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.

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

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

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

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

9. **Grant SAP Number:** 4100047645

10. **Project Number and Title of Research Project:** 7 - Regulation of Nutrient Sensing and Muscle Wasting by Alcohol

11. **Start and End Date of Research Project:** 1/1/2009-6/30/2010

12. **Name of Principal Investigator for the Research Project:** Charles H. Lang, PhD

13. **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:

$ 45,906

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
Kazi	PhD Graduate Student	100	$43,589

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
Lang	PI, Distinguished Professor	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
None		

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:

Grant from the NIH – AAA11290 - $327,260 (total direct costs)

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_____ No___X_____

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:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

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:

This grant supported a graduate student to conduct studies that strengthened the resubmission of a competing renewal application to NIH. In 2011 we were notified that our submitted R01 application received a percentile ranking of 1% and was indeed funded. Additionally, this grant was converted from an R01 application to an R37 MERIT Award as of March 2012.

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

The PRAS40 work in general will not continue because the results of research indicate that this protein does not alter muscle protein synthesis in the anticipated manner and therefore is unlikely to be a potential therapeutic target to combat muscle wasting. However, the methods learned and developed during this funding cycle will be used in the future to address the role of other mTOR complex 1 proteins (e.g., DEPTOR) in regulating muscle protein synthesis.

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			1	
Female				
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other			1	
Unknown				
Total			**1**	

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 grant provided financial support for my PhD graduate student for a period of 1 year. Since the NIH decreased the operating budget for my grant, I would have had to divert funds from research supplies to pay for the student. If this was necessary, it would have substantially slowed the research progress on my NIH grant. Hence, the financial support provided has permitted me to remain competitive to continue my funding from the NIH.

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.

Project Goals, Objects and Specific Aims

The long-term goal of our project was to determine the mechanism by which alcohol impairs cap-dependent translation and protein synthesis in skeletal muscle under basal conditions and in response to the anabolic agent leucine. The specific aims to address this long-term goal are indicated below as sequential experiments. The goal of this entire grant will be achieved by three specific experiments. Experiment 1 will determine the role of altered 4E-BP1 and S6K1 as well as other mTOR-interacting proteins with the scaffold protein raptor as a mechanism for the alcohol-induced decrease in mTOR activity in muscle. The endogenous prevailing level of the mTOR·raptor complex will be isolated from whole muscle or cultured myocytes after alcohol treatment ± leucine by immunoprecipitation of raptor, and then the IP blotted for mTOR, raptor, PRAS40, GβL, Deptor and 4E-BP1. The amount of total and phosphorylated 4E-BP1 bound to raptor will be assessed by Western blot analysis. mTOR kinase activity will be determined and correlated with in vivo-determined rates of protein synthesis in the same muscle. The following endpoints will be determined: a) protein synthesis, b) mRNA translation rate, c) mTOR kinase activity, d) phosphorylation of 4E-BP1, S6K1 and mTOR, and e) protein-protein interactions related to TORC1 formation. Experiment 2 will elucidate the mechanism by which alcohol impairs binding activity of the eIF3 scaffolding complex. Muscle from each treatment group will be homogenized and eIF3 immunoprecipitated. Western blotting will be performed on the IP for total and phosphorylated mTOR, S6K1, eIF4B and eIF4G. We will also examine the temporal correlation between mTOR and S6K1 activation and the binding or dissociation of preinitiation complex components with eIF3 and the 5' cap following stimulation with leucine. In Experiment 3 we will determine whether an alcohol-induced alteration in the binding of PDCD4 to eIF4A impairs eIF4A helicase activity and thereby inhibits translation in skeletal muscle. Hence, PDCD4 will be immunoprecipitated and blotted for PDCD4, eIF4A, and eIF4G. Furthermore, PDCD4 is also a phosphoprotein and a downstream substrate of S6K1. Activation of the mTOR pathway enhances S6K1 kinase activity leading to phosphorylation of PDCD4. As a consequence of this phosphorylation event, PDCD4 is rapidly degraded by the E3 ubiquitin (Ub) ligase complex $SCF^{\beta TRCP}$ (SKP1-CUL1-F-box) which tags its substrates with Ub molecules for proteasome degradation. Finally, using immunoprecipitated PDCD4, we will also Western blot for $SCF^{\beta TRCP}$ which is the endogenous E3 ligase for PDCD4. Collectively, these studies will help provide information related to the molecular mechanism by which alcohol impairs muscle protein synthesis.

Summary of Progress

Materials and Methods

Cell culture. C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's minimum essential medium (DMEM; Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) (all from Mediatech, Herndon, VA) under 5% CO_2 at 37°C. Myoblasts were subcultured and when 100% confluent, the cells were switched to differentiation medium (DM) consisting of DMEM with the above antibiotics-antimycotics and 2% horse serum (Hyclone, Logan, UT) to promote myoblast fusion and differentiation to myotubes. Cells were differentiated for 6 days before experimental manipulation. Myotubes were provided with fresh differentiation medium on day 6 and experiments were performed on day seven. To simulate basal mTOR activity, experiments measuring protein synthesis and the phosphorylation of mTOR substrates were

performed with serum-free DMEM without antibiotics-antimycotics. 5-Aminoimidazol-4-carboximide ribonucleoside (AICAR; Toronto Research Chemicals, Ontario, Canada) when present was added at a final concentration of 2 mM for 8 h. Insulin-like growth factor (IGF) -I when present was used at final concentration of 100 ng/ml for the last 20 min of the experiment. These doses maximally suppress and activate protein synthesis in C2C12 cells, respectivel**y.**

shRNA interference. The lentiviral plasmid pLKO.1-mPRAS40 used was that described by Vander Haar et al and targeted the mouse sequence 5'-GAG CCC ACT GAA ACA GAG ACA-3'; the scramble shRNA was used as a negative control as previously reported with hairpin sequence: CCT AAG GTT AAG TCG CCC TCG CTC TAG CGA GGG CGA CTT AAC CTT AGG; (Addgene, Cambridge, MA). The plasmids were transformed in DH5α cells and isolated. The actual DNA sequence was confirmed at the Pennsylvania State University College of Medicine DNA sequence core facility. Packaging plasmids psPAX2 and envelope protein plasmid pMD2.G were obtained from Torono Lab (Addgene). HEK293FT cells (Invitrogen) were grown in DMEM media; 80-85% confluent plates were rinsed once with Opti-MEM media (Invitrogen) and then incubated with Opti-MEM media for 4 h before transfections. psPAX2 and pMD2.G along with either scramble or pLKO.1-mPRAS40 were added after mixing with Lipofectamine 2000 as per manufacturer's instructions (Invitrogen). Opti-MEM media was changed after overnight incubation with DMEM containing 10% FBS without antibiotics to allow cells to take up the plasmids and recover. Culture media was collected at 36 h and 72 h post-transfection for viral particles. Viral particles present in the supernatant were harvested after a 15 min spin at 1500 x g to remove cellular debris. The supernatant was further filtered using a 0.45 µm syringe filter. Supernatant containing virus was either stored at -80 ºC for long-term storage or at 4 ºC for immediate use. C2C12 cells at 60% confluence were infected twice overnight with 3 ml of viral supernatant containing 8 µg/ml polybrene in serum free - antibiotic free DMEM. Fresh DMEM media containing 10% FBS, antibiotics, and 2 µg/ml puromycin (Sigma, St. Louis, MO) was added the next day. Cells that survived under puromycin selection were either harvested (as stable cells) and stored or used immediately.

^{35}S-methionine labeling. C2C12 myocytes were grown in six-well plates and treated as above. Protein synthesis was measured on Day 3 in myoblasts following seeding or on day 7 following addition of DM in myotubes. For metabolic labeling 10 µCi of radiolabeled ^{35}S-methionine (MP Biologicals, PA) was added to each well of a 6 well plate and radiolabel incorporation into trichloroacetic acid (TCA) precepitable proteins were measured via liquid scintillation as previously described**.**

Multiprobe template production for RNase protection assay. Primer selection for mouse genes of interest was determined using GeneFisher software. The lengths of amplified regions were chosen to allow distinct resolution during electrophoretic separation. Primers were synthesized (IDT, Coralville, IA) with restriction sites for *Eco*RI or *Kpn*I at the 5' end and with three extra bases at the extreme 5' end as follows: for PRAS40- Forward (*5'-GCA GAA TTC* GCC CGA TCG TCA GAT GAG GAG A-3'), Reverse (5'-*CCT GGT ACC* TCA GCT TCT GGA AGT CGC TGG TA-3'); mTOR- Forward (5'-*GCA GAA TTC* GGC CAG TGG ACC AGT TGA GAC A-3'), Reverse (5'-*CCT GGT ACC* CAG CTC AGA CCA GCA GGA CAC A-3'); Raptor- Forward (5'-*GCA GAA TTC* CAT GCA TAG CTG TCG CCG ACA-3'), Reverse (5'-*CCT GGT ACC* ACA ATG AGC GAA CGG TGC GAA-3'); S6K1- Forward (5'-*GCA GAA TTC* GAC CAT GGG GGA GTT GGA CCA T-3'), Reverse (5'-*CCT GGT ACC* CTC CAG AAT GTT CCG CTC TGC TT-3'); 4E-BP1- Forward (5'-*GCA GAA TTC* CGG GGA CTA CAG CAC

CAC TC-3'), Reverse (5'-*CCT GGT ACC* GGG CAG TTG GCT CTG GTT GG-3'), and L32-Forward (5′-GCA GAA TTC CGG CCT CTG GTG AAG CCC AA-3′), Reverse (5′-GCAGGT ACC CCT TCT CCG CAC CCT GTT GTC A-3′). PCR was conducted with HotStarTaq DNA polymerase (Qiagen, Valencia, CA), and mouse total RNA was reverse transcribed with Superscript first-strand synthesis system for RT-PCR (Invitrogen). PCR products were phenol-chloroform extracted, ethanol precipitated, and sequentially digested with *Kpn*I and *Eco*RI (Promega, Madison, WI). Digested products were gel purified, reextracted, and cloned into *Kpn*I/*Eco*RI-digested pBluescript II SK+ (Stratagene, La Jolla, CA). Plasmid DNA was isolated with both QIAprep spin miniprep and plasmid maxi kits (Qiagen). Final constructs were linearized with *Eco*RI, gel purified, and quantitated spectrophotometrically. The template was prepared so that a 2 µl aliquot contained 10 ng each of PRAS40, S6K1, mTOR, 4E-BP1, and raptor, and 20 ng of L32.

RNA extraction and RNase protection assay. Total RNA was extracted from cells using Tri reagent (Molecular Research Center, Cincinnati, OH), exactly as previously described. Concentration, purity, and integrity of the isolated RNA were assessed using a UV/VIS spectrophotometer (Beckman, Fullerton, CA). mRNA expression was determined by RNase protection assay. A 2 µl aliquot of template was prepared with T7 polymerase with buffer (Fermentas, Hanover, MD), NTPs, and tRNA (Sigma-Aldrich), RNasin and DNase (Promega), and [^{32}P]-UTP (Amersham Biosciences, Piscataway, NJ). Unless otherwise noted, the entire RNase protection assay procedure, including labeling conditions, component concentrations, sample preparation, and gel electrophoresis, was as published . Gels were exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA), and data were visualized and analyzed by ImageQuant software (version 5.2; Molecular Dynamics). Signal densities within the linear range for mRNAs were normalized to densities for mouse ribosomal protein L32 mRNA.

Immunoblot analysis and immunoprecipitation. After treatment cells were rinsed 2x with cold DPBS and collected on ice in lysis buffer (20 mM HEPES [pH 7.4], 2 mM EGTA, 50 mM β-glycerophosphate, 0.3% CHAPS, 100 mM KCl, 2 mM EDTA, 50 mM NaF, 0.5 mM PMSF, 1 mM benzamidine, 1 mM sodium orthovanadate, and 2 µg/ml leupeptin). Lysates were sonicated for 10 min and then kept on a rocker for 30 min in the cold prior to being clarified (14000 x g for 10 min at 4°C). A portion of the resulting cell supernatant was used to determine protein concentration via a bicinchoninic acid assay (Pierce, Rockford, IL). Sample buffer (5x) was added and samples were loaded according to total protein content (20 µg) on polyacrylamide gels for separation by SDS-PAGE. Proteins were transferred to Polyvinylidene Fluoride (PVDF) membrane (Biotrace; PALL, Pensacola, FL), blocked in 5% nonfat dry milk, and incubated overnight at 4°C with phosphospecific and total antibodies for raptor (S792), mTOR (S2448), S6K1 (T389), PRAS40 (T246), 4E-BP1 (T37/46), and cleaved caspase-3, poly (ADP- ribose) polymerase (PARP), LC3B, beclin, Atg 7, and p53 (all from Cell Signaling Technology, Inc.; Boston, MA). Antibodies for total pRb, p21, p27, Cyclin-dependent kinase (cdk)2, cdk4, cdk6, MyoD and β-actin (Santa Cruz Biotechnology, CA) and for myosin heavy chain (MHC) (MF-20, Developmental Studies Hybridoma Bank, Iowa City, IA) were also used. Excess primary antibody was removed by washing in 1x TBS + 0.1% Tween 20, and membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Sigma) at room temperature for 1 h. Blots were rinsed with 1x TBS + 0.1% Tween 20 to remove excess secondary antibody and were treated with enhanced chemiluminescence (ECL plus) Western blotting reagents as per the manufacturer's (Amersham; Piscataway, NJ) instructions, and then developed using the Gnome (Bioscience, UK). Uncompressed tiff images were

analyzed using National Institutes of Health (NIH) ImageJ 1.6 software. After development, blots were stripped and incubated with antibodies for total proteins on blots which were probed earlier with phosphospecific antibodies. Antibodies against β-actin or β-tubulin (Santa Cruz Biotechnology) served as an additional control for equal protein loading of samples.

The PRAS40•raptor, PRAS40•eIF3f, and PRAS40•PRAS40 complexes were quantified as described. Briefly, to maintain potential protein-protein interactions, cells were homogenized in CHAPS buffer as described above. The homogenate was mixed on a platform rocker and clarified by centrifugation. An aliquot (normalized to equal total protein) of the resulting supernatant was combined with anti-PRAS40 antibody and immune complexes isolated with a goat anti-rabbit BioMag IgG (PerSeptive Diagnostics, Boston, MA) beads. The beads were collected, washed with CHAPS buffer, precipitated by centrifugation, and subjected to SDS-PAGE as described above. Blots were then probed with appropriate antibodies to study protein-protein interaction and developed with ECL and analyzed as described above.

Cell cycle. Myoblasts were transfected with either a scramble (Control) shRNA or a shRNA targeting PRAS40. Cells were seeded in 10 cm dishes and used at 60% confluence (~24 h post seeding). Cells were trypsinized, washed with DPBS and fixed in cold 70% ethanol overnight at -20°C. Cells were then stained with 100 µg/ml solution of propidium iodide buffer containing 0.1% Triton-X100 and 0.001% DNAse free RNAse at 37°C for 30 min immediately prior to FACS analysis. 10,000 cells per sample were counted and cell cycle phase was measured by propidium iodide staining intensity using a BD FACS-Calibur flow cytometer (Becton Dickinson, Bedford, MA) and ModFit software LT Version 3.2 (Verity Software,Topsham, ME).

Cell size and proliferation. To determine cell size, transfected myoblasts were seeded in 10 cm dishes and used at 60% confluence. For cell number, cells were seeded at similar densities and counted at different time points. Myoblasts were trypsinized and suspended in DMEM with 10% FBS. Cells were then diluted in Isoton II solution (Beckman Coulter, Fullerton, CA) and assayed using the Beckman Coulter Counter and particle size analyzer as per manufacturer's recommendation (Beckman Coulter). Cell size analysis was performed using the AccuComp® Z2 Coulter counter software (Beckman Coulter).

MTT assay. C2C12 myoblasts (~10,000/well) were grown in a 96-well plate for 24 h and then rinsed with PBS to remove the interfering phenol red from the DMEM media. This was followed by the addition of methylthiazoletetrazolium (MTT; 50 µg/100 µl) to cells in each well for 4 h at 37°C. MTT containing PBS was aspirated and 100 µl of DMSO was added to each well to dissolve the resulting formazan and absorbance at 570 nm was read in a plate reader Spectramax (Molecular Devices, Sunnyvale, CA).

DNA isolation and analysis. To analyze nucleosomal DNA fragmentation, 2.5×10^6 C2C12 cells were processed as described by Zhivotovsky *et al.* with minor modifications. 80% confluent cells were trypsinized and centrifuged for 5 min at 4000 rpm to removed media. The cell pellet was resuspended in total DNA extraction buffer (400 mM NaCl; 10 mM Tris-HCl, pH 7.5; 10 mM EDTA with 50 µg/ml RNAse A and 0.2% SDS) and incubated at 37ºC overnight. Proteinase K was added the following day to a final concentration of 50 µg/ml for 4 h at 37ºC. NaCl was then added to the DNA extraction mix to obtain a final concentration of 1.23 M and the extraction mix was left on ice overnight. The following day the mixture was centrifuged at 17,000 rpm for 1 h at 4ºC. The supernatant containing the low molecular weight DNA was removed and extracted 2x with phenol/chloroform followed by a final rinse in chloroform. The extract was

then subjected to ethanol precipitation in 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol at -20 ºC for 1 hour. The samples were centrifuged at 4000 rpm to pellet the DNA and the pellet was washed with 70% ethanol and air dried. The pellet was resuspended in Tris-EDTA buffer (pH 8.0) and the amount of DNA measured spectrophotometrically. Positive control apoptotic DNA from U937 cells was from Roche DNA-ladder kit (Roche Diagnostics Corporation, Indianapolis, IN). DNA (8 µg per sample/lane, and 3 or 5 µg/lane for positive control apoptotic DNA) was loaded onto a 1.5% agarose gel, subjected to electrophoresis (55 V for 2.5 h) in 1x Tris-Borate-EDTA buffer and scanned using the Typhoon fluorescent imager (Molecular Dynamics, NJ).

Cell differentiation. Approximately $0.5x10^6$ myoblasts transfected with scramble or shPRAS40 were seeded in 10 cm plates and photographed daily using a Nikon digital camera (Nikon Corp., Tokyo, Japan) mounted on a binocular microscope using 10x objective lens. Images were composed and edited in Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA). Background was reduced using contrast and brightness adjustments to enhance reprint, and all modifications were applied to the whole image. Similarly treated plates were collected at days 3, 5, 7, and 9 for Western blotting to measure MHC expression as a functional end-point to measure differentiation biochemically as myoblasts were allowed to fuse and form differentiated post-mitotic myotubes.

Cell apoptosis and autophagy controls. Same passage C2C12 myoblasts as PRAS40 KD and Scramble control cells were treated with 2 µM stourosporine in culture media for 4 hr to induced apoptosis and collected in the CHAPS media. To induce autophagy, C2C12 myoblasts were treated with Hanks Buffered Salt Solution (HBSS) for 6 hr and cells collected in CHAPS buffer.

Statistics. Results for individual cell experiments ($n \geq 6$) were replicated in at least three independent experiments and when applicable are presented as means ± SE calculated from the pooled data. Data were analyzed by unpaired Student's t-test in two-group comparisons or with ANOVA and Tukey's posttest in multigroup comparisons to determine treatment effect when ANOVA indicated a difference among the means. GraphPad Prism version 5.0 (GraphPad software, La Jolla, CA) was used for analysis. Differences between groups were considered significant at $P < 0.05$.

Results

Effect of PRAS40 knockdown in C2C12 myotubes. C2C12 stable cell lines deficient in PRAS40 or scramble controls were created using short hairpin RNA (shRNA). shRNA were retrovirally delivered to myoblasts and some of these myoblasts were allowed to differentiate and form myotubes following puromycin selection. shRNA directed towards PRAS40 in myotubes reduced PRAS40 protein levels by greater than 80%, compared to scramble control values. As anticipated PRAS40 knockdown also reduced the PRAS40 mRNA content by ~65% in infected myotubes. In contrast, PRAS40 knockdown did not alter the mRNA content for 4E-BP1, mTOR, S6K1 or raptor, proteins central to the functioning of the mTOR signaling pathway.

Knockdown of PRAS40 in differentiated myotubes did not alter global protein synthesis compared with scramble controls as measured by ^{35}S-methionine incorporation into protein. To determine whether the responsiveness of the PRAS40 knockdown cells to external stimuli was altered, cells were incubated with either an anabolic (IGF-I) or catabolic (AICAR) agent.

Addition of IGF-I to the myotubes increased protein synthesis, whereas, AICAR inhibited protein synthesis. Contrary to expectations, the magnitude of the changes produced by these agents in myotubes was the same in both control and PRAS40 knockdown cells. To confirm protein synthesis data, we performed Western blotting for mTOR and its substrates and binding partners. PRAS40 knockdown cells remained responsive to both types of stimuli and their response was similar and comparable to the scramble controls. For example, IGF-I increased phosphorylation of S6K1 (T389) and PRAS40 (T246), while AICAR increased raptor phosphorylation (S792).

PRAS40 knockdown decreases protein synthesis in C2C12 myoblasts. While the preceding data were obtained from post-mitotic differentiated myotubes (>95%), we also determined whether myoblasts would yield comparable results. In myoblasts, the knockdown of PRAS40 decreased global protein synthesis by ~25% under basal conditions. Despite the decrease in basal protein synthesis in the PRAS40 knockdown cells, the ability of these cells to respond positively or negatively to IGF-I or AICAR, respectively, was unaltered. Contrary to expectations, the decreased protein synthesis observed in PRAS40 knockdown cells under basal conditions was not associated with any difference in phosphorylation state of the mTOR substrates S6K1 and 4E-BP1, compared to the scramble control values or changes in protein-protein interaction of PRAS40-raptor-eIF3 between the two groups. In myoblasts, the ability of IGF-I to stimulate T389 phosphorylation of S6K1 and AICAR to increase S792 phosphorylation of raptor did not differ between scrambled and PRAS40 knockdown cells.

PRAS40 knockdown alters myoblast cell size and proliferation. Vander Haar et al reported that overexpression of wildtype PRAS40 decreased cell size in HEK 293 cells, whereas, knockdown of Lobe (a PRAS40 ortholog in Drosophila) increased cell size. Hence, we hypothesized that knocking down PRAS40 would also increase cell size in myocytes. PRAS40 knockdown increased the diameter (16.8 ± 0.1 μm) of low passage proliferating (~60% confluent) myoblasts compared to scramble control cells (14.0 ± 0.1 μm) as measured using either the Coulter counter particle size analyzer or FACS flow cytometry analysis (data not shown). Mean cell volume was also increased in PRAS40 knockdown cells. However, unexpectedly we found that PRAS40 knockdown cells grew slower compared to time-matched scramble controls, although both cell types were seeded at the same initial density. To exclude anchorage-dependence and altered capacity to attach, cells were seeded and counted 4-8 h after seeding to allow for attachment. An equal number of cells were harvested following trypsinization in both the control and PRAS40 knockdown cells, suggesting no significant difference in the ability of PRAS40 knockdown cells to attach to the culture plates (data not shown). To confirm that the proliferation rate of PRAS40 knockdown cells was slower, we used an independent colorimetric assay based on the conversion of the MTT tetrazolium salt to its formazan product. Consistent with the above presented data, the MTT assay revealed that PRAS40 knockdown cells had a 25% lower rate of proliferation.

PRAS40 and apoptosis. To determine whether increased apoptosis in PRAS40 knockdown cells was responsible for the slower proliferation rate, we isolated low molecular weight DNA and performed an apoptosis DNA laddering assay. There is no difference between the scramble control and the PRAS40 knockdown cells and that neither group of cells were undergoing active apoptosis within the detectable limits of the assay. These findings were confirmed by Western blotting for caspase-3/ PARP cleavage which failed to detect a significant difference between the groups. Myoblasts incubated with staurosporine were used as a positive control and demonstrated increased caspase-3 and PARP cleavage. Collectively, these data suggest that the

decreased protein synthesis and reduced proliferation in PRAS40 knockdown myoblasts cannot be attributed to increased apoptosis.

PRAS40 knockdown inhibits cell cycle progression. To determine the mechanism for the lower proliferation rate in PRAS40 knockdown cells, we stained myoblasts with propidium iodide to study cell cycle events. PRAS40 knockdown myoblasts had a greater proportion of cells in G1/G0 of the cell cycle and fewer cells in active S – phase, compared to control values.

Because PRAS40 knockdown cells were arrested in G1/G0 of the cell cycle we assessed whether proteins regulating cell cycle, especially the G1 – S transition, were concomitantly altered. There was a 25-30% reduction in S807/811 phosphorylation of Rb, consistent with reduced progression from G1 to S phase. In myoblasts with PRAS40 knockdown a 20-30% reduced expression of p21 was also detected in these cells. There was no difference in the other proteins analyzed which regulate cell cycle - p53, cdk 4/6, p27 and cyclin D1.

PRAS40 alters myogenesis. Our data demonstrate the presence of a concomitant delay in proliferation and altered cell cycle in PRAS40 knockdown myoblasts. Since mTOR also regulates autophagy which in turn plays an important role in cell differentiation, we determined the expression of proteins important in regulating autophagy. While there were no changes in the early markers for autophagy including, Atg 7 and Beclin 1, our data indicate that PRAS40KD decreases the ratio of LC3B-II/LC3B-I.

Next we determined whether such changes might be of physiological relevance to skeletal muscle development. In this regard, we seeded the same number of myoblasts and tracked their progression to form myotubes. We observed that control cells reached confluent status earlier than the PRAS40 knockdown and began fusion to form substantial number of myotubes by day 5, whereas PRAS40 knockdown cells only sparsely formed myotubes by day 5. These data suggest that myotube formation and myogenesis is delayed in PRAS40 knockdown cells.

To quantitate these findings, cell lysates were collected at various stages of development of myoblasts and myotubes to measure the expression of myosin heavy chain (MHC) – a protein expressed only in differentiated matured myotubes. While MHC expression was absent in myoblasts (day 3) and there was an initial delay in MHC expression in PRAS40 knockdown cells (days 5 and 7), by day 9 the expression of MHC in both scramble control and PRAS40 knockdown cells were comparable. There were no differences between scramble control and PRAS40KD in their expression of the muscle transcription factor MyoD.

Summary

PRAS40 is an mTOR binding protein which has complex effects on cell metabolism. Our study tests the hypothesis that PRAS40 knockdown (KD) in C2C12 myocytes will increase protein synthesis via up-regulation of mTOR-S6K1 pathway. PRAS40 KD was achieved using lentiviruses to deliver shRNA targeting PRAS40 or a scrambled control. C2C12 cells were used as either myoblasts or differentiated to myotubes. Knockdown reduced PRAS40 mRNA and protein content by greater than 80% of time-matched control values but did not alter the phosphorylation of mTOR substrates, 4E-BP1 or S6K1, in either myoblasts or myotubes. No change in protein synthesis in *myotubes* was detected as measured by the incorporation of ^{35}S-methionine. In contrast, protein synthesis was reduced 25% in *myoblasts*. PRAS40 KD in myoblasts also decreased proliferation rate with an increased percent of cells retained in G1 phase. PRAS40 KD myoblasts were larger in diameter and had a decreased rate of myotube

formation as assessed by myosin heavy chain content. Immunoblotting revealed a 25-30% decrease in total p21 and S807/811 phosphorylated Rb protein considered critical for G1 – S phase progression. Reduction in protein synthesis was not due to increased apoptosis as cleaved caspase-3 and DNA laddering did not differ between groups. In contrast, the protein content of LC3B-II was decreased by about 30% in the PRAS40 KD myoblasts, suggesting a decreased rate of autophagy. Our results suggest that a reduction in PRAS40 specifically impairs myoblast protein synthesis, cell cycle, proliferation, and differentiation to myotubes

The funded research accomplished the goal of better defining the role of PRAS40 in alcohol-induced myopathy. The work contributed to our overall accomplishment of our long-term goals and specific aims by better elucidating the potential role of PRAS40.

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. PRAS40 regulates protein synthesis and cell cycle in C2C12 myoblasts	Abid Kazi and Charles H. Lang	Molecular Medicine *Mol Med* 16:359-371, 2010	May 2010	☐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:

We are currently completing the experiments necessary for the submission of a peer-reviewed paper and the initial submission of this paper is anticipated in the next several weeks.

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. – see next page.*

BIOGRAPHICAL SKETCH

NAME **Lang, Charles H., PhD**	POSITION TITLE **Distinguished Professor and Vice-Chairman**
eRA COMMONS USER NAME (credential, e.g., agency login) CHARLESLANG	

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
Westminister College, New Wilmington, PA	BS	06/1976	Biology
Hahnemann Medical College, Philadelphia, PA	MS	05/1979	Physiology/Metabolism
Hahnemann Medical College, Philadelphia, PA	PhD	05/1981	Physiology/Biochemistry
LSU Medical Center, New Orleans, LA	Post-doc	1981-84	Sepsis/metabolism

A. Personal Statement

This proposal seeks to identify the molecular mechanisms underlying alcoholic myopathy. The PI has over 30 years of experience and leadership in metabolism, cell signaling, and alcohol research. The research in my laboratory has focused on the mechanisms for alcohol-induced changes in protein metabolism in skeletal and cardiac muscle, and the role of cytokines in the regulation of the IGF system in regulating alcoholic myopathy. My laboratory has made several seminal contributions to the understanding of the regulation of protein metabolism in alcoholic myopathy, as well as the role of skeletal muscle as an important component of the innate immune system. I have worked extensively in recent years on translational control by growth factors and nutrients during various catabolic states, including alcohol, sepsis, thermal injury, disuse atrophy, and HIV infection. The physical resources of my laboratory, the department, and the College of Medicine are outstanding. The overall goal of this R01 application is to investigate the mechanisms responsible for alcohol-induced leucine resistance in skeletal muscle. The foundation for our proposed research has been laid during the past 15 years of continuous NIH function for this grant, for which I have served as the PI. In addition, I am currently the PI on an NIH grant related to muscle catabolism during sepsis which is in its 25th year of continuous funding and the Director of a T32 Postdoctoral training grant for surgical residents on organ injury which is in its 10th year. I have been a permanent member of 2 NIH study sections: Surgery, Anesthesiology and Trauma (current) and Alcohol and Toxicology IV. Therefore, I have the requisite experience to design, conduct and analyze the cutting-edge research proposed in the current application, as well as the necessary administrative (budget, staffing, etc) experience. I have published more than 300 peer-reviewed articles in my career, 45 papers since 2006, and > 20 papers have been published in the immediate past funding cycle in support of our alcohol-related research efforts. Therefore, I have also demonstrated the ability to prioritize and bring multiple projects to fruition. These publications attest to my ability to use various in vivo models (rats, transgenic mice) and sophisticated in vitro approaches, where appropriate, to address our specific aims. As seen in my representative publications, I have a long-standing research collaboration with Dr. Hong-Brown, and I have successfully collaborated with numerous researchers both inside and outside our department and College. Dr. Hong-Brown has been a co-investigator on this grant for almost 10 years. As the PI, I will continue to oversee the direction of the grant, and will work with Dr. Hong-Brown in the design, analysis, and interpretation of the proposed experiments. Collectively, our research team has a sustained track record of successful, productive and novel NIH-funded research of high clinical relevance,

and the PI is well positioned have access to several unique reagents to elucidate the mechanisms underlying alcoholic muscle myopathy.

B. Positions and Honors

1984-1989	Physiology	Assistant Professor	LSU Medical Center
1989-1992	Physiology	Associate Professor	LSU Medical Center
1992-1997	Surgery	Professor/Dir. Research	SUNY-Stony Brook
1997-present	Physiology/Surgery	Professor & Vice-Chair	Penn State College of Medicine
2008-present	Physiology	Distinguished Professor	Penn State College of Medicine

Honors and Memberships (selected)

1995, 1998, 2001	NIH Special Emphasis Panel-Research Training Reviews in Trauma and Burn
1993, 95, 97	Ad hoc reviewer for Surgery, Anesthesiology and Trauma Study Section (SAT/NIH)
2000, 01, 06	NIH Special Emphasis Panel-Large-Scale Collaborative Projects Award ("GLUE" grants)
2001	NIGMS, SEP (P50)-Inflammation and Hemostasis Control in Hemorrhagic Shock, Chair
2002	SEP (51)- Treatment of HAART-Associated Metabolic Changes in Patients with HIV
2003	NIAAA, SEP (60)-Alcohol-Induced Immune Dysfunction, IRPG review (Chair)
2003 & 2005	ZRG1 GMA-3 AIDS Clinical & Metabolic Diseases, Member Conflicts (Chair)
2001-2003	NIH Study Section-Alcohol & Toxicology IV (permanent member)
2005	ZRG1 MOSS-D (14) Small Business Muscle, Chair
2004-2007	NIH Study Section-Surgery, Anesthesiology & Trauma (permanent member)
2006	ZGM1 PPBC-5 Recent Center Grant Review: Burn Injury & Alcohol Exposure, Chair
2008	ZRG1 DIG-E29L-Fellowships-Diversity Program, member review committee
2001-2004	Chairman, Endocrinology & Metabolism Section, American Physiological Society
2008-present	NIH Study Section-Surgery, Anesthesiology & Trauma (permanent member)
1991-present	Editorial board-Shock, J Nutrition, and Int J Clin Exp Med
2008-present	Publication Management Committee, J Nutrition
1997-present	Program Advisory Group for NIAAA-funded Alcohol Research Ctr., New Orleans, LA
2010-present	Editor, Am J Physiology: Endocrinology & Metabolism
2008-present	Director, Graduate Program in Molecular Medicine, Penn State College of Medicine

C. Selected Peer-Reviewed Publications (Selected from more than 300 peer-reviewed publications)

Most relevant to the current application *(additional publications can be found in the progress report)*

1. Kazi, A, Hong-Brown, L, SM Lang, CH Lang. Deptor knockdown enhances mTOR activity and protein synthesis in myocytes and ameliorates muscle atrophy. *Molec Med* 17:925-936, 2011.
2. Kazi A, and **CH Lang**. PRAS40 regulates protein synthesis and cell cycle in C2C12 myoblasts. *Molec Med* 16:359-371, 2010.

3. Hong-Brong LQ, CR Brown, DS Huber, AA Kazi, AM Pruznak, **CH Lang**. Alcohol and PRAS40 knockdown decrease mTOR activity and protein synthesis via AMPK signaling and changes in mTORC1 complex interactions. *J Cell Biochem* 109:1172-1184, 2010.
4. **Lang CH**, CJ Lynch, and TC Vary. Alcohol-induced IGF-I resistance is ameliorated in mice deficient for mitochondrial branched-chain aminotransferase (BCATm). *J Nutr*, 140:932-938, 2010.
5. **Lang CH**, AM Pruznak, GJ Nystrom, TC Vary. Alcohol-induced decrease in muscle protein synthesis associated with increased binding of mTOR and raptor: Comparable effects in young and mature rats. *Nutr Metabol* 2009, 6:4doi:10.1186/1743-7075-6-4.