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Global and specific controls of protein synthesis in hibernators

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GLOBAL AND SPECIFIC CONTROLS OF PROTEIN SYNTHESIS IN HIBERNATORS

by

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ABSTRACT

Global and specific controls of protein synthesis in hibernators

by

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Mammalian hibernation is a highly dynamic physiological process that is composed of a series of torpor bouts, wherein hibernators oscillate between periods of torpor and interbout arousal. Although normally vital to homeostasis, many energetically consumptive processes such as translation or protein synthesis are virtually ceased during hibernation. Earlier studies indicated that protein synthesis had fallen to almost negligible levels. Cap-dependent initiation of translation is well regulated by eukaryotic translation initiation factor 4E (eIF4E) and its binding partner eIF4E-binding protein 1 (4E-BP1) when hibernators cycle in and out the torpor state. Herein, I investigated well-characterized regulatory mechanisms of global and specific control of protein synthesis in hibernators, with the ultimate goal of understanding how hibernators regulate protein synthesis in an extremely dynamic physiological process of hibernation.

Given that cap-dependent initiation of translation was actively regulated during hibernation in golden-mantled ground squirrels, *Spermophilas lateralis*, I was particularly intrigued by the role of cap-independent initiation of translation,

more specifically, the role of internal ribosomal entry site mediated initiation of translation (IRESmt), which allows for internal initiation of translation. I utilized quantitative real time PCR (qRT-PCR) to assess the association of both IRES and non-IRES containing transcripts with ribosomes throughout the entire sucrose gradients as a function of torpor state. Data indicate that mRNAs harboring IRES elements are preferentially associated with ribosomes as a torpor bout progresses. Naturally aroused squirrels have a higher IRES preference index than those animals that are prematurely aroused from torpor. Furthermore, data indicate that this change in IRES preference is the result of changes in mRNA association instead of mRNA abundance. Thus, ribosomes are preferentially loaded with IRES-containing transcripts when squirrels arouse from torpor and translation resumes. Differential translation of preexisting mRNAs may allow for the preferential synthesis of key stress proteins critical for survival of physiological hardships of hibernation that are lethal to non-hibernating mammals.

microRNAs (miRNAs) are a class of conserved ~ 22 nucleotide-long non-coding RNA molecules. miRNAs have been demonstrated to play critical roles in controlling protein synthesis by targeting mRNA transcripts for translational repression in many systems, e.g. miRNAs control translation initiation by inhibiting eIF4E. However, it was largely unknown as to what role miRNAs may play during hibernation. I screened for changes in miRNA populations in livers of golden-mantled ground squirrels as a function of torpor state by utilizing a miRNA microarray. Data indicate that there were no changes in the four major miRNA

species (miRNA-122, miRNA-15, miRNA-21, and miRNA-146) as a function of torpor state. These findings suggest that the potential control of protein synthesis exerted by miRNAs may be limited during hibernation.

p53 is a specific regulator of protein synthesis, e. g. p53 activation can cause dephosphorylation of 4E-BP1 and therefore repress translation initiation. However, p53 is best recognized as a transcription factor that regulates the expression of stress response genes involved in a variety of biological progresses. Earlier studies have shown that transcription is significantly depressed during hibernation. However, some reports in the literature revealed transcription factor movements during torpor, e.g. nuclear factor kappa B (NF-κB) enters the nucleus and p53 is excluded from nucleus. The presumption is that transcription factors function during hibernation and that these changes have an impact on the physiology of the animal. An earlier study claimed that nuclear p53 protein concentration was significantly reduced 4-fold in torpid ground squirrels. I exploited a variety of techniques to further study the role of p53 during hibernation. My data reveal that p53 mRNA and protein levels are not related, but that p53 protein concentrations were elevated during late torpor and interbout aroused states. Data also indicate that expression levels of several known regulators of p53 are consistent with the activation of p53 during hibernation. Taken at face value, these data might suggest that p53 works as a transcription factor to induce preferential transcription of target genes. My data indicate that nuclear p53 levels are ~ 2 fold higher in the late torpor state. Furthermore, p53 protein binds to known target genes. However, nuclear run-on data as well as

quantitative assessment of downstream target genes indicate a very modest role for p53 as a transcription factor. These data are important in that formulated assumptions using lessons learned from active steady state conditions should be applied cautiously in the setting of a depressed metabolic state.

My research investigates the global and specific controls of protein synthesis in hibernation by studying the basic roles of IRES mediated initiation of translation, miRNAs, and transcription factor p53 use in hibernating golden-mantled ground squirrels. These data provide compelling evidence that hibernators actively regulate translation initiation and therefore preferentially produce key stress proteins geared towards enhanced survivorship. However, miRNAs likely play a limited role in the control of protein synthesis in hibernating ground squirrels. Finally, my data on p53 metabolism also underscore the value of cautious data interpretation of regulatory mechanisms at a time of suspended regulation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Mammalian hibernation

Mammalian hibernation is an interesting phenomenon and an amazing survival strategy that has intrigued biologists for decades. Many mammals enter a state of metabolic depression known as torpor in response to adverse environmental conditions, such as food scarcity and cold temperatures in winter (Carey et al., 2003). A typical hibernation season consists of periods of torpor wherein the core body temperature (T_b) may be as low as -2.9°C and metabolic rate may be as low as 1/100th of euthermic rates, interrupted by periodic interbout arousals wherein both T_b and metabolic rate return to euthermic values (Barnes, 1989; Wang and Lee, 2000; van Breukelen and Martin, 2002).

Each torpor bout begins with a relatively gradual decline in T_b to near that of ambient temperature. The length of torpor may last 1 to 3 weeks depending on species, ambient temperature, and time of year (Buck and Barnes, 2000; French, 1982; Geiser and Baudinette 1990; Twente et al., 1977). Upon the completion of a torpor bout, squirrels spontaneously arouse and quickly return T_b to euthermic temperatures. Following a 12 to 24 hour interbout-aroused interval, the next torpor bout begins (Figure 1-1, modified from van Breukelen and Martin, 2002). These torpor cycles are repeated ~ 15 times during a typical ~ 6 months hibernation season for golden-mantled ground squirrels, *Spermophilus (Callospermophilus) lateralis* (Geiser and Kenagy, 1988).

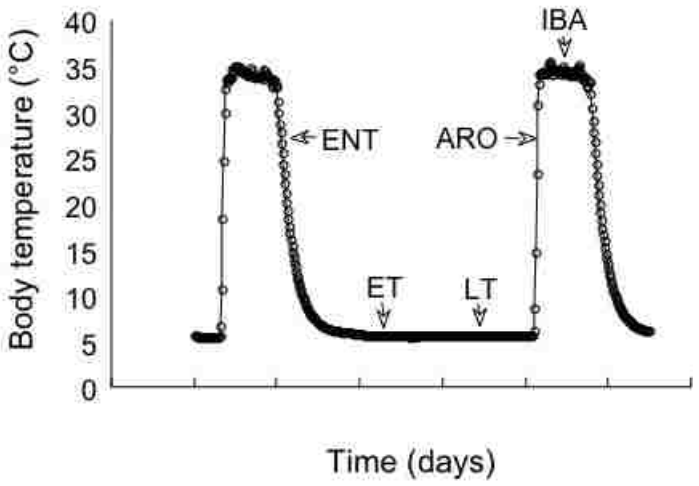

Figure 1-1. Body temperature during a typical hibernation cycle in a golden-mantled ground squirrel housed at 4°C. Each point represents a datum measured every 30 minutes. Points where livers were obtained for this study are marked: ENT, entrance into torpor; ET, early torpor; LT, late torpor; ARO, arousal from torpor; IBA, interbout arousal; not depicted are summer active (SA) animals which were euthermic squirrels collected during the summer to control for seasonal variations (modified from van Breukelen and Martin, 2002).

Protein synthesis during mammalian hibernation

Protein synthesis is one of the more energetically expensive biological processes, representing ~ 20-30% of standard metabolic rate in mammalian liver (Rolfe and Brown, 1997). Since torpid squirrels can only function at ~ 1% of active metabolic rate (Wang and Lee, 2000), it follows that protein synthesis cannot be energetically supported during torpor. Several earlier studies on translation demonstrated that protein synthesis was drastically repressed in torpid ground squirrels. *In vivo* radiolabel incorporation assays showed that protein synthesis was severely diminished to 0.13-0.5% of active euthermic rates during torpor, but was hyperactivated by approximately 1.5 to 2 fold during

interbout arousal compared with summer animals (Zhegunov et al., 1988). Data from polysome distribution assessments indicate that protein synthesis was tightly controlled across a torpor bout. As animals enter torpor, translation initiation was uncoupled from elongation precisely at 18°C, but elongation of nascent polypeptides continued slowly throughout the torpor bout. Intriguingly, during arousal, limited new initiation events occurred at T_b below 18°C, however, the bulk of initiation and elongation were only fully recoupled when T_b reached 18°C (van Breukelen and Martin, 2001).

Control of cap-dependent initiation of translation during hibernation

The translation process may be divided into three distinct steps: initiation, elongation, and termination (Merrick and Hershey, 1996). Initiation is usually the rate-limit step of eukaryotic translation (Kapp and Lorsch, 2004; Gebauer and Hentze, 2004). Not surprisingly, eukaryotic translational control mainly occurs at the phase of initiation by modification of the eukaryotic initiation factors (eIFs) under most circumstances (Reviewed in Kozak, 1992). Most cellular mRNAs are translated by a cap-dependent mechanism wherein ribosomes are recruited to the transcripts through recognition of 5′ cap structure (m^7GpppN, where N is any nucleotide and m is a methyl group) by eIF4E, the cap-binding protein (Reviewed by Gingras et al., 1999; Kapp and Lorsch, 2004). Several translation initiation factors (e.g. eIF2, eIF4B, eIF4E, eIF4GI, eIF4GII and associated binding proteins) were reported to be involved in the regulation of translation in nonhibernators (DeGracia et al., 1996; Gingras et al., 1999; Gradi et al., 1998;

Hershey and Merrick, 2000). Therefore, in order to get a general perspective on translational control during hibernation, van Breukelen et al. (2004), surveyed the key eukaryotic initiation factors for their availability and phosphorylation status in livers of golden-mantled ground squirrels across the torpor bout. Data indicate that modulation of eIF4E activity was the only key control point for regulation of cap-dependent initiation of translation (van Breukelen et al., 2004). The regulation of eIF4E was coordinated through at least two main mechanisms: 1) direct phosphorylation of eIF4E and 2) interaction of eIF4E with its binding proteins, 4E-BP1. Given the role of eIF4E in the regulation of translation initiation, these data suggest that squirrels actively regulate cap-dependent initiation of translation during hibernation. I therefore asked the question: if cap-dependent initiation of translation was regulated, then what happens to the alternative cap-independent initiation during hibernation?

Internal ribosome entry site mediated initiation of translation (IRESmt) during hibernation

IRESmt is a well documented and validated cap-independent translation mechanism (Merrick, 2004). IRES sequences are highly ordered RNA structures located in the 5′-untranslated region (5′-UTR) of an mRNA transcript. IRESes can directly recruit ribosomes to the mRNA molecule and allows protein synthesis to occur in a cap-independent manner (Holcik and Sonenberg, 2005). These IRES sequences were first discovered in 1988 in poliovirus RNA (Pelletier and Sonenberg, 1988). However, a large number of cellular mRNA molecules

also have been experimentally verified to contain IRES elements in their 5′-UTRs. The information about experimentally demonstrated IRES segments are accessible in IRES database (http://iresite.org/).

IRESmt plays a critical role during physiological stresses, including mild hypothermia, hypoxia, and oxidative stress (Holcik and Sonenberg, 2005; Conte et al., 2008; Holcik et al., 2000; Lang et al., 2002; Chappel et al., 2001; Stein et al., 1988) and those stresses are critically important during hibernation. The utilization of IRESmt has been shown to strongly promote expression of stress proteins geared toward enhanced survivorship (Geiser and Kenagy, 1988; Marash et al., 2008). As one might expect, similar to other biologic models, if hibernators use IRESmt, the preferential production of key stress proteins might enable the squirrels to withstand the hardships of hibernation. Therefore, I asked a very fundamental question, if squirrels load their ribosomes with IRES containing transcript during hibernation in order to produce key stress proteins geared toward enhanced survivorship? My findings indicate that, as squirrels arouse from torpor and translation resumes, IRES containing transcripts preferentially associate with actively translating polysomes. Squirrels exploit IRESmt to gain differential translation of preexisting transcripts so as to preferentially produce key stress proteins geared toward enhanced survivorship. Direct comparison of IRES preference indices between naturally aroused and alarm aroused squirrels indicate that naturally aroused animals are poised to experience greater IRESmt, suggesting that squirrels likely prepare for arousal from torpor.

miRNAs and translational control

miRNAs are small non-coding RNAs that negatively regulate gene expression by binding to specific sequences in the 3′-UTR of target mRNAs (Branislav et al., 2006) and 10-30% of protein-coding genes are predicted to be regulated by miRNAs (Branislav et al., 2006). The interaction between miRNAs and target mRNAs may result in either translation inhibition or induction of mRNA cleavage. If miRNAs are perfectly base-paired to the 3′-UTR of the target mRNAs, they will induce target mRNA degradation by direct cleavage of a single phosphodiester bond, which is common in plants. More common in animals is a mechanism wherein miRNAs that are imperfectly base-paired to target mRNAs will inhibit the translation of that mRNA. Complementarity seems to be the key factor in determining the fate of the target mRNA (Branislav et al., 2006).

miRNAs have been demonstrated to play critical roles in a variety of fundamental biological processes, including proliferation, differentiation, development, cell death and interactions between virus and host cells in other systems (Branislav et al., 2006). The miRNA Registry (http://microrna.sanger.ac.uk/sequences/) provides a list of the annotated miRNAs across various species. Although various functions of miRNAs in plants and animals have been revealed recently, however, little is known about their identity and potential functions in hibernating ground squirrels. As miRNAs play important roles in regulation of protein synthesis, I asked whether metabolic depression during hibernation led to changes in cellular miRNA expression. To

address this question, I did a comprehensive screen of miRNA species in liver as a function of torpor state. However, data indicate that there were no changes in the four major miRNA species identified in liver as a function of torpor. One of these, miRNA-122, was robustly expressed in both SA and LT in all four independent miRNA screens. These data suggest limited roles, if any, of miRNAs in liver of hibernating ground squirrels.

p53: more than a transcription factor

The tumor-suppressor protein p53 is best recognized as an important transcription factor that plays a critical role in cellular responses to a variety of stress signals, predominately through its ability of transactivating or transrepressing different target genes (Bode and Dong, 2004). In particular, p53 also functions as a regulator of protein synthesis. The activation of p53 protein can cause a rapid impairment of global protein synthesis through three different mechanisms: (1) p53 stabilizes RNA secondary structure in 5′- UTR and therefore slows or hinders ribosome scanning; (2) p53 forms complexes with ribosome RNAs and proteins and thus directly affects translational machinery; (3) p53 modulates activities of initiation factors, e. g. p53 activation causes dephosphorylation of 4E-BP1 (Ewen and Miller, 1996). This p53-mediated down-regulation of overall protein synthesis occurs at the level of initiation of translation (Horton et al., 2002) and is accompanied by specific cleavages of translation initiation factor eIF4G and eIF4B (Constantinou et al., 2003).

Normally, p53 is highly unstable, with a short half-life of 20 minutes (Davidoff et al., 1992) and is maintained at low levels by ubiquitin-mediated protein degradation (Bode and Donge, 2004). However, p53 ubiquitylation is inhibited during stress exposure and therefore p53 becomes stabilized and activated. The overexpressed p53 protein accumulates in the nucleus, where it functions as a transcription factor to control sets of target genes involved in a variety of biological processes (Nagaich et al., 1999). When no longer needed after a stress response, excess p53 is degraded through the ubiquitin-dependent process to return p53 concentration to normal levels (Joseph et al., 2003). Multiple post-translational modifications are shown to be involved in the processes of p53 stabilization, activation and accumulation in the nucleus and therefore markedly influence the expression of p53 target genes (Bode and Donge, 2004). For example, monoubiquitylation may negatively regulate the transcriptional activity of p53 by preventing it from binding to DNA and is also strongly associated with its nuclear export from the nucleus to the cytoplasm, where p53 would become polyubiquitylated and degraded by 26S proteasome degradation (Rodriguez et al., 2000). As a result, p53 could be a very interesting protein to study in the context of hibernation where transcription is severely depressed. Transcription accounts for as much as 10% of a cellular energy budget (Rolfe and Brown 1997), meaning that transcription is not likely affordable during torpor when metabolic rate is dramatically reduced to as low as 1% of active rates (Wang and Lee, 2000). Earlier studies demonstrated a remarkable decrease in the global transcriptional activity by ~ 90% during torpor when

compared to euthermic values (Bocharova et al., 1992). More recent studies used nuclear run-on assays to demonstrate that the global depression of transcriptional activity was a combination of a two-fold reduction in initiation and a greater than 90% reduction in elongation due to temperature sensitivity in torpid golden-mantled ground squirrels (van Breukelen and Martin, 2002). Given that, a very fundamental question that remains unanswered is what happens to p53 transcriptional activity in the context of hibernation where global depression of transcriptional activity is detected? My study provided compelling insights and also posed interesting questions regarding p53 activity during hibernation. Data indicate that p53 was seemingly activated and accumulated in the nucleus during torpor, and nuclear p53 did bind to known target genes. However, somewhat paradoxically, p53 was not an effective transcription factor as evidenced by cellular [mRNAs] of known p53 target genes.

Experimental System

Many animals throughout the class Mammalia have been used in hibernation research, but a large amount of data were obtained from ground dwelling rodents in the family Sciuridae, such as prairie dogs, marmots, ground squirrels and chipmunks (Carey et al., 2003). My PhD research is focused on using golden-mantled ground squirrel [*Spermophilus (Callospermophilus) lateralis*] as a model to study translational control in response to stress.

Golden-mantled ground squirrels are exceptional hibernators and they usually experience predictable torpor bouts, typically 15 times during a 6 month

hibernation season, when housed in 4°C environmental chambers (Geiser and Kenagy, 1988; van Breukelen and Martin, 2002). During torpor, they may experience body temperatures that may be below 0°C and metabolic rates that may be as low as 1% of active rates (Frank, 1992; Wang, 1979; Wang and Lee, 2000).

Golden-mantled ground squirrels are widely distributed herbivorous rodents found in mountainous areas of western North America. All experimental animals used for my dissertation research were captured in July or early August from southern Nevada, Utah, or California. Some animals were killed immediately as a seasonal control. The remaining squirrels were maintained on a diet of rat chow supplemented with sunflower seeds. In order to precisely measure the T_b throughout the hibernation season, temperature sensitive radiotelemeters (model VM-FH disc; Mini Mitter, Sun River, OR) were surgically implanted into the abdominal cavity of squirrels in October, prior to the hibernation season. Following recovery from surgery, implanted squirrels were housed in an environmental chamber at 4°C and allowed to hibernate. Animals spontaneously stopped feeding, and torpor bouts typically commenced in early November. The T_b of torpid squirrels was ~ 5°C.

Livers were obtained from ground squirrels representing various stages of a torpor bout, snap-frozen in liquid N_2, and stored at -80°C until use. Here are the three reasons I chose liver as research material: 1) hepatic function accounts for ~ 20% of basal metabolic rate, making it a great organ to work on in the context

of severe metabolic depression; 2) liver has fairly homogeneous cell types; 3) most of the previous research from our laboratory has been done in liver.

The University of Nevada, Las Vegas Institutional Animal Care and Use Committee approved all procedures related to the experiments discussed in this dissertation.

Scope of the Study

The research goals of this dissertation are to investigate the global and specific controls of protein synthesis in hibernating ground squirrels by studying the basic roles of IRES mediated initiation of translation, miRNA, and transcriptional factor p53.

In chapter 2, I asked what happens to cap-independent initiation of translation during hibernation when cap-dependent initiation is compromised. More specifically, what is the role of IRESmt, which allows translation initiation to occur independent of the 5′ mRNA cap structure? I employed sucrose gradient fractionation and quantitative real time PCR (qRT-PCR) to assess the association of both known IRES and non-IRES containing transcripts with ribosomes as a function of torpor state. My analyses demonstrated that ribosomes are referentially loaded with IRES-containing transcripts when squirrels arouse from torpor and translation resumes. This differential translation of preexisting mRNAs may allow for preferential production of key stress proteins critical for survival of physiological insults that are lethal to other non-hibernating mammals.

In chapter 3, I investigated the role of miRNAs, as a putative mechanism to explain the depression of protein synthesis during mammalian hibernation. I utilized commercial NCode multi-species miRNA microarrays to screen miRNA species and determine the changes in their concentrations. The results indicate no significant change in the abundance of major miRNA species in liver as a function of torpor state, suggesting the limited importance of miRNAs during mammalian hibernation.

In chapter 4, I extensively studied the transcriptional activity of p53 in hibernators by using a variety of techniques. Data indicate that although p53 is localized in the nucleus and is also binding to the known target genes, it is not a effective transcription factor. This study provides new insight into p53 function during hibernation. A particular interesting question is put forward, if p53 does not work as a robust transcription factor, then what is the possible role of p53 during hibernation? Could p53 work a specific regulator of protein synthesis by modulating initiation factor activities?

Altogether, my research has revealed significant aspects and suggested mechanisms that are fundamental for our understanding of translational control in hibernation.

CHAPTER 2

PREFERENCE OF IRES-MEDIATED INITIATION OF TRANSLATION

Abstract

Mammalian hibernation involves virtual cessation of energetically consumptive processes normally vital to homeostasis, including gene transcription and protein synthesis. As animals enter torpor, the bulk of initiation of translation is blocked at a body temperature of 18°C in golden-mantled ground squirrels [*Spermophilus (Callospermophilus) lateralis*]. Previous data demonstrated regulation of cap-dependent initiation of translation during torpor. We asked what happens to cap-independent, specifically, IRES mediated initiation of translation during hibernation. We analyzed polysome fractions for mRNAs that are known to contain or not to contain IRES elements. Here, we show that mRNAs harboring IRES elements preferentially associate with ribosomes as a torpor bout progresses. Squirrels allowed to naturally complete a torpor cycle have a higher IRES preference index than those animals that are prematurely aroused from torpor. Data indicate that this change in preference is not associated with gene expression, i.e., change is due to change in mRNA association with ribosomes as opposed to mRNA abundance. Thus, although processes like transcription and translation are virtually arrested during torpor, ribosomes are preferentially loaded with IRES-containing transcripts when squirrels arouse from torpor and translation resumes. Differential translation of preexisting mRNAs may allow for the preferential production of key stress

proteins critical for survival of physiological insults that are lethal to other mammals.

Statement of Previously Published Work

The content of this chapter were previously published in the American Journal of Physiology (Pan and van Breukelen, 2011). The copyright release is not required as seen in Appendix 2. Peipei Pan is the first author for the publication. All work was conducted in and financially supported by the van Breukelen laboratory. Peipei Pan solely performed the data collection and the initial writing of the manuscript. Peipei Pan and Frank van Breukelen jointly conducted the statistical analysis and edited the manuscript. Chris Ross assisted in cluster analyses.

Introduction

Many mammals enter a state of metabolic depression known as torpor to survive winter when faced with limited food availability and cold environment (Carey et al., 2003). During torpor, ground squirrels may experience T_b that may be as low as -2.9°C (Barnes, 1984) and metabolic rates that are as low as 1% of active rates (Wang and Lee, 2000). Torpor during the hibernation season is not continuous but rather is interrupted by periodic euthermic arousals, and so the overall energetic savings may be as high as 90% (Wang, 1979). Each torpor bout begins with a relatively gradual decline in core T_b to near that of ambient temperature. A torpor bout may last 1–3 wk depending on species, ambient

temperature, and time of year (Storey, 2001). Upon the completion of a torpor bout, squirrels spontaneously arouse and quickly return T_b to euthermic temperatures. Following a 12- to 24-h interbout aroused period, the next torpor bout begins. These torpor cycles are repeated ~ 15 times during a typical ~ 6-month hibernation season for golden-mantled ground squirrels (Geiser and Kenagy, 1988).

Although normally vital to homeostasis, protein synthesis represents ~ 20-30% of standard metabolic rate (Rolfe and Brown, 1997). This energetic outlay is incompatible with the metabolic rates of mammalian hibernation; translation is severely reduced to near-negligible levels during torpor (0.13–0.5% of euthermic values) and is fully restored during the interbout arousal (van Breukelen and Martin, 2001; 2002). When mRNAs are actively translated, they will associate with multiple ribosomes, forming polyribosomes or polysomes. Polysome analyses revealed that initiation of translation is markedly reduced during the entrance into torpor when T_b reaches 18°C (van Breukelen and Martin, 2001). However, elongation of nascent peptides continues slowly throughout the torpor bout. At the end of the torpor bout, few polysomes remain. As T_b begins to rise, some new initiation occurs, but translational initiation and elongation are only fully recoupled when T_b reaches 18°C. A survey of major regulatory initiation factors that could be involved in the active suppression of initiation of translation revealed one regulatory locus in livers of hibernating ground squirrels (van Breukelen et al., 2004). eIF4E, the cap-binding protein, was regulated during torpor. Regulation of eIF4E, and thus the regulation of cap-dependent initiation

of translation, is known to occur by at least two distinct mechanisms: *1)* direct phosphorylation of eIF4E and *2)* interaction of eIF4E with binding proteins, e.g., 4E-BP1. 4E- BP1 was absent in summer but present in winter in golden-mantled ground squirrels (van Breukelen et al., 2004). In summer, squirrels apparently control initiation of translation through reversible phosphorylation of eIF4E. In winter, squirrels appear to control initiation of translation through reversible binding to 4E-BP1. 4E-BP1 is differentially phosphorylated between the torpid and active states (van Breukelen et al., 2004). Given the role of eIF4E in regulating initiation, these data suggest that squirrels actively downregulate cap-dependent initiation of translation during hibernation.

We asked what happens to cap-independent initiation during hibernation when cap-dependent translation is inhibited. More specifically, what is the role for IRESmt, which allows for translational initiation to occur independent of the commonly used 5′-mRNA cap structure (Holcik and Sonenberg, 2005)? IRESmt is a well-documented and -validated cap-independent translation mechanism (Merrick, 2004). In IRES use, a subset of mRNAs contain higher-ordered RNA structures in the 5′-untranslated region. Theses structures allow for the recruitment of eukaryotic initiation factors (eIF) independent of the 5′,7-methyl guanosine cap of mRNA and eIF4E function (Svitkin et al., 2005). IRESmt is important during physiological stresses, including mild hypothermia, hypoxia, and oxidative stress (Chappell et al., 2001; Conte et al., 2008; Holcik et al., 2000; Holcik and Sonenberg, 2005; Lang et al., 2002; Stein et al., 1988). The utilization of IRESmt promotes differential protein expression geared toward

enhancing survivorship (Marash et al., 2008). Similarly, if hibernators used IRESmt, one might expect the translation of stress proteins that could enable the squirrels to withstand the hardships of hibernation. To determine the role of IRESmt in hibernation, we asked if IRES-containing transcripts are preferentially associated with ribosomes during a torpor bout.

Materials and Methods

Animals and Tissue collection

Adult golden-mantled ground squirrels [*Spermophilus (Callospermophilus) lateralis*] were captured in July and early August from southern Nevada and California. Some animals were killed immediately as a seasonal control [summer active (SA), T_b = ~ 37°C]. The remaining squirrels were maintained on a diet of rat chow supplemented with sunflower seeds. These squirrels were implanted in October with temperature-sensitive radiotelemeters (Minimitter, Sun River, OR) that allowed for the precise determination of torpor state. Following recovery from surgery, implanted squirrels were housed in an environmental chamber at 4°C and allowed to hibernate. Animals spontaneously stopped feeding, and torpor bouts typically commenced in early November. The T_b of torpid squirrels was ~ 5°C. All winter animals were killed in February to early March by CO_2 asphyxiation except for the torpid animals. Torpid animals were killed by decapitation because of their low respiratory rates. Livers were obtained from ground squirrels representing various stages of a torpor bout: euthermic animals killed in summer (SA; *n* = 3); animals during entrance into torpor [at T_b of 10°C

(10.1 ± 0.4), 20°C (19.8 ± 0.2), or 30°C (30.0 ± 1.0); *n* = 3]; animals that were torpid for ~ 2 days (early torpor; *n* = 3); animals that had completed ~ 80% of the predicted torpor bout (typically 7 days; late torpor; *n* = 3); animals that were arousing from torpor spontaneously [natural arousal at T_b of 10°C (10.0 ± 0.1), 20°C (20.4 ± 0.1), or 30°C (31.1 ± 1.1); *n* = 3]; animals that were induced to arouse prematurely in response to gentle shaking [alarm arousal at T_b of 10°C (10.3 ± 0.2), 20°C (20.8 ± 1.0), or 30°C (28.5 ± 1.0); *n* = 3]; and animals that were euthermic between torpor bouts (interbout aroused; *n* = 3). Livers were removed, snap-frozen in liquid N_2, and stored at −80°C until use. All experiments were approved by the University of Nevada Las Vegas Institutional Animal Care and Use Committee.

Choice of transcripts

Transcripts that are experimentally verified in other species to contain an IRES site as well as control transcripts that are known not to participate in IRES-mediated initiation of translation were selected using the IRES database (http://www.rangueil.inserm.fr/IRESdatabase). 28S rRNA was used to normalize for the amount of ribosome. The primers were designed based on the homologous genes from either other ground squirrels or *Mus musculus* queried from GenBank with corresponding accession numbers (Table 1-1). The amplified fragments were sequenced to confirm transcript identity and sequences have been deposited in GenBank. BiP, c-myc, and CaM Kinase II were used as IRES-containing transcripts, while GAPDH and β actin were used as non-IRES

transcripts. Please see Supplemental Data and accompanying Figure S1 and Table S1 for further details on choice of transcripts. We note that IRES use has been well conserved in closely related species. The c-Myc IRES has been experimentally verified in human and mouse (Créancier et al., 2001; Storey 2001); the BiP IRES was verified in human, rat, mouse, Chinese hamster and several invertebrate species (Kim et al., 2000; Le and Maizel,1997; Thoma et al., 2004, Young et al., 2008); and the CaM kinase II IRES was verified in mouse, rat, and rabbit (Fährmann and Kaufhold, 2006; Lin et al., 2005; Pinkstaff et al., 2001).

Table 1-1. Oligonucleotide primers used for qRT-PCR

Gene Name	Forward Primer	Reverse Primer
β-Actin	5'-TGGCATTGTGATGGACTCC-3'	5'-GTAGCCACGCTCAGTCAG-3'
GAPDH	5'-AAGGTCGGAGTGAACGGAT-3'	5'-CTTTGATGTTGGCGGGAT-3'
BiP/GRP78	5'-CATCCCGTGGCATAAAC-3'	5'-TAGACCTTGATTGTAACAGTAG-3'
c-Myc	5'-GACAGCCTTCTGCCAGAAG-3'	5'-TGCGTAATTGTGCTGGTGTG-3'
CaM kinase II	5'-GAGGATGAAGATGCCAAAGC-3'	5'-GCGGATGTAGGCGATGC-3'
28S	5'-CGAAACGATCTCAACCTA-3'	5'-CGGCAGGTGAGTTGTTA-3'

Known non-internal ribosome entry site (IRES) containing transcripts include actin and GAPDH while BiP, c-myc and calmodulin kinase (CaM kinase) II transcripts are known to harbor IRES elements. 28S rRNA was used to normalize for the amount of ribosomes. Amplified fragments were sequenced and sequences were deposited in GenBank.

Polysome fractionation and polysome RNA isolation

Polysomes were isolated using a sucrose density gradient with an ISCO flow system as previously described (van Breukelen and Martin, 2001). The gradients were recovered in 12 one ml fractions. The early fractions contain monosomes and oligosomes whereas polysomes are restricted to later fractions. We pooled the fractions into light (early fractions 2-5) and heavy (later fractions 7-12) pools to test if there were differences in oligosomes vs heavy polysomes.

Quantitative real time PCR (qRT-PCR)

Polysome RNA was isolated from the pooled fractions with TRIzol Reagent (Invitrogen, Carlsbad, CA). Polysome RNA for each sample was used in a reverse transcription reaction to synthesize single stranded cDNA (SuperScript™ III Reverse Transcriptase, Invitrogen, Carlsbad, CA). A total RNA sample was isolated from a single SA animal and used as a standard reference to avoid issues with differential efficiencies of qRT-PCR plates (Figure 2-2). A reference standard curve was constructed for each assayed transcript from this serially diluted and quantified total RNA (dilution factors of 1X, 5X, 25X, 125X, and 625X). qRT-PCR was used to investigate the association of both IRES (BiP, c-myc, and CaM Kinase II) and non-IRES (GAPDH and β actin) containing transcripts with ribosomes in various stages of a torpor bout from golden-mantled ground squirrels with corresponding specific primers (Table 1-1). qRT-PCR was performed on both the polysome fractions and for standard curve construction using the SYBR Green I kit with ROX (Bio-Rad, Hercules, CA). Reactions (1

reaction per animal for both the transcript of interest and 28S rRNA along with the standard curve) were performed on a single plate to reduce interplate variability. Preliminary experiments revealed little difference between replicate plates provided the reaction efficiencies were high. All PCR reactions were run with the following program: 3 min at 95° C, 15 s at 95° C, 30 s at 52° C, and 30 s at 72° C (40 cycles) with a mixture containing 1 μl of cDNA template, 12.5 μl qPCR Supermix, and 200 nM of each primer in a total volume of 25 μl (Bio-Rad, Hercules, CA). Melt curves to ascertain that real specific products were synthesized were obtained using the following program: 1 min at 95° C, 1 min at 55° C, and 80 cycles of 10 s at 55° C with a step of 0.5° C every cycle.

Construction of IRES preference, abundance indices and statistical analyses

Results from qRT-PCR were compared against a reference standard curve made from total RNA of a single summer active (SA) animal (Figure 2-1). The raw critical threshold (C_t) values were then normalized for the amount of starting material, and expressed as the concentration of transcript of interest/28S rRNA ratio (Wong and Medrano, 2005). From earlier work (van Breukelen and Martin, 2001 and references therein), we know that during torpor, there is a dramatic reduction in the quantity of protein synthesis (and therefore heavy polysomes). The normalization to ribosomal RNA removes quantitative changes in translation to the analysis (see discussion). The ratios were log-transformed and the IRES preference index was obtained by summing the log-transformed values from the known IRES containing transcripts and then subtracting the log-transformed

values from the known non-IRES containing transcripts [IRES preference index = ∑log (normalized IRES transcript concentration) - ∑log (normalized non-IRES transcript concentration)]. To make the data on IRES preference more accessible as a single value for comparison, we converted the average values for all animals at a given torpor stage into an RGB color scale. Values are on a scale of 0-255, in which 0 is magenta (lowest IRES preference index value as a % of maximum IRES preference index value) and 255 is green (maximum IRES preference index value). IRES preference indices were analyzed by average linkage cluster analyses (uncentered correlation) for all individual animals to test if there was a switch in the preference as a function of torpor state. Data for both heavy and light fractions on IRES preference index were subjected to paired t-test to determine if there was an effect of fraction pool. Analyses demonstrated no effect of fraction pool e.g. light versus heavy fractions on IRES preference (paired t-test, $P > 0.05$) and values for both pools were summed in all graphical representations.

The IRES abundance index was determined for these mRNAs in a similar fashion to the IRES preference index except qRT-PCR was performed on total RNA as opposed to polysome RNA from these same animals. The results from transcript abundance were subjected to ANOVA with Tukey adjustment for multiple comparisons and differences where $P < 0.05$ were considered significant (Livak and Schmittgen, 2001).

EDTA treatment control

Lysates from summer active (SA) and early arousal (early ARO; $T_b \approx 10^\circ$ C) animals were loaded onto a sucrose gradient and polysomes were isolated as previously described (Schultes et al., 2005; same methodology as used above). Parallel lysates were pre-treated with 30 mM EDTA prior to loading. All experiments were performed as above except total RNA was subjected to analysis by the Bio-Rad Experion system (indicates integrity and concentration; analyses as per manufacturer's directions) to show disruption of RNA from heavy material by EDTA.

Results

Efficiencies of Quantitative Real-time PCR Reactions

We exploited qRT-PCR to determine the quantity of specific mRNAs that associated with ribosomes during torpor. To avoid issues with differential efficiencies of qRT-PCR plates and changes in the quantity of protein synthesis as a function of torpor state, the raw critical threshold (C_t) values were compared against a reference standard curve made from total RNA of a single animal, transformed to the amount of starting material and expressed as the concentration of transcript of interest/28S rRNA ratio. Our reactions had reaction efficiencies of 92.84 ± 2.51% (Figure 2-1).

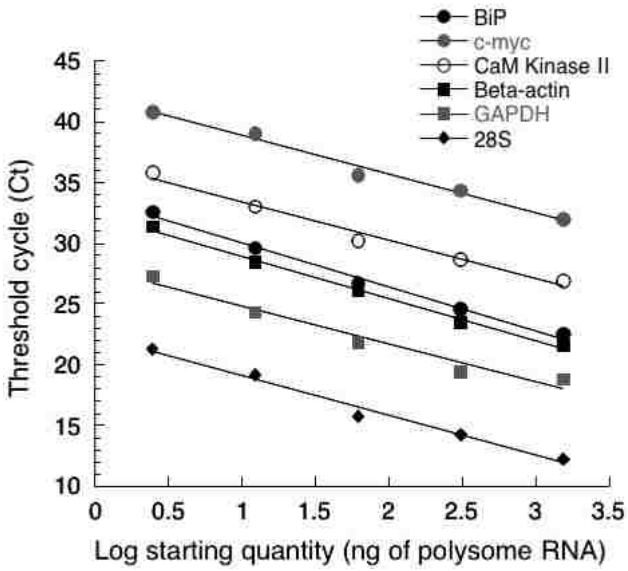

Figure 2-1. Efficiencies of quantitative real-time PCR (qRT-PCR) reactions. To avoid issues with differential efficiencies of qRT-PCR plates and expressing values as $\log_2$ e.g. using raw critical threshold (C_t) values, samples were compared against a reference standard curve made from total RNA of a single animal. Correlation coefficients (r^2) for the standard curve were > 0.98. Reaction efficiency (E) of the reactions was calculated by the equation: $E = 10^{(-1/slope)}-1$. Reaction efficiencies of our PCR reactions across dilutions were 92.84 ± 2.51%. CaM Kinase, calmodulin kinase.

EDTA Treatment

RNA association with proteins is disrupted by pretreatment with EDTA (Cardinali et al., 2003). EDTA treatment disrupted the polysome profile in our experiments (Figures 2-2, 2-3, and 2-4). We demonstrate that EDTA treatment shifted polysome profiles toward monosome and oligosome-bearing fractions for total RNA (Figure 2-2) as well as for specific messages amplified by qRT-PCR (Figure 2-3). We note that, as a result of EDTA treatment, shifts in 28S rRNA coincide with shifts in transcript levels (Figure 2-3). We show sample profiles for early arousing (T_b ~ 10°C) and SA ground squirrels to demonstrate the

differences in RNA distributions as a function of state (Figure 2-4). Such differences were used as a basis for the calculated IRES preference index.

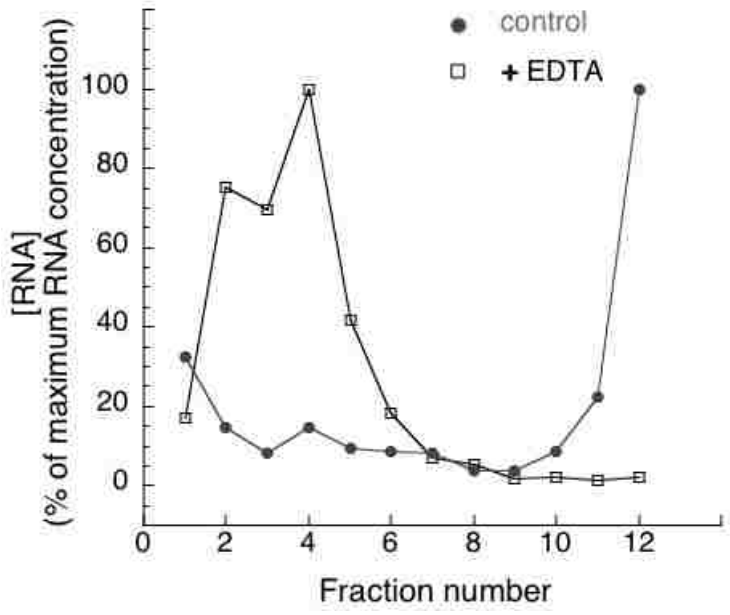

Figure 2-2. EDTA treatment disrupts polysome association with RNA. A lysate from a summer active (SA) animal was loaded onto a sucrose gradient as indicted in the text (control; gray filled circle). A parallel lysate was treated with treated with 30 mM EDTA prior to loading (+ EDTA; black open squares). The RNA concentration in each fraction was determined following exposure to an RNA-specific dye using the Bio-Rad Experion system. Values were normalized to the maximum value for that gradient and expressed as % maximum. Note EDTA treatment shifts the RNA distribution profile towards the lighter fractions indicative of polysome disruption.

Preferential association of IRES-harboring mRNA Transcripts to Ribosomes

As a torpor bout progresses, mRNAs that harbor IRES sequences preferentially associate with ribosomes (Figure 2-5). Squirrels in the summer experience an IRES index that is biased toward cap-dependent initiation of

translation. During winter, squirrels entering torpor have similar indexes as summer squirrels, indicating preference for cap-dependent initiation of translation. However, as a torpor bout progresses, IRESmt becomes more and more dominant until the highest IRES indexes are obtained during the arousal process. This result suggests that, as squirrels arouse from torpor and translation resumes, their ribosomes are preferentially loaded with mRNAs containing IRES elements. To ensure that these changes were bona fide, we performed average linkage cluster analyses (uncentered correlation) on our obtained IRES preference indexes and found two groups (Figure 2-6) demonstrating a shift in the preference of transcripts to associate with the ribosomes as a function of torpor state.

Expression levels of mRNA Transcripts

To detect if the observed changes in IRES preference were the result of a bias of association of IRES participants with ribosomes as opposed to differential expression of mRNA, a similar index was performed using qRT-PCR from total RNA (as opposed to polysome fractions) from these same animals. An abundance index was determined for these mRNAs in a similar fashion to the IRES preference index (Figure 2-7). No significant differences were found in the transcript abundance (ANOVA with Tukey adjustment for multiple comparisons, $P > 0.05$ for all comparisons), demonstrating that the observed effect of association of IRES-containing transcripts to ribosomes was not the result of changes in concentration of mRNAs but rather changes in association.

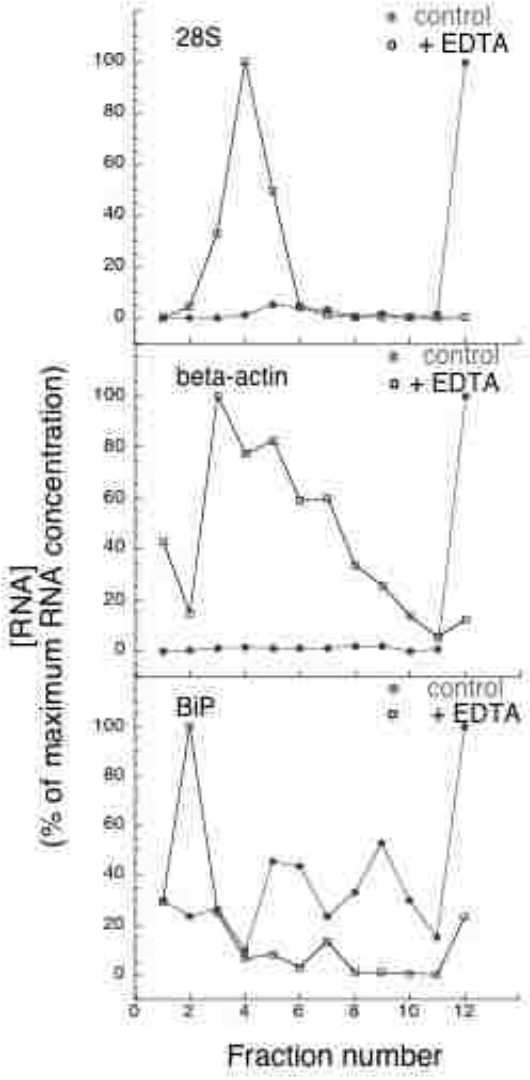

Figure 2-3. EDTA treatment disrupts polysome association with mRNA measured by qRT-PCR. A lysate from a summer active (SA) animal was loaded on a sucrose gradient as indicted in the text (control). A parallel lysate was treated with 30 mM EDTA before loading (EDTA). The abundance of specific RNA species was measured by qRT-PCR. 28S rRNA was used as a loading control for the amount of ribosome. β-ctin is encoded by a non-internal ribosome entry site (IRES) containing transcript. BiP′s transcript harbors an IRES element. Values are indicated as the percentage of the maximum value in the gradient for each RNA species. Note that EDTA treatment shifts the RNA distribution. [RNA], RNA concentration.

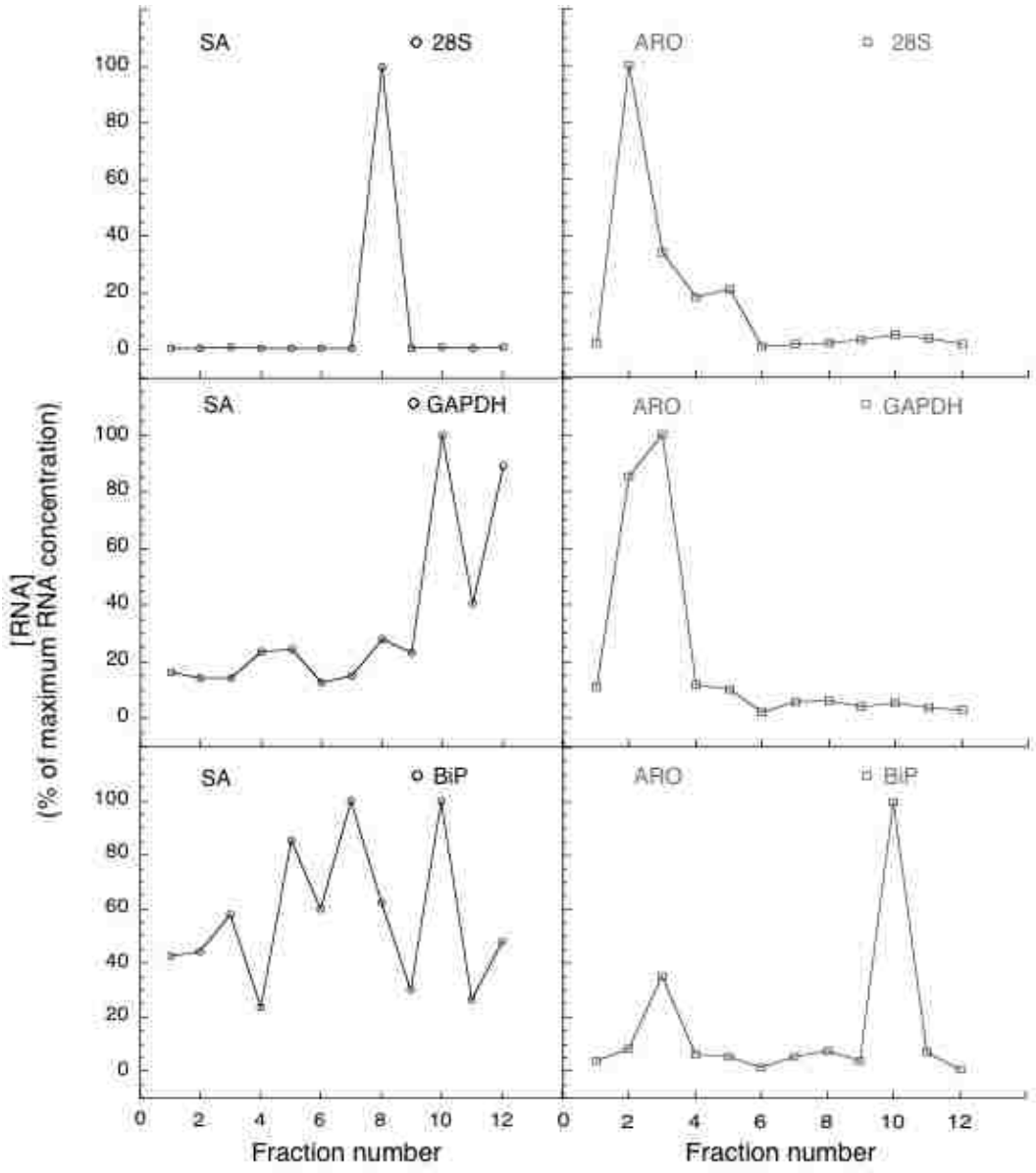

Figure 2-4. IRES-containing mRNAs are relatively more abundant on heavy polysomes in early arousing squirrels. Lysates from SA (left) and early arousing (ARO; body temperature ~ 10°C; right) animals were loaded on a sucrose gradient as indicated in the text. The abundance of specific RNA species was measured by qRT-PCR. Note that, although most of the 28S and non-IRES-containing GAPDH RNA is found in the lighter fractions in the ARO animal, the IRES-containing BiP is found most frequently in a heavy polysome-containing fraction.

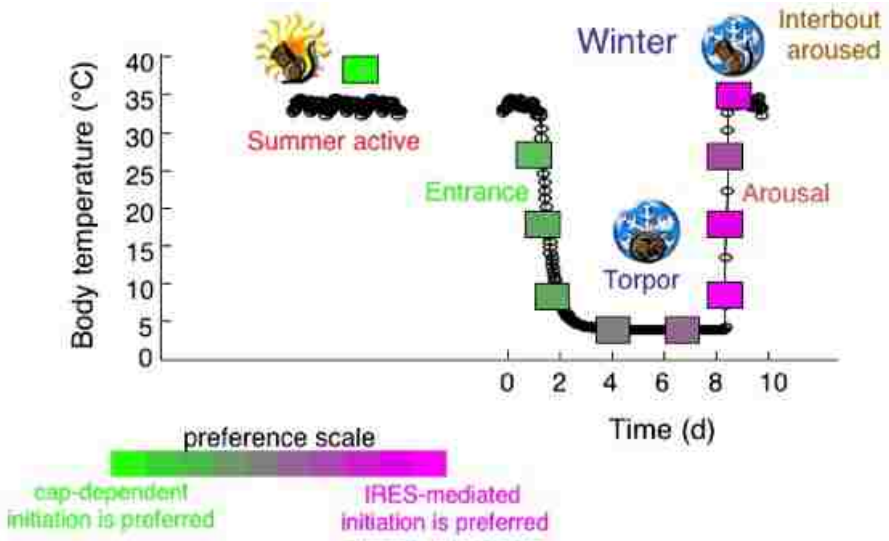

Figure 2-5. IRES-containing mRNAs preferentially associate with ribosomes as a torpor bout progresses. The cartoon depicts the dynamic nature of IRES preference across a typical torpor bout as body temperature (T_b) oscillates during hibernation. Superimposed are color-coded data for a calculated IRES preference index for each sampling point used. In summer, squirrels experience a preference for cap-dependent initiation of translation (indicated by green square). During the winter, squirrels entering torpor have similar IRES preference indexes as summer squirrels. However, by the time a squirrel naturally arouses from torpor and protein synthesis resumes, its ribosomes are preferentially loaded with IRES-containing mRNAs (indicated by magenta square). d, Day.

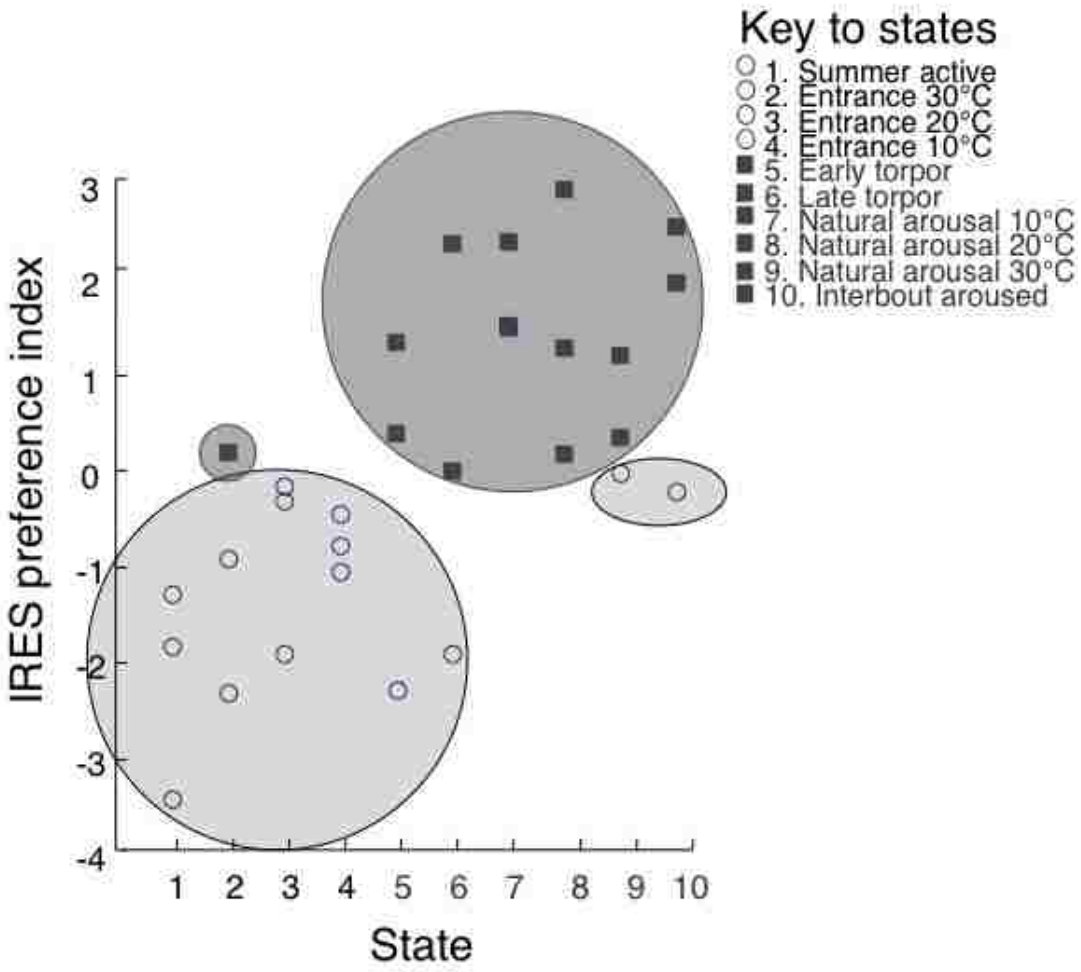

Figure 2-6. IRES preference indexes are clustered into two natural groups. IRES preference index data used in Figure 2-4 were clustered into two groups by average linkage cluster (uncentered correlation) using the Clustering and Tree View software packages. The IRES preference index was plotted for each animal for all states except alarm arousal. Cluster analysis revealed two natural clusters with the two groups generally coinciding with the beginning of the torpor bout represented by open circles and the end of the torpor bout represented by filled squares. These analyses demonstrate that there is a shift in the bias of messages that associate with ribosomes toward IRESmt during the torpor bout. Five data points were outliers of the natural clustering and are indicated by different symbols, e.g., during interbout aroused (IBA; state 10), one datum clusters with the beginning of the torpor bout instead of at the end of the torpor bout.

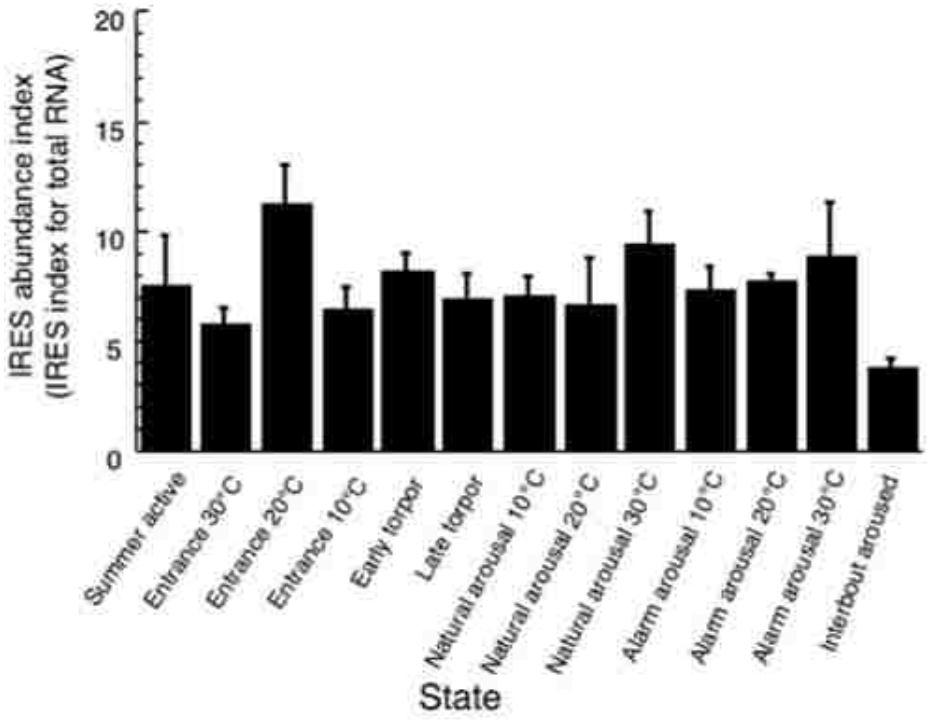

Figure 2-7. IRES-harboring transcript abundance was not changed as a function of torpor bout. IRES abundance index demonstrates that there is no preferential expression of IRES genes during the torpor bout. An abundance index was calculated similarly to the IRES preference index used in Figs. 2-5, 2-6, and 2-8 except the values were obtained using total RNA and not ribosome-associated RNA. Values represent means ± SE, n = 3 animals for each state. There are no significant differences between the various states ($P > 0.05$, ANOVA with Tukey correction for multiple comparisons). These data indicate that the observed changes in IRES preference are the result of preferential translation and not differential gene expression.

Premature arousal from torpor affects the IRES preference index

Normally, golden-mantled ground squirrels spontaneously arouse from torpor in a very predictable fashion. However, squirrels may also be induced to arouse prematurely before the completion of the torpor bout through various disturbances such as shaking, loud noises, or sudden shifts in ambient

temperature (unpublished observations). We alarm-aroused squirrels by gentle shaking before the completion of the torpor bout. Premature arousal from torpor affects the IRES preference index (Figure 2-8). These data indicate that there is a shift during the torpor bout such that animals that spontaneously arouse are poised to experience greater IRESmt (ANOVA, $P < 0.05$). Although animals who had a T_b of 10°C show a significantly reduced IRES preference when alarm aroused, IRESmt may be exploited by the time animals reach T_b of 20°C during the arousal process (Figure 2-8).

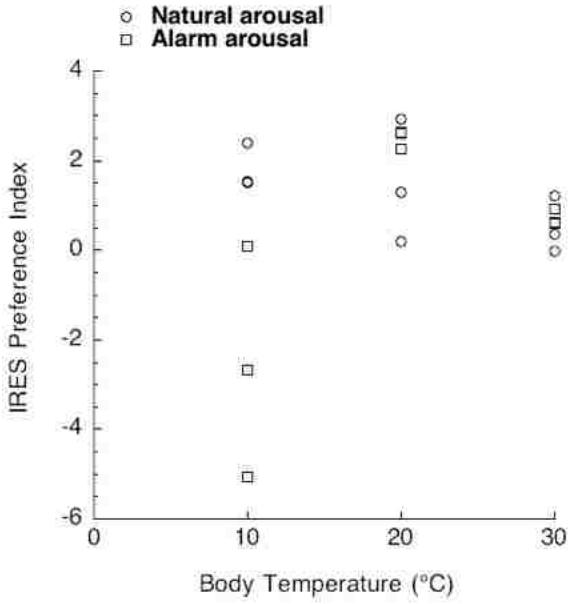

Figure 2-8. Premature arousal from torpor affects the IRES preference index. Animals (n = 3 for each group) were allowed to spontaneously arouse from torpor as a normal function of the torpor cycle (natural arousal) or were forced to arouse prematurely (alarm arousal). The abscissa represents the T_b of the squirrel when sampled. Data from alarm-aroused squirrels at T_b = 10° are significantly different from all other values (ANOVA, $P = 0.045$). No other differences were found. Some data points overlap.

Discussion

To balance energetic outlays with available resources, mammalian hibernators must arrest most processes normally vital to homeostasis. Accordingly, ground squirrels downregulate processes of transcription and translation (van Breukelen and Martin, 2001; 2002). Of note is that, while much of this downregulation is accomplished through passive mechanisms, there is a role for active suppression of cap-dependent initiation of translation (van Breukelen et al., 2004). In this study, we investigated a cap-independent process of initiation, e.g., IRESmt. We analyzed polysome fractions for mRNAs that are known to contain or not to contain IRES elements.

As a torpor bout progresses, mRNAs that harbor IRES sequences preferentially associate with ribosomes (Figure 2-5). As translation resumes when the squirrels' T_b is increased during arousal, the availability of IRES-containing transcripts may have tremendous physiological consequences. One possible explanation for increased association of IRES participants to ribosomes may be differential gene expression, i.e., if there was an increase in mRNA abundance for these IRES-containing transcripts then there may simply be increased association of these transcripts through mass action reaction rates. Although the severely restricted transcription available during torpor would make this possibility unlikely (van Breukelen and Martin, 2002), we determined if there were any changes in mRNA abundance. Our analyses demonstrate that the IRES-harboring transcripts that we examined were not differentially expressed

(Figure 2-7). Rather, the squirrels exploit an available mechanism of IRESmt to gain differential translation of preexisting transcripts as the squirrels begin to arouse from torpor and translation resumes.

The use of IRESmt has been demonstrated in other systems in response to physiological insults and preferentially encodes for stress proteins (Cardinali et al., 2003; Gilbert et al., 2007; Yoon et al., 2006). Indeed, glucose-induced starvation in yeast resulted in IRESmt, which increased survivorship (Geiser and Kenagy, 1988). We contend that the use of IRESmt in hibernators may help provide a protective phenotype by allowing increased expression of stress proteins as squirrels arouse. For instance, during entrance to hibernation, heart rate drops precipitously before any observed change in T_b (Milsom et al., 1999). This reduced blood supply may provide an ischemic insult, and indicators of oxidative stress such as conjugated dienes and ubiquitylated protein concentrations increase during entrance into torpor (reviewed in Ref. Carey et al., 2003). The induction of stress proteins may help to serve to mitigate this damage. A number of known IRES participants increase during hibernation. These include BiP/ GRP78 (Epperson et al., 2004; Mamady and Storey, 2006), connexin 43 (Li et al., 2005), hypoxia-inducible factor (HIF-1; see Ref. Morin and Storey, 2005), and c-Jun (O'Hara et al., 1999). BiP is an endoplasmic reticulum-resident chaperone that may facilitate proper folding of proteins and function during torpor (Epperson et al., 2004; Mamady and Storey, 2006). Connexin 43 is associated with increased conductivity in the heart, and it may play a role in maintaining cardiac rhythms during torpor (Fedorov et al., 2005; Saitongdee et

al., 2000; Schiavi et al., 1999). HIF-1 is a transcription factor and may serve to enhance hypoxia tolerance (Morin and Storey, 2005). c-Jun is a transcription factor critical for efficient axonal regeneration (Raivich et al.,2004). Recent data suggest a need for substantial axonal regeneration during arousal from hibernation (von der Ohe et al., 2007).

One might expect enormous changes in transcript abundance as a result of the tremendous physiological changes inherent to hibernation. However, numerous investigations of differential gene expression examining total mRNA have found relatively few differences in transcript abundance as a function of torpor state (e.g., see Refs. Williams et al., 2005; Yun et al., 2008). This seemingly surprising result might be explained by the notion that transcription of new mRNAs is virtually arrested during torpor (van Breukelen and Martin, 2002). Therefore, a mechanism that allows for the differential translation of pre- existing messages that encode for stress proteins during the torpor cycle is of great importance. We note that, in the examples of IRESmt use in other systems during times of stress, transcription is still available to these organisms. In the hibernator, the switch to IRESmt that we observed is without significant input from transcriptional efforts and may be the only practicable mechanism to express stress proteins.

Availability of eIF4E, the protein responsible for binding the 5′ cap structure of mRNA for recruitment to the ribosome, appears critical for the transition from cap-dependent initiation of translation to IRESmt (Svitkin et al., 2005). A survey of key initiation factors in the liver, including eIF2, eIF4B, eIF4E, eIF4GI, eIF4GII,

and binding proteins 4E-BP1, -2, and -3, revealed regulation of only eIF4E activity during hibernation (van Breukelen et al., 2004). Given the inability to respond to a physiological stress like hibernation with transcription, squirrels apparently evolved a complex regulation of eIF4E to inhibit its function during torpor. 4E-BP1 was absent in summer, present in winter, and differentially phosphorylated between the torpid and active states (van Breukelen et al., 2004). This regulation results in the active inhibition of cap-dependent initiation of translation. Exploitation of 4E-BP1 allows a squirrel to employ IRESmt preferentially. Interestingly, cap-dependent and cap-independent processes may not be competing with one another. DAP5 mediates cap-independent translation in response to cellular stresses in cell lines without affecting overall cap-dependent translation or polysome profiles (Marash et al., 2008). We note that, while there is an increased association of IRES- containing transcripts with ribosomes as a torpor bout progresses, our data cannot address if IRES-containing transcripts outnumber non-IRES-containing transcripts. We suspect that IRES-containing transcripts may represent only a small fraction of total transcript associated with ribosomes but that this fraction is increased during a torpor bout.

We asked if squirrels might prepare for arousal, i.e., must squirrels load up ribosomes with IRES-containing transcripts to arouse from torpor? We investigated this hypothesis by alarm-arousing ground squirrels prematurely from their torpor bouts. During a natural, spontaneous arousal, IRES preference index is relatively high and remains constant throughout the arousal process (Figure 2-

8). In contrast, alarm-aroused squirrels at 10°C demonstrate IRES preference indexes that are similar to animals obtained earlier in the torpor bout, e.g., early torpor or late torpor. However, by the time the squirrels have reached a T_b of 20°C, IRES preference index is indistinguishable from naturally aroused squirrels. Thus, it appears that squirrels are normally prepared for an arousal.

Although hibernation is assumed to be a very effective survival strategy, as many as 40-70% of ground squirrels may die in a given year during the winter (Sherman 1984). Any mechanism that might ameliorate the damage associated with the hardships of torpor should be under significant evolutionary selective pressure. Therefore, it follows that a mechanism that allows for the differential translation of preexisting messages that preferentially encode for stress proteins during the torpor cycle is of great importance. Arousing with a new set of proteins may be the critical adaptation that allows a hibernator to survive physiological insults that are lethal to most other mammals.

Perspectives and Significance

Our main finding is IRES-harboring transcripts are preferentially loaded on polysomes when squirrels arouse from torpor and translation resumes. The use of IRESmt may allow for preferential production of key stress proteins that could conceivably protect squirrels from harsh physiological stresses inherent to the arousal process. An interesting question is if IRESmt use is reserved for stressful periods. For instance, sleep may be a homologous process to mammalian hibernation (e.g., see Ref. Berger 1984). Hibernation and sleep

share similar features, including reduced metabolic rate, decreased core T_b, and energy conservation (Berger 1984). To date, no definitive answer to why animals sleep has been found (Riley et al., 2010). A lack of sleep leads to an outward phenotype of a stressed physiological state, including increased oxidative stress, e.g., lipid peroxidation; altered inflammatory responses; elevated morbidity of neurobehavioral, cardiovascular, and metabolic diseases; increased risks of hypertension, atherosclerosis, and diabetes; and impaired brain function (Change et al., 2008; Copinschi 2005; Dong-Vu et al., 2006; Malhotra and Loscalzo, 2009; Dang-Vu et al., 2006; Mullington et al., 2009; Ogawa et al., 2003; Schultes et al., 2005; Silva et al., 2004; Thase 2006). An interesting outcome of this study is that it begs the question of what the role of IRESmt during sleep may be. Although several investigations of protein synthesis during sleep have revealed relatively minor changes in rates of translation (Ramm and Smith, 1990; Shapiro and Girdowood, 1981), no study that we are aware of has investigated the role of IRESmt during sleep. Could invocation of IRESmt preclude the damages associated with sleep deprivation?

CHAPTER 3

EVIDENCE FOR A RESTRICTED ROLE FOR MICRORNAS IN REGULATION DURING MAMMALIAN HIBERNATION

Abstract

miRNAs are small (~ 22 nucleotides long) non-coding RNAs that are recognized as playing key roles in regulating gene expression and translation in response to environmental stress. However, their roles remain dubious in mammalian hibernation when translation is reduced to near negligible levels. Given the increasingly important regulatory roles of miRNAs, we hypothesized that miRNAs could play a critical role in regulating translation in hibernation. We utilized NCode multi-species miRNA microarray (Invitrogen) to identify miRNA species and determine their expression levels in livers from golden-mantled ground squirrels representing different torpor stages (summer active and late torpor). Here, we show that four major miRNA species: miRNA-122, miRNA-15, miRNA-21, and miR146 have been identified in liver, and there are no significant changes in the their abundance as a function of torpor state. Our findings indicate a limited role for miRNAs during mammalian hibernation.

Introduction

Ground squirrels employ hibernation as a strategy presumably in response to cold environment and food scarcity in winter. The hibernation season is composed of periods of torpor wherein metabolic rate may be as low as 1/100th of the active euthermic rates and core body temperatures may approach that of

the ambient temperature to as low as -2.9°C, interrupted by periodic arousals wherein core body temperature and metabolic rate return to euthermic values. The overall energetic savings for the entire hibernation season may be as high as 90% (Wang 1979).

The significant reduction in metabolic rate in torpor must be achieved by depressing multiple energetically consumptive cellular processes normally vital to homeostasis, such as transcription and translation (Rolfe and Brown 1997). A global reduction of transcriptional activity has been reported from studies of nuclear run-on assays during torpor (van Breukelen and Martin, 2002). Some mRNA transcripts seem to be sequestered in ribonucleoprotein complexes (Knight et al., 2000). Studies on *in vivo* incorporation of radiolabeled amino acids in various organs reported that protein synthesis is dramatically reduced to 0.13-0.5% of euthermic values during torpor, but is hyperactivated by approximately 1.5-2 fold during interbout arousal when compared to active animals (Zhegunov et al., 1988). Polysome analyses demonstrate that the bulk of initiation of protein synthesis ceases during entrance into torpor when T_b reaches 18°C in golden-mantled ground squirrels, but elongation of preinitiated polypeptides continues very slowly throughout the torpor bout. When T_b begins to rise during arousal, initiation of translation resumes and becomes fully recoupled with elongation at precisely 18°C (van Burekelen and Martin, 2001). However, mRNAs harboring IRES elements are preferentially associated with ribosomes as a torpor bout progresses (Pan and van Breukelen, 2011). These data indicate significant regulation of gene expression as animals cycle between prolonged bouts of

torpor and brief euthermic intervals. One possible mechanism for such regulation may be the use of miRNAs.

miRNAs are a class of evolutionarily conserved, small, non-coding RNA molecules found in eukaryotes (Bartel et al., 2004). miRNAs are reported to control the expression of thousands of target mRNAs, with each mRNA targeted by multiple miRNAs (Pillai, 2005). About 1-5% of predicted genes in animals encode miRNAs and they could regulate up to 10-30% of protein-coding genes (Branislav et al., 2006). Previous studies have uncovered various mechanisms by which miRNAs down-regulate their target mRNAs. Mammalian miRNAs can affect the mRNA levels of a large number of transcripts (Lim et al., 2005). Studies on let-7 miRNA by polysome analysis revealed that let-7 miRNA inhibited translation initiation of target mRNA (Pillai et al., 2005). Further, experiments done to pinpoint the step of translation initiation that is affected by let-7 led to the conclusion that only cap-dependent translation is subject to repression. Translation initiated in a cap-independent manner by internal ribosome entry site (IRES) elements or by tethered initiation factors eIF4E and eIF4G (DE Gregorio et al., 2001) were immune to repression by let-7 miRNA (Pillai et al., 2005). This finding suggested that the molecular target of miRNAs lay upstream of the eIF4G recruitment via eIF4E, such as the m^7G cap structure itself or its recognition by eIF4E. Moreover, both miRNAs and target mRNAs were shown to accumulate in specialized cytoplasmic processing bodies, termed P-bodies (Liu and Parker et al., 2005); such relocalization could be a means to assure translational

repression as P-bodies lack ribosomes and initiation factors (Teixeira et al.m 2005 & Pillai, 2005).

Over the past decade, miRNAs are well characterized as negative regulators of gene expression and protein synthesis; however, it would be very interesting to learn how a miRNA acts as a positive regulator. A recent report has shown that miRNAs could lead to translation repression by binding to the AU-rich elements in 3′-UTR of target mRNA in differentiating cells, but in quiescent cells the same binding would result in translation activation (Rusk 2008).

Accumulating evidence has suggested that miRNAs may act as well-conserved key regulators of various biological processes such as cell proliferation and differentiation, early development, programmed cell death, and interactions between virus and host cell (Branislav et al., 2006). To determine the role of miRNAs during hibernation, we ask if miRNAs are differentially expressed as a function of torpor state.

Materials and Methods

Animals and Tissue collection

Adult golden-mantled ground squirrels [*Spermophilus (Callospermophilus) lateralis*] were captured in July and early August from southern Nevada and California. Some animals were killed immediately as a seasonal control [summer active (SA), T_b = ~ 37°C]. The remaining squirrels were maintained on a diet of rat chow supplemented with sunflower seeds. These squirrels were implanted in October with temperature-sensitive radiotelemeters (Minimitter, Sun River, OR)

that allowed for the precise measurement of T_b throughout the entire hibernation season. Following recovery from surgery, implanted squirrels were housed in an environmental chamber at 4°C and allowed to hibernate. Animals spontaneously stopped feeding, and torpor bouts typically commenced in early November. The T_b of torpid squirrels was ~ 5°C. All winter animals were killed in February to early March by CO_2 asphyxiation except for the torpid animals. Torpid animals were killed by decapitation because of their low respiratory rates. Livers were obtained from ground squirrels representing various stages of a torpor bout: euthermic animals killed in summer (SA; *n* = 3); animals that had completed ~ 80% of the predicted torpor bout (typically 7 days; late torpor; *n* = 3); and animals that were euthermic between torpor bouts (interbout aroused; *n* = 3). Livers were removed, snap-frozen in liquid N_2, and stored at −80°C until use. All experiments were approved by the University of Nevada Las Vegas Institutional Animal Care and Use Committee.

Isolation of small RNAs

Total RNAs were isolated from the liver tissues of various stages: SA and LT (n = 3) with TRIzol Reagent (Invitrogen, Carlsbad, CA). The total RNA integrity was assessed afterwards using ~ 0.8% agarose gel electrophoresis. RNA concentration was measured on a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE). Small (≤ 200 nt) cellular RNA molecules, including regulatory RNA molecules such as miRNA and short interfering RNA (siRNA) were isolated using the PureLink miRNA isolation kit (Invitrogen, Carlsbad, CA)

from 5 μg of the above total RNA samples. Isolation was performed as per the manufacturer's instructions. Briefly, small RNA molecules were purified using spin column based centrifugation. 5 μg total RNA samples were added to the spin cartridge with Binding Buffer (L3) and ethanol (total 600 μl) and then centrifuged at 12,000 x g for 1 minute at room temperature. 700 μl 96% ethanol were added to the 600 μl flow through and then transferred to a new spin cartridge to repeat the above centrifugation step. The flow through was discarded. After washing with 500 μl Wash Buffer (W5), the spin cartridge was centrifuged again and the flow through was discarded. Finally, elute the small RNA molecular with 50 μl RNase free water by centrifugation at maximum speed for 1 minute at room temperature.

miRNA labeling

The NCode rapid miRNA labeling system (Invitrogen, Carlsbad, CA) was used to polyadenylate the enriched small RNA molecules from previous step and ligate a DNA polymer with fluorescent Alexa Fluor 3 or Alexa Fluor 5 dye molecules to each tailed RNA molecule for microarray analysis. NCode multi-species miRNA microarray control V2 was added to each tube of sample and used as a positive control (Invitrogen, Carlsbad, CA). Labeling was performed as per the manufacturer's instructions. Dye-swap comparisons were performed for quality control purposes.

Microarray Scanning

The tagged and tailed miRNAs were subsequently hybridized to the NCode multi-species miRNA microarray V2 (Invitrogen, Carlsbad, CA) which consists of ~ 1100 unique probes printed in triplicate targeting the validated mature miRNAs in mirBase 9.0 (http://microrna.sanger.ac.uk) from human, mouse, rat, *Drosophila melanogaster*, *Caenorhabditis elegans*, and zebrafish, to screen for the changes in miRNA populations. The array contains internal controls to control for sample loading and to assess dye performance, labeling efficiency and sample normalization. Hybridizations were performed as per the manufacturer's instructions.

Three pairs of replicate dye-swap microarray hybridization (6 chips) on each pair of miRNA samples were performed respectively. The hybridized array was incubated at 52°C for 20 hours in a sealed hybridization chamber (Argos Technologies, Elgin, IL). Microarray slides were scanned using the GenePix 4000B array scanner and the signal intensities were quantified with the GenePix Pro microarray analysis software (Axon Instruments, Union City, CA).

Statistical Analyses

All statistical analyses were conducted using R version 2.8.1 (R Development Core Team 2008). All comparisons of the fluorescent intensities of miRNA-15, miRNA-21, miRNA-122 and miRNA-146 between SA and LT samples were made with an unpaired student t-test ($P > 0.05$).

Results

miRNA expression levels as a function of torpor state

All miRNAs species were screened via NCode multi-species miRNA microarray in liver of both SA and LT ground squirrels, but only four major miRNA species (miRNA-15, miRNA-21, miRNA-122, and miRNA-146) were clearly identified in reference to the hybridization controls on the microarray (Figure 3-1). One of these, miRNA-122 was robustly expressed in both SA and LT states. The expression levels of all those four miRNAs were quantified, but none of their abundance changed significantly between SA and LT states (Figure 3-2). For all six microarrays, the dye markers were right in place. Alexa Fluor dye controls were used for internal normalization of differences in fluorescent signal intensities.

Figure 3-1. Representative sub-microarray image for identification of miRNA species in liver of golden-mantled ground squirrels using NCode multi-species miRNA microarray V2. Four miRNA species (miRNA-15, miRNA-21, miRNA-122, and miRNA-146) were identified on the array (as shown in each yellow circle). The five Alexa Fluor dye controls printed with different concentrations on the array from high to low (left to right) located as a row in both the top left and bottom right corners of the microarray image, to allow for normalization of differences in fluorescent signal intensities. Three dye markers (typically appear greener than other spots) were located in the middle row on the array, to enable proper alignment of the array in the scanner.

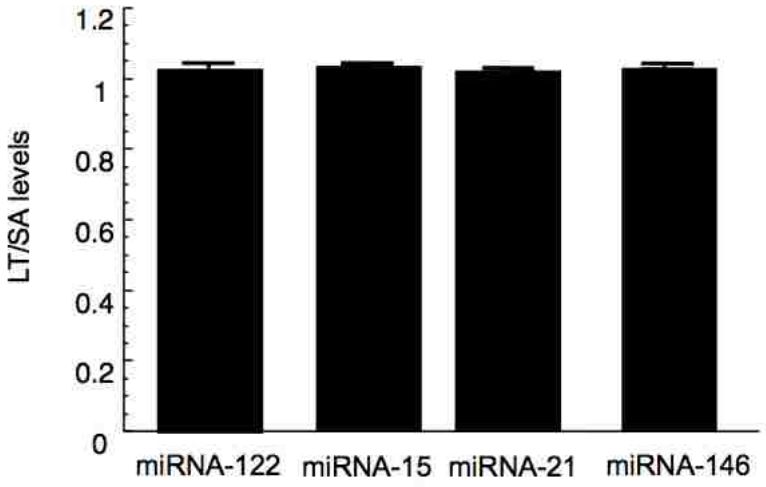

Figure 3-2. The expression levels of four miRNA species in liver of golden-mantled ground squirrels. There were no significant changes in the abundance of four major miRNA species: miRNA-15, miRNA-21, miRNA-122 and miRNA-146 in liver as a function of torpor state (unpaired student t-test, $P > 0.05$). Values represent means ± SE, n = 3.

Discussion

Since the first discovery of miRNAs in *Caenorhabditis elegans* in 1993 (Lee 1993), more than 17,000 different miRNA species have been identified by various computational and experimental methods across multiple species (Wang 2011). The miRNA Registry (http://microrna.sanger.ac.uk/sequences/) provides a list of the annotated miRNAs across various species. miRNAs are recognized as a distinct class of conserved regulators of post-transcriptional gene expression (Wightman et al., 1993). In mammals, there is a high-degree of Watson-Crick base-pairing between miRNA and target mRNA at nucleotides 2-7 at the 5' end of miRNA, termed the "seed match" (Zhao et al., 2007). When only interacting at the site of "seed match", miRNAs can induce degradation of their target mRNAs even though the recognition is through incomplete complementarity.

Translational repression has been noted in several miRNA models (Reinhart et al., 2000; Brennecke et al., 2003; Zeng et al., 2003) and is oftentimes characterized by greater changes in target protein concentration than target mRNA concentration (Branislav et al., 2006). However, as a putative mechanism to explain the depression of protein synthesis, little is know of their roles during hibernation when translation is reduced to near negligible levels. We hypothesized that miRNAs could play an important role in regulating translation in hibernation. In this study, we employed NCode multi-species miRNA microarrays to screen all miRNA species and determined their expression levels

in liver from animals representing different torpor stages. We found there was no significant difference in the abundance of four major miRNA species: miRNA-15, miRNA-21, miRNA-122 and miRNA-146 as a function of torpor state. There appears to be a limited role for miRNAs in regulating cellular processes including translation in liver during hibernation.

Our identified mRNAs include miRNA-15b, miRNA-21, miRNA-122, and miRNA-146. miRNA-15b is involved in the regulation of cell cycle progression (Xia et al., 2009). In collaboration with other miRNAs, miRNA-15b may function as a tumor suppressor (Klein et al., 2010), or regulate cellular ATP levels in cardiac myocytes (Nishi et al., 2009). Another study found that the expression levels of miRNA-15 remain constant in heart, kidney, liver and skeletal muscle of euthermic and hibernating ground squirrels, *S. tridecemlineatus* (Morin et al., 2008).

miRNA-21 is an anti-apoptotic factor involved in tumor progression (Carletti 2010), an inhibitor of angiogenesis through the depression of RhoB in mouse endothelial cells (Sabatel et al., 2011) and plays a critical role in the development of heart disease (Thum 2008). The expression level of miRNA-21 in the kidney was significantly increased by ~ 2 fold, but remain unchanged in heart, liver and skeletal muscle of hibernating ground squirrels when compared to euthermic values (Morin et al., 2008). The authors conclude the up-regulation of anti-apoptotic miRNA-21 might be associated with a potential repression of apoptosis in torpor, which is in accordance with an overall elevation in the expression levels of anti-apoptotic proteins in intestinal mucosa of torpid ground squirrels, *S.*

tridecemlineatus (Fleck and Carey 2005). However, the tissue-specific changes make such a role somewhat dubious.

miRNA-122 is the most abundant miRNA in liver representing about 70% of total miRNA population in this organ (Chang et al., 2004), which is important for normal liver function (Krutzf 2005, Girard 2008). miRNA-122 is also reported to play a role in fatty acid metabolism in liver (Esau 2006, Kojima 2011). A expression level of miRNA-122 could result in a dramatic reduction of fatty acid synthesis but an increase in fatty acid oxidation in mouse liver (Esau 2006). miRNA-122 is also known as an anti-apoptotic factor involved in inhibition of cancer progression (Coulouarn 2009). In this study, we found the expression level of miRNA-122 remain unchanged in liver as a function of torpor state even though it was robustly expressed. However, it was reported that miRNA-122 transcript levels were reduced by ~ 20% in the skeletal muscle of hibernating ground squirrels (Morin et al., 2008). The limited depression and the tissue specificity again shed doubt as to its global importance to the hibernating phenotype.

miRNA-146 is involved in induction of apoptosis by inhibiting the expression level of nuclear factor κB (NFκB) (Paik et al., 2011) and plays a important role in innate immune response as well (Taganov et al., 2006). We found the expression levels of miRNA-146 remain unchanged as a function of torpor state in liver of ground squirrels. In addition, miRNA-146 levels were not changed significantly either between euthermic and hibernating state in skeletal muscle of

ground squirrel, *S. tridecemlineatus*. The limited transcription may suggest an equally limited role for miRNAs.

miRNAs have been shown to exhibit tissue specific expression patterns (Lagos-Quintana, 2002). For example, miRNA-122 is identified as the most abundant miRNA species in liver (Chang et al., 2004). Initially, it was thought that expression was limited to liver, but some expression has also been found in heart (Tang et al., 2007). Although our study indicate no significant changes in four major miRNA species in liver of ground squirrels as a function of torpor state, the expression levels of miRNA-24 and miRNA-122 were reduced by ~ 30% and 20%, respectively, in skeletal muscle of hibernating ground squirrels, *S. tridecemlineatus* (Morin et al. 2008). We should note that miRNA-122 found in the skeletal muscle of hibernators had not previously been found in skeletal muscle. Combined with the limited changes in abundance, one may question if the technique of using PCR to amplify these miRNAs may have led to spurious results. Interestingly, a novel ground squirrel specific miRNA (mirdeep-192) was also predicted (Liu et al., 2010).

All miRNAs share common biogenesis pathways and reaction mechanisms (Lee et al, 2003). miRNA genes are first transcribed by RNA polymerase II into the stem-loop primary-miRNA, which will be further processed by RNAse III drosha before being transported into the cytoplasm for subsequent processing by another RNAase III, dicer, to generate the mature form (Tomari et al., 2004). Dicer protein levels were shown to be greatly increased by ~ 3 fold in the heart, but decreased by ~ 60% in the kidney in hibernating state when compared to

euthermic values. Besides these findings, no differences were found in the skeletal muscles between hibernating and euthermic animals (Morin et al., 2008). Although Morin et al., concluded that miRNAs may play a critical role in translational regulation during mammalian hibernation, the limited changes in miRNA abundance and the apparent paradoxes (e.g., Dicer protein level was deceased by ~ 60% in kidney, but miRNA-1 and miRNA-21 both increased significantly in kidney during torpor) likely means a limited role of miRNAs in translational control during mammalian hibernation.

In conclusion, our study shows that the four major miRNA species are not differentially expressed in liver of ground squirrels as a function of torpor state. Although some miRNA species were nominally differentially expressed in various organs in ground squirrels *S. tridecemlineatus*, no critical target mRNAs have been identified thus far. Overall, these data suggest that miRNAs play a limited role in regulating cellular processes during hibernation.

CHAPTER 4

P53 METABOLISM DURING MAMMALIAN HIBERNATION

Abstract

Protein synthesis is severely diminished during torpor in golden-mantled ground squirrels. Transcription factor p53 plays a critical role in translational regulation and sits on the crossroads of a variety of intracellular pathways. Here, I ask what role p53 may have in torpor. Could p53 help foster differential transcription like internal ribosome entry site mediated initiation of translation (IRESmt) led to differential translation? Data from qRT-PCR analyses indicate p53 mRNA levels decline significantly in torpid squirrels as compared to euthermic animals between bouts of torpor. Data from western blot analysis indicate that p53 protein level is unchanged in torpid squirrels as compared to summer active animals, but is significantly elevated by ~ 2.5 fold in euthermic animals between bouts of torpor. The expression levels of several p53 regulatory proteins are consistent with the activation of p53 protein in both torpid and interbout aroused squirrels. In addition, immunohistochemical data indicate that p53 protein accumulates in the nucleus in torpid animals. Finally, using chromatin immunoprecipitation and qRT-PCR based nuclear run-on assays, I have found that nuclear p53 protein binds directly to the response elements in its known target genes and sort of differentially regulates their expression as a function of torpor state. Take together, these results suggest that p53 is not working as an effective transcription factor during hibernation and it may serve

different functions, e.g. p53 could exert a different role in the cytoplasm than it does in the nucleus.

Introduction

Many mammals respond to adverse environmental conditions such as low temperatures and food scarcity by entering a hypometabolic state known as torpor (Carey et al., 2003). Torpor is characterized by T_b that may be as low as -2.9°C (Barnes, 1989) and drastically depressed metabolic rate that may be as low as 1/100th of active rate (Wang and Lee, 2000) in ground squirrels. The overall energetic savings achieved over the hibernation season may be as high as 90% for ground squirrels (Wang, 1979). Ground squirrels typically cycle through 15 bouts of torpor during the 6-9 months long hibernation season (Carey et al., 2003; Frank et al., 2008; Geiser and Kenagy, 1988). During entry into torpor, core T_b is decreased slowly to near that of ambient temperatures. The duration of a typical torpor bout might range from 1 to 3 weeks, largely determined by species, ambient temperature, and time of year (Buck and Barnes, 2000; French, 1982; Geiser and Baudinette 1990; Twente et al., 1977). Upon the completion of a torpor bout, squirrels spontaneously arouse and quickly return to euthermic T_b (~ 36°C) in about 2 to 3 hours (van Breukelen and Martin, 2002; Utz et al., 2007). After arousing, squirrels maintain euthermic body temperatures for 12 to 24 hours followed by torpor reentry.

In the previous chapters, I demonstrated that the use of IRESmt appears to induce preferential expression of stress proteins geared toward enhanced survivorship under conditions in which normal cap-dependent translation is

repressed (Pan and van Breukelen, 2011; Marash et al., 2008). It should be noted that p53 protein has been shown to play a key role in translational control through three distinct mechanisms: 1) p53 protein stabilizes mRNA secondary structure in 5′-UTR of target mRNA transcripts and therefore hinders ribosome scanning; 2) p53 directly affects translation machinery by interacting with ribosomal proteins and RNAs, e.g. p53 complex: p53-5.8S rRNA-28S rRNA-S9-S13; 3) p53 modulates activities of initiation factors, e.g. eIF4E and 4E-BP1 (Ewen and Miller,1996). p53-mediated down-regulation of global protein synthesis occurs at the level of translation initiation (Horton et al., 2002) and is accompanied by specific cleavages of translation initiation factors eIF4GI and eIF4B (Constantinou et al., 2003). Previous studies have shown that p53 mRNA can be translated via IRES mediated translation into two distinct protein isoforms in a cell phase dependent manner (Yang et al., 2006; Ray et al., 2006). The IRES segments for full-length p53 and amino-terminal-deleted p53 (ΔN-p53) isoforms are active at G2/M and G1/S transitions, respectively. It is important to note that, ΔN-p53 may facilitate G1/S transition by significantly inhibiting full-length p53 activity and ΔN-p53 is resistant to Mdm2-mediated protein degradation (Ray et al., 2006; Courtois et al., 2002; Yin et al., 2002).

p53 protein is also widely known as a transcription factor that plays a key role in response to a variety of cellular stresses, such as DNA damage, hypothermia, hypoxia and most recently, metabolic stress (Figure 4-1; Hirao et al., 2000; Koumenis et al., 2001; Puzio-Kuter, 2011; Maddocks et al., 2011). Having a very short half-life of ~ 20 minutes (Maltzman and Czyzyk, 1984), p53 usually

presents at undetectable levels under normal circumstances. These low levels are primarily maintained via ubiquitin-mediated proteolysis and the activity of E3, ubiquitin ligase, Mdm2 (Haupt et al., 1997). In addition, Mdm2 and another novel negative regulator of p53, Mdm4 work synergistically to inhibit p53 activities under various circumstances (Francoz, et al., 2006). Mdm2 is also known to inhibit p53 translational activity through binding to ribosomal protein L26 (RPL26), which can bind to p53 mRNA transcript to enhance its translation (Ofir-Rosenfeld et al., 2008; Takagi et al., 2005). In response to stress signals, p53 ubiquitylation is suppressed and the half-life of p53 protein is drastically extended (Maltzman and Czyzyk, 1984), resulting in a marked accumulation of p53 protein in the nucleus (Fritsebe et al., 1993; Hall et al., 1993). Usually, those nuclear p53 proteins are transcriptionally inactive, refractory to integration into the transcriptional complex (Wolff et al., 2001). Therefore, it is widely believed that p53 protein becomes activated as a transcription factor through multiple post-translational modifications such as phosphorylation, acetylation, ubiquitylation, SUMOylation, NEDDylation and metylation (Sakaguchi et al., 1998; Brooks and Gu, 2003; Li et al., 2003; Gostissa et al., 1999; Xirodimas et al., 2004; Chuikov et al., 2004).

Transcription accounts for as much as 10% of standard metabolic rate in most mammalian cells (Rolfe and Brown, 1997). This energetic cost is much higher than the available 1% metabolic rate to hibernator. As a result, transcription initiation was reduced two fold in torpid ground squirrels and transcription elongation is essentially arrested at low temperatures typical of torpor (van

Breukelen and Martin, 2002). However, some reports in the literature revealed transcription factor movements during torpor, e.g. nuclear factor kappa B (NF-κB) enters the nucleus and p53 is excluded from nucleus. The presumption is that transcription factors function during hibernation and that these changes have an impact on the physiology of the animal. An earlier study claimed that nuclear p53 protein concentration was significantly reduced 4-fold in torpid ground squirrels (Fleck and Carey, 2005). Taken at face value, these data might suggest that p53 works as a transcription factor to induce preferential transcription of target genes. To determine the role of p53, I asked the question whether p53 is working as an effective transcription factor and differentially regulate various target genes in the context of hibernation where global transcriptional activity is significantly suppressed.

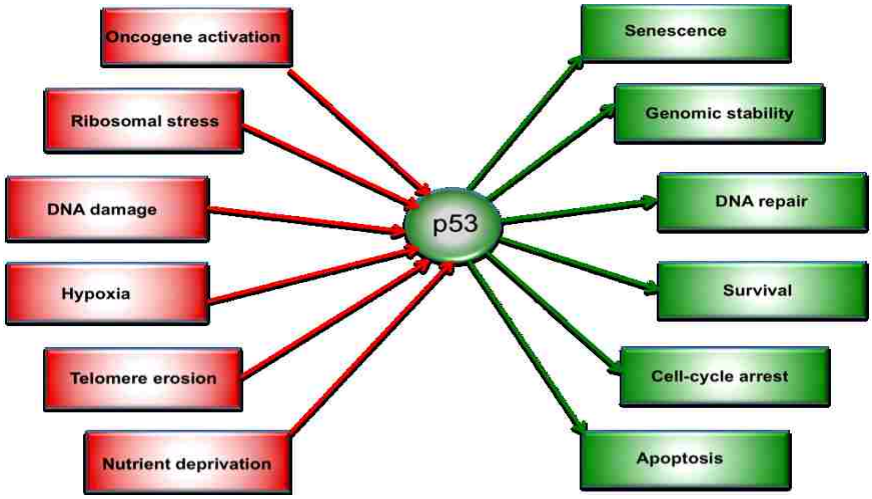

Figure 4-1. A schematic diagram represents p53 activation and the functions of p53 (modified from Figure 1-1 in Vousden and Lane, 2007).

Materials and Methods

Animals and Tissue collection

Golden-mantled ground squirrels (*Spermophilus lateralis*) were captured in July or early August from Southern Nevada, California, or Utah. Some squirrels were used immediately as a summer active control. The remaining squirrels were surgically implanted with temperature sensitive radiotelemeters (model VM-FH disc; Mini Mitter, Sun River, OR), which allow for precise measurement of T_b throughout the hibernation season. Implanted squirrels were housed in an environmental chamber at 4°C and allowed to hibernate. The T_b of torpid squirrels was ~ 5°C. All animals were killed by CO_2 asphyxiation except for the torpid animals. Torpid animals were killed by decapitation because of their low respiratory rates. Livers were obtained from ground squirrels representing various stages of a torpor bout: squirrels killed in summer used as a seasonal control [summer active (SA); n = 3]; animals that had completed ~ 80% of the predicted torpor bout [typically 7 days; late torpor (LT); n = 3]; and animals that were euthermic between torpor bouts [interbout aroused (IBA); n = 3]. Livers were removed, snap-frozen in liquid N_2, and stored at −80°C until use. All experiments were approved by the University of Nevada Las Vegas Institutional Animal Care and Use Committee.

Quantitative real time PCR (qRT-PCR)

Total RNA was extracted from approximately 50 mg liver tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Quality assessment of total RNA was performed using ~ 0.8% of RNA agarose gel electrophoresis. RNA concentration was measured on a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE). Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) was used to generate single stranded cDNA from 0.5 μg of total RNA with oligo dT and random primers, as per the manufacturer's instructions. qRT-PCR was run with PerfeCTa SYBR Green SuperMix Reaction Mixes (VWR, Radnor, PA) using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The changes in the mRNA levels of p53, p53 binding protein 2 (p53bp2), p53-inducible nuclear protein 1 (p53INP1), and p53-inducible nuclear protein 2 (p53INP2) transcripts with actin as a reference gene were determined with corresponding specific primers listed in Table 4-1. All samples were run in triplicate. All qRT-PCR reactions were run as follows: 2 min at 95° C, 15 s at 95° C, 30 s at 52° C, and 30 s at 72° C (36 cycles) with a mixture containing 1 μl of cDNA template, 12.5 μl qPCR Supermix, and 200 nM of each primer in a total volume of 25 μl (VWR, Radnor, PA), as per the manufacturers' instructions. Data were collected during the 30s-52°C extension step. Melt curves were performed using the following program: 1 min at 95° C, 1 min at 55° C, and 80 cycles of 10 s at 55° C with a step of 0.5° C every cycle. Melting curve analyses showed no primer-dimers and non-specific products. The efficiency of the primers was controlled as described previously (Pan and van Breukelen, 2011).

Table 4-1. Oligonucleotide primers used for qRT-PCR to detect the mRNA expression levels of p53 and its regulators.

Gene Name	Forward Primer	Reverse Primer
p53	5′-GTTCCGAGAGCTGAATGAGG-3′	5′-TCTGAGTCAGGCCCTTCTGT-3′
p53bp2	5′-CGTCATTTATGCACTGTGGG-3′	5′-CTAGCAAGTTGCGTGGAACA-3′
p53INP1	5′-GAGTCCTGTCCAATGGAGGA-3′	5′-TGCTGGGATGTTCAATGAGA-3′
MDM4	5′-CTCGCTCTCGCACAGGATCAC-3′	5′-GCCAGCTACATCCCACTCCTC-3′
GAPDH	5'-AAGGTCGGAGTGAACGGAT-3'	5'-CTTTGATGTTGGCGGGAT-3'
actin	5′-CACTGGCATTGTGATGGACTCCG-3′	5′-GTAGCCACGCTCAGTCAGGATC-3′

p53, p53 binding protein 2 (p53bp2), p53-inducible nuclear protein 1 (p53INP1), and mouse double minute 4 (MDM4) genes were used in qRT-PCR studies. Housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin was used as internal controls. Amplified fragments were sequenced and sequences were deposited in GenBank.

Protein Sample Preparation

About 100 mg of frozen liver samples were pulverized in liquid nitrogen and then homogenized in 300 μl ice-cold lysis buffer A or B using a glass Teflon homogenizer (DuPont, Wilmington, DE). Buffer A contained 50 mM Tris HCl, pH 7.5, 150 mM KCl, 1 mM DTT, 1 mM EDTA, 50 mM glycerolphosphate, 1 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 0.1 mM orthovanadate, and 50 nM okadaic acid. Samples were centrifuged at 10,000 x g for 10 min at 4°C to remove insoluble cellular debris. Buffer B contained 50 mM Tris HCl, pH 8.3, 20% glycerol, 2% SDS, and 0.4 M β-mercaptoethanol. Samples were centrifuged at 10,000 x g for 30 min at 4°C to remove insoluble cellular debris.

The supernatant was collected, aliquoted and stored in -80°C until further analysis.

Western blotting

Sample protein concentrations were determined by a modified Lowry assay.

p53 blotting. 80 μg total protein samples extracted with lysis buffer A were heat denatured at 100°C for 5 min. Samples were cooled on ice for 5 min and then electrophoresed on 12% (30:0.5 acrylamide:bisacrylamide) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to hydrophobic polyvinylidene difluoride membrane (PVDF, Immobilon-P; Millipore, Bedford, MA). Nonspecific protein-binding sites were blocked overnight at 4°C in 10 mM Tris HCl, pH 8, and 150 mM NaCl (Tris-buffered saline; TBS) blocking solution containing 0.5% Tween-20 (TTBS) and 3% dry milk. After one 5 min wash in TBS, two successive 5 min washes in TTBS, and a final wash in TBS, membranes were incubated overnight at 4°C with goat anti-p53 polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-1313) at a dilution of 1: 200 in TTBS with 3% milk and goat anti-actin polyclonal HRP-conjugated antibody at a dilution of 1:5000 in TTBS with 1% milk (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-1615; as a control). Washes were performed as described above. The membrane probed with p53 primary antibody was incubated with donkey anti-goat IgG-HRP secondary antibody at a dilution of 1: 5000 (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-2020). Washes were performed as described above before detection was performed by

enhance chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) using Typhoon 9410 Variable Mode Imager (GE Healthcare, Piscataway, NJ).

MDM4, MDM2, and RPL26 blotting. The same protocol described above was used, but 50 μg total protein samples extracted with lysis buffer B were used for all three antibodies. Primary antibody incubations were performed with a goat polyclonal antibody to MDM4 diluted 1: 200 (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-14738), followed by incubation with donkey anti-goat IgG-HRP secondary antibody at a dilution of 1: 5000 (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-2020); a rabbit polyclonal antibody to phospho-MDM2 (Ser 166) antibody diluted 1: 1000 (Cell Signaling Technology, Beverly, MA; # 3521S), followed by anti-rabbit secondary antibody at a dilution of 1: 5000 (Amersham Life Science, Arlington Heights, IL; # 356501); a rabbit polyclonal antibody to RPL26 antibody diluted 1: 1000 (Cell Signaling Technology, Beverly, MA; # 2065S), followed by anti-rabbit secondary antibody at a dilution of 1: 5000 (Amersham Life Science, Arlington Heights, IL; # 356501).

Immunohistochemistry

Frozen livers were embedded in Tissue-Tek OCT compound (VWR Scientific, Bridgeport, NJ) and equilibrated to -20°C for 20 min. Frozen sections were cut at a thickness of 7 μm with a Vibratome Ultrapro 5000 cryostat (St. Louis, MO) and subsequently mounted on glass slides coated with freshly prepared 0.01% poly-L-lysine (Sigma, St. Louis, MO). Sections were fixed with 1: 1 methanol/ acetone

(v/v) solution for 10 min at -20°C and then rehydrated in PBS solution (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) for 10 min at room temperature. The fixed slides were immersed in blocking solution (3% milk in PBS) overnight at 4°C. After three 5 min washes in PBS, the sections were incubated with goat anti-p53 polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-1313) diluted 1: 200 in blocking solution overnight at 4°C. After three 5 min washes in PBS, the sections were reacted with Alexa Fluor 488 conjugated donkey anti-goat IgG antibody (Invitrogen, Carlsbad, CA, cat. no. A-11055) diluted 1: 000 in blocking solution for 1 hour at room temperature. After another three 5 min washes in PBS, the sections were counterstained with propidium iodide (1μg/ml; Enzo life science, Farmingdale, NY) to label nuclei. Finally, tissue sections were visualized with a laser scanning confocal fluorescent microscopy (Carl Zeiss, Jena, Germany).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using the MAGnify chromatin-immunoprecipitation system (Invitrogen, Carlsbad, CA), as per the manufacturer's instructions. Briefly, 150 mg pulverized liver tissue were homogenized in PBS buffer and cross-linked in 1% formaldehyde for exactly 10 min at room temperature. The crosslinking was quenched with addition of 2.5 M glycine to a final concentration of 0.125 M. The liver cells were lysed to release chromatin from the nuclei. Chromatin DNA was sheared to generate fragments of around 200 to 500 bp in length using a probe sonicator (Branson Sonifier 450,

VWR Scientific, San Francisco, CA; Duty Cycle: Constant, Output Control: 3, Output: 5, 30s ON plus 2 min OFF, 16 cycles on ice). 100 μl of chromatin DNA was reserved as an input control (Input DNA). Dynabeads Protein A/G were pre-coupled with 10 μg goat anti-p53 polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-1313). Sheared chromatin DNA was incubated overnight with antibody-conjugated beads at 4°C with rotation. The binding was reversed by incubating 15 min at 55°C. Immunoprecipitated chromatin DNA (ChIP DNA) was purified and quantified by PCR reactions with the corresponding primers listed in Table 4-2. All primers were ordered from Integrated DNA Technologies (IDT, San Diego, CA). PCR reactions were performed with 5 μl of both ChIP and Input DNA samples as templates. The PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide.

The concentrations of ChIP DNA were measured by using Hoechst 33258 dye. Briefly, 5 μl aliquots were added to 40 μl assay buffer (10 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM EDTA, and 100 ng/ml Hoechst stain 33258), equilibrated to 30°C, and read using a spectrofluorometer (Shimadzu, Kyoto, Japan) with excitation/emission wavelengths of 350/ 455 nm. The fluorescence readings were compared against a standard curve (r^2 = 0.995) constructed from serial dilutions of herring sperm DNA (Sigma, St. Louis, MO). The concentrations of Input DNA were measured on a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE).

Table 4-2. Oligonucleotide primers used for ChIP-PCR analysis.

Gene Name	Forward Primer	Reverse Primer
MDM2	5′-GCTCTACCACTCAAACACATCACAGC-3′	5′-TGAGTTCAAAGCCAGCCTCAGC-3′

Murine double minute 2 (MDM2) is a known p53 target gene. Amplified fragments were sequenced and sequences were deposited in GenBank.

Nuclear preparation

Nuclear preparations were performed essentially as described previously (van Breukelen and Martin, 2002). Briefly, About 1 g of frozen liver tissues were gently homogenized on ice using a Dounce homogenize in lysis buffer (10 ml 10 mM Hepes, pH 7.6, 25 mM KCl, 0.30 mM spermine, 1 mM spermidine, 1 mM EDTA, and 40% glycerol) with ten strokes of A (loose fitting) pestle and two strokes of B (tight fitting) pestle. After coarse filtering through a cloth towel, the homogenate was centrifuged at 800 x g for 10 min at 4 °C. The pellet was resuspended in 3 ml ice-cold resuspension buffer (10 mM Hepes, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, and 10% glycerol), and carefully laid over a 0-35% gradient of iodoxanol (Optiprep; Nycomed Pharma, Oslo, Norway) prepared in the resuspension buffer. The gradient was centrifuged at 10,000 x g for 20 min at 4 °C. The pellet containing the nuclei appeared on the tube wall at the outer side of the rotor, which was collected and washed in 15 ml washing buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 10 mM NaCl). The resulting suspension was centrifuged at 800 x g for 10 min at

4°C. The final pellet was resuspended with an equal volume of ice-cold storage buffer (50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA and 40% glycerol) and stored at -80°C until further use.

The DNA concentrations in the nuclei were measured using Hoechst 33258 dye. Briefly, 5 μl aliquots of isolated nuclei were incubated in 1 ml extraction buffer (1 M NaCl, 1 N NH_4OH, and 0.2% Triton X-100) for 10 min at 37°C. 12.5 μl of the resulting DNA solution was added to 750 μl assay buffer (10 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM EDTA, and 100 ng/ml Hoechst stain 33258), equilibrated to 30°C, and read using a spectrofluorometer (Shimadzu, Kyoto, Japan) with excitation/emission wavelengths of 350/ 455 nm. The fluorescence readings were compared against a standard curve (r^2 = 0.996) constructed from serial dilutions of herring sperm DNA (Sigma, St. Louis, MO).

qRT-PCR Based Nuclear Run-on Assay

Nuclear run-on assays were titrated to ensure the transcriptional activity was within the linear range and the optimal conditions for nuclear run-on assays were determined to be 5 min at 25°C as described previously (van Breukelen and Martin, 2002). All assays were performed in triplicate. Briefly, for the 0 min assay, one 20 μl aliquot of nuclei lysate was added to a 190.25 μl cold mixture of reaction buffer (47.8 mM Tris- maleate, pH 7.6, 71.6 mM KCl, 19.1 mM $(NH_4)_2SO_4$, 2.3 mM magnesium acetate, 1.9 mM $MnCl_2$), 14.3 mM dithiothreitol, 47.8 units/ml RNasin (Promega, Madison, WI), NTP mix (310 μM ATP, 310 μM UTP, 620 μM CTP, 620 μM GTP), 157μl TRIzol LS (Invitrogen, Carlsbad, CA)

with 1 μl 20 mg/ml glycogen. For the 5 min assay, another 20 μl aliquot containing the same amount of nuclei was incubated at 25°C for 5 min in a 52.25 μl mixture containing the same amount of reaction buffer, dithiothreitol, RNasin, and NTP mix. The reaction was stopped by adding 157μl TRIzol LS with 1 μl 20 mg/ml glycogen.

Total RNAs were immediately extracted from the both solutions containing TRIzol reagents (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) was used to generate single stranded cDNA from 2 μl of total RNA with oligo dT and random primers, as per the manufacturer's instructions. qRT-PCR was run with PerfeCTa SYBR Green SuperMix Reaction Mixes (VWR, Radnor, PA) using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The primers used are listed in Table 4-3 and actin was used as a reference gene. All qRT-PCR reactions were run as follows: 2 min at 95° C, 15 s at 95° C, 30 s at 60° C, and 30 s at 72° C (36 cycles) with a mixture containing 1 μl of cDNA template, 12.5 μl qPCR Supermix, and 200 nM of each primer in a total volume of 25 μl (VWR, Radnor, PA), as per the manufacturers' instructions. Data was collected during the 30s-60°C extension step. Melt curves were performed using the following program: 1 min at 95° C, 1 min at 55° C, and 80 cycles of 10 s at 55° C with a step of 0.5° C every cycle. Melting curve analyses showed no primer-dimers and non-specific products. The efficiency of the primers was controlled as described previously (Pan and van Breukelen, 2011).

Statistical Analyses

The qRT-PCR data was analyzed to show the relative changes in gene expression levels based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and subjected to ANOVA followed by the Tukey multiple comparisons tests. Relative band intensities observed by western blotting and immunohistochemical data determined with confocal microscopy were quantified densitometrically using Image Quanta software (Molecular Dynamics, Sunnyvale, CA) and subjected to ANOVA followed by the Tukey multiple comparisons tests. ChIP data was analyzed by ANOVA as well. The nuclear run-on data was statistically analyzed according to a previously described method by LinReg PCR (11.0) software (Ruijter et al., 2009). Results with *P* values < 0.05 were considered statistically significant. R software was used for all data analyses (R Development Core Team, 2008).

Result

Protein and mRNA expression levels of p53 as a function of torpor state

I used qRT-PCR to measure p53 mRNA expression levels in liver of ground squirrels representing various states with primers listed in Table 4-1. Data indicate that p53 mRNA level was reduced by ~ 60% in LT, but fully restored during IBA as compared to SA values (Figure 4-2, A). I also measured p53 protein levels by western blot with an antibody specific for p53 protein (Figure 4-2, B). Although p53 mRNA level was drastically reduced during LT, western blot

analyses indicate that p53 protein abundance remained unaltered in LT, but was significantly elevated by ~ 2 fold during IBA (Figure 4-2, B and C).

mRNA or protein expression levels of several known p53 regulators as a function of torpor state

p53 is tightly controlled by multiple regulatory proteins including p53bp2, p53INP1, Mdm2, Mdm4, and ribosomal protein L26 (RPL26). The mRNA expression levels of p53bp2, p53INP1, and Mdm4 were also measured by qRT-PCR with primers listed in Table 4-1. The protein expression levels of Mdm2, Mdm4, and RL26 were measured by western blot with specific antibodies against those proteins. p53bp2 binds to DNA-binding domain of p53 and therefore enhances its binding to specific consensus sequences on target genes (Iwabuchi et al., 1998). Data indicate that p53bp2 mRNA levels were significantly increased by ~ 2.6 fold during LT and IBA (Figure 4-3, A). p53INP1 can either decrease p53 activity or promote the ubiquitin-mediated proteolysis of Mdm2 by stabilizing homeodomain-interacting protein kinase 2 (HIPK2) (Tomasini et al., 2003; Wang et al., 2001). Data indicate that p53INP1 mRNA levels remained unaltered as a function of torpor. Mdm4 can bind p53 and inhibit p53 transactivation (Finch et al., 2002). Data indicate that Mdm4 mRNA level remained unchanged in LT, but was significantly reduced by ~ 60% during IBA (Figure 4-3, A). However, Mdm4 protein levels were significantly reduced by 30-70% in LT and IBA (Figure 4-3, C). Mdm2 is an important negative regulator of p53 via ubiquitin-mediated proteolysis (Kubbutat et al., 1997). Data indicate that

Mdm2 protein abundance was significantly reduced by 30-40% in LT and IBA (Figure 4-3, C). RPL26 binds p53 mRNA and enhances its translation (Takagi et al., 2005). Data indicate that RPL26 protein levels were significantly elevated nearly 2 fold in both LT and IBA (Figure 4-3, C).

Nuclear p53 protein level is increased as a function of torpor

Using immunohistochemical analysis followed by quantitative confocal microscopy, I investigated the subcellular localization of p53 protein as a function of torpor state in liver of golden-mantled ground squirrels. The O.C.T-embedded liver sections were incubated with anti-p53 antibodies followed by an Alexor Fluor 488 conjugated secondary antibody. Immunohistochemical data were quantified by measuring the total green fluorescence in the nucleus and whole cells (100 clearly identified cells were used for quantification in each group) and the results were shown as percentage of nuclear versus total p53 localization. Representative photographs of immunolocalization of p53 protein are shown in Figure 4-4 (A).

Immunohistochemical analysis revealed a ~ 2 fold increase in the nuclear p53 protein levels in LT when compared to SA value (Figure 4-4, B). Although p53 protein abundance remained unaltered in LT, these results indicate a subcellular shuttle of p53 protein from the cytoplasm to the nucleus. The ratio of nuclear to total p53 proteins remained unchanged between LT and IBA, however total p53 protein levels were significantly elevated by ~ 2 fold (Figure 4-2, C), therefore,

there is a robust increase in the net amount of nuclear-localized p53 protein during IBA.

Nuclear p53 binds to its target genes as a function of torpor state

Data indicate p53 protein accumulates in the nucleus as a function of torpor state (Figure 4-4). Nuclear localization of p53 is critical for its potential transcriptional activity. To function as a transcription factor, p53 has to actively bind to its target genes. I performed chromatin immunoprecipitation (ChIP) assay to determine the binding of nuclear p53 protein to the response elements in its target genes. Data indicate p53 binds to DNA (Figure 4-5, A). To confirm that p53 directly binds to its target genes rather than binding through its interacting proteins, I performed PCR reactions with both immunoprecipitated DNA (ChIP DNA) and input DNA (1% of the sonicated chromatin) as templates with primers specific for regions of Mdm2 promoter as listed in Table 4-2. The results indicate p53 binds to its known target genes as a function of torpor state (Figure 4-5, B).

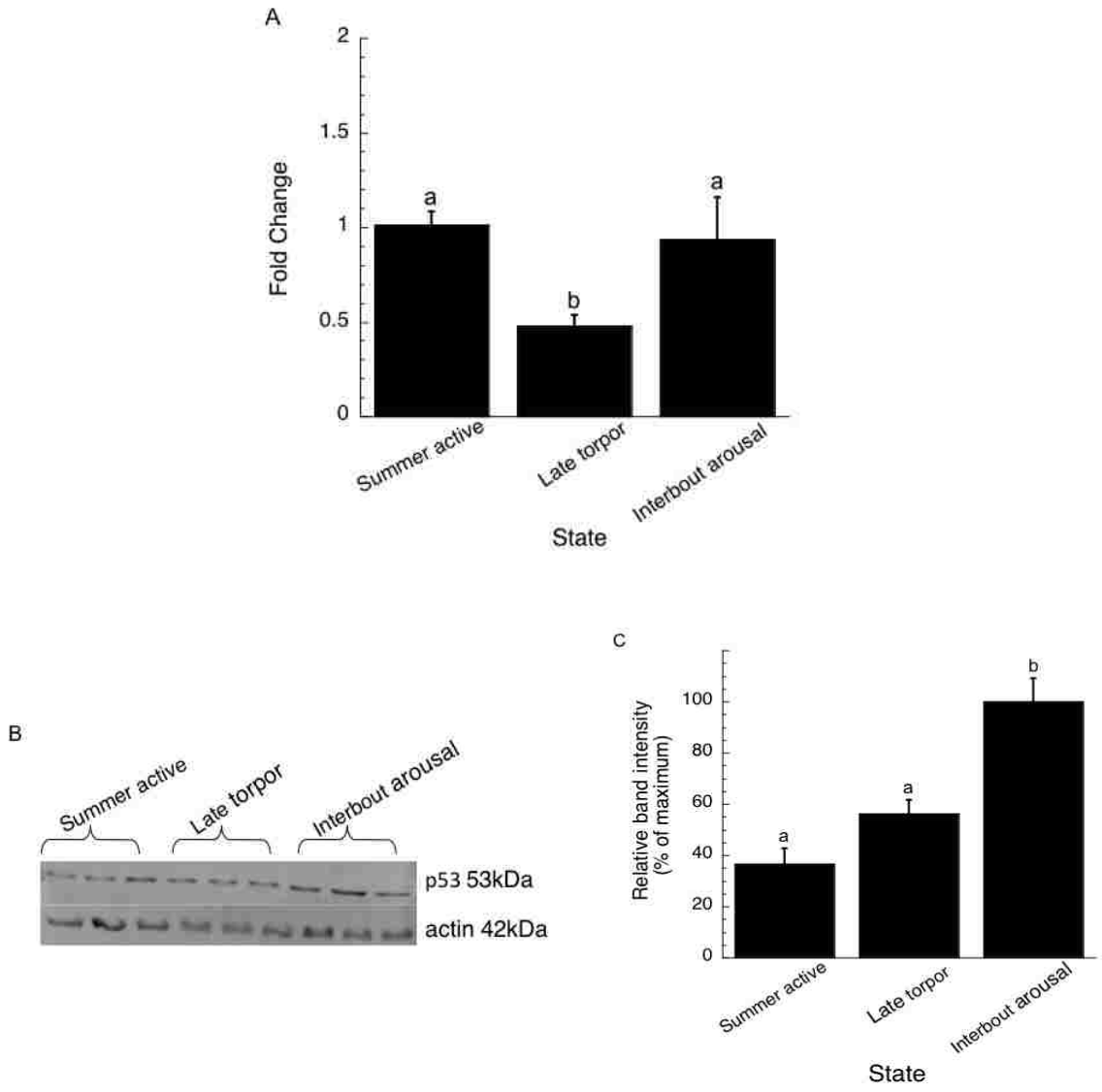

Figure 4-2. mRNA and protein expression levels of p53 in liver as a function of torpor state. (A) mRNA levels of p53 were measured by qRT-PCR. Data indicate that p53 mRNA level was significantly reduced by ~ 70% in LT as compared to SA values. Relative expression values are shown as fold change ± SE for triplicates in reference to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). p53 protein levels were measured by western blot as described in Materials and Methods. (B) Western blotting probed with antibodies against p53. (C) p53 protein level was normalized by actin protein level and shown in the bar graph. p53 protein expression level remained unaltered in LT, but was significantly increased by ~ 2.5 fold during IBA. n = 3 animals were used for each state. Groups with different letter superscripts are significant different ($P < 0.05$, ANOVA with Tukey correction for multiple comparisons). Different letters indicate significantly different means, $P < 0.05$.

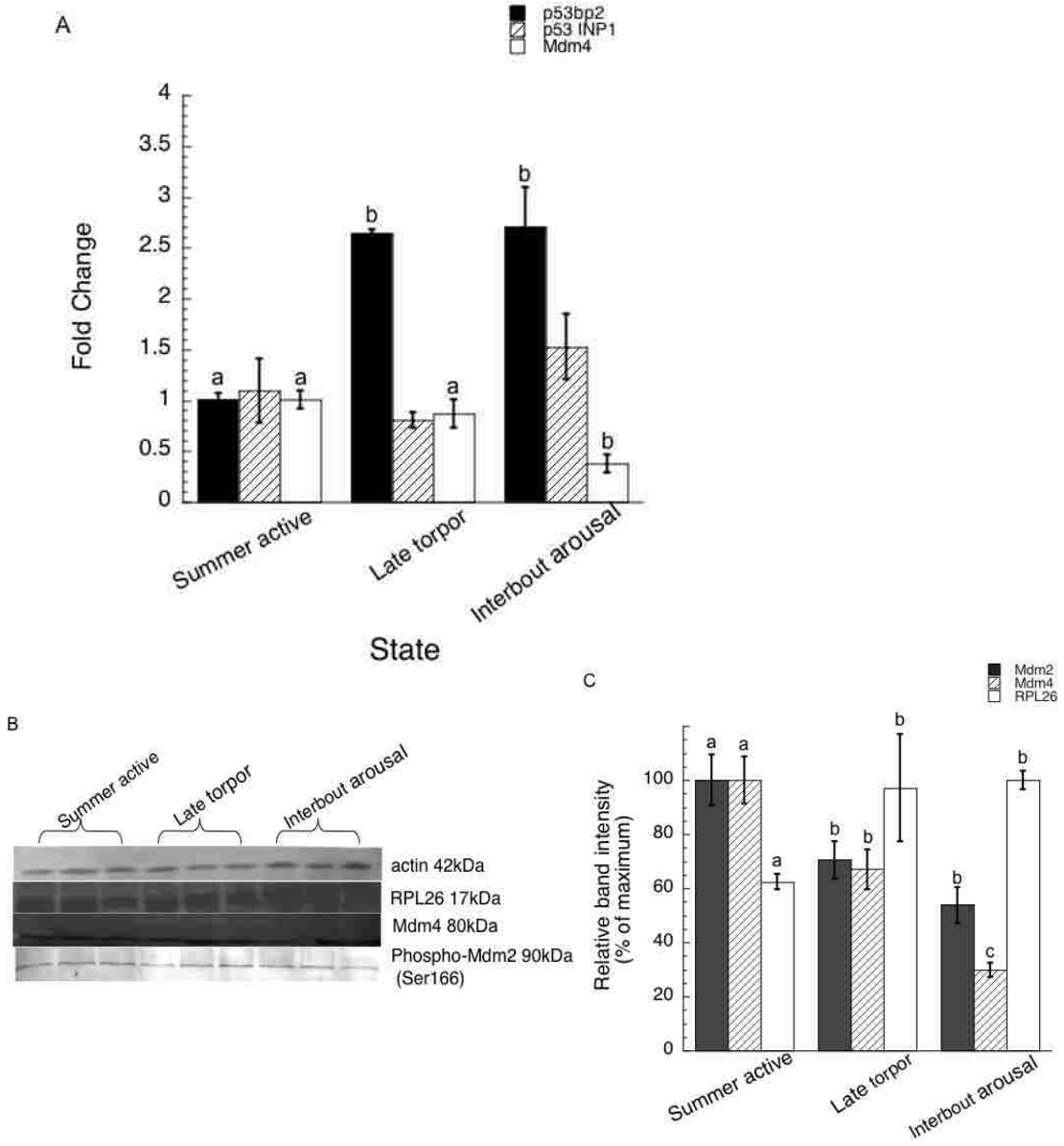

Figure 4-3. mRNA or protein expression level of several known p53 regulators in liver as a function of torpor state. (A) mRNA levels of p53 binding protein 2 (p53bp2), p53 inducible nuclear protein 1 (p53INP1), mouse double minute 4 (Mdm4) were measured by qRT-PCR. Relative expression values are shown as fold change ± SE for triplicates in reference to housekeeping gene GAPDH or actin. (B) Western blots probed with antibodies against Mdm2, Mdm4, and RPL26 antibodies. To control for equal loading, the blots were probed with actin antibody. (C) Quantitative analyses of Mdm2, Mdm4, and RPL26 protein expression were normalized to actin protein levels. Data of relative immunoreactive band intensity are expressed as a percentage of maximum intensity. Values shown in the bar graph represent mean ± SE (n = 3 animals were used per group). Groups with different letter superscripts are significant different ($P < 0.05$, ANOVA with Tukey correction for multiple comparisons).

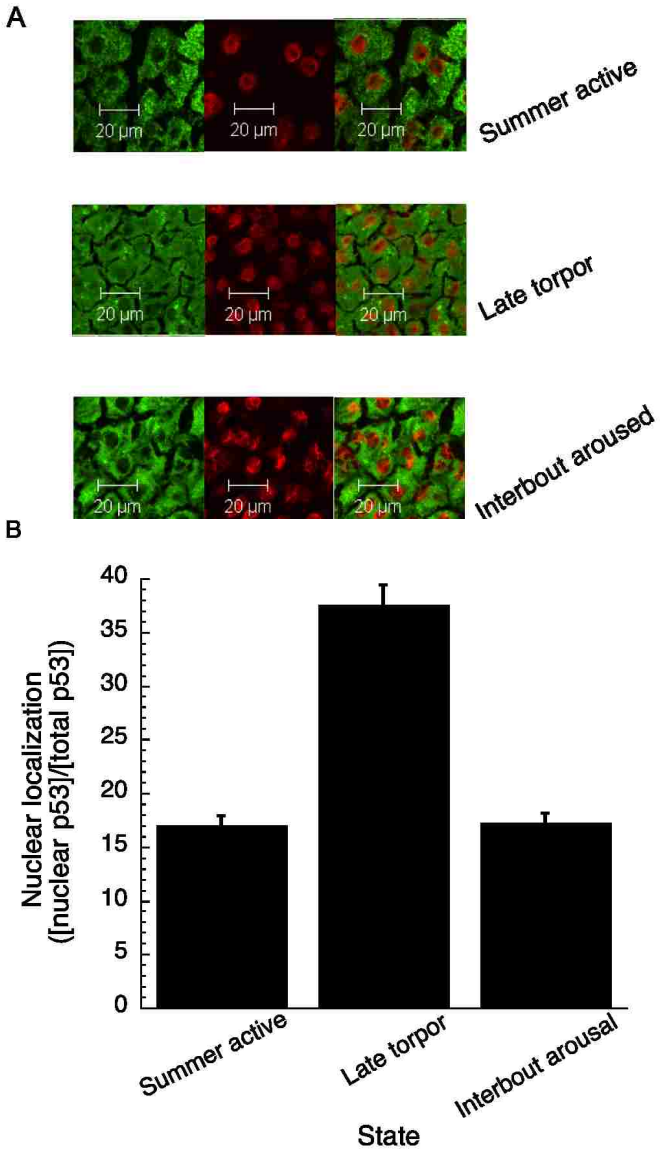

Figure 4-4. Immunohistochemical analysis of subcellular localization of p53 protein by confocal laser scanning microscopy showing a translocation of endogenous p53 protein from the cytoplasm to the nucleus. (A) Representative confocal photograhs are shown as single and overlay images. 7 μm O.C.T-embedded liver tissue sections were incubated with anti-p53 antibody followed by an Alexa Fluor 488 conjugated secondary antibody (green color) (left panels). DNA in the tissue sections was counterstained with propidium iodide(PI) to identify the nucleus (red color) (middle panels). Merge images were generated by superimposing p53 staining onto DNA staining (right panels). Scale bar = 20 μm. (B) The bar graph shows quantification of fluorescent intensity in the nucleus and whole cells to determine p53 localization as a function of torpor state. One hundred clearly identified cells were examined for each group. n = 3 animals were used for each torpor state. Different letters indicate significantly different means, $P < 0.05$.

p53 moderately differentially regulates its targets genes as a function of torpor state

p53 proteins bind to its known target genes as a function of torpor state (Figure 4-5). To find out whether p53 can selectively discriminate and differentially regulate various target genes as a transcription factor, I performed qRT-PCR based nuclear run-on assays with primers for known p53 target genes (Table 4-3). The results reveal distinct expression patterns of an array of known p53 target genes as a function of torpor state (Table 4-4). On average, the run-on mRNA expression of known up-regulated p53 target genes were elevated by ~ 1.5 fold during LT and IBA as compared to SA values (Figure 4-6, C). However, there were no differences in the run-on mRNA expression of known down-regulated p53 target genes amongst the groups (Figure 4-6, B; Table 4-4). To further test if p53 is an *effective* transcription factor, I performed qRT-PCR to measure the cellular mRNA concentrations of target genes tested in the nuclear run-on assays as listed in Table 4-3. The results indicated mRNA levels of those target genes were significantly reduced during torpor, with an exception of GADD45A, whose mRNA levels was significantly increased by ~ 1.5 fold in LT (Figure 4-6, C). However, mRNA levels of down-regulated mRNAs were very similar to those of up-regulated ones (Figure 4-6, B). These results indicate that p53 is ineffective in modulating up and down-regulate target genes, suggesting a very limited role of p53 in directing differential transcription during torpor.

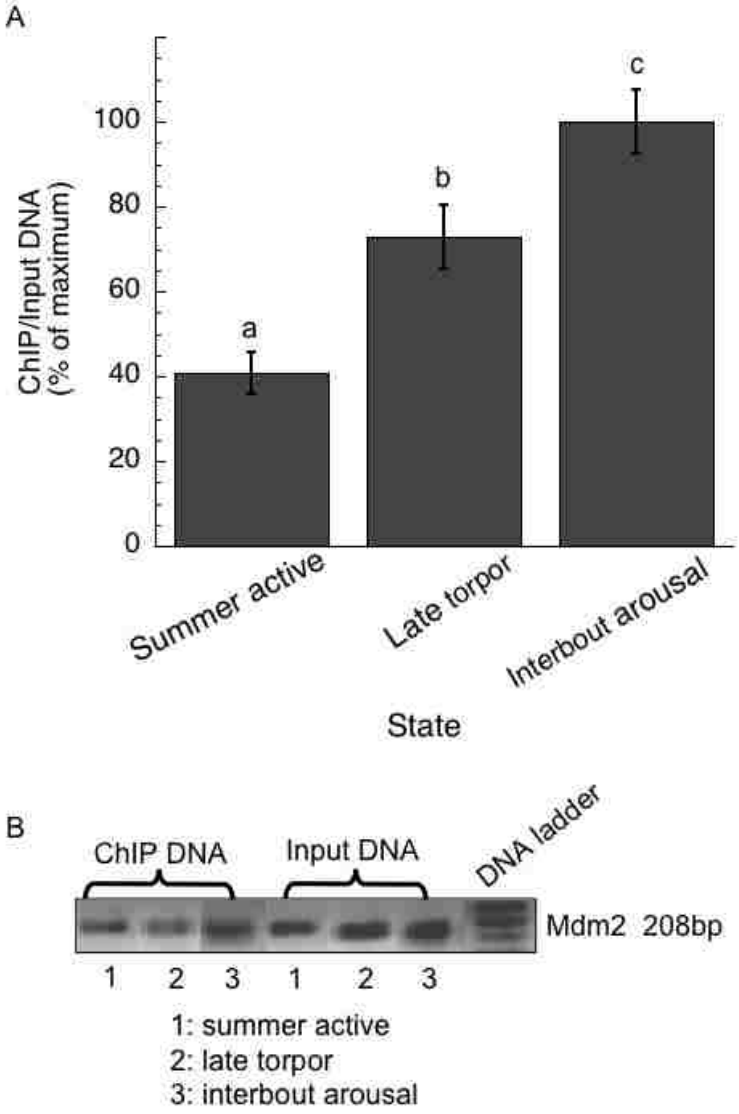

Figure 4-5. ChIP analysis of the association of p53 protein and its target genes as a function of torpor state. ChIP analyses were performed as reported in "Materials and Methods' section. DNA from liver tissue in each torpor state was immunoprecipitated in the presence of primary antibody against p53 protein and quantified by using Hoechst 33258 dye. Results are reported as a ratio of concentration of immunoprecipitated DNA (ChIP DNA) to concentration of Input DNA for each torpor state (% of maximum) and were shown in the bar graph (A). PCR reactions were performed with primers (listed in Table 4-2) for a known p53 target gene Mdm2 using both Input DNA [A fraction (1%) of the sonicated chromatin] and ChIP DNA as templates (B). n = 3 animals were used for each torpor state. Different letters indicate significantly different means, $P < 0.05$.

Table 4-3. Oligonucleotide primers used for qRT-PCR based nuclear run-on assays.

Gene Name	Forward Primer	Reverse Primer
actin	5′- CACTGGCATTGTGATGGACTCCG -3′	5′- GTAGCCACGCTCAGTCAGGATC-3′
GADD45A	5′-AGCATTTACCCGCTTTGCCCA-3′	5′-CAGAGCCACATCCCTGTCGTC-3′
BAX	5′-TGTGGAGCAGATTTGGGGAGTGA-3′	5′-CTCGCTCAGCTTCTTGGTGGAAG-3′
Pig3	5′-GGCACTGGAAGATTGGGGACTC-3′	5′-GGAGGTGAGCCAAACTTCTGGG-3′
DDB2	5′-TGTCAGGGTCTTCAGCAGTCCTTT-3′	5′-ATATCTCCCCCTTTGGAACCCACA-3′
maspin	5′-AGGATGTGGAGGATGAGTCGATGG-3′	5′-GGGAAGGGAGAGTTTGACTTTGGC-3′
IGFBP3	5′-GAACAGGGTGTGCTGCTATGGG-3′	5′-CAAGTCTCTAGGTTGGCTGCGG-3′
DRAM	5′-TCTGGCTTTTGTCTGCGGTGTG-3′	5′-CCATGCGTATGTGGCACGTCAA-3′
SLC38A2	5′-TGAAGAAAGCCGAGATGGGAAGGT-3′	5′-GCAGCTTGCTTGGTGGGATAGG-3′
CDC25C	5′-GAACCCCAGAGCTACTGCCCTA-3′	5′-TCAGTTGCCGCTTTCCTTCCTG-3′
ODC1	5′-AGCTCTTCCTCGTGTTACCCCC-3′	5′-TCAAATCCTGTCCCAATGGCAGC-3′
CRYZ	5′-TCAGTACCAGAACAATCTCGGGGG-3′	5′-GCTCGAAAGGCAGTAAAGTACGGG-3′

Known up-regulated p53 target genes include growth arrest and DNA damage inducible gene (GADD45A), Bcl-2 associated X gene (BAX), p53 inducible gene 3 (PIG3), insulin-like growth factor-binding protein 3 (IGFBP3), Serpin peptidase inhibitor, clade B (ovalbumin), member 5 (maspin), DNA damage-binding protein 2 (DDB2), and DNA-damage regulated autophagy modulator 1 (DRAM). Known down-regulated p53 target genes include crystallin, zeta (quinone reductase) (CRYZ), cell division cycle 25 homolog C (CDC25C), Sodium-coupled neutral amino acid transporter 2 (SLC38A2), and Ornithine decarboxylase 1 (ODC1). actin was used as a housekeeping gene control. Amplified fragments were sequenced and sequences were deposited in GenBank.

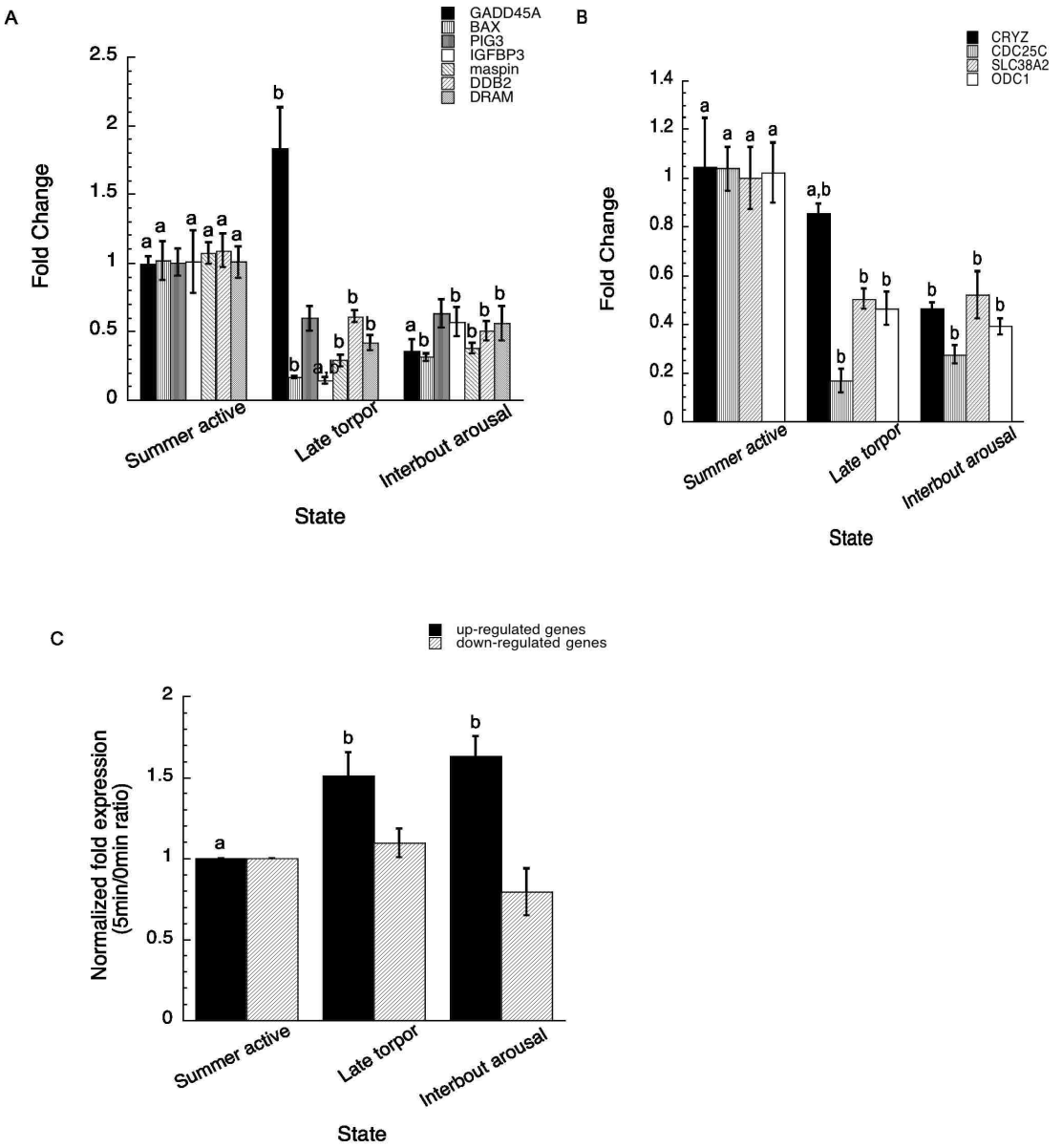

Figure 4-6. p53 differentially regulates its target genes as a function of torpor state in liver of golden-mantled ground squirrels. For (A) and (B), total RNA samples extracted from liver of ground squirrels representing various states were used for qRT-PCR analysis as described in 'Materials and Methods' section. (A) The mRNA expression of known p53 up-regulated genes as a function of torpor state. The mRNA levels of all but GADD45 were significantly reduced as a function of torpor state. GADD45 mRNA abundance was increased by ~ 1.5 fold in LT. (B) The mRNA expression of known p53 down-regulated genes as a function of torpor state. (C) qRT-PCR based nuclear run-on assay measured the transcription rate of both known up-regulated and down-regulated p53 target genes as a function of torpor state. Values shown in the bar graph represent mean ± SE (n = 3 animals were used per group). Statistical analysis has been done using ANOVA ($P < 0.05$). Different letters indicate significantly different means.

Table 4-4. Relative fold change in the mRNA abundance of p53 target genes over a 5 min *in vitro* transcription elongation period.

	Genes	Summer active	Late torpor	Interbout aroused
Up-regulated target genes	GADD45A	1.00 ± 0.08	1.51 ± 0.20	1.65 ± 0.38
	BAX	1.00 ± 0.14	0.91 ± 0.17	1.75 ± 0.33
	PIG3	1.00 ± 0.23	1.98 ± 0.50	1.87 ± 0.48
	IGFBP3	1.00 ± 0.09	1.73 ± 0.29	1.43 ± 0.20
	maspin	1.00 ± 0.03	1.75 ± 0.21	2.17 ± 0.23
	DDB2	1.00 ± 0.25	1.11 ± 0.30	1.18 ± 0.32
	DRAM	1.00 ± 0.08	1.59 ± 0.23	1.36 ± 0.18
Down-regulated target genes	CRYZ	1.00 ± 0.14	1.13 ± 0.17	0.64 ± 0.13
	CDC25C	1.00 ± 0.15	1.04 ± 0.18	0.52 ± 0.09
	SLC38A2	1.00 ± 0.11	1.32 ± 0.25	1.19 ± 0.20
	ODC1	1.00 ± 0.04	0.90 ± 0.08	0.82 ± 0.11
	actin	1.00 ± 0.03	0.97 ± 0.06	0.98 ± 0.09

Known up-regulated p53 target genes include growth arrest and DNA damage inducible gene (GADD45A), Bcl-2 associated X gene (BAX), p53 inducible gene 3 (PIG3), insulin-like growth factor-binding protein 3 (IGFBP3), Serpin peptidase inhibitor, clade B (ovalbumin), member 5 (maspin), DNA damage-binding protein 2 (DDB2), and DNA-damage regulated autophagy modulator 1 (DRAM). Known down-regulated p53 target genes include crystallin, zeta (quinone reductase) (CRYZ), cell division cycle 25 homolog C (CDC25C), Sodium-coupled neutral amino acid transporter 2 (SLC38A2), and Ornithine decarboxylase 1 (ODC1). actin was used as a non-target gene control. Values in the table were represented as means ± SE. Summer active seasonal variation control was arbitrarily set to 1 (n = 3 animals were used per group).

Discussion

p53 sits on the crossroads of a variety of cellular processes (Figure 4-1). I asked if p53 metabolism was altered as a function of torpor state. The results of qRT-PCR revealed that p53 mRNA level was drastically reduced by ~ 60% in LT, but was fully restored during IBA (Figure 4-2). However, data from western blot analyses indicate that p53 protein level remained unaltered in LT, but was elevated ~ 2 fold during IBA (Figure 4-2, B and C). The differences between p53

mRNA and protein levels underscore the poor relationship that may exist between mRNA concentration and protein concentration (Gygi et al., 1999).

I also examined the mRNA or protein expression levels of several p53 regulators, including p53bp2, p53IN1, Mdm2, Mdm4, and RL26 (Figure 4-3). Taken together the data are consistent with a seeming activation of p53 during torpor. Indeed, other studies in the mammalian hibernation literature oftentimes cite such data in support of activity (e.g. Fleck and Carey, 2005; Allen and Storey, 2011).

Importantly, the above data, while consistent with activation, do not actually address activity *per se*. As a transcription factor, p53 accumulates in the nucleus where it can be activated to regulate a cascade of target genes involved in multiple biological processes. Previous studies suggested that nuclear p53 protein levels were fourfold less in hibernating ground squirrel as compared to summer active animals (Fleck and Carey, 2005). However, there were technical issues with this study. These authors only examined nuclear fractions and did not control for the amount in the cytosol. I carried out immunohistochemistry analysis to study subcellular localization of p53 protein as a function of torpor state. Data indicate that p53 protein accumulates in the nucleus and there is a moderate 15% increase of p53 protein localization during LT and IBA (Figure 4-4, B). Since total p53 protein level was significantly increased by ~ 2 fold during IBA (Figure 4-3, B), there is an even greater increase in the net amount of nuclear p53 proteins during IBA as compared to SA values.

Even if p53 were localized to the nucleus, some p53 proteins might be transcriptionally inactive (Wolff et al., 2001). To further elucidate the role of p53 as a transcription factor, I used ChIP assay to demonstrate the direct binding of nuclear p53 protein to its target genes. p53 binding to its target genes was clearly increased during LT and IBA (Figure 4-5).

These data suggest that p53 can bind DNA. However, the data do not indicate if transcriptional initiation was affected by the p53 transcription factor. I exploited a nuclear run-on assay to determine if p53 binding affected the transcription initiation status of known p53 target genes. Nuclear run-on assays quantify *in vivo* initiation of transcription because initiation of new transcripts is negligible following cell lysis (Weber et al., 1977; Groudine, et al., 1981; Hofer and Darnell, 1981; Lohr and Ide, 1983). Known p53 up regulated and p53 down-regulated genes were amplified following a nuclear run-on assay. The data indicate a very modest role for p53 in regulating transcription. Run-on mRNA concentrations for genes known to be upregulated by p53 were increased in abundance by ~ 1.5 fold during both LT and IBA (Figure 4-6, C; Table 4-3). Importantly, such changes are not reflected in total mRNA levels for the tissue (Figure 4-6, A).

An important lesson from these data is that cautious data interpretation is required when interpreting data from a depressed metabolic state. Traditional interpretations would have suggested activation by p53 as evidenced by known regulators of p53 metabolism, p53 protein concentrations, p53 nuclear localization, p53 binding to DNA, and even p53 effects on initiation of

transcription of known upregulated target genes. However, by exploiting a systems level analysis of function, I was able to ascertain that p53 does not defend the mRNA concentrations of these target genes. Indeed, the data are similar for known p53 down-regulated target genes. Thus, p53 transcription factor function during torpor is limited at best. An interesting question is if p53 is not being used to regulate transcription, could it possibly be used elsewhere in one of its myriad of known regulatory roles?

CHAPTER 5

CONCLUSIONS

Summary

Inherent to mammalian hibernation is a need to reduce energy consumption. Translation represents an energetically expensive process that is normally vital to homeostasis. I studied mechanisms associated with translational control. The data presented here provide valuable insight into the mechanisms that allow an animal to hibernate.

Preference of IRES mediated initiation of translation as a function of torpor state

Previous studies indicate that cap-dependent initiation of translation was inhibited as squirrels enter into torpor (van Breukelen and Martin, 2001). Regulation of cap-dependent translation initiation is mediated via eIF4E and its binding protein 4E-BP1 (van Breukelen et al., 2004). I asked the question of what happens to cap-independent initiation of translation, more specifically IRESmt? The second chapter demonstrated that mRNAs that harbor IRES sequences in their 5′-UTR are preferentially loaded on ribosomes as a torpor bout progresses (Figure 2-4). The increased association of IRES participants to ribosomes is a result of differential translation rather than simply due to differential gene expression (Figure 2-6). Squirrels exploit an available mechanism of IRESmt to gain differential translation of preexisting mRNA transcripts as the squirrels begin to arouse from torpor and translation resumes.

Interestingly, there is a significant difference in the IRES preference index between spontaneous aroused and alarm aroused animals, strongly suggesting that squirrel might prepare for arousal (Figure 2-8). The use of IRESmt may allow for preferential production of key stress proteins that could conceivably protect squirrels from harsh physiological stresses inherent to the arousal process geared toward enhanced survivorship.

miRNAs play limited role in translational control during hibernation

miRNAs have been demonstrated to play key role in multiple fundamental biological processes including translational control. In the third chapter, I utilized a miRNA microarray to screen for changes in miRNA abundance as a function of torpor state. I found four major miRNAs species expressed in liver, but there were no significant changes in expression levels of those miRNAs as a function of torpor state. Studies for miRNA expression levels detected by qRT-PCR and deep sequencing also found few substantial changes in miRNA expression levels (Morin et al., 2008; Liu et al., 2010). These findings support the idea that miRNA might play a very limited role in translational control during mammalian hibernation.

p53 metabolism during mammalian hibernation

p53 plays a key role in translational control as a specific negative regulator (Reviewed in Ewen and Miller, 1996). Additional data indicate that p53 can act as a transcription factor upon stress exposure (Wei et al., 2006). In the fourth

chapter, I examined mRNA and protein concentrations of p53 as a function of torpor state. Immunohistochemical data revealed p53 was stabilized and accumulated in the nucleus. Data from ChIP and nuclear run-on assay indicate that nuclear p53 proteins are actively binding to downstream targets genes, resulting in moderate transcriptional output. Very importantly, cellular [mRNAs] of known target genes are not defended. These data underscore the limited role of p53 despite a seeming activation during hibernation. These findings suggest that any changes in p53 metabolism may be a simple consequence of poorly controlled processes during hibernation as opposed to a deliberate effect of preferential gene expression.

Future studies

Accumulating evidence has shown that post-translational modifications, including ubiquitylation, phosphorylation, acetylation and sumolylation, might play important roles in regulating p53 stability and transcriptional activity under different circumstances (Kubbutat et al., 1998; Bode and Dong, 2004; Sakaguchi et al., 1998; Brooks and Gu, 2003; Melchior and Hengst, 2002). For instance, polyubiquitylation leads to protein degradation and nuclear export of p53, while monoubiquitylation results in nuclear export of p53. It would be interesting to elucidate the role of post-translational modifications of p53 in respect to its transcriptional activity in mammalian hibernation wherein most of those modifications are not well controlled.

An interesting idea is if neonatal programming is what allows a hibernator to express the hibernation phenotype on a seasonal basis as is what is seen in golden-mantled ground squirrels (van Breukelen and Martin, 2002). If true, an identification of mechanisms that allow for the expression of that neonatal program would be very informative. I propose that epigenetic control mechanisms such as methylation or acetylation could be key to this regulation. I propose to use deactylases or methylases to explore their effect on patterns of hibernation use.

APPENDIX 1

ABBREVIATIONS

4EB-BP1	Eukaryotic initiation factor 4E-binding protein 1
ARO	Arousal
BAX	Bcl-2 associated X gene
CRYZ	crystallin, zeta (quinone reductase)
CDC25C	cell division cycle 25 homolog C
DDB2	DNA damage-binding protein 2
DRAM	DNA-damage regulated autophagy modulator 1
eIF4E	Eukaryotic initiation factor 4E
ENT	Entrance into torpor
ET	Early torpor
GADD45A	growth arrest and DNA damage inducible gene
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IBA	Interbout arousal
IGFBP3	insulin-like growth factor-binding protein 3
IRES	Internal ribosomal entry site
IRESmt	Internal ribosomal entry site mediated initiation of translation
LT	Late torpor
maspin	Serpin peptidase inhibitor, clade B (ovalbumin), member 5
Mdm2	Mouse double minute 2
Mdm4	Mouse double minute 4
ODC1	Ornithine decarboxylase 1
p53bp2	p53 binding protein 2
p53INP1	p53 inducible nuclear protein 1
p53INP2	p53 inducible nuclear protein 2
PIG3	p53 inducible gene 3
RPL26	Ribosomal protein L26
SA	Summer active
SLC38A2	Sodium-coupled neutral amino acid transporter 2
T_b	Body temperature

APPENDIX 2

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APPENDIX 3

SUPPLEMENTAL DATA

Choice of transcripts

To date, there are no methods to reliably predict the existence of an IRES element based on the primary nucleotide sequence of the 5′-UTR (Pestova et al., 2001). Experimentally, a bicistronic construct encoding for two different luciferases with an intervening sequence containing a putative IRES element may be used to verify bona-fide IRES element use (Kozak, 2005). Despite a lack of conservation of nucleotide sequence, IRES use has been well conserved in closely related species. The c-Myc IRES has been experimentally verified in human and mouse (Créancier et al., 2001; Storey, 2001); the BiP IRES was verified in human, rat, mouse, Chinese hamster and several invertebrate species (Kim et al., 2000; Le and Maizel, 1997; Thoma et al., 2004; Young et al., 2008); and the CaM kinase II IRES was verified in mouse, rat, and rabbit (Fährmann and Kaufhold, 2006; Lin et al., 2005; Pinkstaff et al., 2001). As far as we are aware, there are no verified IRES elements that are species specific. We blasted the 5′-UTR regions of our chosen transcripts using the 5′-UTR of the congeneric species *Spermophilus tridecemlineatus* against other species. The putative IRES containing sequences of ground squirrel share 72% - 89% identity to the experimentally verified IRES containing sequences of the other species. We note that all species experience similar divergences in their 5′-UTR regions (Figure S1). Such similarities are not found in those genes that have been experimentally verified to lack an IRES element e.g. actin and GAPDH. For

instance, even though actin protein is highly conserved amongst species, the identity in the 5′ UTR was only 51%.

Method for Sequence Alignment

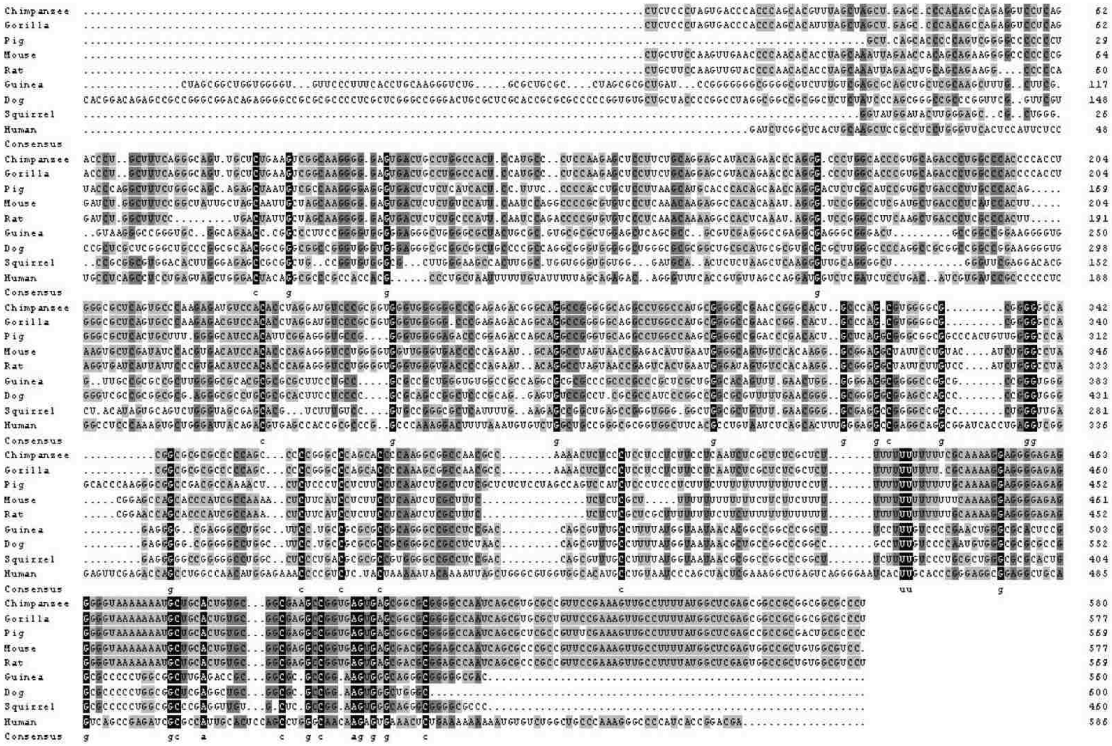
Sequences were retrieved from NCBI GenBank and Ensembl. Sequences were aligned using ClustalX (edition 2.1; Figure S1). Percent identity is listed in Table S1. BiP/GRP78 5′ UTR sequences are from human, chimpanzee, gorilla, macaque, mouse, rat, pig, cat, and squirrel. c-myc 5′ UTR sequences are from human, chimpanzee, gibbon, marmoset, rat, mouse, pig, sheep, and cat. CaM kinase II 5′ UTR sequences are from human, mouse, squirrel, guinea pig, rabbit, pika, and tree shrew. Sequences for β actin 5′ UTR are from human, chimpanzee, gorilla, mouse, rat, guinea pig, squirrel, dog, and pig. Sequences for GAPDH 5′ UTR are from human, gorilla, mouse, squirrel, guinea pig, rabbit, pig, and cat.

Table S1. Sequence similarities of chosen non-IRES and IRES transcripts

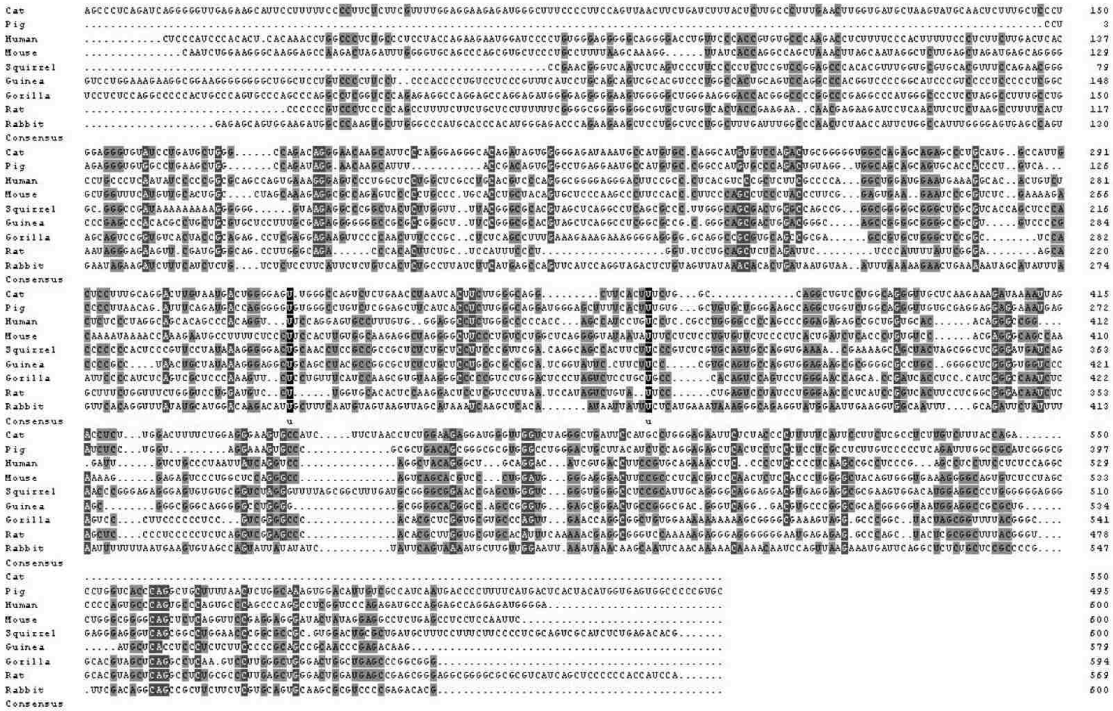
Gene Name	Sequences Similarity and Identity (%)
Non-IRES	
β-actin	51.24
GAPDH	43.63
IRES	
BiP/GRP78	75.1
c-myc	72.14
CaM kinase II	89.03

Percent identity of aligned 5′ UTR sequences for species indicated in the supplemental data text.

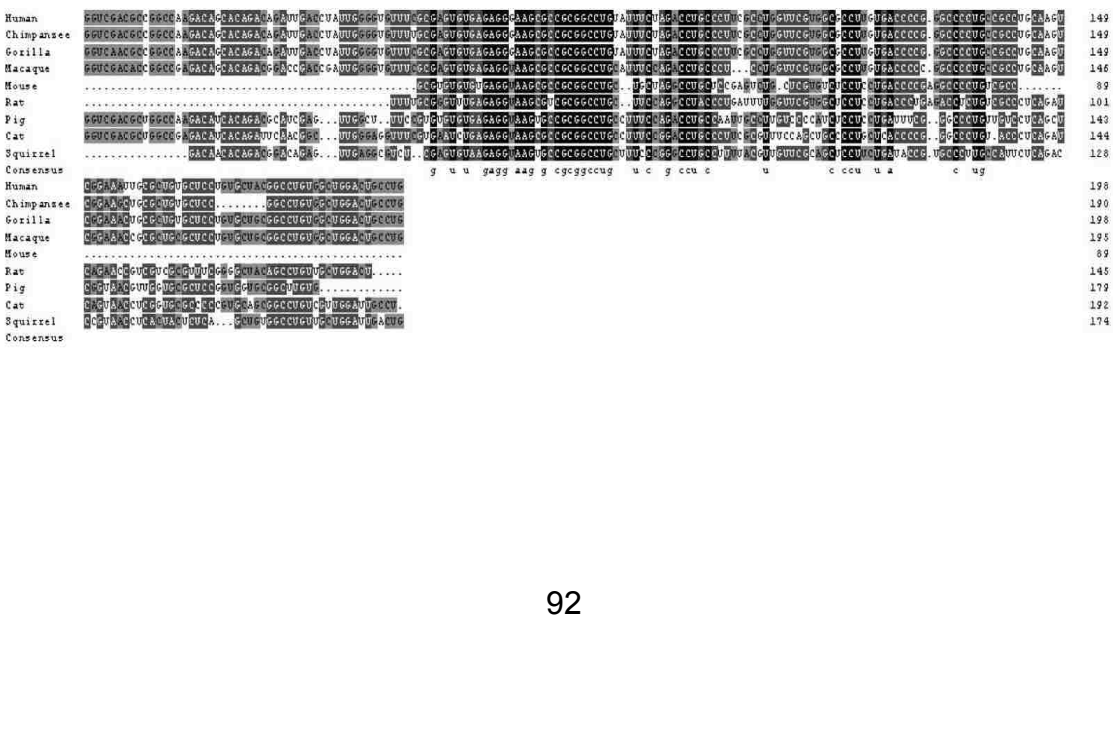
Sequence alignment of GAPDH



Sequence alignment of beta-actin



Sequence alignment of BiP/GRP78



Sequence alignment of c-myc

Sequence alignment of CaM Kinase II

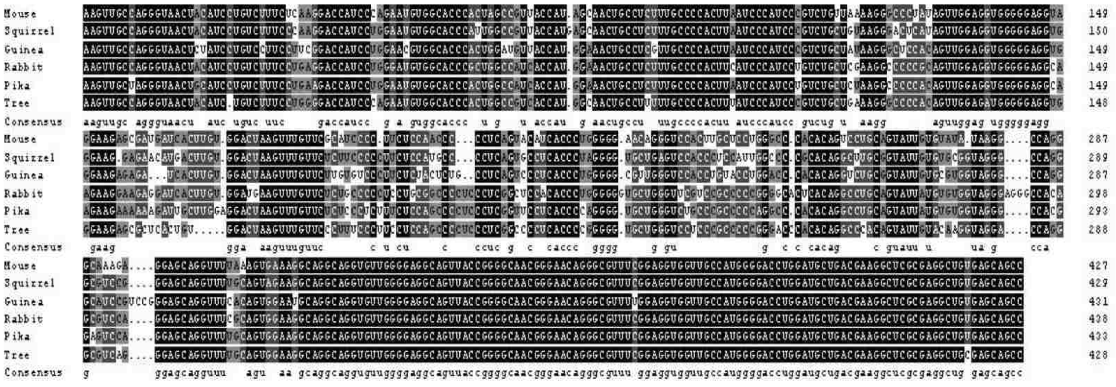


Figure S1. Sequence alignment of available mammalian c-myc, BiP/GRP78, CaM kinase II, β-actin, GAPDH 5′ UTR sequences showing conservation. Deletions are denoted by dots.

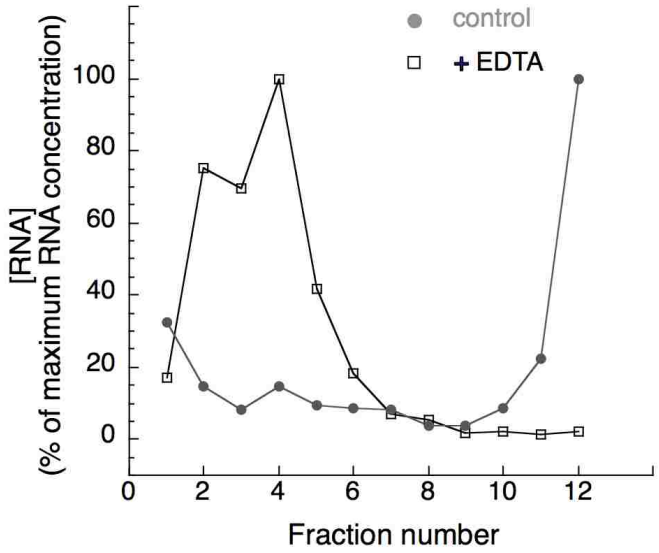


Figure S2. EDTA treatment disrupts polysome association with RNA. A lysate from a summer active (SA) animal was loaded onto a sucrose gradient as indicted in the text (control; gray filled circle). A parallel lysate was treated with treated with 30 mM EDTA prior to loading (+ EDTA; black open squares). The RNA concentration in each fraction was determined following exposure to an RNA-specific dye using the Bio-Rad Experion system. Values were normalized to the maximum value for that gradient and expressed as % maximum. Note EDTA treatment shifts the RNA distribution profile towards the lighter fractions indicative of polysome disruption.

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