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RESEARCH PAPER

Characterization of the hypothalamic transcriptome in response to food deprivation reveals global changes in long noncoding RNA, and cell cycle response genes

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Abstract The hypothalamus integrates energy balance information from the periphery using different neuronal subtypes within each of the hypothalamic areas. However, the effects of prandial state on global mRNA, microRNA and long noncoding (lnc) RNA expression within the whole hypothalamus are largely unknown. In this study, mice were given either a 24-h fast, or ad libitum access to food. RNA samples were analyzed by microarray, and then a subset was confirmed using quantitative real-time PCR (QPCR). A total of 540 mRNAs were either up- or down-regulated with food deprivation. Since gene ontology enrichment analyses identified several categories of mRNAs related to cell cycle processes, ten cell-cycle-related genes were further analyzed using QPCR with six confirmed to be significantly up-regulated and one down-regulated in response to 24-h fasting. While 22 independent microRNAs were differentially expressed by microarray, secondary analysis by QPCR failed to confirm significant changes with fasting. There were 622 lncRNAs identified as differentially expressed, and of three tested by QPCR, two were confirmed. Overall, this is the first time that expression of hypothalamic lncRNAs has been shown to be responsive to food deprivation. In addition, this study is the first to identify a list of lncRNAs with high expression in RNA extracted from hypothalamus. Individual contributions from specific miRNA, lncRNA and mRNAs to the food deprivation response can now be further studied at the physiological and biochemical levels.

Keywords microRNA · lncRNA · mRNA · Fasting · Gene ontology · Hypothalamus

Introduction

The hypothalamus integrates central nervous system control of energy balance including responses to both food deprivation and re-feeding. Within the hypothalamus, multiple neuronal subtypes respond with both cellular signaling (for example, leptin and insulin signals) and gene regulatory responses involving protein-coding genes (mRNA), microRNAs (miRNA) and long noncoding RNAs (lncRNA). According to the Mouse Genome Informatics: Gene Expression Database, there are 2630 mRNAs with postnatal expression in the midbrain (http://www.informatics.jax.org/, Smith et al. 2014). Of these, up to 588 are transcription factors (Good 2010). In addition, miRNAs also play a role in gene/protein regulation by repressing gene expression through an interaction with the mRNA transcript that results in either degradation or blocked translation of the mRNA. Finally, lncRNAs are emerging as key posttranscriptional regulators of mRNA, including

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regulation of transcription (both as scaffolds for chromatin modifying proteins and transcription factors, and as interfering moieties), splicing, mRNA decay and translation, as well as microRNA decoys (Rinn and Chang 2012; Yoon et al. 2013).

Differentially expressed hypothalamic mRNAs have been identified in selected rodent-based studies using the food deprivation/feeding paradigm. In a study by Poplawski et al. (2010), 48-h food deprivation in mice was used, in conjunction with a mouse expression array. This study found that global glucose metabolism was altered in response to this fast. In another study that uses laser microdissection in conjunction with rat whole-genome arrays, over 3000 differentially expressed mRNAs in the rat arcuate nucleus of the hypothalamus (Paulsen et al. 2009).

microRNAs have been reported to be differentially expressed in several brain disorders, including neuronal cancers, and neurodegeneration (Fassan et al. 2011; Roshan et al. 2009). There are also studies that demonstrate a role for microRNAs in more normal physiological processes, such as learning, synaptic plasticity and neuroadaptation (McNeill and Van Vactor 2012). A recent study using hypothalamic tissue from *ob/ob* mice, a genetically obese and leptin-deficient strain has identified changes in miR-200a, miR-200b and miR-429 (Crepin et al. 2014). Furthermore, within other tissue types, studies report an association of microRNA levels in adipose tissue, under high-fat diet or obesity, and in brown fat undergoing differentiation (Martinelli et al. 2010; Sun et al. 2011; Takanabe et al. 2008). Together, the evidence from these studies suggests that expression of hypothalamic microRNA may also change in response to normal changes in energy availability.

Long noncoding RNAs (lncRNA) are distinguished from microRNAs as they are more than 200 nucleotides in length (for reviews, see Kornfeld and Bruning 2014 and Kung et al. 2013). Similar to microRNAs, lncRNAs have been reported to be functionally associated with adipogenesis (Sun et al. 2013). In addition, studies also demonstrated that transcription of lncRNAs occurs in response to food supply and insulin/insulin-like growth factor levels (Ellis et al. 2014; Hellwig and Bass 2008). These indicate that lncRNAs might be involved in the energy balance regulation. In the brain, several papers have detailed the importance of lncRNA in neuronal differentiation and brain development (Aprea et al. 2013; Lin et al. 2014), but adult expression patterns under stress-type conditions such as fasting have not been reported.

The study reported herein utilized microarray platforms with the ability to capture exon-specific mRNA, microRNA and lncRNA data from the same samples. This allowed for full characterization of the whole hypothalamic transcriptome, including mRNA, microRNA and long noncoding RNA, in response to two prandial states, 24-h fasting and *ad lib* fed. As the global expression patterns have not been reported for either microRNA, nor for long noncoding RNAs in response to short-term fasting, these results characterize the simultaneous changes in all three subsets of the transcriptome, and help to further identify specific RNA targets in the fasting and fed response states.

Experimental procedures

Animals

The Institutional Animal Care and Use Committee at the Virginia Tech approved all studies. C57Bl/6 mice were purchased from The Jackson Laboratory as matched littermates, and only male mice were used in this experiment so that estrous cycles did not have to be taken into account during fasting. All mice were maintained under 12-h light/dark cycle with free access to food and water except as noted during experimentation. At the age of 8 weeks, mice were randomly separated to either a food-deprived ($N = 3$, microarray; $N = 4$–6, QPCR) or *ad lib* group ($N = 3$, microarray; $N = 4$–6, QPCR), and food was removed for deprived mice at 9 a.m. (Fig. 1a). After 24-h food deprivation, mice were euthanized and brains were collected. The hypothalamic region was isolated from the whole brain using a Zivic-Miller brain slicer, and taking a slice containing only the region between the optic chiasm and the mammillary bodies. That slice was further dissected to contain only the region between the cerebral peduncle on either side of the hypothalamus and to the top of where the third ventricle terminates, between the hypothalamus and thalamus. The slice was put into *RNAlater*® solution (Ambion, USA) for RNA analysis. For a limited number of animals, gastrocnemius was collected to compare the sensitivity of our arrays for detecting tissue-specific microRNAs.

RNA isolation

The RNA was extracted using the *mir*Vana™ PARIS™ Kit (Ambion, USA) according to the manufacturer's procedure without modifications. Enrichment procedure for small RNAs was performed for microRNA study. RNA integrity was tested after extraction using Experion™ system (Bio-Rad, USA).

Microarray and statistical analysis

The microRNA analysis was done in triplicate ($N = 3$) using individual mouse samples and the Affymetrix

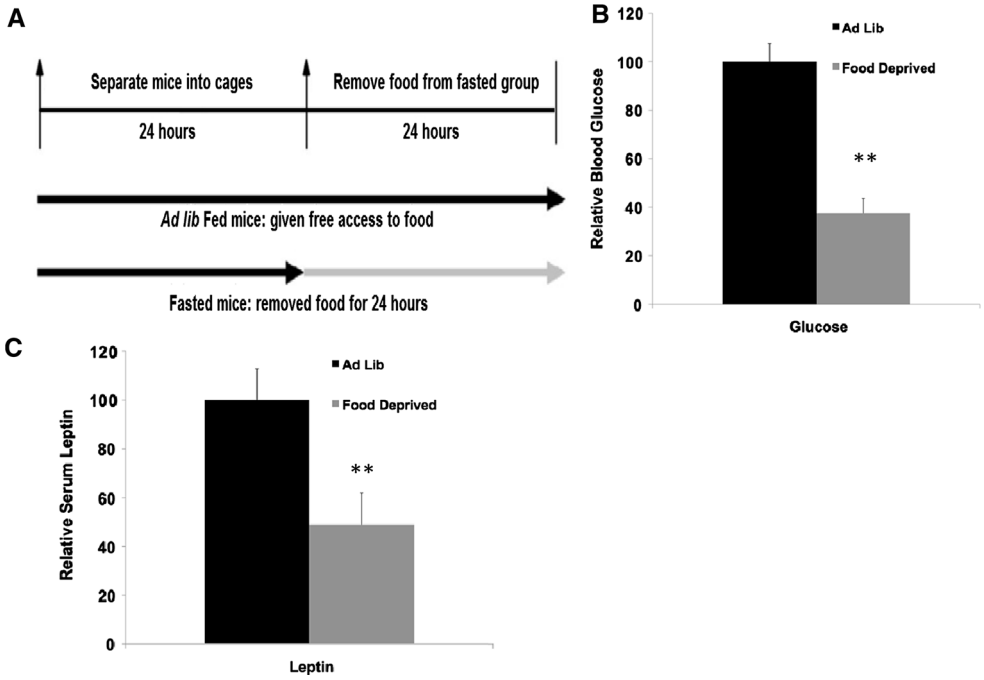

Fig. 1 Experimental design and confirmation of food deprivation conditions. **a** Experimental design of the experiment, showing the timeline for the *ad lib* and food-deprived groups of animals. **b** Serum glucose levels, relative to *ad lib* fed. **c** Serum leptin levels, relative to *ad lib* fed $^{**}P \leq 0.01$

GeneChip® microRNA 1.0 array. The Affymetrix microRNA array raw data were extracted using the Affymetrix microRNA QC tool. Global normalization was used to normalize the raw data. The log2 values of the expression levels for each microRNA were processed, and Student's *t* test was performed between *ad lib* fed and food-deprived groups. To survey for candidate microRNA with differential expression, statistical criteria of *P* value $\leq$0.05 and fold change of $\geq$1.3 were used to identify differentially expressed microRNAs.

mRNA analysis was performed in triplicate with the same individual mouse samples group as were analyzed for the microRNAs. These were done using the Affymetrix GeneChip® Mouse Exon 1.0 ST array. Global normalization was used to normalize the raw data. The log2 values of the expression levels for each mRNA were processed, and Student's *t* test was performed between *ad lib* fed and food-deprived groups. To survey for candidate mRNA with differential expression, statistical criteria used were a *P* value $\leq$0.05 and fold change of $\geq$1.3.

Exon array-based lncRNA analysis was performed with the Affymetrix GeneChip® Mouse Exon 1.0 ST array data using Noncoder (Gellert et al. 2013), a Web interface designed for lncRNA analysis with the Affymetrix GeneChip® Mouse Exon 1.0 ST array. The CEL files used for mRNA analysis were uploaded to Noncoder, and data were processed using RMA normalization. The log2 value of the expression level for each lncRNA was processed, and the Student's *t* test was performed between *ad lib* fed and food-deprived groups. To survey for candidate lncRNA with differential expression, only lncRNAs with more than one probe set were used for further statistical comparisons, with cutoff of *P* value $\leq$0.05 and fold change of $\geq$1.3.

Quantitative PCR (QPCR) analysis

To perform independent confirmation of statistically significant changes in transcript abundance, RNA from 4 to 6 additional mice was obtained for all confirmatory analyses, with microRNA expression levels measured utilizing the Taqman microRNA assay (Applied Biosystems, Foster City, CA). For each microRNA tested, 5 ng of small RNA-enriched samples from $N = 4$–6 mice per assay was used. This number of individual mice used is based on experimental data and results from our laboratory and is sufficient to detect a 1.5-fold or better differences in expression (Vella et al. 2007). Reverse transcription and QPCR were performed according to the assay manual. The expression level of sno-202 RNA was used as the normalization control in microRNA analysis, as this NC RNA has previously been shown to be most effective in a normalization analysis (Brattelid et al. 2011). In our hands, we also found sno-202 to be consistently expressed between tissues and conditions. All QPCR results were compared between groups using Student's *t* test with *P* value $\leq$0.05 for statistical significance.

The expression levels of mRNA and lncRNA were measured using designed primers and the iTaq™ SYBR® Green Supermix with Rox (Bio-Rad, Hercules, CA). New total hypothalamic RNA was isolated from $N = 5$–6 mice, and 2 μg of total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, Wisconsin) and then subjected

to reverse transcription using M-MLV Reverse Transcriptase (Promega, Madison, Wisconsin). A 20 ng aliquot of cDNA was then used for QPCR analysis using the ABI 7900 system (Applied Biosystems, Foster City, CA). The expression level of β-actin was used as the normalization control. All QPCR results were compared between groups using Student's *t* test with *P* value ≤0.05 for statistical significance. For lncRNA analysis, QPCR products were then sequenced to confirm that the sequence of the amplicon was unique to the lncRNA region.

Leptin and Glucose measurement

Whole blood was collected immediately after each mouse was euthanized. Blood glucose level was measured using FreeStyle Freedom Lite® Blood Glucose Monitoring System (Abbott Laboratories, USA). The serum was collected from the whole blood sample by centrifuging at 1000×*g* for 10 min at 4 °C. Serum leptin level was measured using the Mouse Leptin Quantikine ELISA kit (R&D System, USA) according to the manufacturer's instructions and using manufacturer-provided standards to produce the standard curve. Blood glucose and leptin level were compared between groups using Student's *t* test with *P* value ≥0.05 for statistical significance.

GO and STRING analysis

Gene ontology analysis was performed using GeneCodis3 (Carmona-Saez et al. 2007; Nogales-Cadenas et al. 2009; Tabas-Madrid et al. 2012). Genes passing the cutoff of microarray analysis were used for the GO Biological Process, GO Molecular Function, GO Cellular Component and KEGG pathways analysis. For the settings of statistical parameters, the minimum number of genes was set to two, and a hypergeometric statistical test and FDR *P* value correction were used. The results were listed with the multiple-testing-corrected, hypergeometric *P* value.

STRING version 10 was used in its online format to generate an interactive network mode using the all cell cycle genes tested for QPCR (Franceschini et al. 2013). The confidence view was used with one expansion of the protein interactions network.

Results

Leptin and glucose levels

Following 24-h food deprivation (Fig. 1a), serum leptin and blood glucose levels for each treatment group showed a significant reduction, as would be expected from fasting conditions (Fig. 1b, c).

Overall transcriptome changes

Twenty-four-hour food deprivation resulted in changes in the overall hypothalamic transcription of varying magnitude. Using DNA microarrays and the statistical selection criteria of a *P* value of ≤0.05 and fold change of ≥1.3, a subset of the candidate differentially expressed genes was identified. As shown in Table 1, the majority of detectable changes were found within the mRNA and lncRNA transcriptome, while the fewest changes were found within the microRNA transcription.

mRNA microarray analysis and QPCR validation

The data from the Affymetrix exon arrays (Supplemental Data File 1, excel list) were first used to identify differentially expressed hypothalamic mRNAs between *ad lib* fed and 24-h food-deprived animals. The log2 scatter plot comparison (Fig. 2a) suggested that there is no dramatic transcriptome adjustment in response to food deprivation, while volcano plot analysis (Fig. 2b) indicated that there were still some significant changes between the two conditions. In all, there were 540 candidate differentially expressed mRNAs identified, with 298 mRNAs found to be up-regulated and 242 mRNAs down-regulated with food deprivation (Table 1). The up- and down-regulated mRNAs identified using the array data are shown in Supplemental Table 1. The top ten mRNAs with the highest hypothalamic expression levels overall are shown in Supplemental Table 2. None of differentially expressed mRNAs were in the list of those with the highest hypothalamic expression. A subset of the differentially expressed candidate genes was selected for further analysis based on probable role in energy balance regulation, or brain function, and *P* value. As shown

Table 1 Comparison of microRNA, lncRNA and mRNA array results

	microRNA	lncRNA	mRNA
Total detected	536	12,521	16,294
Number of those significantly up-regulated by fasting	16	421	298
Number of those significantly down-regulated by fasting	6	201	242

Total detected RNA species, and those found to be significantly up- or down-regulated by food deprivation, compared to ad lib feeding, based on the criteria of at least 1.3-fold (either direction) different, and $P \leq 0.05$

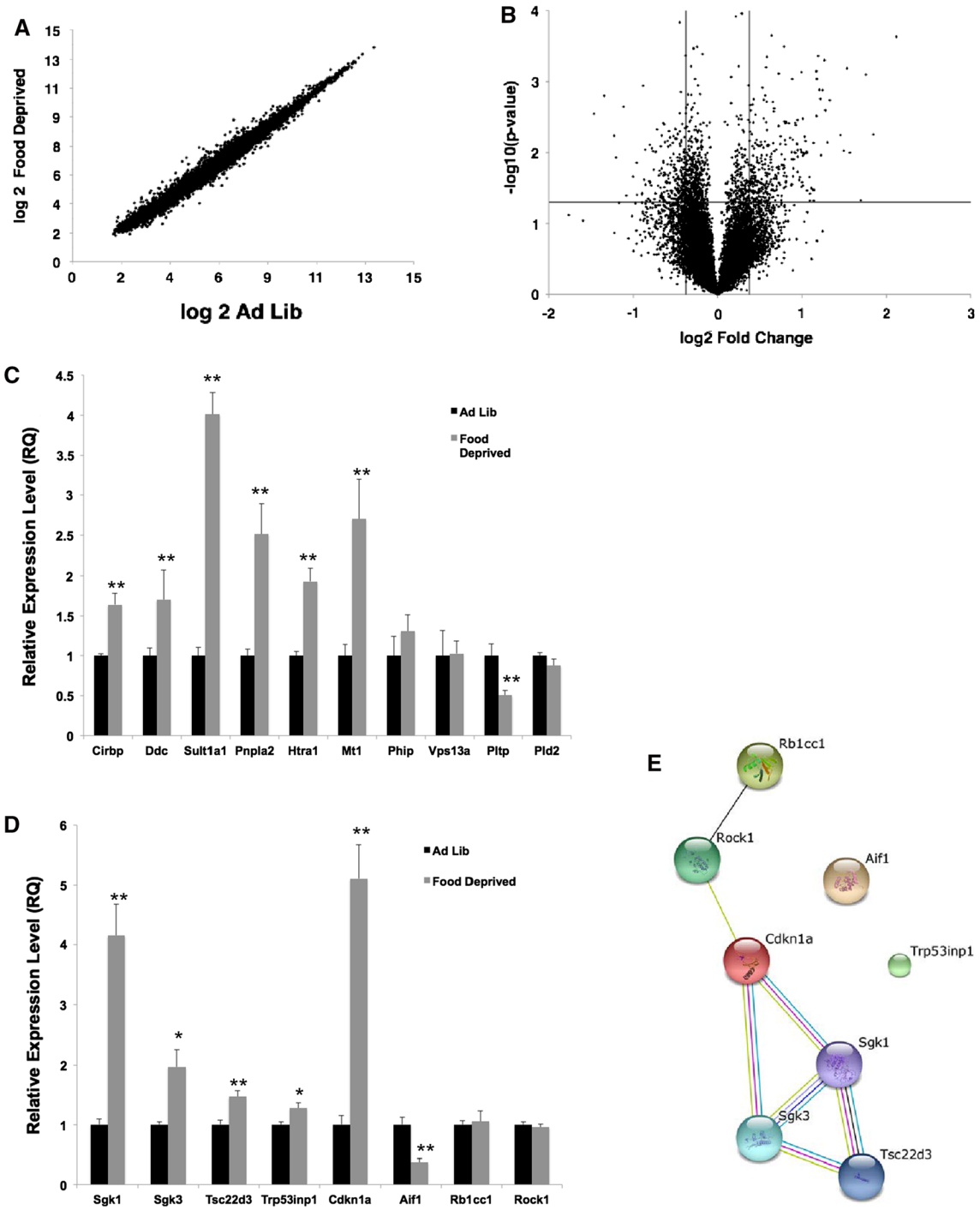

Fig. 2 mRNA array plots and QPCR analysis. **a** log2 scatter plot of hypothalamus mRNA array data from Affymetrix array. Data were filtered using a *P* value <0.05 and 1.3-fold change. **b** Volcano plot showing *P* value versus log2 expression level for data from Affymetrix array. *Lines* indicate where significance cutoff values were made. **c** QPCR analysis of selected mRNAs that were differentially expressed in food-deprived versus *ad lib* mice. **d** mRNAs were selected from cell cycle GO categories, and their expression levels tested by QPCR using $N = 5$–6 new samples for each group. All mRNA levels are reported as relative to levels in *ad lib* fed animals and normalized to the housekeeping gene β-actin. **e** String v10 was used to generate a network using the proteins tested in 7A as input. The network (interactive network mode) was expanded by one level. Only the names of the proteins input are shown. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$

in Fig. 2c, d, most genes chosen using these criteria were confirmed to be significantly differentially expressed in independent samples isolated from *ad lib* and food-deprived animals. Of ten genes selected as differentially regulated with 24-h fasting, seven of these changes were confirmed by QPCR using an independent test set of animals.

Gene ontology (GO) analysis of the 540 differentially regulated candidate genes between *ad lib* fed and food-deprived mice was performed, and the top five GO terms for each of the major component or process category are shown in Table 2. Close examination of the entire dataset of significant categories (P value ≤ 0.05) revealed that cell-cycle-related categories were found once in the top five list, and then multiple times in the remaining significant categories. These additional categories of cell-cycle-related terms are shown in Table 3. A subset of genes found in cell cycle categories was further analyzed using QPCR. Of eight cell cycle category genes analyzed, six were confirmed to be significantly differentially regulated in new independent hypothalamic mRNA samples from *ad lib* and food-deprived animals (Fig. 2e). Using STRING (Search Tool for Retrieval of Interacting Genes/proteins) (Szklarczyk et al. 2011) analysis, four of these comprise a network of protein products involved in cell cycle control, and all of the four, except rock 1, showed significantly different expression by QPCR (Fig. 2f) using the independent test set.

microRNA microarray analysis and QPCR validation

microRNA expression analysis was performed in triplicate on the Affymetrix microRNA microarray platforms (Supplemental Data File 2, excel list). Scatter plot analysis showed that the overall expression level differences between the 24-h fasting and *ad lib* treatment groups were similar and suggestive of few differentially regulated microRNAs, especially within the significance criteria (Fig. 3a, b). A total of 536 microRNAs were detected with the microarrays, but only 22 candidates for differential expression were identified using the relatively loose statistical criteria of $P \leq 0.05$ and fold change ≥ 1.3, with 16 up-regulated and 6 down-regulated (Table 1 and Supplemental Table 3). A subset of these candidate microRNAs was chosen for further analysis based on p value and whether they had been found to be involved in any aspect of energy balance in previous studies, including those using other tissues than brain. These criteria identified six for

Table 2 GO analysis (mRNA arrays)

GO term	Gene count (significant)	Gene count (reference)	P value
Biological process			
GO:0006810: transport	56	1620	8.73E−07
GO:0007049: cell cycle	26	533	3.41E−05
GO:0016310: phosphorylation	29	689	8.99E−05
GO:0048812: neuron projection morphogenesis	7	36	0.000192
GO:0006974: response to DNA damage stimulus	17	308	0.000447
Cellular component			
GO:0016020: membrane	178	5723	4.76E−24
GO:0005886: plasma membrane	103	2782	1.23E−17
GO:0005634: nucleus	144	4867	7.00E−17
GO:0005737: cytoplasm	141	5026	1.33E−14
GO:0016021: integral to membrane	143	5398	6.95E−13
Molecular function			
GO:0005515: protein binding	100	2999	3.92E−16
GO:0046872: metal ion binding	91	2802	5.08E−14
GO:0000166: nucleotide binding	64	1999	8.20E−10
GO:0005524: ATP binding	47	1421	6.59E−08
GO:0003677: DNA binding	51	1635	1.10E−07
KEGG pathways			
KEGG:04670: leukocyte transendothelial migration	9	117	4.27E−05
KEGG:04724: glutamatergic synapse	9	123	6.32E−05
KEGG:04010: MAPK signaling pathway	10	263	0.004306
KEGG:04144: endocytosis	9	213	0.003344
KEGG:04062: chemokine signaling pathway	12	177	8.61E−06

All significantly differentially expressed mRNAs (529 total) were used in this analysis. The top five GO terms for each category are shown. The number of genes significant for that category (gene count, significant), the number of total genes in the reference category (gene count, reference) and P value for the GO term are shown

Table 3 Cell cycle category GO terms

GO term	Gene count (significant)	Gene count (reference)	*P* value
Cell cycle category			
GO:0007049: cell cycle (BP)	26	533	3.41E−05
GO:0001938: positive regulation of endothelial cell proliferation (BP)	7	48	0.000652
GO:0001936: regulation of endothelial cell proliferation (BP)	2	4	0.031983
GO:0050768: negative regulation of neurogenesis (BP)	3	14	0.029825
GO:0043066: negative regulation of apoptotic process (BP)	14	340	0.017967
GO:0006915: apoptotic process (BP)	16	504	0.043501

List of all significant GO terms related to cell cycle categories is shown. The number of genes significant for that category (gene count, significant), the number of total genes in the reference category (gene count, reference) and *P* value for the GO term are shown

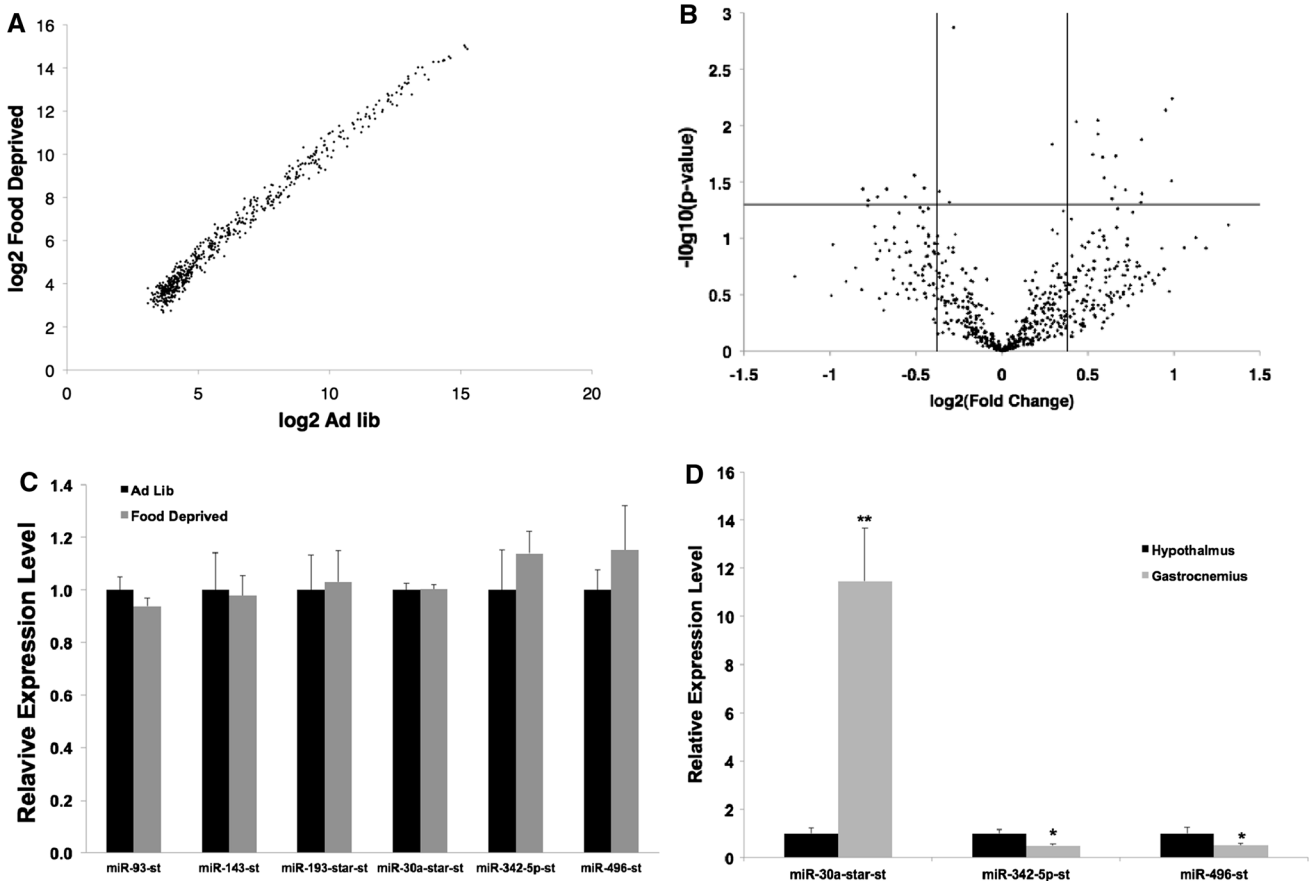

Fig. 3 microRNA array plots and QPCR analysis. **a** log2 scatter plot of hypothalamus microRNA array data from Affymetrix array. Data were filtered using a *P* value <0.05 and 1.3-fold change. **b** Volcano plot showing *P* value versus log2 expression level for data from Affymetrix array. *Lines* indicate where significance cutoff values were made. **c** microRNA QPCR analysis in hypothalamic tissue of *ad lib* versus food-deprived mice. microRNAs were selected using the cutoff criteria, and expression level tested in $N = 4$–6 new samples for each group. All microRNA levels are reported relative to levels in *ad lib* fed animals and normalized to the sno-202 microRNA. **d** microRNA expression in skeletal muscle versus hypothalamus. $*P \leq 0.05$; $**P \leq 0.01$

further expression analysis using independent samples. However, QPCR analysis failed to confirm that any of these chosen microRNAs were significantly different in an independent analysis between *ad lib* and food-deprived treatment groups (Fig. 3c). Nevertheless, differences could be detected comparing skeletal muscle versus hypothalamic microRNA QPCR for three of these microRNAs (Fig. 3d), indicating that detection should have been

possible with these probe sets had differences existed. For additional verification of the system, *ad lib* fed mice were used to compare hypothalamic and skeletal muscle (gastrocnemius) microRNAs (Supplemental Figure 1A), revealing the exploded comet shape suggestive of the expected significant differences in expression levels of specific microRNAs between the two tissue types. Specific microRNAs were then analyzed by QPCR using total RNA from both hypothalamus and skeletal muscle of both *ad lib* and fasted mice. As shown in Supplemental Fig. 1b, let-7d was highly expressed in both tissues, with similar expression levels under all conditions. Expression of the skeletal muscle-specific microRNA, miR-1, was significantly higher in skeletal muscle than in hypothalamus tissue, in both *ad lib* and fasted mice, as expected (Supplemental Figure 1C). Conversely, the nervous system-specific microRNA, miR-124a, was highly expressed in hypothalamic RNA isolated from both fed and fasted mice, when compared to its detection at a significantly lower level in gastrocnemius RNA isolated from either mice in either treatment group (Supplemental Figure 1D). The 10 highest expressing hypothalamic microRNAs in ad lib fed mice are shown in Supplemental Table 4. None of the microRNAs with the highest hypothalamic expression showed evidence of differential expression, but these results provide information on the hypothalamic microRNAome, overall, with highly expressed microRNAs warranting future exploration into their roles.

lncRNA microarray analysis and QPCR validation

The Affymetrix exon arrays were also used to identify hypothalamic lncRNAs which were differentially expressed between *ad lib* fed and 24-h fasted animals. Only lncRNAs with two or more probe sets were used in the analysis, giving a total of 12,521 lncRNAs that could be detected by Noncoder (Table 1) (Supplemental Data File 3, excel list) (Gellert et al. 2013). Scatter plot analysis showed that the overall expression level differences between food-deprived and *ad lib* treatment groups were similar. Employing the statistical criteria of $P \leq 0.05$ and a fold change of ≥ 1.3, 421 candidate lncRNAs were found to be up-regulated by 24-h fasting compared to *ad lib* feeding, whereas 201 lncRNAs were down-regulated (Table 1; Fig. 4a, b). A list of the differentially expressed lncRNAs is shown in Supplemental Table 5. Three of the candidate lncRNAs with the highest fold change accompanied by highly significant *p* values between *ad lib* and food-deprived groups were selected for further analysis by QPCR. As shown in Fig. 4c, two lncRNAs, AK038506 and AK049914, were confirmed to be significantly differentially expressed, both on the array platform, and using QPCR in independent samples isolated from *ad lib* and food-deprived animals. The top ten lncRNAs with the highest hypothalamic expression levels overall are shown in Supplemental Table 6. Similar to the microRNAs, none of the lncRNAs with the highest hypothalamic expression showed evidence of differential expression, but provide information on the hypothalamic lncRNAome, and again those that might warrant further exploration.

Discussion

The results of this study yield two major findings. First, after a 24-h fast, the hypothalamic microRNA response is minimal, when compared to the lncRNA or mRNA response. Statistically significant changes in microRNA expression levels could not be confirmed by QPCR in an independent test set, and to date, this is the only global hypothalamic analysis of the microRNA transcriptome in response to food deprivation. Second, and in contrast to the microRNA results, there are multiple lncRNAs and mRNAs differentially expressed following food deprivation. While the lncRNAs are not well characterized, and pathway analysis is not available for this subset of RNA, the mRNAs dataset revealed a high number of differentially expressed mRNAs within the cell cycle categories following gene ontology analysis. As noted, multiple pathways, including phosphorylation, stress response and specific enzyme categories, are also significantly changed when the mRNA datasets were compared between these conditions. This is the first study to fully characterize the transcriptome of the hypothalamus in 24-h fasting, compared to the *ad lib* fed animals.

Given the previous published studies showing differential regulation of microRNAs in brain, adipose and other tissues conditions such as cancer or obesity, it was surprising to find so few differentially expressed microRNAs via microarray analysis and none that could be confirmed by further QPCR analysis (Fassan et al. 2011; Martinelli et al. 2010; McNeill and Van Vactor 2012; Roshan et al. 2009; Sun et al. 2011; Takanabe et al. 2008). Although current database information suggests that there are only 105 microRNAs expressed in the midbrain above the detection threshold (http://www.microrna.org), we detected 536 microRNAs using the Affymetrix microarrays. Our data confirmed that the let-7 family, miR-124a, miR-125 family and miR-138 were highly represented in the hypothalamic microRNAome, and found several others such as miR-709 and miR-690 which have not previously been shown to be highly expressed in this region (Bak et al. 2008; Meister et al. 2013; Olsen et al. 2009). Moreover, we were able to demonstrate that the microarrays could detect many statistically significant differences between hypothalamus and skeletal muscle. However, only 22

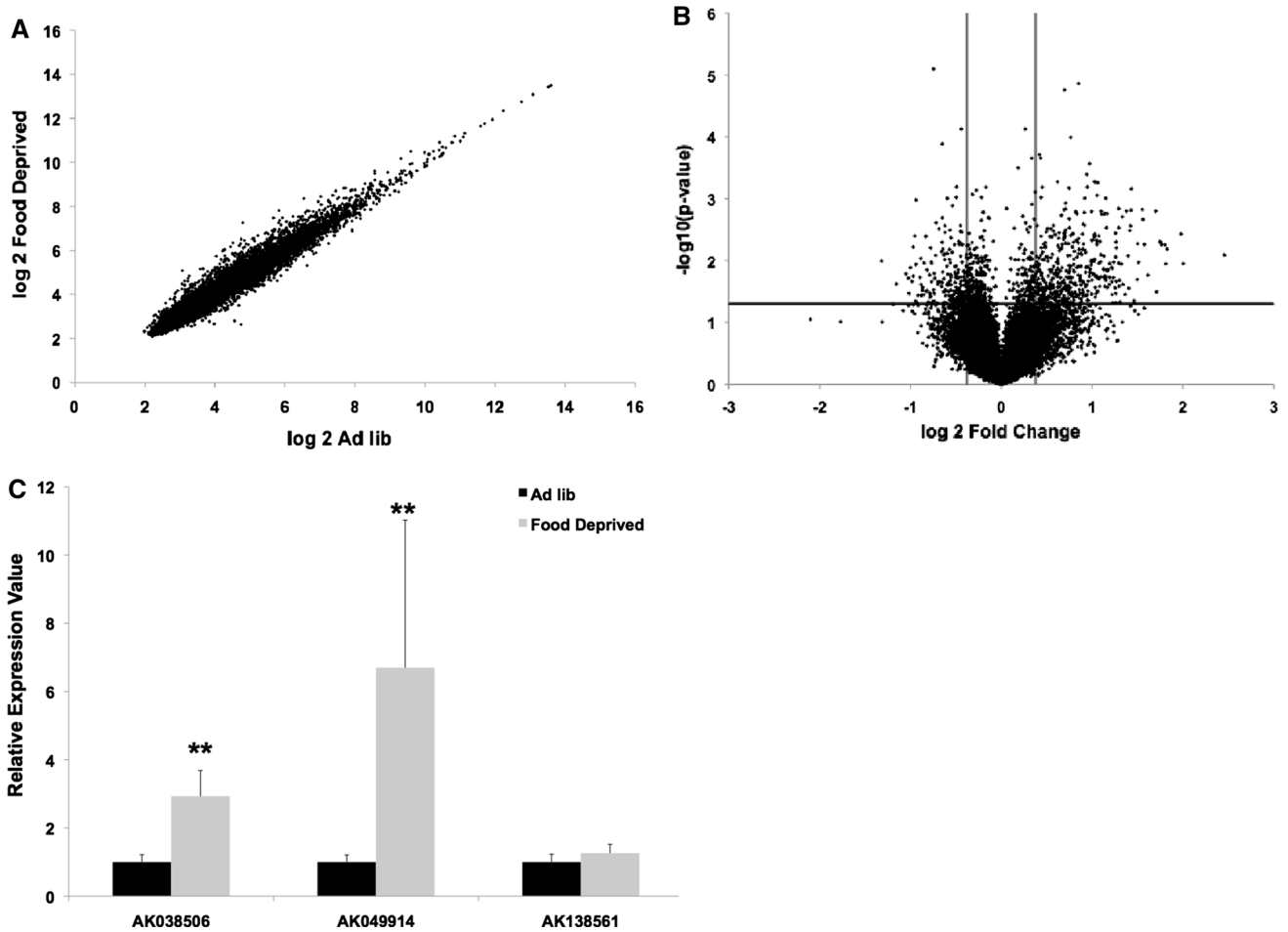

Fig. 4 lncRNA array plots and QPCR analysis. **a** log2 scatter plot of hypothalamus lncRNA array data. Data were filtered using a *P* value ≤0.05 and 1.3-fold change. **b** Volcano plot showing *P* value versus log2 expression level for data from lncRNA results. *Lines* indicate where significance cutoff values were made. **c** lncRNA QPCR analysis using hypothalamic tissue of *ad lib* versus food-deprived mice. All lncRNA levels are reported relative to levels in *ad lib* fed animals and normalized to the sno-microRNA $^{**}P \leq 0.01$

candidate differentially expressed microRNAs were identified between the 24-h fasted and ad lib fed states in the hypothalamus, and none of the changes were confirmed to be statistically significant in an independent set of samples using QPCR. In a study by Sangiao-Alvarellos and colleagues which examined hypothalamic miRNAs in high-fat-fed, versus chronically restricted, low-fat-fed animals (fed at 65 % of control, but not completely food restricted), ten differentially expressed miRNAs were identified (Sangiao-Alvarellos et al. 2014). However, only one of these significant differences was repeatable in a later figure of the same paper with 48-h fast (Sangiao-Alvarellos et al. 2014). None of the microRNAs from Sangiao-Alvarellos and colleagues were differentially expressed in our samples, and thus, the findings by Sangiao-Alvarellos and colleagues are therefore consistent with ours, in few hypothalamic miRNAs changing in response to negative energy balance.

It is possible that statistically significant miRNA expression may have been detected at earlier time points but this beyond the scope of the current study. In addition, as this study examined the transcriptome of the whole hypothalamus, rather than region-specific transcriptomes, the possibility exists that region-specific changes were missed. For example, deletion of the dicer gene within hypothalamic POMC neurons was shown to lead to obesity in adulthood (Schneeberger et al. 2012). Given this finding and the dicer's global role in processing microRNAs, it is likely that examining of nuclei-specific microRNAs may still reveal differential expression patterns with fasting or other conditions that change energy availability in the body.

Hypothalamic lncRNAs have not been previously implicated in any condition involving energy availability, making this study the first to both characterize the top differentially regulated lncRNAs and provide a list of

highly expressed lncRNAs for this tissue. Noncoding RNAs, such as lncRNA, are of increasing interest, having predicted roles in transcriptional regulation during brain development and disease (Clark and Blackshaw 2014; Qureshi and Mehler 2013). This study identified more than 12,000 lncRNAs that are expressed within whole hypothalamus and is the first of its kind to do so for mouse hypothalamus undergoing *ad lib* feeding or fasting. To date, only one other study in canines has examined hypothalamic lncRNAs, and this was done in nonfasting male beagles (Roy et al. 2013). The study identified 57 lncRNAs in the canine hypothalamus, although none appear to have any overlap with those found in this study using our criteria. Other studies, not using hypothalamic tissue, have implicated lncRNAs in various nutritional states, including those found in the gut in germ free, versus microbiota (free-living) conditions, and those identified in diabetics (SNP association, without verification, Liang et al. 2015; Wessel et al. 2015). Additionally, lncRNAs have been shown to enhance brown and white adipocyte differentiation, participate in pancreatic beta-cell function and possibly protect against diet-induced obesity, as shown for SRA, the steroid receptor RNA activator (Kameswaran and Kaestner, 2014; Liu et al. 2014; Sun et al. 2011; Sun et al. 2013; Xu et al. 2015; You et al. 2015). The most relevant to our work would be SRA, as a whole body knockout of this lncRNA protected against diet-induced obesity (Liu et al. 2014). However, our results confirm theirs showing low expression in hypothalamus, with SRA not being found differentially expressed in our results.

Of the more than 12,000 identified, more than 600 lncRNAs were found differentially expressed by microarray analysis and can now be further studied by QPCR or other methods their possible roles in energy balance. Of the two lncRNAs confirmed by QPCR analysis to be differentially expressed with fasting, neither has been previously characterized for any tissue or condition. AK038506, which we have found to be significantly increased with fasting, was originally identified in adult male mouse hypothalamic cDNA during global cloning studies (Carninci et al. 2005). It is a 1933-nucleotide sequence with only minimal (less than 5 % overlap) to other GeneBank sequences by nucleotide BLAST (data not shown). AK049914, which is also significantly increased with fasting, is a 4061-nucleotide sequence, originally found in adult male mouse hippocampus cDNA, and sequence analysis indicates that this lncRNA appears to overlap within the third and fourth exons of the apolipoprotein D gene (Apod) (Carninci et al. 2005). Furthermore, there is a 289 bp sequence (NC_000082.6: 31308226 to 31308521) that has a very high similarity (97 % identities based on the BLAST search) to a sequence localized about 200 bp upstream to itself (NC_000082.6: 31309821 to 31310117), but still within the two exons of Apod. These together indicate a potential link of transcription activities between AK049914 and Apod. In fact, it has been reported that Apod is up-regulated by calorie restriction in whole brain, skeletal muscle and heart (Yan et al. 2012). A study from 1994 linked the apolipoprotein D gene to obesity and fasting insulin status (Vijayaraghavan et al. 1994). A more recent study has explored the finding that several Alzheimer's disease-associated genes, including apolipoprotein E, have lncRNAs within their genes (Holden et al. 2013). These findings are consistent with the up-regulation of AK049914 during fasting and open the door for future studies on the role of lncRNAs in energy balance regulation, hypothalamus function and diseases such as Alzheimer's disease.

In this study, animals were food-deprived for 24 h, in order to analyze the effects of food deprivation on the whole transcriptome. The mRNAs were studied as part of the whole transcriptome, with interesting findings detected in cell-cycle-related gene expression, through both GO and STRING network analyses. In particular, p21/Cdkn1a was one of the most significant food deprivation up-regulated genes, and this finding was confirmed by QPCR. p21/Cdkn1a controls progression of cells through the cell cycle, by blocking cyclin-dependent kinase activity, with two published reports linking p21/Cdkn1a activity, obesity and adipose tissue (Inoue et al. 2008; Nakatsuka et al. 2012). Interestingly, the link between fasting-induced up-regulation of p21/Cdkn1a has previously been made for hypothalamic tissue as well. In a 2010 study of C57Bl/6 mice undergoing a 48-h food deprivation, p21/Cdkn1a expression was significantly up-regulated (Poplawski et al. 2010). More recently, in a 2013 paper, liver and hypothalamus of mice fasted 24 and 48 h showed significant induction of a p21/Cdkn1a promoter construct (Tinkum et al. 2013). Further examination of our dataset revealed that there were many genes with GO categories related to cell cycle processes. Based on this, we looked into whether differential regulation of other cell-cycle-related genes could be confirmed by QPCR and were able to confirm six out of eight of these genes in follow-up studies on new tissue samples. The apparent relationship between fasting and cell cycle regulation by this dataset is an interesting one, given that studies suggest that the onset of hypothalamic adult neurogenesis is related to high levels of leptin or ciliary neurotrophic factor (Kokoeva et al. 2005). However, up-regulation of p21/Cdkn1a by food deprivation, which lowers serum leptin levels, is consistent with a blockage in cell cycle and reduced proliferation and the opposite of what is seen with leptin and/or CNTF exposure (Cheng 2013). At this time, more studies would be needed to determine whether differential regulation of the p21/Cdkn1a and other cell cycle regulation-associated genes

result in apoptosis, cell cycle arrest or some other event following food deprivation.

It is known, through work using several different models, that calorie restriction can increase individual longevity, and at least one study has examined hypothalamic gene expression in response to a 60 % restricted diet (Fu et al. 2006). In that study, nine genes were differentially regulated by calorie restriction in aging mice, but none overlapped with those in our study. Calorie restriction can reduce levels of oxidative stress and protect proteins, lipids and DNA form oxidative damage, thereby possibly contributing to cellular protection against diabetes, cardiovascular disease, cancers and neurodegenerative diseases (Mattson 2005; Mattson and Wan 2005; Sohal and Weindruch 1996). While an acute fast, such as 24-h food deprivation, is a different treatment than long-term calorie restriction, biological processes category such as "response to DNA damage stimulus" (GO:0006974), "oxidation–reduction process" (GO:0055114) and "lipid biosynthetic process" (GO:0008610) was found among the top groups of GO terms. Likewise, stress-related mRNAs such as Sult1a1, Cirbp and Tsc22d3 were also confirmed to be differentially regulated by QPCR (De Leeuw et al. 2007; Maglich et al. 2004; Szklarczyk et al. 2012). Our findings present new directions to study the potential relationship between the acute and chronic effects of calorie restriction.

In summary, there is little evidence of a global microRNA response following food deprivation of 24 h in mouse hypothalamic tissue, even though microRNAs were detectable in hypothalamus and those tested were differentially expressed when compared in skeletal muscle. It is possible that physiological stressors other than food deprivation may result in differential microRNA expression, or the use of whole hypothalamus in our analysis was not appropriate to detect the select, nuclei-specific microRNAs that were differentially expressed following food deprivation. The study, however, was designed to fully characterize the whole transcriptome, including lncRNAs, of which over 600 found to be differentially expressed. In addition, close to 50 % of all of the mRNA species represented on the array were also detectable in hypothalamic tissue, and over 500 of them were differentially regulated in response to food deprivation. Our confirmation of a relationship between food deprivation and the differential regulation of mRNAs within the cell cycle control gene ontology categories now provides new insight into hypothalamic plasticity and adult hypothalamic neurogenesis (Kokoeva et al. 2005). These data also suggest new directions of research aimed at clarifying role of lncRNA in the fields of obesity, caloric restriction and nutrigenomics, specifically in identifying possible hypothalamic gene regulatory pathways activated by food deprivation signals.

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Compliance with ethical standards

Conflict of interest Hao Jiang, Thero Modise, Richard Helm, Roderick V. Jensen and Deborah J. Good declare that they have no conflict of interest.

Ethical standard All institutional and national guidelines for the care and use of laboratory animals were followed.

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