expressed G-protein subunit \( \text{G}_\alpha \) (GNAS), transcription factor \( \text{MEF}2\text{D} \), adenylate cyclase 3 (\( \text{ADCY}3 \)), and phosphorylase, glycogen, liver (\( \text{PYGL} \)).

To determine genes that are differentially expressed across the four collection points, the test statistic \( p \)-values computed by DESeq for each set of genes were independently filtered [14] by restricting to those with a mean of at least 100 normalized counts at one collection point, and with a 50% change between the means of any two collection points. After this filtering, there were 2,631 candidate genes that were differentially expressed in BAT. The Benjamini-Hochberg algorithm [15] with a false discovery rate of 0.05 was used to select the final set of 2,083 differentially expressed genes in BAT (Table S2).

**Mitochondrial-targeted proteins**

Due to the abundance of mitochondria in BAT, the transcriptome is greatly enriched in mRNAs from the mitochondrial genome (Table 1) and the nuclear genome encoding mitochondrial-targeted proteins. Out of 835 nuclear genes identified as coding for mitochondrial proteins, 170 were differentially expressed in BAT. Table 2 shows some of the differentially expressed genes encoding mitochondrial proteins such as the thermogenic uncoupling protein 1 (\( \text{UCP}1 \)), acyl-
CoA synthetase long-chain family member 1 (ACSL1), pyruvate dehydrogenase kinase, isozyme 4 (PKD4), carnitine/acylcarnitine translocase (SLC25A20), and carnitine palmitoyltransferase 1A (CPT1A) and 2 (CPT2).

Examples of differentially expressed genes coding for mitochondrial proteins that are more highly expressed during torpor and/or IBA are shown in Figure 4. One of the most dramatic of these is KMO (kynurenine 3-monooxygenase), whose expression is ten times higher in torpor and IBA compared to April. KMO catalyzes the first step in the conversion of kynurenine to quinolinic acid - a precursor of the hypothalamus, and cortex).

Figure 2. Twenty most abundant mRNAs in ground squirrel BAT (black) shown along with their respective levels in WAT (gray). Measurements shown on the x-axis are the mean of the upper-quartile normalized counts of reads from the four collection points. Abbreviations: ACeT2A2, acetyl-CoA acyltransferase 2; ACADL, acyl-CoA dehydrogenase, long chain; ACADVL, acyl-CoA dehydrogenase, very long chain; ADCK3, aarF domain containing kinase 3; ACO2, aconitase 2; ATP5A1, ATP synthase, alpha subunit 1; ATP5B, ATP synthase, beta polypeptide; CLASP2, cytoplasmic linker redox cofactor nicotinamide adenine dinucleotide (NAD) used mitochondrial proteins that are more highly expressed during torpor and IBA are shown in Figure 4. One of the most dramatic of these is KMO (kynurenine 3-monooxygenase), whose expression is ten times higher in torpor and IBA compared to April. KMO catalyzes the first step in the conversion of kynurenine to quinolinic acid - a precursor of the hypothalamus, and cortex).

Figure 3. Seasonal expression of four genes that are highly enriched in BAT versus WAT. Error bars represent standard error of the mean. Abbreviations: ADCY3, Adenylate cyclase 3; GNAS, G-protein subunit G; MEF2D, myocyte enhancer factor 2D; PYGL, phosphorylase, glycogen, liver. doi: 10.1371/journal.pone.0085157.g003

Adrenergic signaling

Adrenergic signaling controls high-throughput lipolysis and ultimately heat-generation in BAT (reviewed in [1]). Beta-adrenergic receptor genes ADRB1 and ADRB3 show opposite patterns of expression, with ADRB3 much higher in April (Figure 5). Changes in hibernator adrenergic receptor type are discussed in Kramarova et al. [18]. The beta-adrenergic receptors are coupled to the G-protein stimulatory subunit GNAS. Although not differentially expressed, GNAS was expressed at extremely high levels at all time points (Figure 3) with the highest number of reads of any mRNA in BAT. Activation of natriuretic peptide receptors can induce effects similar to the beta-adrenergic pathway [19]. It is interesting that all three natriuretic peptide receptor genes are expressed in BAT (NPR1, NPR2, NPR3) at levels similar to the beta-adrenergic receptor genes, but only NPR3 is differentially expressed, with higher values in torpor and IBA (Table S2).

Once activated by the beta-adrenergic receptors, GNAS stimulates the production of cAMP by activating membrane-bound adenylyl cyclases. We found several types of adenylyl cyclase genes are expressed in BAT: ADCY3 (62% of ADCY mRNAs), ADCY6 (24%), ADCY4 (5%), ADCY9 (5%), ADCY10 (2%), and very small transcript levels of ADCY5, ADCY7, ADCY8, and ADCY2. Only ADCY4 is differentially expressed, with the highest expression in April. ADCY3 is very BAT specific (Figure 3), with low expression in the other tissues available for comparison (heart, skeletal muscle, WAT, hypothalamus, and cortex).

The cAMP produced by the adenylyl cyclases activates protein kinase A, which is composed of the catalytic subunit PRKACA and the regulatory subunit PRKAR2B. Genes for both of these protein kinase A subunits are differentially expressed with highest levels of expression in torpor and IBA (Figure 5). cAMP is converted back to AMP by phosphodiesterases. Several phosphodiesterase genes were differentially expressed in BAT, most abundant of which were PDE3B.
lipolysis, glycogen utilization, and lipid transport

The catalytic subunit of protein kinase A, PRKACA, phosphorylates and activates PYGL (glycogen phosphorylase, liver), PNPLA2 (patatin-like phospholipase domain containing 2, also known as ATGL) and LIPE (hormone sensitive lipase). PYGL transcripts are abundant (Figure 3) although not differentially expressed. PYGL breaks down glycogen to produce glucose, which is required to support increased nonshivering thermogenesis in BAT [22]. PNPLA2 liberates the first free fatty acid from triglycerides, and the PNPLA2 gene is differentially expressed with highest values in torpor and IBA (Figure 6). LIPE cleaves the next fatty acid from the diacylglycerol; it has high mRNA levels in BAT, but is not differentially expressed.

Transcripts for perilipins PLIN2, PLIN4, and PLIN5, which regulate the access of PNPLA2 and LIPE to triacylglycerol (TAG) droplets [23,24], are abundant in BAT and differentially expressed, with higher expression in torpor and IBA. In addition, PLIN1 mRNA is abundant in BAT, but is not differentially expressed. The strong expression of PLIN4 (Figure 6) is notable considering its low expression in BAT in mice [25].

Once free fatty acids are cleaved from TAG they must be transported to the mitochondria. Fatty acid binding protein FABP3 is differentially expressed (Figure 6), with mean expression over three times higher in torpor and IBA than April. FABP4 was very highly expressed in BAT but did not meet our differential expression criteria. Mitochondrial import of fatty acids can be facilitated by the mitochondrial carnitine/acycarnitine translocase (SLC25A20) and by carnitine palmitoyltransferases CPT1A and CPT2, all three of which are up-regulated during hibernation (Figure 6).

In addition to generating free fatty acids from intracellular stores, BAT also utilizes lipids from the circulation. CUBN (cubilin) is a receptor for apolipoprotein-A1 and high-density lipoprotein [26], and albumin [27], so it may be important for lipid import in BAT. CUBN is differentially expressed showing much higher levels in October, torpor, and IBA.

Table 2. Differentially expressed nuclear genes encoding BAT mitochondrial proteins showing highest expression in October, Torpor, or IBA.

| Gene | Name/description | Genotype | Fold change over April | Number of normalized reads April | Number of normalized reads October | Number of normalized reads IBA |
|------|------------------|----------|------------------------|----------------------------------|----------------------------------|-------------------------------|
| KMO  | kynurenine 3-monooxygenase (kynurenine 3-hydroxylase) |          | 12.2                   | 48                               | 96                              | 454                           |
| UCP3 | uncoupling protein 3 |          | 6.0                    | 153                              | 131                             | 690                           |
| ABCD3| ATP-binding cassette, sub-family D (ALD), member 3 |          | 2.3                    | 4602                             | 3808                            | 10708                         |
| MCART1| mitochondrial carrier triple repeat 1 |          | 2.3                    | 1889                             | 4085                            | 4316                          |
| ACSL1| acyl-CoA synthetase long-chain family member 1 |          | 2.2                    | 14295                            | 20827                           | 30772                         |
| DECR1| 2,4-dienoyl CoA reductase 1 |          | 2.2                    | 6414                             | 14081                           | 12121                         |
| PREP | prolyl endopeptidase |          | 2.2                    | 890                              | 1235                            | 1521                          |
| UCP1 | uncoupling protein 1 |          | 2.2                    | 74907                            | 111962                          | 165776                        |
| SLC25A20| solute carrier family 25 (camilnine/acycarnitine translocase), member 20 | | 2.1                    | 4923                             | 8229                            | 10021                         |
| ACADM| acyl-CoA dehydrogenase, C-4 to C-12 straight chain |          | 2.0                    | 12577                            | 24105                           | 23427                         |
| MARCH5| membrane-associated ring finger (C3HC4) 5 |          | 2.0                    | 862                              | 1376                            | 1701                          |
| TIMP50| translocase of inner mitochondrial membrane 50 homolog (S. cerevisiae) | | 2.0                    | 1041                             | 1601                            | 2045                          |
| CPT1A| carnitine palmitoyltransferase 1A (liver) |          | 1.9                    | 11994                            | 14985                           | 22769                         |
| HADHA| hydroxacyl-CoA dehydrogenase/3-keboacy-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit | | 1.8                    | 25333                            | 36671                           | 44564                         |
| HADHB| hydroxacyl-CoA dehydrogenase/3-keboacy-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit | | 1.8                    | 42827                            | 65428                           | 75912                         |
| PDK4 | pyruvate dehydrogenase kinase, isozyme 4 |          | 1.7                    | 5968                             | 1575                            | 10244                         |
| PINK1| PTEN induced putative kinase 1 |          | 1.7                    | 2368                             | 4071                            | 3069                          |
| SUC2A2| succinate-CoA ligase, ADP-forming, beta subunit |          | 1.7                    | 11440                            | 18296                           | 19453                         |
| CPT2 | carnitine palmitoyltransferase 2 |          | 1.6                    | 6166                             | 7619                            | 9973                          |
| CRLS1| cardiolipin synthase 1 |          | 1.5                    | 4082                             | 5511                            | 6322                          |
| ETFDH| electron-transferring-flavoprotein dehydrogenase | | 1.5                    | 11969                            | 16228                           | 18390                         |

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membrane [28], are both highly BAT-specific but not differentially expressed. These proteins may also be associated with endocytotic lipid import in BAT. The related REEP4 is differentially expressed but at relatively low levels. In this context it is worth noting that the gene coding CD36, a protein known to be important in fatty acid transport, is highly expressed in BAT, but not differentially.

Two phospholipases, PLA2G4C (phospholipase A2, group IVC) and PLA2G16 (phospholipase A2, group XVI) are differentially expressed in BAT. PLA2G4C has a particularly dramatic rise in expression at the torpor timepoint (two-fold higher than IBA, three-fold higher than April). PLA2G4C is known to preferentially cleave arachidonic acid from the second phosphatidylcholine site [29]. It may be present in the mitochondria [30], where its long chain fatty acid products activate UCP1 [31]. It is thus interesting that SCGB1A1 (secretoglobin, family 1A, member 1), which is BAT-specific within our data, inhibits phospholipase A2 activity, suppresses inflammatory responses [32], and interacts with cubulin [33]. SCGB1A1 mRNA levels are significantly higher in torpor and IBA compared to April and October.

Figure 7 is a model showing the involvement of highly expressed and/or differentially expressed ground squirrel BAT genes involved in adrenergic signalling, lipolysis and heat generation during hibernation.

Transcription factors

One of the most highly expressed genes in BAT is MEF2D, a MADS-box transcription factor. MEF2D is not differentially expressed but is highly expressed in BAT versus other ground squirrel tissues that are available for comparison including WAT (Figure 3). MEF2D has recently been shown to positively regulate mitochondrial transcription [34], and is known to play a crucial role in regulating muscle-specific gene expression through histone modification [35].

A number of differentially expressed transcription factors showed unusual patterns of expression in BAT. Many of them have very low expression at torpor only to rebound at IBA, suggesting they are involved in rapid changes in gene expression between these two phases of hibernation. Some of these genes are highlighted in Table 3 and Figure 8. One example is NR4A1 (nuclear receptor subfamily 4, group A, member 1, also known as NUR77), which is known to be involved in the control of UCP1 expression [36] and is transcriptionally activated by MEF2D [37]. The related NR4A2 and NR4A3 genes have similar expression patterns as NR4A1 (very low in torpor) but do not meet our criteria for differential expression. Another differentially expressed transcription factor is BHLHE40 (class E basic helix-loop-helix protein 40), which plays a critical role in circadian rhythms [38].

A constellation of other transcription factor genes, that show lower expression in torpor than IBA, include ATF3 (activating transcription factor 3), NFIL3 (nuclear factor, interleukin 3

Figure 4. Differentially expressed genes encoding representative mitochondrial proteins in BAT. Error bars represent standard error of the mean. Transcript levels for all genes shown have an FDR<0.05. The letters above each bar represent post hoc pair-wise comparisons to determine significance between collection points. Any collection point not connected by the same letter is significantly different (FDR<0.05). Abbreviations: ACSL1, acyl-CoA synthetase long-chain family member 1; HADHA and HADHB, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit and beta subunit; KMO, kynurenine 3-monoxygenase; UCP1 and UCP3, uncoupling protein 1 and 3.

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Figure 5. Differentially expressed genes encoding representative proteins in the adrenergic signaling pathway in BAT. Error bars represent standard error of the mean. Transcript levels for all genes shown have an FDR<0.05. The letters above each bar represent post hoc pair-wise comparisons to determine significance between collection points. Any collection point not connected by the same letter is significantly different (FDR<0.05). Abbreviations: ADORA1, adenosine A1 receptor; ADRB1 and ADRB3, beta-adrenergic receptors 1 and 3; PDE3B, phosphodiesterase 3B, cGMP-inhibited; PRKACA, protein kinase A catalytic subunit; PRKAR2B, protein kinase A, cAMP-dependent, regulatory subunit, type II, beta.

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regulated), FOS (FBJ murine osteosarcoma viral oncogene homolog), JUNB (jun B proto-oncogene), and EGR1 (early growth response 1). Reduced expression of these genes has also been shown to be associated with reduction of inflammation in patients undergoing gastric bypass surgery and omentectomy [39]. Several of these same genes have also been associated with circadian rhythms and/or the CREB pathway. A transcriptional regulator showing reduced expression during torpor is salt-inducible kinase 1 (SIK1; Figure 8). SIK1 has been shown to repress the CREB pathway, but when stimulated by cAMP it relocates to the cytoplasm [40], linking CREB to the adrenergic pathway.

Genes with high April expression

Most of the data presented above focuses on genes that are more highly expressed at the torpor and IBA time points. The interpretation of expression patterns in BAT during hibernation is much easier because of the extreme demands of thermogenesis during arousals. However, many of the differentially expressed genes show their highest expression levels during April. It is more difficult to characterize the pathways and processes involving these gene products, but some of the more extreme cases are highlighted in Table 4 and Figure 9.

The most differentially expressed gene in our entire dataset is ASS1 (argininosuccinate synthase 1) whose expression plummets 169-fold from the April to the October time point and stays low during hibernation. Less dramatically ASL (argininosuccinate lyase) is also significantly higher in April compared to the other time points. ASS1 is the first enzyme in the urea cycle that acts outside of the mitochondria, catalyzing the formation of arginino-succinate from citrulline and aspartate. ASL in turn converts the arginino-succinate to

### Table 3. Transcription factors/regulators in BAT.

| Genes   | Torpor level | IBA to IBA | Differentially significantly expressed in lower than fold | Torpor level | Number of normalized reads |
|---------|--------------|------------|----------------------------------------------------------|--------------|---------------------------|
|         | BAT          | IBA        | Torpor level                                             | IBA          | April October Torpor IBA  |
| RASD1   | Yes          | Yes        | 10.9                                                     | 373          | 409 27 295                |
| NR4A1   | Yes          | Yes        | 9.7                                                      | 521          | 568 50 487                |
| BHLHE40 | Yes          | Yes        | 5.4                                                      | 184          | 224 44 239                |
| JUN     | Yes          | Yes        | 4.8                                                      | 343          | 392 89 425                |
| SIK1    | Yes          | Yes        | 4.3                                                      | 802          | 580 101 439               |
| NFIL3   | Yes          | No         | 4.2                                                      | 390          | 220 53 221                |
| ATF3    | Yes          | Yes        | 4.0                                                      | 47           | 67 45 180                 |
| EGR1    | Yes          | Yes        | 3.7                                                      | 543          | 711 80 294                |
| FOS     | Yes          | Yes        | 2.3                                                      | 102          | 184 37 86                 |
| JUNB    | Yes          | Yes        | 2.0                                                      | 147          | 184 41 82                 |
| TSC22D3 | No           | No         | 1.4                                                      | 699          | 373 138 190               |
| MEF2D   | No           | No         | 1.1                                                      | 54501        | 72957 61852 67403         |

(The letters above each bar represent post hoc pairwise comparisons to determine significance between collection points. Any collection point not connected by the same letter is significantly different (FDR<0.05). Abbreviations: CPT1A, carnitine palmitoyltransferase 1A; FABP3, fatty acid binding protein 3; PLIN2 and PLIN4, perilipin 2 and 4; PNPLA2, patatin-like phospholipase domain containing protein 2; SLC25A20, mitochondrial carnitine/acylcarnitine translocase.

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Figure 6. Differentially expressed genes encoding representative proteins involved in lipolysis or fatty acid transport in BAT. Error bars represent standard error of the mean. Transcript levels for all genes shown have an FDR<0.05. The letters above each bar represent post hoc pairwise comparisons to determine significance between collection points. Any collection point not connected by the same letter is significantly different (FDR<0.05). Abbreviations: CPT1A, carnitine palmitoyltransferase 1A; FABP3, fatty acid binding protein 3; PLIN2 and PLIN4, perilipin 2 and 4; PNPLA2, patatin-like phospholipase domain containing protein 2; SLC25A20, mitochondrial carnitine/acylcarnitine translocase.

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Figure 7. Model highlighting differentially expressed genes involved in fuel utilization and heat generation in BAT during hibernation. The role of the gene products in various metabolic processes in a brown adipocyte is shown. Genes with abbreviations in red meet the criteria for differential expression showing highest mRNA levels during the hibernation phases of torpor or interbout arousal. Abbreviations of genes that are not differentially expressed but are highly expressed, and/or tissue-specific genes in BAT, are shown in black. Molecules that serve as sources of fuel are labeled in green. Gene name abbreviations are defined in Table S1. Abbreviations: Ad, adenosine; NE, norepinepherine; NEFA, non-esterified fatty acids; TAG, triacylglycerol.

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Table 3. Transcription factors/regulators in BAT.
arginine. The low expression of these genes during the hibernation season may be important for repressing the urea cycle and perhaps for preserving metabolites in the citric acid cycle.

There are 63 ribosomal proteins which are more highly expressed in April compared to torpor and IBA time points. In striking contrast, the only ribosomal proteins with lower expression in April compared to any other time point were mitochondrial ribosomal proteins. Taken together these results form a consistent pattern of suppressed nuclear ribosome and mitochondrial ribosome production during the hibernation season. The function of other genes with higher expression in April is less clear. For example, FCN1 (ficolin-1) is at least 64-fold higher in April compared to the other time points. The eleven genes with at least a 6-fold change from April to any other time point were shown in Table 4.

### Discussion

Understanding the optimization of fat catabolism and heat generation in BAT of the naturally hibernating thirteen-lined ground squirrel was the subject of this study. We performed deep sequencing of the BAT transcriptome at various times of the year to determine the identity and abundance of mRNAs that contribute to the hibernating phenotype.

![Figure 8. Differentially expressed genes encoding BAT transcription factor/regulator mRNAs that are low in torpor.](image-url)

**Figure 8.** Differentially expressed genes encoding BAT transcription factor/regulator mRNAs that are low in torpor. Error bars represent standard error of the mean. Transcript levels for all genes shown have an FDR<0.05. The letters above each bar represent post hoc pair-wise comparisons to determine significance between collection points. Any collection point not connected by the same letter is significantly different (FDR<0.05). Abbreviations: BHLHE40, class E basic helix-loop-helix protein 40; EGR1, early growth response 1; FOS, FBJ murine osteosarcoma viral oncogene homolog; NFIL3, nuclear factor, interleukin 3 regulated; NR4A1, nuclear receptor subfamily 4, group A, member 1; SIK1, salt-inducible kinase 1.

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### Table 4. Differentially expressed BAT genes showing highest expression in April.

| Gene | Name/description | April to Torpor or IBA fold change | Number of normalized reads April | October | Torpor | IBA |
|------|------------------|-----------------------------------|---------------------------------|---------|--------|-----|
| ASS1 | argininosuccinate synthase 1 | 84.7 | 1355 | 8 | 9 | 16 |
| FCN1 | ficolin | 64.5 | 5674 | 77 | 88 | 56 |
| ACSF2 | acetyl-CoA synthetase family member 2 | 23.4 | 1711 | 93 | 68 | 73 |
| BCAT2 | branched chain amino acid transaminase 2, mitochondrial | 18.7 | 731 | 40 | 37 | 39 |
| SLC1A5 | solute carrier family 1 | 16.1 | 507 | 37 | 13 | 28 |
| IGBP5 | insulin-like growth factor binding protein 5 | 15.3 | 4725 | 360 | 271 | 309 |
| ALDH1A1 | aldehyde dehydrogenase 1 family, member A1 | 7.8 | 2699 | 491 | 278 | 346 |
| IGLL1 | immunoglobulin lambda-like polypeptide 1 | 7.8 | 1452 | 61 | 16 | 187 |
| GSTM5 | glutathione S-transferase mu 5 | 7.6 | 921 | 126 | 121 | 85 |
| IGLL5 | immunoglobulin lambda-like polypeptide 5 | 6.9 | 1496 | 51 | 14 | 218 |
| HP | haptoglobin | 6.3 | 4732 | 1950 | 749 | 489 |

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### Regulation of BAT metabolism

Adrenergic signaling controls heat-generation in BAT. We found genes encoding the beta-adrenergic receptors ADRB1 and ADRB3 show opposite expression patterns during the year (Figure 5). The receptors are coupled to the G-protein stimulatory subunit (G_s) encoded by GNAS. Overall GNAS has the highest number of reads of any mRNA in BAT. Although our read counts are not corrected for the expected bias from the length of the transcript, the GNAS transcript is not unusually long (approximately 2000 bases). By examining the distribution of reads along the genomic region containing the GNAS locus we confirmed that virtually all of the reads come from the shorter, active form [41]. The massive disparity in transcript levels for GNAS (Figure 3) versus the beta-adrenergic receptors ADRB1 and ADRB3 (Figure 5) is consistent with stoichiometric results for the protein products [42]. GNAS is epigenetically imprinted in the mouse, and its expression is important in lipid clearance, energy metabolism, and thermogenesis [43-45].

Although several studies suggest that the alpha-2 (inhibitory) adrenergic receptors are present in BAT of some species, we found very few counts from ADR2A in BAT (maximum of 6 counts in April). The absence of alpha-2 adrenergic receptors in other hibernating species (for example the golden hamster) provides some indirect evidence that the alpha-2 adrenergic receptors are not present in hibernator BAT in significant
Brown Adipose Gene Expression during Hibernation

Depolarization and ion flux

All cells maintain some concentration differences across their membranes which usually result in a membrane potential. For example, WAT has a resting potential of about -30 mV, while BAT is about -48 mV but can be -60 mV in the absence of bicarbonate [1]. Like neural and muscle cells, BAT can depolarize, although the purpose of this depolarization remains unknown. The depolarization of BAT occurs in at least three stages [1]: (A) a release of intracellular calcium, (B) a Cl- current, and (C) Na+ and K+ currents.

We found a few differentially expressed genes in BAT that may relate to stage (A) above. PLCD3, phospholipase C, delta 3, is higher in torpor and IBA with moderately high expression. This enzyme hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol and inositol 1,4,5-trisphosphate (IP3). PLCB4, phospholipase C, beta 4, is moderately but not differentially expressed, as are PLCL2, phospholipase C-like 2, PLCG1, phospholipase C, gamma 1, and PLCB3, phospholipase C, beta 3. CIB2, calcium and integrin binding family member 2, which is very similar to calcineurin B and calmodulin, is differentially expressed with higher levels in October, torpor and IBA.

The chloride current, stage (B), may involve CLIC5, chloride intracellular channel 5, which is differentially expressed in BAT with highest expression in torpor and IBA. It may be notable that CLIC5 can also conduct Na+ and K+ currents [51]. These ion currents may facilitate the transport of carbon dioxide in the form of bicarbonate out of the cell therefore preventing acidosis. They could also explain the otherwise puzzling expression of the genes coding acid-sensing ion channels ASIC1 and ASIC4, which are both more highly expressed during the hibernation season. In addition, the H+K+ exchanging ATPase ATP4A shows an unusually extreme expression pattern, with very low values during the IBA time point compared to torpor.

Depolarization could also be associated with endocytosis during arousal. MYO5B (myosin VB) is differentially expressed with low expression in April, and has a known role in membrane recycling [52]. In this context it is also worth noting the genes GBF1 (gap junction beta 1) and CGNL1 (cingulin-like 1) are significantly lower in April. It has long been known that BAT adipocytes are linked by gap junctions [53], which presumably synchronize the depolarization throughout the tissue.

Expression of neural and inner-ear related genes

TECTB (tectorin beta) was both differentially expressed and highly BAT-specific. This extracellular protein is known to be important in the tectorial membrane of the inner ear [54]. Other BAT-expressed genes associated with the inner ear include OTOF (otoferlin), USH1C (Usher syndrome 1 C), and SLC12A7 (solute carrier family 12, member 7). OTOF is highly expressed in BAT during torpor and has been described to have a role in neurotransmitter release of cochlear inner hair cells [55]. It is possible that OTOF expression occurs in innervating axons of BAT rather than the brown adipocytes themselves (reviewed in [56]). SLC12A7 is highly expressed in peripheral nerves and is important for the survival of cochlear...
Studies in mice have emphasized the role of PPARGC1A (peroxisome proliferator-activated receptor gamma, coactivator-related 1) in this process [60,61]. We found the PPARGC1A gene to be differentially expressed in the BAT of ground squirrels, with the highest values during IBA (Table S2). The depth of transcriptomic detail provided by our study identified many of the genes involved in pathways for thermogenesis, lipolysis, beta-adrenergic activation, and also suggested novel patterns of gene activity. While some classic markers of BAT in mice such as UCP1 and CIDEA are highly expressed in our ground squirrel samples, others such as FGF21 [62], PRDM16 [63], and ZIC1 [64] are either absent at all collection points, or present only at low levels and not differentially expressed. It may be that PRDM16 is most important in the initial development of BAT, and that its expression is not necessary in the adult ground squirrel. Several other highly BAT-specific genes in ground squirrels including REEP6, OBP2B, TECTB, SCGB1A1, do not show similar expression patterns in a comparison of BAT and WAT in mice [GEO dataset GDS2813].

Materials and Methods

Animals

The thirteen-lined ground squirrel, Ictidomys tridecemlineatus, is our model for mammalian hibernation. This species is abundant and unprotected in Minnesota where they are known to damage agricultural crops (http://www.dnr.state.mn.us/mammals/thirteenlinedground squirrel.html). The squirrels were live-trapped on private property with permission near Paynesville, Minnesota by slowly pouring water into burrows and capturing the animal in a butterfly net when it emerges. Animals were housed in the designated animal facility at the University of Minnesota Medical School Duluth and all experimental procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee (protocol #1103A97712).

Following capture squirrels are kept individually in plastic top-load rat cages filled with aspen shavings. Diet consists of standard rodent chow (Purina, #5001) and water ad libitum. A total of 12 male and 12 female thirteen-lined ground squirrels were used in these experiments. The squirrels were kept at room temperature in a 12:12 light/dark cycle at 23 °C and fed standard rodent chow and water ad libitum from April through October. Cardboard tubes from paper towel rolls were provided as enrichment from April through October. During the hibernation season (November-March), the squirrels were moved into an artificial hibernation chamber and kept in constant darkness at 5-7°C with no food provided. All animals were deeply anesthetized with 5% isoflurane and killed by decapitation to minimize pain and discomfort at the time of sacrifice.

Collection points

The collection points for these experiments were chosen to uncover the most meaningful comparisons in ground squirrel BAT across the hibernation season. Four collection points were used: pre-hibernation (October active), torpor, IBA, and post-hibernation (April active) (Figure 1). Three males and three females were sacrificed at each collection point. Animal state at each point was verified by rectal body temperature and animal behavior (Torpor: 6-8°C/inactive; Active and IBA: 35-37°C/active). All animals were sacrificed between 10 am and 3 pm.

Visceral white adipose tissue (WAT) was dissected to collect only the retroperitoneal fat pad [65]. BAT samples were taken from the axillary pad. The axillary BAT pad is located in the left and right axilla along the lateral aspect of the rib cage between the musculature of the anterior and posterior axillary folds. The BAT pads are dissected from the surrounding tissues, cleaned of any contaminating tissue, and flash frozen in liquid nitrogen. Three samples were sequenced for each time point. Each sequenced sample used RNA pooled from two individuals, one male and one female.

Illumina sequencing of phase-specific BAT RNA

Total BAT RNA was isolated from ground squirrels at the following four times/activity states: April active; October active; January torpor; January IBA. RNA was purified by standard procedures used in our lab [10]. Generation of the ground squirrel BAT transcriptome was performed at the Biomedical Genomics Center (BMGC) at the University of Minnesota Twin Cities. Total RNA integrity was assessed using capillary electrophoresis and quantified using fluorimetry (Agilent BioAnalyzer 2100), generating an RNA Integrity Number (RIN). To pass the initial Quality Control (QC) step, at least 1 microgram of BAT RNA required a RIN of 8 or greater before it is converted to Illumina sequencing libraries.

Library creation

From each BAT replicate 1 microgram of total RNA (equal RNA from 1 female and 1 male) was converted to poly(A)+ RNA using oligo-dT coated magnetic beads, fragmented and then reverse transcribed into cDNA. The cDNA is fragmented, blunt-ended, and ligated to indexed (barcoded) adaptors and amplified using 15 cycles of PCR. Final library size distribution was validated using capillary electrophoresis and quantified using fluorimetry (PicoGreen) and by Q-PCR.
Brown Adipose Gene Expression during Hibernation

Cluster generation and sequencing

Truseq libraries were hybridized to a paired-end flow cell and individual fragments were cloned amplified by bridge amplification on the Illumina cBot. Libraries were clustered at a concentration of 12pM. Once clustering was complete, the flow cell was loaded on the HiSeq 2000 and sequenced using Illumina’s SBS chemistry. Upon completion of a read, a 7 base pair index read is performed. Samples were run for 100 cycles with a minimum of 10 million single reads per sample.

Base call (.bcl) files for each cycle of sequencing were generated by Illumina Real Time Analysis (RTA) software. The base call files and run folders are then exported to servers maintained at the Minnesota Supercomputing Institute. Primary analysis and de-multiplexing are performed using Illumina’s CASAVA software 1.8.2, resulting in de-multiplexed FASTQ files.

Bioinformatic analysis

Each Illumina HiSeq read was 100 bases. BAT sequences were mapped to contigs constructed from other Illumina reads from this project and from brain cortex and hypothalamus [10], and skeletal muscle, heart, and white adipose tissue RNA sequence data recently determined by the Andrews Lab. A preliminary set of contigs was assembled by the Trinity suite of programs [11]. Trinity was also used to predict coding domain subsequences from these contigs. Any contig containing a predicted coding domain was then trimmed to this domain plus up to 100 bases on both ends of the domain. These sequences were then compared to the NCBI RefSeq human mRNA sequences using NCBI Blastn [12]. Before matching reads to the contigs, mitochondrially-encoded reads were screened using the thirteen-lined ground squirrel mitochondrial genome sequence [13] and NCBI’s megablast program [12] due to the high density of mitochondrial genomes in BAT. Genes encoding 835 nuclear-encoded mitochondrial proteins were identified using annotations by NCBI for RefSeq entries and using Gene Ontology gene associations [66]. Original sequence data is available from the NCBI Sequence Read Archive under accession SRS396817.

Differential gene expression

The data used to determine if a gene was differentially expressed across the hibernation season consisted of the counted number of RNA sequences that were positively identified with each identified gene in each sample. Samples consisted of three replicates of each of the following four phases: April active; October active; January Torpor; January IBA. Each replicate consists of RNA extracted from one male and one female ground squirrel. Replicates are true replicates and not repeated measures because different individuals were included in each group. Data was normalized to the upper-quartile across all BAT samples [67].

Analysis by DESeq

Differential gene expression was estimated using DESeq v1.6.1, an R program language package available through the Bioconductor platform [68]. DESeq estimates the variance-mean dependence in count data from high-throughput sequencing assays and tests for differential gene expression [69]. Upper-quartile normalized sequence counts for each gene in each sample were compiled in a tab-delimited text document that was read into R v2.14.1 (copyright 2004-2011, The R Foundation for Statistical Computing). Other than using upper-quartile normalization, analysis proceeded as described by Anders and Huber [69].

Supporting Information

Table S1. Distinct transcripts in ground squirrel BAT that mapped to human mRNAs. Table provides the mean (n=3) and standard error for each of the four collection points of all 14,573 genes that are expressed in brown adipose tissue. Gene names listed are the official HGNC and human UNIPROT designations. APR: April collection point; IBA: Interbout arousal collection point; OCT: October collection point; TOR: Torpor collection point; std. error: standard error. (XLS)

Table S2. Differentially expressed BAT genes. Table provides the mean (n=3) and standard error for each of the four collection points of all 2,083 genes that are differentially expressed in brown adipose tissue. The p-value generated from the DESeq analysis of deviance is also provided for each gene. A gene was considered differentially expressed and included in this Table if at least one collection point had at least 100 total counts, there was at least a 50% change between the collection points with the highest and lowest counts, and the FDR was less than 0.05. The p-value cutoffs for significance (FDR<0.05) was obtained from the Benjamini-Hochberg method. Gene names listed are the official HGNC and human UNIPROT designations. APR: April collection point; IBA: Interbout arousal collection point; OCT: October collection point; TOR: Torpor collection point; std. error: standard error. (XLS)

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Author Contributions

Conceived and designed the experiments: MH RGM MTA. Performed the experiments: MH RGM. Analyzed the data: MH RGM MTA. Contributed reagents/materials/analysis tools: MH RGM MTA. Wrote the manuscript: MH MTA.
Brown Adipose Gene Expression during Hibernation

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