Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Pennsylvania State University

2. **Reporting Period (start and end date of grant award period):** 1/1/2009 – 12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** John T. Anthony, MPA

4. **Grant Contact Person's Telephone Number:** 814-935-1081

5. **Grant SAP Number:** 4100047645

6. **Project Number and Title of Research Project:** 5 - Molecular Targets for Preventing Loss of Skeletal Muscle Mass

7. **Start and End Date of Research Project:** 1/1/2009 – 6/30/2012

8. **Name of Principal Investigator for the Research Project:** Leonard S. Jefferson, PhD

9. **Research Project Expenses**.

   9(A) Please provide the amount of health research grant funds spent on this project for the entire duration of the grant, including any interest earned that was spent:

   $ 436,295

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Tuckow	Graduate Assistant	100	$22,260
Kelleher	Graduate Assistant	100	$21,075
Kutzler	Research Technologist	39	$18,674
Lacko	Research Technologist	40	$24,916
Stanley	Mass Spectrometrist	45	$43,475

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Jefferson	Principal Investigator/Advisor	5

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
Participated in Animal cage washing equip	All of our researchers who have animals at the Animal Research Facility and the Hershey Center for Applied Research Facility are benefitted by the new tunnel and cage washer system.	$191,814

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes___X_____ No_____

If yes, please indicate the source and amount of other funds:

5 R01 DK15658-41 "Regulation of Skeletal Muscle Metabolism" $225,000 DC (current year)

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes____X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Regulation of Skeletal Muscle Metabolism	✓NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	7/10	$1,930,267	$1,713,122
Macronutrient Regulation of Alternative Pre-mRNA Splicing	✓NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	11/12	$1,299,349	Pending

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

Competitive renewal of NIH R01 DK01568, "Regulation of Skeletal Muscle Metabolism" will be sought to enable continuation of the project beyond 2016.

12. Future of Research Project. What are the future plans for this research project?

Future plans are to continue to elucidate signaling pathways and effector mechanisms that contribute to disuse atrophy of skeletal muscle with the aim of identifying molecular targets for intervention to prevent tissue wasting.

13. **New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes___X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male			2	
Female				
Unknown				
Total			**2**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic			2	
Unknown				
Total			**2**	

	Undergraduate	Masters	Pre-doc	Post-doc
White			2	
Black				
Asian				
Other				
Unknown				
Total			**2**	

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No___X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

The project provided a direct impact on research capacity and quality by contributing to the

training of two PhD graduate students and the employment of two highly skilled research support staff. Its indirect impact is major since it enabled a successful competitive renewal of a five-year NIH R01 award.

This project also participated in the purchase of a LYNX model tunnel cage washer with a stainless steel utility cabinet enclosure, LYNX model automatic cage dryer, LYNX model automatic bedding dispenser, and LYNX model cage and rack washer with a stainless steel utility cabinet enclosure. This purchase replaced the twenty-five-year-old existing equipment at the Animal Research Facility. LYNX also provided training for animal care technicians, the facility manager, and the maintenance crew. LYNX was able to provide custom built equipment to fit our facility and the existing area with minor renovation and redesign of the space. LYNX also has an excellent service record for repair and maintenance of their products.

The tunnel and rack washer benefits Penn State Hershey researchers Basic Science departments, including Biochemistry and Molecular Biology, Cellular and Molecular Physiology, Comparative Medicine, Microbiology and Immunology, Neural and Behavioral Sciences, and Pharmacology. The cage washer also benefits Clinical Science departments, including Medicine and Surgery, as well as the Penn State Hershey Cancer Institute.

All of our researchers who have animals at the Animal Research Facility and the Hershey Center for Applied Research Facility (HCAR) are benefitted by the new tunnel and cage washer system. It is estimated that sixty-five investigators currently conduct research at the Animal Research and HCAR Facilities.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No____X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No_____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant application's strategic plan). Summarize the progress made in achieving these goals, objectives and aims for the entire grant award period. Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Originally, the project was initiated with the following three specific aims:

Specific Aim #1: To validate that an acute resistance exercise bout results in increased eIF2Bε protein expression in human skeletal muscle. Based on results obtained by the Principal Investigator's laboratory using a rat model of resistance exercise, we hypothesize that eIF2Bε protein expression in human skeletal muscle will increase 16 h after resistance exercise without a concomitant change in mRNA abundance and that the protein and mRNA abundance for the remaining four eIF2B subunits (α, β, γ, and δ) will not change.

Specific Aim #2: To demonstrate that in vivo modulation of eIF2Bε expression directly affects muscle protein synthesis and muscle fiber size. Based on available evidence, we postulate that overexpression of eIF2Bε in rat skeletal muscle *in vivo* for 7 days will lead to an increase in global protein synthesis and subsequent hypertrophy of myofibers. In contrast, *in vivo* knockdown of eIF2Bε mediated by shRNA will lead to decreased protein synthesis and muscle fiber atrophy.

Specific Aim #3: To validate the role of miR-133 in the translational repression of eIF2Bε mRNA. MicroRNA-133a (miR-133a) and miR-133b are microRNAs known to be expressed in skeletal muscle and are also predicted (*in silico)* to target multiple target sites or "microRNA recognition elements" (MREs) in the 3'-untranslated region (3'-UTR) of eIF2Bε. Thus, we seek to demonstrate that these miRNAs target eIF2Bε and lead to its translational repression in a cell culture model.

Aim #1 of the initial project was designed to validate a resistance exercise-induced expression of eIF2Bε in human skeletal muscle. In these studies, investigators from The U.S. Army Research Institute of Environmental Medicine Military Performance Division (USARIEM-MPD) collected muscle biopsy samples to examine the effects of physical training on the acute-exercise induced response of metabolic and growth factor biomarkers relating to bone and muscle turnover. A graduate student, Mr. Alexander Tuckow, visited the USARIEM-MPD and performed Western blot and quantitative real-time PCR analysis on the muscle samples that had been previously collected and homogenized as part of the USARIEM protocol H06-07 ("Maximizing the Osteogenic Properties of Plyometric Exercise to Induce Optimal Structural Adaptations in Bone and Muscle: Effects on Endocrine and Cellular Controls"). During the study, muscle biopsies were collected from the vastus lateralis muscle from thirty-five young men and women (18-35 yrs) before, immediately after, and two hours after an acute exercise test. The targets that were to be examined included specific endpoints of the mTORC1 signaling pathway (p70S6K1, rpS6, and 4E-BP1), upstream regulators of mTORC1 signaling (Akt, FKBP38, and REDD1), components of the mTORC1 signaling complex (raptor and mTOR), two subunits of the eIF2B guanine nucleotide exchange factor (eIF2Bdelta and eIF2Bepsilon), and three control proteins (actin, GAPDH, and tubulin). Initially, Western blot analysis of expression of actin, GAPDH, tubulin, FKBP38, raptor, Akt, and REDD1 were performed. Unfortunately, the results were highly variable, even within samples taken from a single individual. Thus, in some cases expression of the target protein was unchanged in response to exercise and in some cases it exhibited increased or decreased expression. Moreover, the intensity of the signal varied dramatically from one set of samples to another. Upon reviewing the protocol used by personnel at USARIEM-MPD, it was discovered that the method used for homogenization was the likely cause of the variability. Mr. Tuckow re-homogenized the samples and re-ran some of the Western blots. However, re-homogenization did not improve the reproducibility of the analysis. Unfortunately, there were no remaining muscle biopsies available for further analyses. Consequently, no useable data were obtained from the study.

Aim #2 of the initial project was designed to examine the effect of overexpression of eIF2Bε on skeletal muscle protein synthesis. In preparation for the studies proposed for this aim, we optimized the technique of in vivo electro gene transfer delivering plasmid DNA to rat tibialis anterior muscles. We demonstrated successful transfection of the majority of muscle fibers

utilizing a plasmid expressing a green fluorescent protein (GFP) and visualizing a transverse section of the muscle via fluorescence microscopy. Utilizing the same electro gene transfer technique we were able to achieve a consistent overexpression of eIF2Bε protein in the tibialis anterior muscle (~2-fold mean increase above endogenous expression in the contralateral tibialis anterior) 24 h post-transfection. An assay measuring the guanine nucleotide exchange activity of eIF2B revealed a ~23% increase in eIF2B activity in muscles overexpressing the eIF2Bε subunit compared to contralateral controls 24 h post-transfection, demonstrating functional significance. Rates of protein synthesis showed a trend toward an increase when eIF2Bε was overexpressed. With regard to knockdown of eIF2Bε, we have generated two new shRNA constructs, that are to be tested in a cell culture model to verify targeting of eIF2Bε prior to *in vivo* animal experiments.

Unexpectedly, longer-term experiments revealed difficulty in maintaining plasmid-based expression of eIF2Bε at 5-7 d post-transfection despite co-transfection with other plasmids (e.g., GFP) that continue to be expressed. Moreover, protein expression driven by other plasmids, such as the ribosomal protein S6 kinase S6K1, was also maintained at 5-7 d post-transfection. The basis for the downregulation of eIF2Bε expression at later times after transfection is unknown, but it appears to be due to enhanced degradation of the protein (see later paragraph).

Following up on our experiments examining the overexpression of eIF2Bε *in vivo*, we utilized the electroporation technique to express the FLAG-eIF2Bε construct in skeletal muscle of septic rats. Using a rat model of chronic sepsis in which both protein synthesis and eIF2B activity were previously shown to be impaired, we expressed the catalytic epsilon subunit in one tibialis anterior muscle of control and septic rats and were able to demonstrate a rescue of eIF2B activity and protein synthesis in the muscle transfected with FLAG-eIF2Bε in spite of the septic condition. The data from the healthy and septic rat studies were published in the paper entitled "Ectopic expression of eIF2Bε in rat skeletal muscle rescues the sepsis-induced reduction in guanine nucleotide exchange activity and protein synthesis" (*Am J Physiol Endocrinol Metab* 299: E241–E248, 2010. First published May 18, 2010; doi:10.1152/ajpendo.00151.2010.)
Abstract: Eukaryotic initiation factor 2B (eIF2B) is a guanine nucleotide exchange factor (GEF) whose activity is both tightly regulated and rate-controlling with regard to global rates of protein synthesis. Skeletal muscle eIF2B activity and expression of its catalytic ε-subunit (eIF2Bε) have been implicated as potential contributors to the altered rates of protein synthesis in a number of physiological conditions and experimental models. The objective of this study was to directly examine the effects of exogenously expressed eIF2Bε in vivo on GEF activity and protein synthetic rates in rat skeletal muscle. A plasmid encoding FLAG-eIF2Bε was transfected into the tibialis anterior (TA) of one leg, while the contralateral TA received a control plasmid. Ectopic expression of eIF2Bε resulted in increased GEF activity in TA homogenates of healthy rats, demonstrating that the expressed protein was catalytically active. In an effort to restore a deficit in eIF2B activity, we utilized an established model of chronic sepsis in which skeletal muscle eIF2B activity is known to be impaired. Ectopic expression of eIF2Bε in the TA rescued the sepsis-induced deficit in GEF activity and muscle protein synthesis. The results demonstrate that modulation of eIF2Bε expression may be sufficient to correct deficits in skeletal muscle protein synthesis associated with sepsis and other muscle-wasting conditions.

Based on our observation of the very rapid loss of the exogenously expressed eIF2Bε, we subsequently pursued potential mechanisms of degradation of the protein. We experimentally

verified that the mRNA for the FLAG-eIF2Bε was still present in transfected muscles despite the absence of expression of the protein, indicating regulation at the level of translation or degradation. Following up on these results, we obtained evidence that eIF2Bε protein expression was elevated in the presence of the proteosome inhibitor MG-132. This evidence was provided by a study of the effects of the cholesterol lowering drug simvastatin on eIF2B subunit expression in C2C12 myoblasts. The results of that study were recently published in the paper entitled, "Simvastatin represses protein synthesis in the muscle-derived C_2C_{12} cell line with a concomitant reduction in eukaryotic initiation factor 2B expression," (*Am J Physiol Endocrinol Metab* 300:E564-E570, 2011. First published January 11, 2011; doi:10.1152/ajpendo.00383.2010)

Abstract: Statins are a widely prescribed class of cholesterol lowering drugs whose use is frequently associated with muscle-related ailments. A number of mechanisms have been implicated in statin-induced myotoxicity including alterations in both protein synthesis and protein degradation. The objective of the present study was to explore the mechanisms(s) contributing to the statin-induced reduction in protein synthesis in the muscle-derived C_2C_{12} cell line. Cells were treated with 10µM simvastatin or vehicle alone for 24h in 1% serum. Cells exposed to simvastatin exhibited reduced rates of protein synthesis, as evidenced by [^{35}S] methionine and [^{35}S] cysteine incorporation into protein. The reduction in protein synthesis occurred with a concomitant decrease in expression and activity of eukaryotic initiation factor 2B (eIF2B), a regulated and rate-controlling guanine nucleotide exchange factor known to affect global rates of protein synthesis. The reductions in protein synthesis and eIF2B expression were prevented by coincubation with mevalonate. Simvastatin treatment also resulted in a proteasome-sensitive reduction in the protein expression of all the subunits of the eIF2B heteropentameric complex. Finally, increased phosphorylation of the catalytic ε-subunit at Ser^{535} was observed, an event consistent with an observed reduction in eIF2B activity. These results suggest that repression of eIF2B expression and activity may contribute, at least in part, to the statin-induced reduction in protein synthesis.

Finally, in the course of performing the studies described above, we generated plasmid constructs (cloned from human skeletal muscle) containing the 5'-untranslated region (UTR), the 3'-UTR, or both the 5'- and 3'-UTR of the eIF2Bε mRNA with a firefly luciferase coding region to investigate their potential role in the translational regulation of eIF2Bε. Several cell lines (HEK293, Rat2, C2C12) were transfected with the various eIF2Bε-luciferase fusion constructs to examine the effects of manipulating the mTOR pathway (e.g., serum and leucine deprivation followed by replenishment; treatment with rapamycin) on expression of the constructs. The luciferase activity of the eIF2Bε reporter constructs was not altered in the context of these experiments. Additionally, we investigated the role of several microRNAs predicted to target the 3'-UTR of eIF2Bε by utilizing the luciferase construct with co-transfected pre-miRNAs. Co-transfection of the microRNAs investigated did not dramatically affect luciferase reporter activity of the eIF2Bε-3'-UTR construct, nor did they affect expression of endogenous eIF2Bε protein as determined by Western blotting.

The studies described above suggested that degradation of eIF2Bε occurs through a proteasome-dependent process. We therefore extended the previous findings to identify amino acid residues in eIF2Bε that are subject to ubiquitination. In these studies, C2C12 myoblasts were transfected with a plasmid expressing eIF2Bε bearing a FLAG epitope at the N-terminus. The cells were

then serum starved for 16 h and treated with or without the proteasome inhibitor MG-132 for 8 h. Interestingly, in the absence of MG-132 the expression of FLAG-eIF2Bε was nearly identical to that of the endogenous eIF2Bε expression. In contrast, with the addition of the proteasome inhibitor, there was a dramatic elevation in FLAG-eIF2Bε protein expression. Furthermore, additional bands were identified in Western blots of lysates from cells treated with MG-132 that were presumed to be mono- and multi-ubiquitinated FLAG-eIF2Bε respectively. To provide more direct evidence that eIF2Bε is ubiquitinated, lysates from C2C12 myoblasts transfected with two plasmids, one expressing FLAG-eIF2Bε and the other expressing HA-Ubiquitin (HA-Ub), were subjected to co-immunoprecipitation using a monoclonal antibody directed against eIF2Bε. Immunoprecipitation of eIF2Bε enriched the abundance of higher molecular weight bands detectable by Western blot. Probing the membrane with anti-HA (i.e., ubiquitin) antibody revealed a smear at molecular weights greater than the major eIF2Bε band. The smear is characteristic of polyubiquitinated proteins and represents HA-ubiquitin proteins that co-immunoprecipitated with eIF2Bε. Immunoprecipitation of HA(Ub) was also performed to examine the co-immunoprecipitation of eI2Bε with ubiquitin. HA(Ub) immunoprecipitation followed by Western blot for eIF2Bε revealed several distinct bands as well as higher molecular weight smearing. The three most distinct bands were presumed to be eIF2Bε with one, two, and three ubiquitins. None of these eIF2Bε bands were detected in HA immunoprecipitates from cells that were not transfected with HA-Ubiquitin.

Having established the co-immunoprecipitation of eIF2Bε with HA-Ub using either approach (i.e., immunoprecipitation using eIF2Bε or FLAG antibody to pull down the modified protein as well as immunoprecipitation using anti-HA(Ub) antibodies and detection of the eIF2Bε protein), experiments were next directed towards identification of residues modified by ubiquitination using tandem mass spectrometry. In these studies, C2C12 myoblasts were co-transfected with pFLAG-eIF2Bε and pRK5-HA-Ubiquitin and treated with the proteasome inhibitor MG-132 for eight hours, allowing for the accumulation of ubiquitin-modified proteins. The lysates were subjected to FLAG immunoprecipitation followed by HA immunoprecipitation, and the sample was subjected to SDS-PAGE and two major band(s), corresponding to approximate molecular masses of 75-100 kDa and 100-250 kDa, respectively, were excised and analyzed by LC-MS/MS. The analyses resulted in 67.2% overall coverage of the rat eIF2Bε protein sequence. Five unique lysine residues containing the -GG modification indicative of ubiquitination were identified in the ~75-100 kDa excised gel slice: Lys56, Lys98, Lys136, Lys212, and Lys500. Two of these residues (Lys136 and Lys212) were also identified as ubiquitinated peptides in the highest molecular weight gel slice, although the overall peptide coverage was lower in that analysis so we cannot rule out the possibility that other lysines are ubiquitinated in the highest molecular weight species. Finally, four of the five ubiquitinated lysine residues and the regions surrounding them are highly conserved in the mouse, rat, and human proteins whereas Lys500 is not conserved in the human protein.

In addition to the ubiquitin modifications, the LC-MS/MS analysis revealed several phosphorylated residues among the rat eIF2Bε peptides. Three serine residues (Ser527, Ser535, and Ser539) were identified that have been previously reported and characterized. In addition to the known sites of phosphorylation, three novel residues were identified as phosphorylated residues: Ser22, Ser125, and Thr317. A fourth residue was contained among the contiguous serine and threonine residues (#325-327) but the exact location of the phosphorylated residue

was not clear from the spectra. Each of the novel phosphorylation sites identified (Ser22, Ser125, Thr317) are conserved among rat, mouse, and human eIF2Bε proteins. A manuscript describing the results of these studies has been submitted for publication: Tuckow, A.P., Kazi, A.A., Kimball, S.R., and Jefferson, L.S. "Identification of ubiquitin-modified lysine residues and novel phosphorylation sites on eukaryotic initiation factor 2B epsilon."

Aim #3 of the initial project was designed to investigate the role of microRNAs (miRNAs) in the translational control of eIF2Bε expression. This aim was based on our prior studies showing that resistance exercise induces expression of eIF2Bε through a mTORC1 signaling pathway dependent translation control mechanism. While an exhaustive list of miRNAs expressed in mature skeletal muscle has not been published and new miRNAs are still being cloned, several miRNAs have been experimentally verified to be expressed as well as to play important roles in the translational regulation of target genes in skeletal muscle. In particular, miR-1-1, miR-1-2, miR-133a, miR-133b and miR-206 are induced during myotube formation of C2C12 myoblasts via the myogenic transcription factors myogenin and MyoD (1). In one of the few studies investigating skeletal muscle in animals, miR-1, miR-133a, and miR-206 were verified to be expressed in mouse soleus and plantaris and both miR-1 and -133a were downregulated after 7 d of functional overload induced hypertrophy (2). Based on the known expression and importance of these miRNAs in muscle in combination with results from several *in silico* prediction algorithms implicating the muscle-specific miR-133 in targeting eIF2Bε, experiments were performed to validate or refute their involvement. The results of these experiments are included herein since they have not as yet been published in a scientific journal.

First, we utilized several *in silico* prediction algorithms to determine which miRNAs might target the eIF2Bε mRNA paying particular attention to the miRNAs involved in muscle adaptations described above. According to miRNAMap (3), 144 experimentally verified miRNAs were predicted to target the 3'-UTR of eIF2Bε at the time the experiments were performed. Among those predicted were miR-133a and miR-133b which are known to be particularly important in skeletal muscle (2, 4). Further evidence was provided by another prediction algorithm, MicroInspector (5) which predicted ~110 miRNAs targeting eIF2Bε, again including the skeletal muscle expressed miR-133a and miR-133b. Additionally, StarMir (6), RNAhybrid (7), and rna22 (8) all predicted multiple putative miR-133 MREs within the 3'-UTR of eIF2Bε. Interestingly, some algorithms yielded consistent predictions with regard to the location of MREs in the 3'-UTR while others revealed unique sites for miR-133 binding. A graphical representation of predicted miR-133 MREs and their location in the eIF2Bε 3'-UTR is presented in Figure 1.

In order to establish the level of expression of miR-133a in various cell lines, small RNAs were isolated, reverse transcribed, and subjected to semi-quantitative end-point PCR. Several cell lines were chosen first to confirm the expression of miR-133a in C2C12 myotubes (as expected) but also in the hopes that miR-133a would not be expressed in other cell lines. The absence of expression of a given miRNA in a cell line makes that line particularly suitable for introduction of pre-miR molecules that are subsequently processed to mature miRNAs. In contrast, if the cell line expresses high amounts of that particular miRNA, it may be more suitable to transfect antagomiR-miRs to reduce expression of the miRNA and examine the effect on target protein or reporter expression.

The results showing expression of the 5S rRNA (which serves as a small RNA control), miR-133a, and miR-200c in multiple cell lines are presented in Figure 2. Note that miR-133a expression is specific to the two skeletal muscle-derived cell lines (C2C12 myoblasts and differentiated myotubes) and exists in greater abundance in the differentiated myotubes. The absence of miR-133a in HEK-293 and Rat2 cells allows functional analysis of miR-133a expression by transfecting pre-miR-133a into cells that do not normally express the miRNA. The miR-200c was not detectable in any of the cell lines tested here, but was abundant in MCF-7 cells (data not shown). Although miR-200c is perhaps the strongest candidate miRNA for targeting eIF2Bε, the function of miR-200c on eIF2Bε expression was not pursued because it was not expressed in muscle derived cells.

To directly examine whether or not mir-133 targets the 3'-UTR of eIF2Bε, firefly luciferase reporter constructs harboring either a miR-133 MRE (pGL3-miR-133 sensor) or the full-length 3'-UTR of human eIF2Bε gene (pmiR-eIF2Bε-3'-UTR) were co-transfected in several cell lines with control (scrambled) pre-miR molecules or pre-miR-133. The miR-133 sensor reporter, which contains a single perfectly complementary site for miR-133 downstream of the firefly luciferase coding region, was successfully repressed by co-transfection of pre-miR-133 (Figure 3A,B, and C). Importantly, despite expression of endogenous miR-133 in myoblasts (as shown in Figure 2), the addition of pre-miR-133 repressed the miR-133 reporter plasmid in C2C12 myoblasts as effectively as the HEK293 and Rat2 cell lines. These results demonstrate successful delivery of the pre-miR-133 molecule into each of the three cell lines examined as well as downstream functionality as evidence by the repression of the luciferase activity from pGL3-miR-133. In contrast, the pre-miR-133 miRNA precursor had no effect on either luciferase activity from the pmiR-eIF2Bε-3'-UTR plasmid or on eIF2Bε protein abundance (Figure 3). Similar results were obtained utilizing an additional pGL3-eIF2Bε-3'-UTR reporter construct which employs a different promoter. The results strongly suggest that miR-133 does not directly target the 3'-UTR of eIF2Bε for translational repression.

At the time these miRNA experiments were performed, another algorithm was introduced that takes into consideration the context of the miRNA target site with regard to the predicted secondary structure of the target mRNA. Whereas most of the miRNA:target prediction algorithms at the time relied either heavily or entirely on sequence complementarity (i.e., Watson-Crick base-pairing of miRNA to the target mRNA sequence), the new algorithm, referred to as PITA (Probability of Interaction by Target Accessibility (9)), calculates the difference between the energy gained by formation of the duplex of miRNA to target sequence in the mRNA (ΔG_{duplex}) and the energy lost by unpairing the nearby secondary structure of the target sequence (ΔG_{open}). In other words, ΔG_{open} is a "penalty" for the energy required to gain access to a highly structured region of the mRNA target, whereas a target site in a completely open or unfolded region would be scored entirely on the free energy of hybridization of the miRNA to mRNA target nucleotides. The final score is thus calculated as follows:
$\Delta\Delta G = \Delta G_{duplex} - \Delta G_{open}$

According to the authors, a general recommendation regarding PITA predictions is that a score $\Delta\Delta G <= -10.0$ is a “very likely” target (i.e., a more negative score represents a greater likelihood of interaction). The authors presented a systematic evaluation and comparison of the PITA

algorithm to existing algorithms and provided convincing evidence that the PITA algorithm should be a stronger prediction of true miRNA targets.

Because of the large number of miRNAs that are predicted to target eIF2Bε, the decision to pursue additional miRNAs was based on the following three criteria to keep the experiments relatively stringent: 1) the miRNA must be expressed in skeletal muscle; 2) the miRNA must have been shown to be dysregulated in models of cardiac hypertrophy (very little literature on miRNA expression during *skeletal* muscle hypertrophy was available); and 3) the predicted miRNA:eIF2Bε target duplex exhibited favorable prediction scores that were conserved in both the mouse and human genes (rat miRNA target predictions were not available for the PITA algorithm). For example, miR-93, -133, -149, and miR-181b are downregulated during cardiac hypertrophy due to trans-aortic banding (10, 11) as well as in transgenic mice expressing activated calcineurin A which exhibit cardiac hypertrophy (11). Furthermore, overexpression of miR-93 and miR-181b in cardiomyocytes leads to atrophy of the myocytes (11). That the upregulation and downregulation of these miRNAs have opposite effects with regard to cardiomyocyte or myocardial size suggests a crucial role in targeting genes that act as growth repressors. Since overexpression of eIF2Bε has been implicated in cardiac hypertrophy (12), it is a logical extension that if miRNA(s) that target and therefore repress eIF2Bε expression are downregulated, relief in the repression of eIF2Bε expression might be implicated in the resulting cardiac hypertrophy. Accordingly, several miRNAs were selected based on the PITA prediction algorithm along with the aforementioned criteria for functional assay utilizing the eIF2Bε 3'-UTR reporters pmiR-eIF2Bε-3'-UTR and pGL3-eIF2Bε-3'-UTR. The miRNAs analyzed, their corresponding predicted target locations within the eIF2Bε 3'-UTR, and the PITA prediction scores are presented in Figure 6-4.

Despite the favorable scores predicted by the PITA algorithm, no remarkable changes were observed in either pmiR-eIF2Bε-3'-UTR reporter activity or eIF2Bε protein abundance in HEK 293 cells upon transfection of pre-miR-30e*, -93, -149, or -181b (Figure 5A). In some experiments, several pre-miRs were co-transfected simultaneously based on the concept that multiple miRNAs may target different regions of the same mRNA. In the event that one miRNA exhibits only a small effect with regard to repression, multiple miRNAs may act cooperatively to target the same gene and repress protein expression to a more dramatic extent (13). Under the conditions examined herein, the pool of four miRNAs did not repress eIF2Bε reporter activity or protein expression (Figure 5A). Similar results were obtained with transfection of the pre-miR miRNA precursors with both of the 3'-UTR reporters (pmiR-eIF2Bε-3'-UTR and pGL3-eIF2Bε-3'-UTR) and in both C2C12 and HEK293 cells.

The binding of miRNAs to their cognate mRNAs may be dependent on the conditions of the cell. For example, the presence of additional *trans*-acting factors on the 3'-UTR (e.g., other miRNAs or RNA binding proteins) may prohibit the miRNA from targeting what might be a legitimate MRE within the target mRNA. For example, binding of the RNA binding protein Dnd1 to the 3'-UTR of several miR-430 and miR-372 targets prohibits interaction of those miRNAs with the target (i.e., relieves the miRNA repression) in primordial germ cells of zebrafish (14). Another pertinent example for conditional miRNA repression occurs in an mTOR-dependent manner. In synaptoneurosomes isolated from rat brain, the translation of the Limk1 mRNA is repressed by miR-134. However, upon stimulation with BDNF, the translational repression is relieved.

Intriguingly, BDNF fails to relieve miR-134 mediated repression in the presence of rapamycin (15). Such examples in combination with the evidence for mTORC1-specific translational stimulation of eIF2Bε prompted an additional experiment to investigate whether the previously examined miRNAs might target eIF2Bε in a rapamycin-sensitive manner. To address this possibility, HEK 293 cells were transfected with luciferase reporters only (i.e., no pre-miR molecules), a negative control pre-miR, or a pool of five pre-miRs (pre-miR-30e*, -93, -133, -149, and -181b). Eighteen hours post-transfection, subsets of each of the transfected cells (in growth medium) were treated with or without rapamycin (100 nM) for 6 hours prior to harvest. As shown in Figure 5B, rapamycin treatment decreased the phosphorylation of eIF4E-BP1, suggesting proper inhibition of mTORC1. However, no differences were observed in the pmiR-eIF2Bε-3'-UTR reporter activity under any of the conditions. In addition to the data presented here, three other miRNA precursors (-30a*, -125b, and -378) were tested based on various bioinformatics predictions but none resulted in any remarkable changes in eIF2Bε-3'UTR reporter activity (data not shown). These results strongly rule out the involvement of these particular miRNAs in the translational regulation of eIF2Bε (at least mediated by the 3'-UTR).

In order to study a more general model (i.e., not specific to individual miRNAs) that might more closely reflect the previously described mTORC1 sensitive translational regulation of eIF2Bε (16, 17), several new plasmid constructs were generated by cloning the 5’-UTR, 3’-UTR, or both the 5’- and 3’-UTR of the human eIF2Bε mRNA into a firefly luciferase reporter vector essentially placing these untranslated regions in their appropriate respective location in relation to the firefly coding sequence. These constructs were designed to investigate their potential role in the translational regulation of eIF2Bε. Several cell lines (HEK293, Rat2, C2C12) were transfected with the various eIF2Bε-luciferase fusion constructs to examine the effects of manipulation of the mTORC1 signaling pathway on expression of the constructs.

First, we tested the role of the 3'-UTRs of both eIF2Bδ and eIF2Bε in a cell model that has low basal mTORC1 activity (induced by a total of 2.5 h of serum and leucine deprivation) followed by the rapid activation of mTORC1 (replacing the final 0.5 h of deprivation with serum and leucine replenishment in a subset of cells, restoring both growth factor and leucine as inputs to the mTORC1 pathway). This model is based on one established in our laboratory and similar to that used to demonstrate a shift of the eIF2Bε mRNA into polysomes in Rat2 fibroblasts (17). As shown in Figure 6, the firefly luciferase activity from eIF2Bδ and eIF2Bε 3'-UTRs reporter plasmids was not altered by acute manipulation of the mTORC1 pathway in either HEK 293 (Panel A) or Rat2 (Panel B) cells.

There are two important considerations in interpreting the results from the 3'-UTR reporters. First, the half-life of the firefly luciferase may prevent the total 2.5 h deprivation from reducing firefly luciferase protein content. The cells were previously incubated in growth medium and thus likely have quite a bit of luciferase expressed. The 0.5 h treatment, even if stimulating luciferase reporter expression, may have been masked by already high luciferase protein levels. To address this concern, a second set of experiments was performed using a longer period for both deprivation and replenishment to magnify the disparity in mTORC1 signaling between the two conditions. Second, the 3'-UTR is only one potential site for regulatory elements. Thus, the second set of experiments was performed with the newly constructed plasmids containing each combination of the 5'- UTR alone , 3'-UTR alone , or 5'/3'-UTRs in combination of eIF2Bε.

The results of the experiments clearly demonstrate the sufficiency of serum and leucine deprivation and repletion on altering mTORC1 activity as evidenced by changes in S6K1 phosphorylation (right panel of Figure 7A). However, none of the reporters demonstrated enhanced translation as evidenced by the firefly/renilla ratios (Figure 7A). Conversely, the trend was similar for all of the reporters where the firefly/renilla ratios are actually lower following replenishment (significantly so for both the empty pGL3 control plasmid and the 5'/3'-UTR reporter plasmid). Upon closer examination of the data, the general trend of the results under the current conditions indicated that serum and leucine replenishment enhanced translation of *both* the experimental pGL3 reporters as well as the pRL-SV40 renilla "control" plasmid (Figure 7B). The luciferase activity from the pRL-SV40 was in fact increased to a greater extent than the experimental firefly luciferase reporters, leading to the observed decrease in firefly/renilla ratio. The fact that both the pGL3-based and pRL-SV40 plasmids followed the same pattern suggest that all plasmids were similarly regulated under these conditions. Furthermore, the pGL3 empty control vector firefly/renilla ratio was significantly reduced although the plasmid contains no known regulatory elements. Indeed, if the 5'- and/or 3'-UTRs of eIF2Bε conferred translational regulation, one would expect firefly reporter activity to be relatively repressed in the deprivation conditions but dramatically *enhanced* in the replenishment conditions which would be demonstrated by a dramatic increase in the firefly/renilla ratio. Unfortunately, none of the plasmids tested resulted in such a phenomenon.

Difficulties in the computational prediction of miRNA targets remain an enormous challenge (18). Interestingly, several experimentally validated miRNA targets (i.e., those miRNA:target mRNA pairs that demonstrate legitimate functional repression) do not necessarily score highly with the PITA prediction algorithm. Examples of experimentally validated miRNA:target pairs include miR-21:PDCD4 (19-22)(ddG = -2.69); miR-133:SRF (4)(two sites: ddG = -4.64 and -0.38); miR-133:PTBP2 (23)(ddG = -3.27); and miR-133:ERG (24) which was not predicted at all by the PITA algorithm. More recent studies combining sophisticated proteomics approaches with functional overexpression of a miRNA and analysis of the repressed targets revealed that the PITA algorithm performed no better than other algorithms using simple seed match parameters (25, 26). Indeed, both false-positives as well as false-negatives are still a weakness inherent to prediction algorithms due to our poor understanding of the very diverse nature of miRNA:mRNA interactions.

Great strides are being made with respect to our understanding of the function of miRNAs and other small non-coding RNAs. The most recent evidence suggests that the majority of miRNA targets undergo mRNA cleavage (25-27). Additional complexity in predicting targets arises from exceptions to what were previously considered "golden rules" for target prediction. For example, restricting miRNA target prediction to the 3'-UTR of genes (as most algorithms have in the past) would lead to a potentially large number of overlooked yet functionally relevant miRNA:mRNA target interactions. Indeed, recent evidence has demonstrated the presence of functional miRNA recognition elements in the 5'-UTR (28, 29) and coding regions (30-32) of target genes. One important consideration is that the role of the coding region of the eIF2Bε gene was not directly examined in these studies, at least with regard to firefly luciferase reporter activity. Still, in the experiments where pre-miRs were transfected and eIF2Bε protein abundance was determined, no obvious changes were observed. Furthermore, no specific enhancement of translation was noted from luciferase reporters bearing the 5'- and 3'-UTRs of human eIF2Bε (either singly or in

combination) upon mTORC1 stimulation. In conclusion, the regulatory elements within the eIF2Bε mRNA conferring its translational regulation remain unresolved.

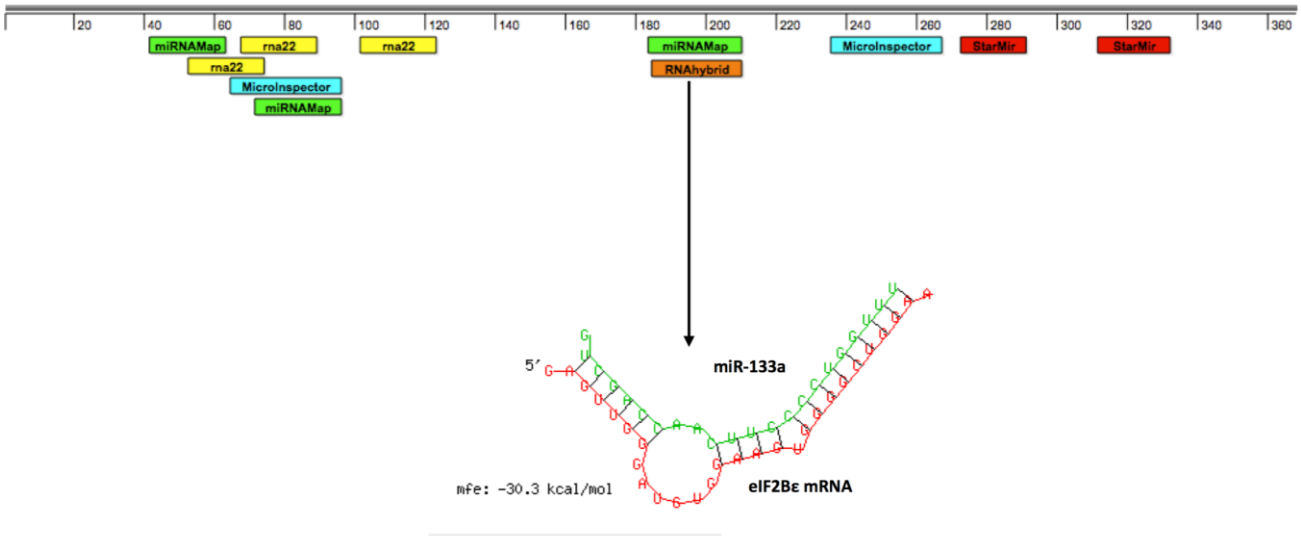

Figure 1. Predicted miR-133 target sites within the 3'-UTR of eIF2Bε.

The results of several microRNA target prediction algorithms are presented and indicated multiple potential target sites for miR-133 in the human eIF2Bε 3'-UTR (NM_003907). The hatched horizontal line represents the 368 nt 3'-UTR sequence with tick marks every 20 nt. The locations of the target sites and the algorithm(s) predicting the respective target locations are depicted by the colored boxes below the line. Algorithms used include miRNAMap, rna22, StarMir, MicroInspector, and RNAhybrid. The arrow points to an RNAhybrid prediction of hybridization of miR-133a to the eIF2Bε mRNA (starting at position 185) with a minimum folding energy of -30.3 kcal/mol for the miRNA/target duplex.

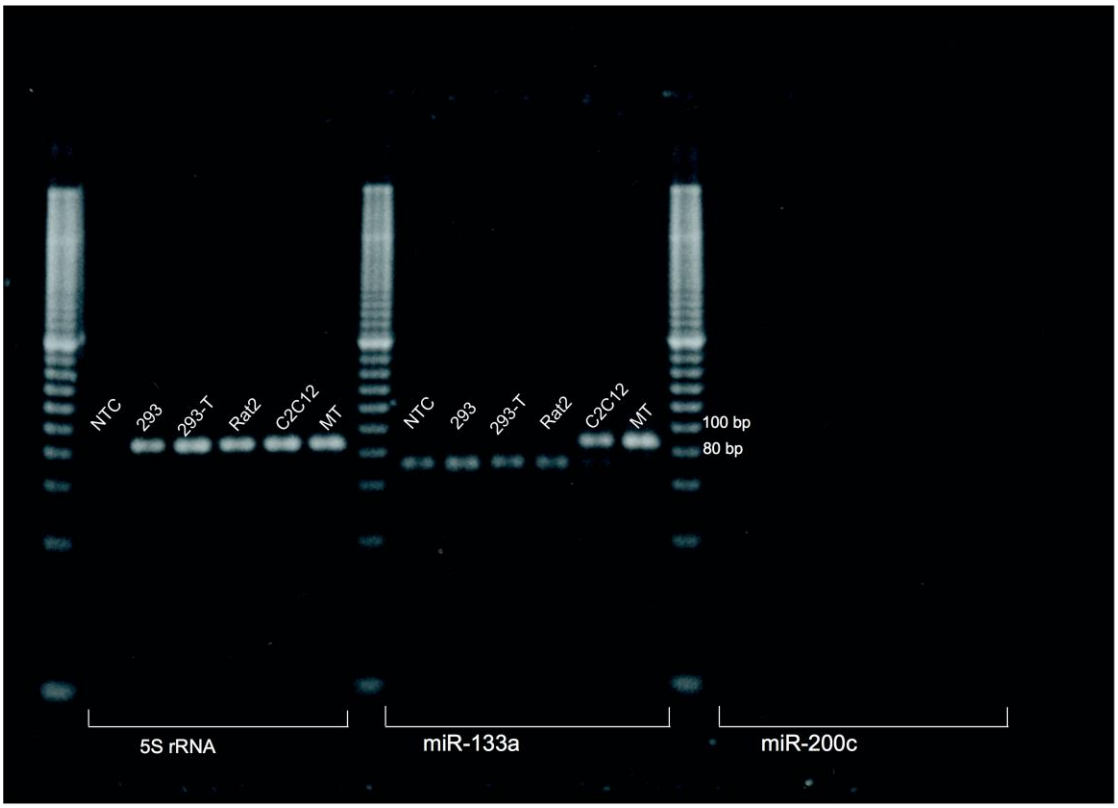

Figure 2. End-point PCR analysis of 5S ribosomal (r)RNA and microRNA expression in various cell types: miR-133a expression is restricted to muscle-derived cell lines**.**

MicroRNAs were isolated from HEK-293, HEK-293-T, Rat2 fibroblasts, C2C12 myoblasts, and C2C12 myotubes (MT) differentiated for five days. Briefly, $3x10^6$ cells were pelleted and miRNAs extracted with the mirVana miRNA isolation kit (Ambion, Austin, TX) and 25 ng of small RNAs were amplified for 25 cycles with the mirVana qRT-PCR kit (Ambion). Reverse transcription and PCR Primers specific for 5S ribosomal RNA (rRNA), miR-133a, and miR-200c were used to detect the presence and relative abundance of each RNA in the various cell types for subsequent experiments. 10 µl of the final 25 µl reaction volume was loaded and subjected to electrophoresis on a 15% Criterion TBE gel (Bio-Rad, Hercules, CA). The gel was stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA). Lanes 1, 8, and 15 contain the EZ Load 20 bp Molecular Ruler (Bio-Rad). Amplicons are expected to be 90 bp. NTC = no template control (water). The 5S rRNA is typically used as an endogenous small RNA control. The lower band in cells not expressing miR-133a is primer dimer).

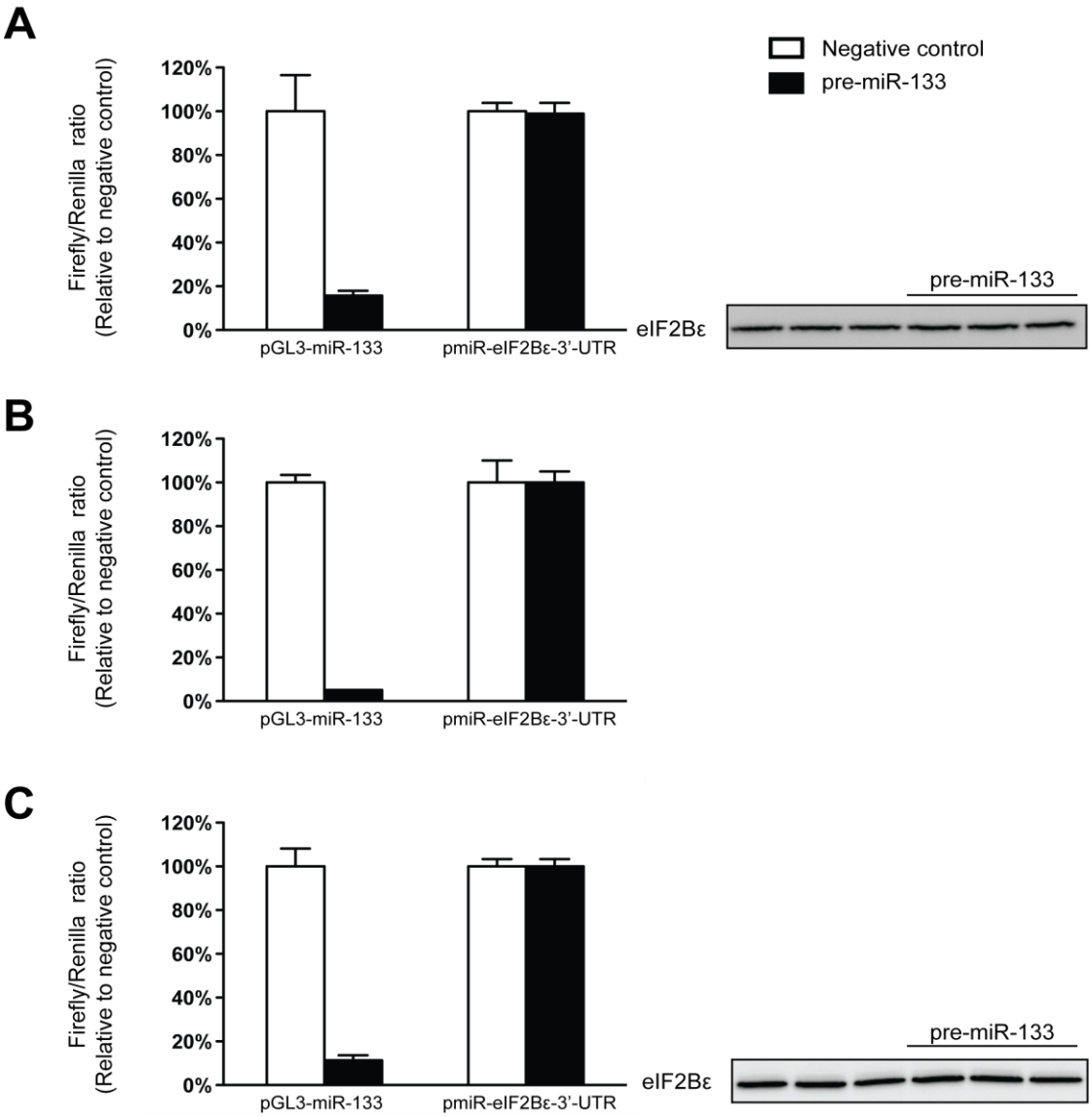

Figure 3. Functional assessment of eIF2Bε as a potential target for miR-133 mediated repression**.**

Firefly luciferase plasmids (pGL3-miR-133 sensor (3 μg) or pmiR-eIF2Bε-3'-UTR (0.1 μg)) were co-transfected with a control *renilla* luciferase plasmid (pRL-SV40; 0.1 μg) and either a negative control pre-miR (open bars) or pre-miR-133a (solid bars) at a final concentration of 100 nM in various cell lines. Dual luciferase reporter assays were performed on the lysates of the transfected cells 24 h post-transfection. Depicted in each panel are the ratio of firefly/renilla luciferase reporter luminescence and Western blots of the lysates for endogenous eIF2Bε expression in **(A)** HEK 293, **(B)** Rat2 fibroblasts, or **(C)** C2C12 myoblasts. While pre-miR-133 successfully repressed the pGL3-miR-133 sensor plasmid indicating successful transfection of the pre-miR-133 molecule, neither pmiR-eIF2Bε-3'-UTR reporter activity nor eIF2Bε protein abundance were affected by pre-miR-133. Luminescence results (presented as means ± SEM) represent three biological replicates and are expressed as a percent of the negative control pre-miR transfected cells for each firefly reporter. The Western blots were probed with anti-eIF2Bε antibody and represent the lysates of cells transfected with pGL3-miR-133 sensor and either the negative control pre-miR or pre-miR-133 (three biological replicates each). Western blots were not performed on Rat2 lysates. Similar results were obtained with the pGL3-eIF2Bε-3'-UTR reporter.

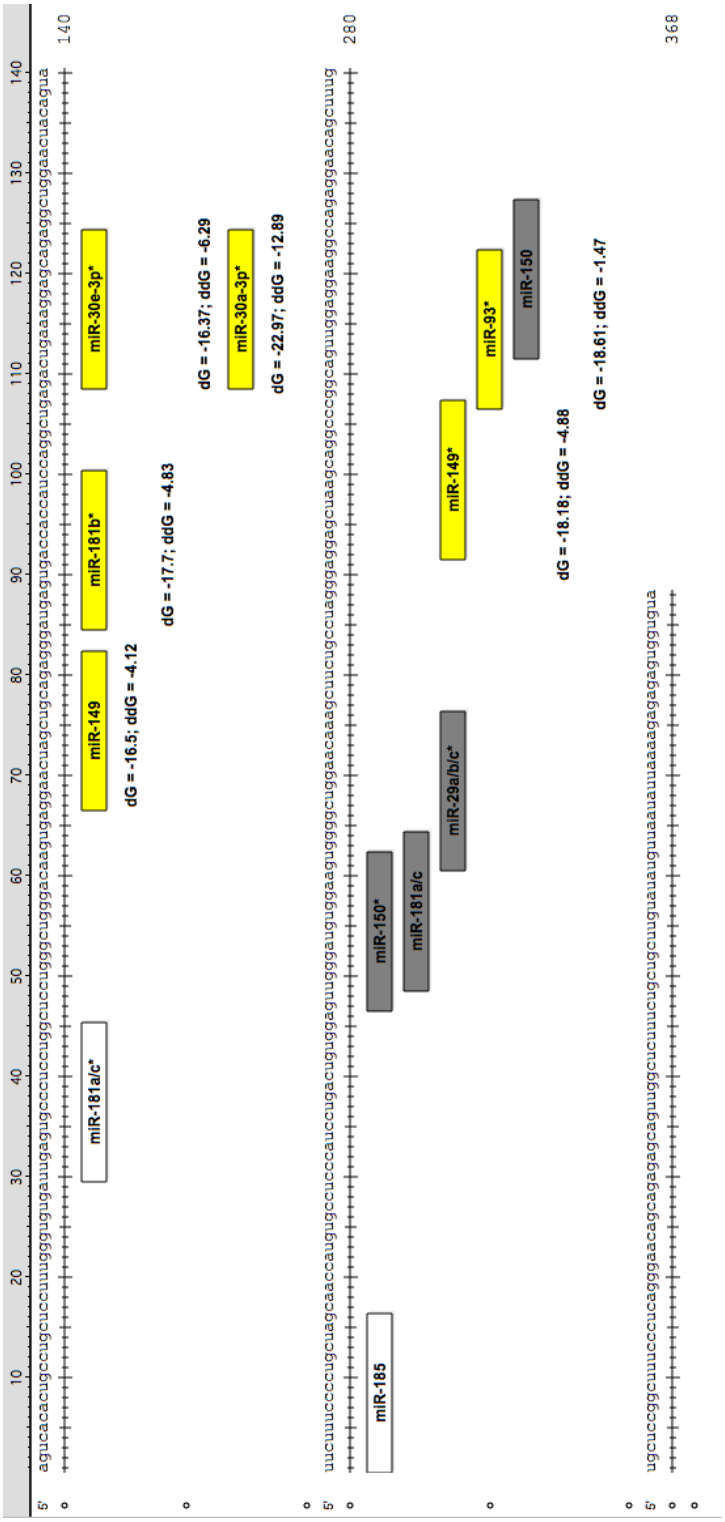

Figure 4. Putative miRNA target sites within the 3'-UTR of eIF2Bε as predicted by the PITA algorithm.

The results of the PITA target prediction algorithm (9) as described in the text are presented and indicate multiple potential target sites for miRNAs in the human eIF2Bε 3'-UTR (NM_003907). The 368 nt 3'-

UTR sequence is displayed as are the locations of selected miRNA recognition elements (yellow boxes) with the corresponding prediction scores beneath. The six sites listed were experimentally tested with the 3'-UTR luciferase reporters to determine whether they target eIF2Bε.

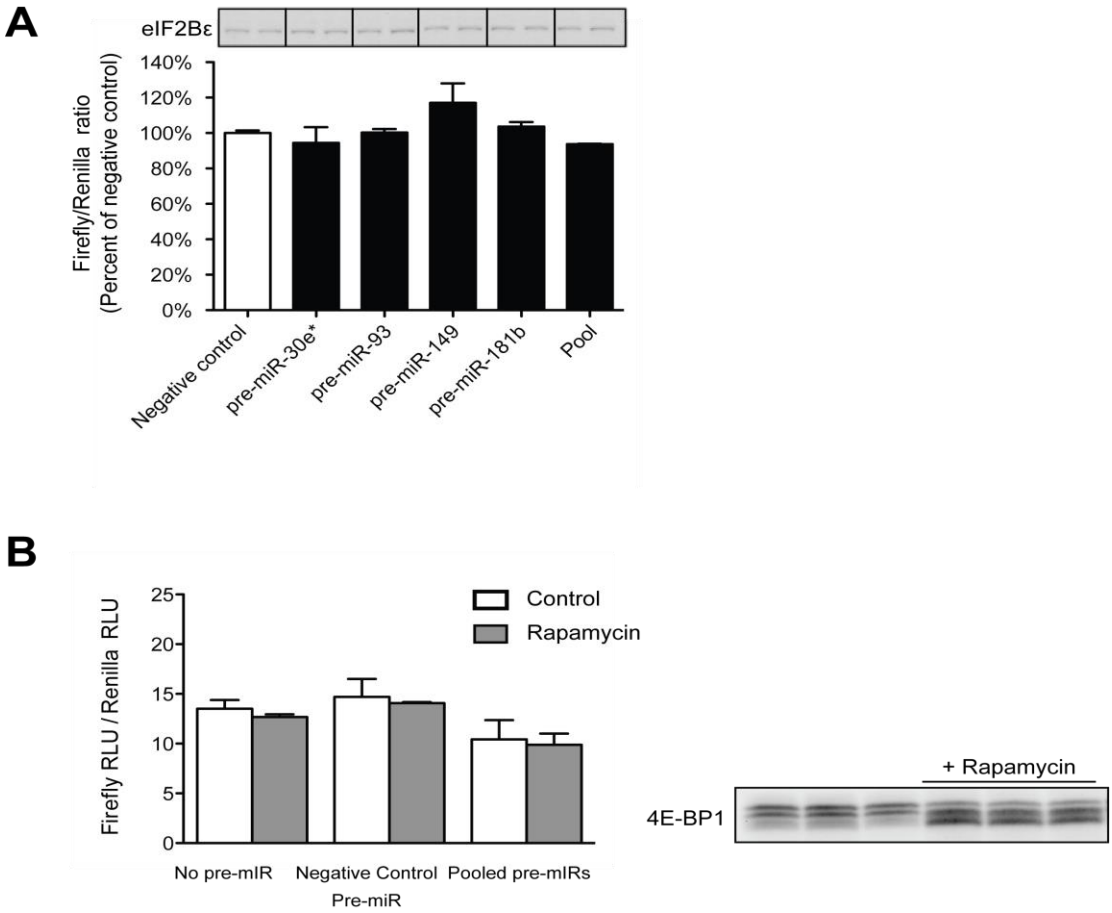

Figure 5. Functional assessment of PITA predicted miRNAs individually, pooled, and in the absence or presence of rapamycin on eIF2Bε-3'UTR reporter activity.

(A) HEK 293 cells were transfected with pmiR-eIF2Bε-3'-UTR (0.05 μg), a control *renilla* luciferase plasmid (pRL-SV40; 0.2 μg), and either a negative control pre-miR (open bars), 100 nM of the individual pre-miR indicated, or a pool of 25 nM each pre-miR-30e*, -93, -149, and -181b. Dual luciferase reporter assays were performed on the lysates of the transfected cells 24 h post-transfection. A Western blot for eIF2Bε is aligned above each bar depicting two of the three replicates. **(B)** HEK 293 cells were transfected with reporters only (i.e., no pre-miR), a negative control pre-miR, or a pool of five pre-miRs (pre-miR-30e*, -93, -133, -149, and -181b). Eighteen hours post-transfection, subsets of each of the transfected cells in growth medium were treated with rapamycin (100 nM) or an equivalent volume of ethanol vehicle as a control for 6 hours prior to harvest. Depicted in the graphs of both panels are the ratio of firefly/renilla luciferase reporter luminescence (presented as mean ± SEM of three biological replicates). The Western blot in panel B (15% Criterion gel) was probed with anti-4E-BP1 antibody and represents the lysates of the transfected cells in the absence or presence of rapamycin (three biological replicates each) in order to verify the effect of rapamycin on the inhibition of mTORC1. No significant differences existed among any of the treatment groups by one-way ANOVA. Similar results were obtained with the pGL3-eIF2Bε-3'-UTR reporter.

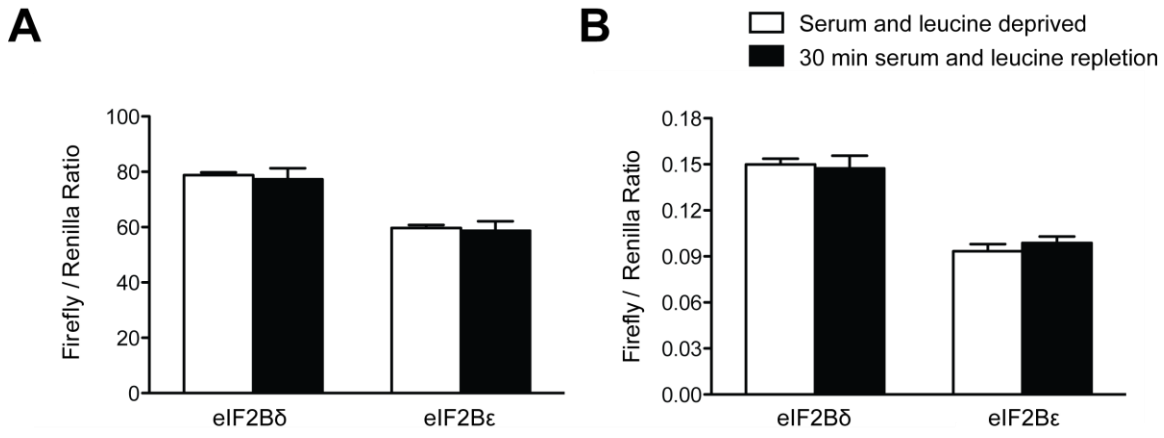

Figure 6. Luciferase activity of eIF2Bδ and ε 3'-UTR reporters is unaltered by acute serum and leucine deprivation or replenishment.

(A) HEK 293 cells and **(B)** Rat2 fibroblasts were transfected with pRL-SV40 (a renilla luciferase control plasmid) and either pmiR-eIF2Bδ-3'-UTR or pmiR-eIF2Bε-3'-UTR and incubated in growth medium. HEK 293 cells received 1.0 μg pRL-SV40 and 4.0 μg pmiR-reporter plasmid. Rat2 fibroblasts received 2.5 μg each pRL-SV40 and pmiR-reporter plasmid. The day following transfections, all cells were washed once in DMEM lacking serum and leucine then incubated in the medium lacking serum and leucine for 2 hours. The 2 h serum and leucine deprivation protocol has been established by the laboratory to repress mTORC1 signaling in both cell types. Following 2 hours of serum and leucine deprivation, subsets of cells were either maintained in the serum and leucine-free DMEM for 30 additional minutes (deprived) or the medium was changed to replete growth medium (i.e., DMEM containing leucine and 10%FBS) for the final 30 minutes prior to harvest to rapidly activate the mTORC1 pathway. Dual luciferase reporter assays were performed on the lysates and the ratio of firefly/renilla luminescence is reported. Data represent means ± SEM of three biological replicates.

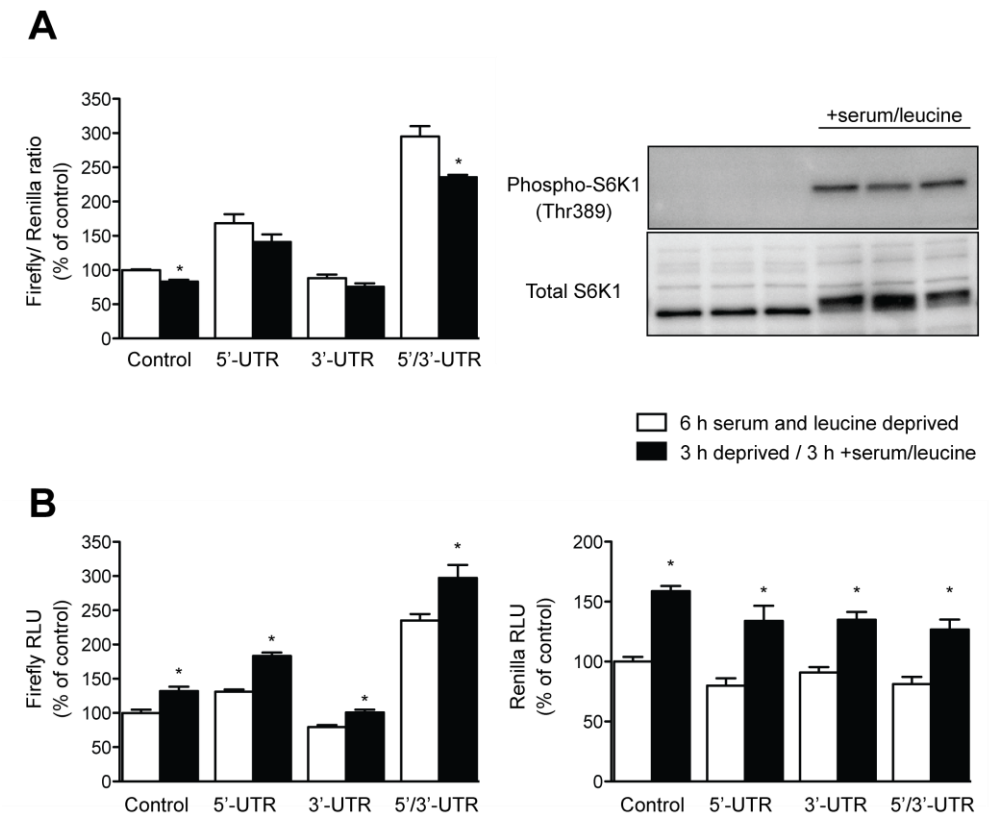

Figure 7. The untranslated regions of human eIF2Bε do not confer mRNA specific translational regulation in response to changes in mTORC1 signaling.

C2C12 myoblasts were transfected with pRL-SV40 (0.1 μg; a renilla luciferase control plasmid) and a pGL3 firefly luciferase reporter (3.0 μg) as indicated in the figure. The experimental firefly luciferase reporters consisted of pGL3 plasmids containing the 5'-, the 3'-, or both the 5'- and 3'-UTR (5'/3'-UTR) of human eIF2Bε. The pGL3 vector served as a control reporter. Transfected cells were incubated in growth medium for 18 h at which point all cells were washed once in DMEM lacking serum and leucine then incubated in medium lacking serum and leucine for 3 hours. Following the 3 hour serum and leucine deprivation, subsets of cells were either maintained in the serum and leucine-free DMEM for an additional 3 h (deprived) or the medium was changed to replete growth medium (i.e., DMEM containing leucine and 10%FBS) for the final 3 h prior to harvest. Dual luciferase reporter assays were performed on the lysates. **(A)** The ratio of firefly/renilla luminescence is presented along with representative Western blots using phospho-specific (Thr389) or total S6K1 antibodies from a 10% Criterion gel to demonstrate the effectiveness of repression and activation of mTORC1 in the deprived and replenished (+serum/leucine) conditions, respectively. **(B)** The experimental firefly (left panel) and control renilla reporter activities in each condition are presented. Note that the firefly/renilla ratios are significantly lower following replenishment for both the pGL3 (Control) plasmid and the 5'/3'-UTR reporter. The general trend indicated that replenishment enhanced translation of both the experimental pGL3 reporters as well as the pRL-SV40 renilla control plasmid. The luciferase activity from the pRL-SV40 was increased to a greater extent than the firefly, leading to a decrease in firefly/renilla ratio. Data represent means ± SEM of two independent experiments each with three biological replicates and are expressed as a ratio of the control (pGL3) plasmid in the 6 h deprivation condition. * $P < 0.05$ for deprivation vs. replenishment condition by unpaired t-test for the respective reporter. Similar results were obtained with Rat2 fibroblasts.

In April, 2011, we received additional funds to extend the project to include an examination of mechanisms involved in a phenomenon that is referred to as anabolic resistance. The Specific Aims of these additional studies were:

1) Establish optimal time point at which anabolic resistance manifests in an experimental model of inactivity-induced wasting of skeletal muscle.
2 Define the composition of the meal that best illustrates anabolic resistance.
3) Optimize conditions for ectopic expression of DNA plasmid constructs in skeletal muscle in vivo.

The rationale for these additional studies was as follows: 1) The loss of muscle mass observed with limb immobilization in humans is primarily attributed to insensitivity of nutrient-induced stimulation of protein synthesis (a phenomenon referred to as anabolic resistance); 2) Mitogen- and nutrient-induced stimulation of muscle protein synthesis is modulated in part through the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway; and 3) Signaling through mTORC1 regulates cap-dependent mRNA translation in part through initiation factor (eIF) 4E binding protein 1 (4E-BP1). We, therefore, hypothesized that nutrient-induced stimulation of protein synthesis is attenuated in skeletal muscle of an immobilized hindlimb due to repression of mTORC1 signaling. The results obtained for these additional studies were published in a paper entitled, "The mTORC1 signaling repressors REDD1/2 are rapidly induced and activation of p70S6K1 by leucine is defective in skeletal muscle of an immobilized hindlimb" (*Am J Physiol Endocrinol Metab* November 27, 2012; doi:10.1152/ajpendo.00409.2012) Abstract: Limb immobilization, limb suspension, and bed rest cause substantial loss of skeletal muscle mass, a phenomenon termed disuse atrophy. In order to acquire new knowledge that will assist in the development of therapeutic strategies for minimizing disuse atrophy, the present study was undertaken with the aim of identifying molecular mechanisms that mediate control of protein synthesis and mTORC1 signaling. Male Sprague-Dawley rats were subjected to unilateral hindlimb immobilization for 1, 2, 3 or 7 days or served as non-immobilized controls. Following an overnight fast, rats received either saline or L-leucine by oral gavage as a nutrient stimulus. Hindlimb skeletal muscles were extracted 30 min post-gavage, and analyzed for the rate of protein synthesis, mRNA expression, phosphorylation state of key proteins in the mTORC1 signaling pathway, and mTORC1 signaling repressors. In the basal state, mTORC1 signaling and protein synthesis were repressed within 24h in the soleus of an immobilized compared to a non-immobilized hindlimb. These responses were accompanied by a concomitant induction in expression of the mTORC1 repressors REDD1/2. The nutrient stimulus produced an elevation of similar magnitude in mTORC1 signaling in both the immobilized and non-immobilized muscle. In contrast, phosphorylation of p70S6K1 on Thr229 and Thr389 in response to the nutrient stimulus was severely blunted. Phosphorylation of Thr229 and Thr389 in response to the nutrient stimulus was severely blunted. Phosphorylation of Thr229 by PDK1 is a prerequisite for phosphorylation of Thr389 by mTORC1, suggesting that signaling through PDK1 is impaired in response to immobilization. In conclusion the results show an immobilization-induced attenuation of mTORC1 signaling mediated by induction of REDD1/2 and defective p70S6K1 phosphorylation.

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32. Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 2008;455(7216):1124-8.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White

_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X_ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication, listed in the table, in a PDF version 5.0.5 format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Ectopic expression of eIF2Bε in rat skeletal muscle rescues the sepsis-induced reduction in guanine nucleotide exchange activity and protein synthesis	Alexander P. Tuckow, Thomas C. Vary, Scot R. Kimball, and Leonard S. Jefferson	American Journal of Physiology - Endocrinology and Metabolism	March 2010	☐Submitted ☐Accepted ✓Published
2. Simvastatin represses protein synthesis in the muscle- derived C2C12 cell line with a concomitant reduction in eukaryotic initiation factor 2B expression	Alexander P. Tuckow, Sarah J. Jefferson, Scot R. Kimball and Leonard S. Jefferson	American Journal of Physiology - Endocrinology and Metabolism	June 2010	☐Submitted ☐Accepted ✓Published
3. The mTORC1 signaling repressors REDD1/2 are rapidly induced and activation of p70S6K1 by leucine is defective in skeletal muscle of an immobilized rat hindlimb	Andrew R. Kelleher, Scot R. Kimball, Michael D. Dennis, Ruud J. Schilder, and Leonard S. Jefferson	American Journal of Physiology - Endocrinology and Metabolism	November 2012	☐Submitted ✓Accepted ☐Published
4. Identification of ubiquity-modified lysine residues and novel phosphorylation sites on eukaryotic initiation factor 2B epsilon.	Alexander P. Tuckow, Abid A. Kazi, Scot R. Kimball, and Leonard S. Jefferson	Biochimica et Biophysica Acta	December 2012	☐Submitted ✓Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X_____ No_____

If yes, please describe your plans:

Elucidation of mechanisms involved in eIF2Bε degradation should result in a number of published articles.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?

Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

   If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

   If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Leonard S. (Jim) Jefferson	Evan Pugh Professor and Chair
eRA COMMONS USER NAME (credential, e.g., agency login) JJEFFERSON	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Eastern Kentucky University, Richmond, KY	B.S.	05/61	Chemistry/Biology
Harvard University, Cambridge, MA	---	06-08/62	Special Studies
Vanderbilt University, Nashville, TN	Ph.D.	05/66	Physiology/Biochemistry
Cambridge University, Cambridge, England	Postdoc	02/66-06/67	Biochemistry

A. Personal Statement

As Principal Investigator of the research project proposed in this competing renewal application, I will continue to provide leadership, vision, and direction to the work of a laboratory group that over a number of years has contributed many important advances to our understanding of the regulation of protein synthesis in skeletal muscle. In collaboration with my long-standing colleague and co-investigator, Dr. Scot R. Kimball, I have led a research effort that has revealed the importance of the initiation of mRNA translation and its regulatory signaling pathways in the acute response of skeletal muscle protein synthesis to hormones, nutrients, and exercise. Working together, Dr. Kimball and I purified, characterized, and cloned a number of mammalian initiation factors and subsequently applied the knowledge gained from those efforts to develop functional assays for the various factors. After establishing the functional assays, our efforts were directed toward delineation of the signaling pathways that control the initiation factors and this in turn led to the recognition of the central role played by the mammalian target of rapamycin complex 1 (mTORC1) in the regulation of mRNA translation and protein synthesis in skeletal muscle. Collectively, these efforts have produced a number of novel findings that have moved the field forward. They have also enabled Dr. Kimball and me to gain a wealth of experience that prepares us well to lead the proposed project.

B. Positions and Honors

Employment: Department of Physiology, College of Medicine, The Pennsylvania State University: Instructor, 1967-68; Assistant Professor, 1968-72; Associate Professor, 1972-75; Professor, 1975-87; Department of Cellular and Molecular Physiology, Professor and Chairman, 1988-present; Senior Associate Dean for Research and Graduate Studies, 1990-2001; Evan Pugh Professor, 1996-present; Executive Director Henry Hood Research Program, 1997-2000; Interim Vice Dean for Research, 2001.

Other Work Experience: Diabetes Research and Training Center, Vanderbilt University School of Medicine, Visiting Professor of Physiology, 1977.

Honors and Awards: Elliot P. Joslin Award, American Diabetes Association, 1966; Distinguished Alumni Award, Eastern Kentucky University, 1974; Established Investigator Award, American Diabetes Association, 1976-81; Lilly Award, American Diabetes Association, 1979; Outstanding Alumnus Award, Eastern Kentucky University, 1986; MERIT Award, NIH, 1986-94; David Rumbough Scientific Award, Juvenile Diabetes Foundation International, 1990; Evan Pugh Professor of Physiology, 1996-present; Solomon A. Berson Distinguished

Lectureship, American Physiological Society, 1999; Keynote Lecture, 11th International Conference on the Biochemistry of Exercise, 2000; Circle of Distinguished Alumni, Vanderbilt University, Department of Molecular Physiology and Biophysics, 2002.

C. Peer-reviewed Publications or Manuscripts (from a total of 295)

Most relevant to the current application

1. Kubica, N., Bolster, D. R., Farrell, P. A., Kimball, S. R., and Jefferson, L. S. Resistance exercise increases muscle protein synthesis and translation of eukaryotic initiation factor 2Bε mRNA in a mammalian target of rapamycin-dependent manner. J. Biol. Chem. 280:7570-7580, 2005.
2. Krawiec, B. J., Frost, R. A., Vary, T. C., Jefferson, L. S., and Lang, C. H. Hindlimb casting decreases muscle mass in part by proteasome-dependent proteolysis but independent of protein synthesis. Am. J. Physiol. 289: E969-E980, 2005.
3. Kubica, N., Crispino, J. L., Gallagher, J. W., Kimball, S. R., and Jefferson, L. S. Activation of the mammalian target of rapamycin complex 1 is both necessary and sufficient to stimulate eukaryotic initiation factor 2Bε mRNA translation and protein synthesis. Int. J. Biochem. Cell Biol. 40(11): 2522-2533, 2008. (PMC2601645)
4. McGhee, N. K., Jefferson, L. S., and Kimball, S. R. Elevated corticosterone associated with food deprivation upregulates expression in rat skeletal muscle of the mTORC1 repressor, REDD1. J. Nutr. 139(5): 828-834, 2009. (PMC2714387)
5. Tuckow, A.P., Vary, T.C., Kimball, S.R., and Jefferson, L.S. Ectopic expression of eIF2Bε in rat skeletal muscle rescues the sepsis-induced reduction in guanine nucleotide exchange activity and protein synthesis. Am. J. Physiol. (*Endocrinol. Metab.*), 299:E241-E248, 2010. (PMC2928514).
6. Schilder, R. J., Kimball, S. R., Marden, J. H., and Jefferson, L. S. Body weight-dependent troponin T alternative splicing is evolutionarily conserved from insects to mammals and partially impaired in skeletal muscle of obese rats. J. Exptl. Biol. 214: 1523-1532, 2011. (PMC3076076).
7. Schilder, R., Kimball, S. R., and Jefferson, L. S. Cell-autonomous regulation of fast troponin T pre-mRNA alternative splicing in response to mechanical stretch. Am. J. Physiol. *(Cell Physiol.)*, 303:C298-307, 2012. (PMC3423020).
8. Kelleher, A.R., Kimball, S.R., Dennis, M.D., Schilder, R., and Jefferson, L.S.. The mTORC1 signaling repressors REDD1/2 are rapidly induced and activation of p70S6K1 by leucine is defective in skeletal muscle of an immobilized rat hindlimb Am. J. Physiol., In Press, 2012.

Additional recent publications of importance to the field

1. Shah, O. J., Iniguez-Lluhi, J. A., Romanelli, A., Kimball, S. R., and Jefferson, L. S. The activated glucocorticoid receptor modulates presumptive autoregulation of ribosomal protein S6 protein kinase, p70 S6K. J. Biol. Chem. 277: 2525-2533, 2002.
2. Bolster, D. R., Crozier, S. J., Kimball, S. R., and Jefferson, L. S. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. J. Biol. Chem. 277: 23977-23980, 2002.
3. Bolster, D. R., Kubica, N., Crozier, S. J., Williamson, D. L., Farrell, P. A., Kimball, S. R., and Jefferson, L. S. Immediate response of mammalian target of rapamycin (mTOR)-mediated signaling following acute resistance exercise in rat skeletal muscle. J. Physiol. 553: 213-220, 2003. (PMC2343483).
4. Kimball, S. R., Do, A. N. D., Kutzler, L., Cavener, D. R., and Jefferson, L. S. Rapid turnover of the mTOR complex 1 (mTORC1) repressor REDD1 and activation of mTORC1 signaling following inhibition of protein synthesis. J. Biol. Chem. 283(6): 3465-3475, 2008. (PMC2654224)

5. Winter, J. Fox, T., Kester, M., Jefferson, L.S., and Kimball, S.R. Phosphatidic acid mediates activation of mTORC1 through the ERK signaling pathway. Am. J. Physiol., 299: C335-C344, 2010. (PMC2928642).
6. Tuckow, A. P., Jefferson, S. J., Kimball, S. R., and Jefferson, L. S. Simvastatin represses protein synthesis in the muscle-derived C2C12 cell line with a concomitant reduction in eukaryotic initiation factor 2B (eIF2B) expression. Am. J. Physiol. *(Endo. Metab.)* 300: E564-E570, 2011. (PMC3064004).
7. Winter, J. N., Jefferson, L. S., and Kimball, S. R. The ERK and Akt signaling pathways function through parallel mechanisms to promote mTORC1 signaling. Am. J. Physiol. 300:C1172-80, 2011. (PMC3093949).
8. Dennis, M.D., Jefferson, L.S., and Kimball, S.R. Role of p70S6K1-mediated phosphorylation of eIF4B and PDCD4 in the regulation of protein synthesis. J. Biol. Chem., Accepted, 2012.