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Characterization of the LKB1-MO25-STRAD AMPKK Complex in Adult Mouse Skeletal Muscle

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Characterization of the LKB1-MO25-STRAD AMPKK Complex in
Adult Mouse Skeletal Muscle

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

Characterization of the LKB1-MO25-STRAD AMPKK Complex in Adult Mouse Skeletal Muscle

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Master of Science

In liver tissue, the AMP-activated protein kinase kinase (AMPKK) complex was identified as the association of LKB1, MO25 α/β , and STRAD α/β proteins; however, this complex has yet to be characterized in skeletal muscle. In this report, we demonstrate the expression of the LKB1-MO25-STRAD AMPKK complex in adult skeletal muscle, confirm the absence of mRNA splice variants, and report the relative mRNA expression levels of these complex-forming proteins. To facilitate this characterization we used control (ctrl) and muscle-specific LKB1 knockout (LKB1^{-/-}) mice. LKB1 detection in untreated ctrl and LKB1^{-/-} muscle lysates revealed two protein bands at approximately 50 and 60 kDa; although, only the heavier band was significantly diminished in LKB1^{-/-} samples (ctrl: 55 \pm 2.5 AU; LKB1^{-/-}: 13 \pm 1.5 AU; $p < 0.01$), suggesting that LKB1 is not represented at 50 kDa as cited previously. Detection of LKB1 at the higher molecular weight was further confirmed following purification of the AMPKK complex using polyethylene glycol (PEG) (ctrl: 43 \pm 5 AU; LKB1^{-/-}: 8.4 \pm 4 AU; $p < 0.01$). Following ion-exchange-fast protein liquid chromatography (FPLC) the low protein band was undetectable in ctrl and LKB1^{-/-} fractions. Mass spectrometry of PEG-treated ctrl lysates confirmed LKB1 protein detection in the 60 kDa protein band while none was detected in the 50 kDa band. Co-immunoprecipitation assays demonstrated associations between all combinations of LKB1, MO25, and STRAD in LKB1-positive samples, confirming proper complex formation. Quantitative-PCR revealed significantly reduced expression of MO25 α and STRAD β in LKB1^{-/-} muscle. Lastly, detection of CaMKK α/β protein in ctrl and LKB1^{-/-} muscle lysates confirmed the presence of another AMPKK in muscle. Interestingly, CaMKK β protein is increased in LKB1^{-/-} muscle (ctrl: 19 \pm 4.3 AU; LKB1^{-/-}: 47 \pm 9.2 AU; $p < 0.05$) without an increase in mRNA levels, suggesting compensation for null LKB1 expression. In all, these findings confirm the presence of the LKB1-MO25-STRAD complex in adult skeletal muscle, suggest a novel post-translational modification of LKB1, and identify a potential compensatory mechanism for loss of LKB1 protein in skeletal muscle.

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Introduction

In skeletal muscle, the AMP-activated protein kinase (AMPK) is highly expressed and plays an integral role in regulating the energy status of the myofiber[1]. AMPK is activated in myofibers during conditions of energy imbalance, such as contraction, hypoxia, and hypoglycemia[2]. When activated, AMPK functions to conserve ATP by inhibiting the mTOR-mediated protein synthesis pathway[3-6], while countering the intracellular energy deficit by enhancing glucose uptake and fatty-acid oxidation to facilitate ATP production[7, 8]. In doing so, active AMPK initiates translocation of GLUT-4 and CD36 transporters to the plasma membrane and inhibits the action of acetyl CoA carboxylase (ACC), thus facilitating carnitine-mediated transport of fatty-acids into the mitochondrial matrix[7-10]. Under chronic AMPK activation, increases in metabolic enzymes and mitochondrial biogenesis have been observed [11-14]. AMPK is ubiquitously expressed, but is more prominent in tissues of high energy output/demand (e.g. liver and muscle tissue)[15]. Due to its global expression and ability to enhance glucose uptake, fatty acid oxidation, and insulin sensitivity, AMPK has become a popular target for pharmaceutical research to help those suffering from metabolic syndrome, obesity, and type II diabetes mellitus.

AMPK is activated by a number of different stimuli; however in every case, complete activation requires phosphorylation at residue T172 on the AMPK α subunit by an upstream AMPK kinase (AMPKK). To date, four AMPKKs have been identified: LKB1 [16-18], Ca²⁺/Calmodulin dependent protein kinase kinase (CaMKK) [19-21], TGF- β activated kinase-1 (TAK-1) [22, 23], and ataxia telangiectasia mutated (ATM) kinase [24, 25]. Originally, LKB1 was thought to be the primary AMPKK in mammalian cells [26, 27]; although recent research suggests the other AMPKKs may play a larger role than initially thought in specific tissues.

LKB1 is a tumor-suppressor gene, that when mutated causes Peutz-Jeghers Syndrome [28, 29]. When in association with the scaffold-like, armadillo-repeat protein, mouse protein-25 (MO25) and the pseudokinase, STE-20 related adaptor protein (STRAD), LKB1 phosphorylates AMPK α at its regulatory T172 site [16-18]. The ubiquitously-expressed protein phosphatase 2C (PP2C) counters LKB1 phosphorylation by de-phosphorylating AMPK α at the same T172 site [30-32]. Regulation of these countering enzymes is dependent upon the position of the AMPK β N-terminal myristoyl group and the AMP: ATP ratio. When ATP levels are replete, ATP molecules occupy the AMPK γ -nucleotide binding sites and the AMPK β myristoyl group is positioned in proximity to a hydrophobic binding pocket on the AMPK α kinase domain; which is thought to maintain auto-inhibition of AMPK and enable PP2C activity. During conditions that increase the AMP: ATP ratio, AMP will replace ATP binding on the AMPK γ subunit and initiate a positional shift in the β -subunit myristoyl group to enable T172 phosphorylation by LKB1 and protection from PP2C de-phosphorylation. AMP binding also enhances AMPK activity via a presumed allosteric mechanism[33]. Therefore, AMP is required for maximal AMPK activation by LKB1 and protection against deactivation by PP2C.

LKB1 is coded by a gene approximately 23 kb long; it contains 10 exons, nine of which are coding sequences. Mouse LKB1 protein contains 436 residues (human LKB1: 433 residues) in which its catalytic region comprises residues ~44-309. Unique to LKB1 is that its catalytic region poorly resembles the catalytic region of other protein kinases[34]. LKB1 not only phosphorylates and activates AMPK α 1/2, but has also been reported to phosphorylate and activate 12 other kinases belonging to the AMPK subfamily [18, 35].

Furthermore, LKB1 is poly-phosphorylated at twelve identified phosphorylation sites; eight sites are phosphorylated by upstream kinases and three sites are auto-phosphorylated with

one site arguably phosphorylated either by autophosphorylation or an upstream kinase. Upstream kinases include protein kinase A (PKA), ribosomal S6 protein kinase (RSK), protein kinase C zeta (PKC- ζ), possibly the ataxia-telangiectasia mutated (ATM) kinase, and recently, from the Src family of tyrosine kinases, fyn kinase[34, 36-41]. Interestingly, LKB1 has a structural T-loop analogous to AMPK, although phosphorylation of the LKB1 T-loop is not required for activation[42]. Additionally, LKB1 is isoprenylated with a farnesyl group at the C-terminal consensus sequence: Cys-Lys-Gln-Gln[43].

It has been demonstrated that both farnesylation and phosphorylation are required for LKB1 mediated regulation of cell polarity; however, the role of all twelve putative phosphorylation sites remains somewhat unresolved[34]. In the presence or absence of LKB1 phosphate groups, both isoforms of STRAD and MO25 are able to interact with each other and with LKB1[44]. Recently, in endothelial cell lines, phosphorylation of LKB1 at S307 and S428 by PKC ζ was shown to be required for proper cytoplasmic localization via the nuclear exportin proteins, CRM1 and Exportin-7[38, 45]. However, under the same conditions, skeletal and cardiac muscle cell lines failed to show the same correlation[46]. Phosphorylation of LKB1 at tyrosines 261 and 365 by Fyn kinase does appear to be important for the proper subcellular localization of LKB1 in skeletal muscle and adipocytes *in vivo*. When Fyn is knocked out, antagonized, or if the phosphorylation sites on LKB1 are mutated, LKB1 remains localized in the cytoplasm and is able to constitutively phosphorylate AMPK and other members of the AMPK protein family[41, 47].

STRAD is a true pseudokinase, meaning it has no identified substrates; yet STRAD is able to bind ATP and exhibits structural motifs characteristic of common protein kinases. STRAD was actually the first identified substrate of LKB1, being phosphorylated at T329 and

T419. From its first discovery, STRAD protein has been implicated as a crucial regulator of LKB1 activity and proper subcellular localization[29]. Just after the discovery of STRAD, MO25 was discovered as a key interacting protein in the LKB1-STRAD complex. Structurally, MO25 resembles the Armadillo Repeat Proteins (ARPs) like β -Catenin and Importin- α which can function as scaffold-like proteins for protein complexes[48]. Accordingly, MO25 functions to stabilize the LKB1-STRAD association as LKB1 interacts only weakly with STRAD in the absence of MO25. In the presence of MO25, the activity of the LKB1-STRAD complex increases 10-fold [16, 18].

To our knowledge the characterization of the LKB1-STRAD-MO25 AMPKK complex in adult skeletal muscle has yet to be described. Therefore, the purpose of this report is to verify the presence of the LKB1-MO25-STRAD complex in skeletal muscle, confirm the absence of mRNA splice variants, and report the relative mRNA expression levels of these complex forming proteins in adult mouse skeletal muscle. To facilitate this characterization we used control (ctrl) and muscle-specific LKB1 knockout ($LKB1^{-/-}$) mice. By enhanced understanding of the LKB1-MO25-STRAD AMPKK complex in skeletal muscle, new insights into AMPK activation are possible, potentiating the development of better treatments for those suffering from metabolic pathologies like obesity and type II diabetes mellitus.

Materials and Methods

Animal Care and Generation of $LKB1^{-/-}$ Mouse

The experimental protocols were sanctioned by the Institutional Animal Care and Use Committee at Brigham Young University. Animal husbandry and development of the muscle specific $LKB1^{-/-}$ mouse using the Cre/LoxP system of gene splicing were described previously [49]. Genotyping DNA obtained from mouse ear snips validated successful incorporation of the

Cre recombinase transgene and floxed LKB1 gene into the mouse genome. Ctrl mice exhibited the floxed LKB1 gene, but no Cre recombinase; thus, enabling LKB1 expression. Furthermore, Western blot analysis of mouse muscle samples verified the presence or absence of LKB1 protein detection in ctrl or LKB1^{-/-} mice, respectively.

Muscle Extraction

Both muscle-specific LKB1^{-/-} [49] and ctrl FVB mice were anesthetized with pentobarbital sodium (0.08 mg/g body weight) into the peritoneal cavity. Upon loss of reflex activity, mouse gastrocnemii, quadriceps, hamstrings, triceps, and pectoral muscles were surgically removed, flash frozen between two aluminum blocks in liquid nitrogen, wrapped in aluminum foil, and stored at -93°C until needed.

AMPKK Purification

In order to characterize the mouse LKB1-MO25-STRAD AMPKK complex, a modified purification method developed by Hawley et al. in 1996[50], which incorporated methods developed by Carling et al. in 1989[51], and which have been modified in our lab previously[52], was used to purify the protein complex. Muscle samples were powdered under liquid nitrogen using a mortar and pestle. Powdered muscles were weighed and homogenized in 9 volumes of ice cold (4°C) homogenization buffer (50 mM Tris-HCl, 250 mM Mannitol, 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1 mM EGTA, 0.02% Brij-35, 10% Glycerol, 1 mM DTT, 1 mM Benzamidine, 1 µg/ml Soybean Trypsin Inhibitor, 0.1 mM Phenylmethanesulfonyl Fluoride, pH 7.4). The homogenate was centrifuged for 10 minutes at 3000xg. Polyethylene glycol 6000 (PEG, 25% m/v, CalBioChem, La Jolla, CA) was added to the supernatant to yield a 6% PEG concentration. The solution was well mixed and allowed to stand for 10 minutes on ice. The 6% PEG homogenate was centrifuged for 10 minutes at 30,000xg and the supernatant was

collected into a clean centrifuge container. Sufficient 25% PEG was added to yield a 10% PEG homogenate solution; the solution was mixed well and allowed to stand on ice for 10 minutes. The 10% PEG homogenate solution was centrifuged for 20 minutes at 30,000xg and the supernatant was discarded. The pellet was re-suspended in homogenization buffer (0.50ml/g muscle) by applying a rotating pestle to the pellet on the bottom of the centrifuge tube. The re-suspended PEG precipitate solution was centrifuged again for 10 minutes at 30,000xg and the supernatant was collected. The solution collected from this protocol is termed the PEG lysate.

The PEG lysates were applied to a HiTrap DEAE FF Sepharose (5ml) ion exchange chromatography column (GE Healthcare Life Sciences, Piscataway, NJ). The column was washed with homogenization buffer (0.5ml/min) until a baseline absorbance reading of less than 5 AU was obtained. The re-suspended PEG precipitate was injected into the column and washed with 10mL of homogenization buffer while 1mL fractions were collected. Elution of the AMPKK complex with 0.2 M NaCl homogenization buffer was performed for 80 minutes while collecting 1mL fractions. In all, a total of 50-one milliliter fractions were collected.

Protein Detection/Western Blot Analysis

Detection of LKB1 in experimental fractions and lysates was assessed using the Western blot/ immunodetection procedure described previously [49]. Aliquots (12.5 μ L) of collected fractions were diluted in 2X sample loading buffer (12.5 μ L: 50mM Tris-HCl, pH 6.8, 10% Glycerol, 2% SDS, 2% β -mercaptoethanol, and 0.1% bromophenol blue) and loaded (25 μ L/lane) onto a 10% Tris-Cl gel (Bio-Rad Criterion System, Hercules, CA). Following the membrane blocking step with 5% nonfat dried milk, the PVDF membranes were probed with an anti-LKB1 antibody (Upstate, cat#: 07-694, Charlottesville, VA, 1:5000; Santa Cruz Biotechnology, Inc, (M-18) cat#: sc-5640, (D-19) cat#: sc-5638, Santa Cruz, CA, 1:2000) in 1% BSA with

0.01%NaN₃. After overnight incubation, the membranes were washed with TBST and then incubated with an anti-rabbit IgG streptavidin-horseradish peroxidase (HRP) conjugate antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:5000) diluted in 1% nonfat dried milk in TBST. The remaining protocol was followed as described in the aforementioned citation[49] and all Western blot images were analyzed semi-quantitatively using AlphaEase software (Genetic Technologies, Miami, FL).

For CaMKK α/β detection in untreated 5% muscle homogenate, isoform-specific antibodies were used under the same procedure (α -CaMKK α (R-73): cat #: sc11370, 1:2000; α -CaMKK β (L-19): cat#: sc9629, 1:1000; Santa Cruz Biotechnology, Inc, Santa Cruz, CA).

Immunoprecipitation Assays

In order to confirm protein-protein interactions between all possible combinations of LKB1, MO25, and STRAD, immunoprecipitation followed by Western blot analysis was performed. Immunoprecipitation assays were carried out using ExactaCruzTM Immunoprecipitation/Western blot kits purchased from Santa Cruz Biotechnology, Inc (Cat #: sc-45038, sc-45040, sc-45041, sc-45043; Santa Cruz, CA). Ten microliters/sample of packed volume immunoprecipitation matrix was aliquoted into a 1.7 mL microcentrifuge tube. Packed volume refers to the volume of matrix in the tube after being centrifuged down at 13,000xg for 30 seconds. Following centrifugation, the supernatant was removed and washed with 1 mL of ice-cold 1x PBS solution. The matrix was centrifuged down again, supernatant removed, and replaced with 1mL fresh, ice-cold 1x PBS solution. Using the appropriate antibody (α -LKB1 (M18): cat #: sc-5640; α -STRAD (N13): cat #: sc-34102, Santa Cruz Biotechnology, Inc, Santa Cruz, CA; α -MO25: Custom made, Affinity BioReagents, Inc, Golden, CO), 1.5 μ g/sample was added to the matrix and allowed to rotate end-over-end for at least 2 hours at 4°C.

After antibody incubation, the matrix was washed three times in ice-cold 1x PBS in the same manner described above. Following the third wash, enough 1x PBS was added to enable 50 μ L aliquots of the desired number of samples under analysis. While vortexing the matrix after every two aliquots, 50 μ L of the matrix suspension was aliquoted into the desired number of 0.65 mL microcentrifuge tubes. The aliquots were spun down to determine equal loading amounts of matrix and the supernatants were suctioned off. Twenty microliters of sample were added to corresponding tubes and allowed to rotate over-night at 4°C. Note: the cohesive and adhesive properties of the matrix/sample solution kept the solution in the bottom of tube, even while inverted, during incubation. This ensured most efficient diffusion of matrix in the sample homogenate.

The following day, samples were centrifuged and 20 μ L of supernatant was collected into separate 0.65 mL microcentrifuge tubes for Western blot analysis. The matrix was washed three times in the same homogenization buffer used in the AMPKK purification protocol. Western blot analysis was performed on both pellet and supernatant samples as described above with the additional step of heating the samples at 90°C for 5 minutes after the appropriate volume of loading buffer was added. Note: the 2X loading buffer prepared for the pellets was diluted to 1X using dH₂O.

Mass Spectrometry

Due to the elusiveness of identifying the protein bands representing LKB1 and STRAD proteins in Western blot assays, mass spectrometry was used to resolve these uncertainties. Immunoprecipitation of LKB1 from ctrl PEG lysate was performed as described above. Following electrophoresis, a coomassie blue (Pierce Biotech, cat#: 24590, Rockford, IL) stain was applied to the gel for 24 hours. The gel was washed 3 x 1 hour to resolve the bands. The

bands at presumed LKB1 and STRAD molecular weights were cut from the gel and submerged in deionized-dH₂O in a 1.7 mL microcentrifuge tube. The sample was shipped to the Harvard based, Taplin Mass Spectrometry Facility, for tryptic-digest mass analysis using an orbitrap mass spectrometer.

Reverse Transcriptase-Polymer Chain Reaction (RT-PCR)

To test for the existence of variable splice-variant isoforms of LKB1, MO25 α/β , and STRAD α/β mRNA expressed in skeletal muscle, RT-PCR was used to study the gene coding sequences by gel electrophoresis and cycle sequencing. Mouse quadriceps muscles were harvested as described above; however, immediately following extraction, muscle samples were submerged in 1mL RNAlater solution (Ambion Inc, Austin, TX) to maintain the integrity of tissue RNA. Extraction of total tissue RNA was performed using RNeasy Fibrous Tissue Mini kit (Qiagen, Valencia, CA) according to product instructions. Nano-drop spectrophotometry was used to determine the approximate total RNA concentration (1 AU at A₂₆₀ = 40 ng/ μ L RNA) and RNA purity (A₂₆₀/A₂₈₀ > 1.9). Total RNA integrity was confirmed by detection of sharp and discrete 28S and 18S RNA bands from total RNA samples run on a borate buffered 1% agarose electrophoretic gel. After ensuring the extraction of viable RNA, reverse transcription followed by PCR was performed according to the LongRange 2Step RT-PCR kit instructions (Qiagen, Valencia, CA).

Primers

Custom primers for RT-PCR, qPCR, and DNA sequencing were purchased from Invitrogen (Carlsbad, CA) and can be found in [Table 1](#).

DNA Sequencing

To confirm mRNA expression of the LKB1, MO25 α/β , and STRAD α/β genes and to test for variant splice isoforms, DNA sequencing and alignment of the RT-PCR products were performed. All DNA sequencing was performed using the dideoxy (Sanger) method by the BYU DNA Sequencing Center. Sequence alignments were performed using Bioedit software (Ibis Therapeutics, Carlsbad, CA).

Quantitative PCR (qPCR)

To assess the relative mRNA expression levels of LKB1, MO25 α/β , STRAD α/β , and CaMKK β genes between ctrl and LKB1^{-/-} mouse muscle, qPCR was performed using the following procedure: Primer sets for qPCR were designed for each gene (LKB1, MO25 α , MO25 β , STRAD α , STRAD β , CaMKK β and 18s-reference gene) yielding an amplicon length between 100-300 bp ([Table 1](#)). Total RNA from mouse quadriceps muscles was harvested and determined viable using the same protocol described in *RT-PCR*. Subsequent cDNA synthesis was prepared using SuperScriptTM III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA) according to product instructions. Concentration and purity of cDNA were assessed using nano-spectrophotometry (1 AU at A₂₆₀ = 50ng/ μ L, A₂₆₀/A₂₈₀ > 1.6, respectively). Serial dilutions of cDNA used in the optimization assays were made by first adding 1 μ L of cDNA to 29 μ L of RNase/DNase-free water, followed by vortexing and centrifugation. Then, 10 μ L of this dilution was added to 20 μ L of RNase/DNase-free water, vortexed and spun down. The process was repeated with the more diluted sample four additional times to make 6 serial dilutions total. Serial dilutions were only used within a 6 hour period.

Optimization and gene expression protocols were performed using the SsoFastTM EvaGreen® Supermix according to product instructions (Bio-Rad, Hercules, CA). Optimal

primer concentrations were determined for each gene of interest with a reproducible efficiency of 95-105% and $r^2 > 0.98$ based off the standard curve derived from the cDNA serial dilutions. Quantitative PCR assays were run using a C1000™ Thermal Cycler/CFX96™ Real-Time System purchased from Bio-Rad (Hercules, CA).

Quantitative PCR Data Analysis

Using GraphPad software (La Jolla, CA) the relative fluorescence readings for all 50 cycles were fit to a Boltzmann sigmoidal curve for each gene. Interpolation of 40 additional fluorescence readings was calculated between each cycle from the data. Then, the second derivative was calculated for each point to reveal the inflection point at which PCR amplification reached maximal acceleration. The exact cycle number that correlated to this point was defined as the threshold cycle (C_T) for that particular gene.

Averages of the 18S reference gene C_T were subtracted from the C_T values of LKB1, MO25 α/β , STRAD α/β , and CaMKK β yielding the ΔC_T . Average ΔC_T s for each gene triplicate were calculated for each cDNA sample and then averaged again to yield the overall average ΔC_T for each gene. Each gene triplicate average ΔC_T for ctrl and LKB1^{-/-} samples was subtracted from the overall ctrl average ΔC_T yielding the $\Delta\Delta C_T$. Normalized gene expression was calculated by using the $2^{(\Delta\Delta C_T)}$ formula for each $\Delta\Delta C_T$ value. Averages and student t-test calculations were performed on all normalized expression values to demonstrate statistical significance.

Results

LKB1 is detected primarily at a molecular weight of approximately 60 kDa in adult skeletal muscle as opposed to 50 kDa, as cited extensively in the literature. Based on its amino acid sequence, the putative molecular weight of LKB1 is approximately 50 kDa. Western blot

analysis using two anti-LKB1 antibodies (Anti-LKB1 #1: Upstate, cat#: 07-694, kinase domain epitope; Anti-LKB1 #2: Santa Cruz Biotech, cat#: sc-5640, C-terminal epitope) of untreated ctrl and LKB1^{-/-} muscle homogenates detected protein bands representing LKB1 at approximately 50 and 60 kDa, as previously cited [17, 50, 53]. Western blot analysis using a third LKB1 antibody (Anti-LKB1 #3: Santa Cruz Biotech, cat #: sc-5638, N-terminal epitope) only detected the heavier protein band. Two different protein molecular weight standards were used (BioRad, cat#: 161-0373; Fermentas, cat#: SM0441) which depicted 50 kDa at slightly different levels. While the lighter protein band migrated at or above the 50 kDa standard (depending on the protein standard), the heavy band representing LKB1 was markedly higher than either 50 kDa protein standard ([Fig 1A](#)).

LKB1 protein detected at the higher molecular weight in ctrl tissue lysates was significantly more intense than LKB1^{-/-} tissue lysates, according to densitometric analysis (ctrl: 55±2.5 AU; LKB1^{-/-}: 13±1.5 AU; p<0.01, n=6, [Fig 1B](#)). The small amount of LKB1 detected in LKB1^{-/-} tissue homogenates is an artifact of non-muscular LKB1 expressing tissue that was extracted and homogenized with the mouse skeletal muscle (e.g. nerve, adipose, and connective tissue) or incomplete LKB1 gene splicing by the Cre recombinase. LKB1 protein detected at 50 kDa was not significantly different between LKB1^{-/-} and ctrl tissue lysates. Based on this evidence, we propose that LKB1 is primarily represented at a molecular weight of about 60 kDa in skeletal muscle due to a novel post-translational modification; and while we cannot completely rule out LKB1 representation at 50 kDa, we assume other non-specific antibody interactions are taking place.

Following the PEG purification procedure, the heavier protein bands were significantly more intense in the ctrl PEG lysates compared to the LKB1^{-/-} PEG lysates (ctrl PEG lysate: 43±5

AU; LKB1^{-/-} PEG lysate: 8.4±4 AU; p<0.01, n=6, [Fig. 1C](#)). Protein band intensity at 50 kDa in ctrl and LKB1^{-/-} PEG lysates were markedly different, but shy of statistical significance (p=0.07, n=6). Following FPLC fractionation, LKB1^{-/-} fractions displayed markedly reduced heavy protein band intensity compared to ctrl fractions; while protein bands at 50 kDa were undetectable in both fraction sets ([Fig. 1D](#)). These data further suggest LKB1 protein is not significantly represented by the 50 kDa protein band but is the artifact of non-specific protein-antibody interactions.

Tryptic-digest mass spectrometric analysis of extracted 60 kDa protein bands obtained from gel electrophoresis of ctrl PEG lysates revealed 12 different peptide fragments positively aligned with the LKB1 amino acid sequence ([Fig. 2](#)); two or more positively aligned peptide fragments confers significant detection. No LKB1 peptide fragments were detected for the 50 kDa band under analysis, possibly due to diminishment of the 50 kDa band following PEG purification. Regardless, this observation confirms LKB1 representation at a higher molecular weight than originally expected.

In attempt to identify a novel post-translational modification of LKB1, more mass spectrometric analysis of the 60 kDa protein band was performed. Other than detection of highly phosphorylated S31 which was previously established[34], no other post-translational modifications were detected. It remains unclear what post-translational modification is responsible for the increased molecular weight of LKB1.

The LKB1-MO25-STRAD AMPKK complex is present in adult murine skeletal muscle.

Co-immunoprecipitation followed by Western blot detection of all possible combinations of LKB1, MO25, and STRAD proteins revealed positive associations in purified lysates ([Fig. 3](#)). Some LKB1^{-/-} fractions showed positive protein-protein associations and detection of LKB1

protein presumably from the non-skeletal muscle tissue inadvertently harvested and purified with muscle extracts or incomplete gene deletion by the Cre recombinase. In all cases, positive protein detection in LKB1^{-/-} fractions was markedly reduced compared to ctrl fractions; illustrating disruption of the LKB1-MO25-STRAD complex formation in LKB1^{-/-} mice.

Identification of STRAD protein was initially elusive using Western blot detection with the anti-STRAD (N-13) antibody. Protein identification was confirmed by tryptic-digest mass spectrometric analysis of the excised, putative STRAD protein-coomassie stained gel band. Two different peptide fragments were positively aligned with the mouse STRAD α amino acid sequence confirming positive and significant identification (Data not shown).

Co-immunoprecipitation/Western blot detection assays were performed using antibodies non-specific for the α/β isoforms of MO25 and STRAD proteins. Hence, determining the relative composition of MO25 and STRAD isoforms in the AMPKK complex was not assessed in this project. The data produced from these assays suggest that the LKB1, MO25, and STRAD proteins comprise an AMPKK complex in skeletal muscle.

CaMKK α/β protein is detected in untreated ctrl and LKB1^{-/-} muscle homogenates by Western blot analysis. Interestingly, CaMKK β protein detection was significantly increased in LKB1^{-/-} untreated homogenates compared to ctrl (ctrl: 19 \pm 4 AU; LKB1^{-/-}: 47 \pm 9; p<0.05; n=6, [Fig 4B](#)). No difference in CaMKK α protein detection was observed ([Fig 4A](#)). This data suggests that rested and untreated LKB1^{-/-} muscle may compensate for loss of LKB1 by up-regulating CaMKK β protein expression; thus underscoring the predominant and acute role LKB1 plays in skeletal muscle.

RT-PCR followed by DNA cycle sequencing confirmed the expression of LKB1, MO25 α/β , and STRAD α/β mRNA in mature mouse skeletal muscle. Custom, gene-specific primers ([Table 1](#))

for LKB1, MO25 α/β , and STRAD α/β were used to amplify sections of cDNA ranging in size from 300-1000+ base pairs in length and spanning multiple exons. Successful PCR products were sequenced and aligned with their respective mRNA sequences established by the online National Center for Biotechnology Information (NCBI) database. NCBI accession codes for each gene used in sequence alignment experiments can be found in [Table 1](#). For every PCR product detected, only a single band at the putative amplicon size was observed in the agarose gel following electrophoresis (data not shown); suggesting no variant splice isoform expression of LKB1, MO25 α/β , and STRAD α/β mRNA in mouse skeletal muscle. Every gene sequence was confirmed by amplification of at least two overlapping PCR products spanning the length of the coding sequence; all of which were identified by a single band on agarose gels.

Quantitative-PCR revealed the relative expression profiles of LKB1, MO25 α/β , STRAD α/β , and CaMKK β in LKB1^{-/-} and ctrl muscle. As expected, there was a small amount of LKB1 gene expression detected in the LKB1^{-/-} samples due to non-muscular tissue harvested along with skeletal muscle or incomplete LKB1 gene splicing by Cre recombinase. The non-muscle derived LKB1 mRNA accounted for 0.31 fold expression compared to ctrl (p<0.01, n=6). Of the four other genes tested, there was significantly decreased expression of MO25 α (ctrl: 1.0 \pm 0.06 AU; LKB1^{-/-}: 0.66 \pm 0.05 AU; p<0.01, n=6, [Fig 5](#)) and STRAD β (ctrl: 1.0 \pm 0.07 AU; LKB1^{-/-}: 0.79 \pm 0.06 AU; p<0.05, n=6, [Fig 5](#)) mRNA in LKB1^{-/-} muscle compared to ctrl. There was no difference in STRAD α , MO25 β , and CaMKK β mRNA expression between ctrl and LKB1^{-/-} tissues.

Discussion

The purpose of this study was to verify the presence of the LKB1-MO25-STRAD complex in skeletal muscle, confirm the absence of mRNA splice variants, and report the relative

mRNA expression levels of these complex forming proteins in adult mouse skeletal muscle. Previous studies described a procedure to purify the AMPKK complex from rat liver tissue [17, 50]. We followed a shortened version of the same protocol to successfully purify the AMPKK protein complex from mouse skeletal muscle. In our purified samples we detected the same protein constituents comprising the AMPKK complex as determined in rat liver tissue: LKB1, MO25, and STRAD [17]. Co-immunoprecipitation assays followed by Western blot or mass spectrometry assays confirmed associations between all combinations of these proteins. In doing so, we confirmed the accuracy of the AMPKK purification protocols previously cited and provide supporting evidence that LKB1 is a primary AMPK phosphorylating protein in adult mouse skeletal muscle.

According to its amino acid sequence, LKB1 has a putative molecular weight of approximately 50 kDa. One of the first papers published on the AMPKK complex in rat liver tissue reported the kinase component as a polypeptide of approximately 60 kDa[50]. In a later paper published by the same group, two Western blot protein bands are identified as LKB1 which are separated by approximately 8-10 kDa; the heavier band migrating as a doublet [17]. Our data confirms this observation. The mass contributed by all 12 identified phosphorylation sites and farnesylation site cannot account for this 8+ kDa molecular weight difference between LKB1 bands. Treatment with protein phosphatases-1 γ and -2A1 were unsuccessful at reconciling the molecular weight difference in these bands[17], and treatment with protein phosphatase-2C reduced the slower-migrating LKB1 doublet to a single band[53]. This data confirms the different phosphorylation states of LKB1 and suggests other post-translational modifications besides phosphorylation are responsible for the 8kDa difference between LKB1 protein bands.

Recognizing the increasing role of ubiquitin in protein regulation, particularly in the nucleus where LKB1 is localized, and its molecular weight of approximately 8.5 kDa, we tested LKB1 for the presence of mono-ubiquitination using the Taplin Mass Spectrometry facility at Harvard. The data from this analysis further confirmed representation of LKB1 protein at 60 kDa; however, no mono-ubiquitinated peptides were observed. Due to the small amount of LKB1 analyzed by mass spec, the possibility of mono-ubiquitination should not be ruled out entirely; more analysis is necessary to determine if ubiquitin is involved in the 8 kDa molecular weight difference in detectable LKB1 protein bands.

Ubiquitin has been demonstrated to play a major role in skeletal muscle regulation. For a review of ubiquitin function in skeletal muscle, consult the following references [54-57]. Evidence linking ubiquitin to the LKB1-AMPK axis is demonstrated in the finding that AMPK and ten of the twelve other AMPK-related protein kinases possess structural ubiquitin-associated (UBA) domains located C-terminal to the kinase domain. The exact function of the UBA domains characterizing the AMPK related kinases is not well understood; but they are required for LKB1-mediated phosphorylation and activation. The same group also demonstrated that the UBA domains do not appear to act as docking sites for the LKB1-MO25-STRAD AMPKK complex[58].

Mice that are deficient in ubiquitin C-terminal hydrolase-L3 (UCH-L3^{-/-}), an enzyme that catalyzes the de-ubiquitination of proteins, are resistant to high fat diet induced obesity and insulin resistance. UCH-L3^{-/-} mice have higher energy expenditure and as a result, lower white adipose tissue mass. Mediating these effects is increased skeletal muscle AMPK activity and subsequent increased fatty acid oxidation. This data provides strong evidence for the role of

ubiquitin in AMPK regulation and that UCH-L3 acts to inhibit AMPK[59]. Further work to resolve the role of ubiquitin in AMPK regulation is still needed.

Despite increasing evidence for CaMKK and TAK-1 AMPKK activity, our data confirms LKB1 as a primary upstream kinase of AMPK in skeletal muscle[16, 26]. Some researchers suggest that the three AMPKKs preferentially phosphorylate different AMPK α subunits which have different primary regulatory roles. In LKB1-null skeletal and cardiac muscle, AMPK α 2 activity is predominantly lost, even in response to AICAR and phenformin, while AMPK α 1 activity is only marginally decreased[60, 61]. These data imply that LKB1 predominantly acts on the AMPK α 2 subunit. Since AMPK α 2 is the predominant AMPK α subunit expressed in skeletal muscle[62], it seems likely that LKB1 is the main AMPKK acting on this subunit. Furthermore, in HeLa cells, which innately lack LKB1, increasing the AMP: ATP ratio or administering AICAR failed to increase AMPK activation, also suggesting that LKB1 is the AMPKK responsible for primarily phosphorylating the AMPK α 2-subunit in response to AMP accumulation [17]. Our data confirms LKB1 mRNA and protein expression required to fulfill this role.

McGee et al. demonstrated that CaMKK α/β protein concentration and activity, and activating TAK-1 phosphorylation increase in overloaded ctrl and LKB1^{-/-} muscle. These increases in concentration, activity, and phosphorylation correlate to an increase in AMPK α 1 T172 phosphorylation in both muscle genotypes. As a result, increased phosphorylation of TSC2, a regulatory protein in the TORC1-mediated protein synthesis pathway and target of AMPK, was observed in overloaded muscle in both genotypes. Downstream targets of TORC1 were also phosphorylated. Chronically activated AMPK also induces increased expression of GLUT4 and other mitochondrial genes[63]; although these genes were unchanged in ctrl and

LKB1^{-/-} overloaded muscle. This evidence suggests that AMPK α 1 in skeletal muscle is preferentially phosphorylated by CaMKK α / β or TAK-1, which increases in concentration and activity during overload treatment in order to participate in cell growth regulation; but is not as involved in regulating metabolic adaptations as AMPK α 2[27].

While the three AMPKKs may preferentially phosphorylate specific AMPK α subunits, evidence for redundancies and cross talk between AMPKKs still exists. In 2010, activation of the CaMKK-AMPK axis was demonstrated in skeletal muscle exposed to a six-day lipid emulsion treatment. The treatment correlated to increased AMPK and acetyl-CoA carboxylase (ACC) phosphorylation, increased ACC β and adiponectin receptor 1 mRNA levels, and increased PPAR γ -coactivator-1 α (PGC-1 α) protein levels. Significantly greater association between CaMKK and AMPK was also observed, while association between LKB1 and AMPK remained unchanged; furthermore, the levels of ATP were unchanged in the lipid emulsion and control treated mice[64]. Unfortunately, the AMPK α subunit was not specified in this study; however, the data demonstrates the ability of CaMKK to also regulate AMPK-mediated lipid metabolism and mitochondrial gene expression and biogenesis pathways, and not just cell growth. Certainly, our data showing increased CaMKK β protein levels in LKB1^{-/-} muscle may be indicative of cellular compensation to activate AMPK and potentiate its metabolic effects.

While CaMKK β protein expression was significantly increased in LKB1^{-/-} muscle, CaMKK β mRNA expression remained unchanged. Therefore, the increase in CaMKK β protein is the result of either enhanced CaMKK β mRNA translation or decreased proteosomal degradation of CaMKK β protein in LKB1^{-/-} muscle. Further work is required to elucidate the mechanisms responsible for increased CaMKK β protein content in LKB1^{-/-} skeletal muscle.

Some researchers suggest that CaMKK and TAK-1 are AMPKKs employed to prepare the cell for an upcoming energy imbalance which might be the case in the lipid emulsion treated muscle[26]. While further work is required to uncover the full function of CaMKK and TAK-1 as AMPKKs in skeletal muscle, LKB1 remains the primary AMPKK responsible for AMPK α 2 activation under acute increases in the AMP: ATP ratio.

Previous studies have demonstrated that formation of the heterotrimeric LKB1-MO25-STRAD protein complex is imperative for proper LKB1 localization out of the nucleus into the cytoplasm [29, 44, 65]. Interestingly, both STRAD and MO25 can freely cross the nuclear membrane. In the nucleus, association with STRAD α facilitates LKB1 binding with the nuclear export proteins, CRM1 and Exportin-7. Likewise, at the cytosolic face, association with STRAD α prevents LKB1 from associating with the nuclear import protein, Importin- α . STRAD β , however, has not been demonstrated to participate in LKB1 shuttling. The function of MO25 was originally thought to facilitate LKB1 nuclear translocation as its structure resembles the family of nuclear transport proteins, karyopherins; however, no data supports this case. MO25 simply enhances the association between STRAD α and LKB1[66].

In addition to coordinating proper sub-cellular localization, association with MO25 and STRAD is also responsible for optimal LKB1 activity [16-18]. This was confirmed by resolution of the LKB1-MO25 α -STRAD α and MO25 α -STRAD α crystal structures. Remarkably, the pseudokinase, STRAD α binds ATP and adopts the active, “closed” kinase conformation, but is yet unable to phosphorylate downstream targets due to the substitution of crucial catalytic amino acid residues. Association with MO25 α enhances the STRAD α -ATP interaction and vice versa. Interaction with MO25 α or ATP is essential for maintaining STRAD α in the closed conformation; and subsequently essential for LKB1 activation. Mutated STRAD α , incapable of

binding ATP or MO25, is unable to activate LKB1; although, complex formation still occurs[67]. Analysis of the trimeric protein structure reveals STRAD α , in its closed conformation, binding LKB1 as a pseudo-substrate. Association with STRAD α and MO25 α maintains the closed and active conformation of LKB1. MO25 α actually interacts with and stabilizes the LKB1 activation loop[68]. These reports suggest LKB1 kinase activity is regulated by allosteric conformational shifts dependent on its association with MO25 and STRAD proteins.

Collectively, the crystal structure data and nuclear transport data reveal two redundant mechanisms of LKB1 activation: proper subcellular localization and structural shifts that render LKB1 in the active and closed kinase conformation. Both regulatory mechanisms are dependent on association with MO25 and STRAD proteins. This data is consistent with previous reports of enhanced LKB1 activity and proper localization when in complex with MO25 and STRAD [16-18, 69].

As mentioned, the gene sequencing data of this study revealed no alternate splice variants of LKB1, MO25, and STRAD proteins. In a previous report, an alternate splice variant of LKB1 mRNA was identified in rodent and human tissues. The two splice isoforms exhibit differential ninth exon incorporation into the mRNA, which leads to a long and short version of LKB1. The LKB1-long splice variant (LKB1_L) is the predominant form of LKB1 mRNA translated in cells. Translation of the LKB1-short splice variant (LKB1_S) mRNA results in substitution of the previously identified C-terminal 63 amino acid residues with an alternate 39 residues. Both isoforms are widely expressed and able to phosphorylate AMPK and other members of the AMPK-protein family. It is unclear what specific roles these splice variants may play; although, male mice lacking LKB1_S in the testis were reproductively sterile, suggesting a crucial role of

LKB1_S in gametogenesis [70]. Our gene sequencing data only detected the LKB1_L isoform suggesting the LKB1_S isoform is not expressed in mouse skeletal muscle.

In a study by Marignani et al, 11 novel splice variants of STRAD α mRNA were reported; although all were identified in human cancer cell lines and all of them disrupted proper LKB1 activity and localization due to lack of complex formation[69]. To our knowledge there have been no reports of alternative splice variants of MO25 α/β proteins. Likewise, our data confirmed no splice variant expression of STRAD and MO25 mRNA in mouse skeletal muscle.

While LKB1^{-/-} tissue exhibited significant decreases in MO25 α and STRAD β gene expression, the role LKB1 may play in regulating gene expression remains unclear. More experimentation using the muscle-specific LKB1 knockout mouse is necessary in this regard.

In this study, specific characteristics of the LKB1-MO25-STRAD AMPKK complex in skeletal muscle have been elucidated. Our results confirm the presence of the LKB1- MO25-STRAD AMPKK complex in mouse skeletal muscle, verify the mRNA coding sequence of each protein expressed and the absence of alternative splice variant expression, and reveal the relative mRNA expression profile of LKB1, MO25 α/β , STRAD α/β , and CaMKK β genes comparing ctrl and LKB1^{-/-} muscle. Notably, this report provides evidence that LKB1 is actually detected at a molecular weight of approximately 60 kDa and not 50 kDa in skeletal muscle. We propose that an unidentified post-translation modification is responsible for this increase in molecular weight. Determining the identification of this protein modification in correlation with further characterization of the LKB1-MO25-STRAD AMPKK complex in skeletal muscle may facilitate new insights into the LKB1-AMPK axis of metabolic regulation and possibly provide new avenues for anti-diabetic drug design.

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Grant Information

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Table 1

Custom primer sequences for (A) PCR and DNA sequencing and (B) quantitative PCR assays.

A.)

Forward Primers		bp start	NCBI Accession
Gene	Primer (5'-3')		
LKB1	CTTGAAAAGAATTGGCGCTC	31	NM_011492
	GGCGAGCTGATGTCGGTGGG	125	
	CCACCGCATCGACTCCACCG	160	
MO25α	GTC AAGCAGGTAGAGTGCCG	376	NM_133781
	ACGGGGACGGAGACGAAGCA	325	
MO25β	AGATGCAGACTCCAGATTGGCG	119	NM_026908
	TCGACATGAGCCACTTGCCA	735	
STRADα	ATGTCTTTTCTTGCCAAT	128	NM_028126
STRADβ	CTGGAGAGTCGCTGGCCTTG	249	NM_172656
	CTGTTGGCAGCTGGCTTTGG	755	
	AAGCACGGACAAAGGCATAG	1006	
	TGGTGCAGCTGTGTTGCAG	1409	

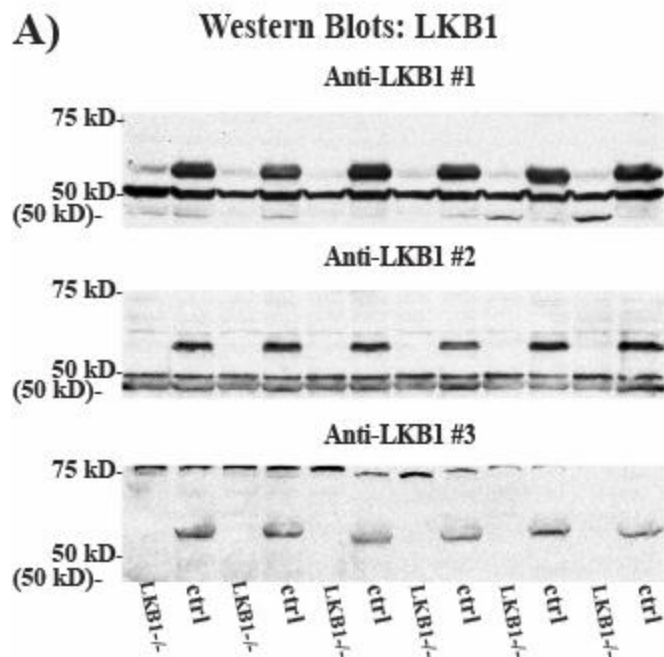
Reverse Primers		bp start
LKB1	ACAGCGAAAGGGTGCAGGGC	699
	TCCACAGCGAAAGGGTGCAGG	702
	AGGTACGAGCGCCTCAGCCA	1045
	GTCCCGAAGTGAGTGGACAG	1483
MO25α	AGCTACCAGGGTGCCGAGGAG	673
	ACGTTCTCCAACCTGGACTG	1604
MO25β	ACACACTGGGCAAGGGAGAC	1345
	AATTGTGGCGGTCCAGGATCA	1009
STRADα	ATCGTGGACTGCACGCTGCC	713
	CCTGAGCTCACACAACAGGA	1561
STRADβ	AGCCAGCTGCCAACAGTGAA	770
	CGGCTGCACTGATGTGCTGA	1068
	TCCTGGCTTTCTTCTTTCATCTG	1508
	CGAGGAAGTCGTGCGGGTGC	1831

B.)

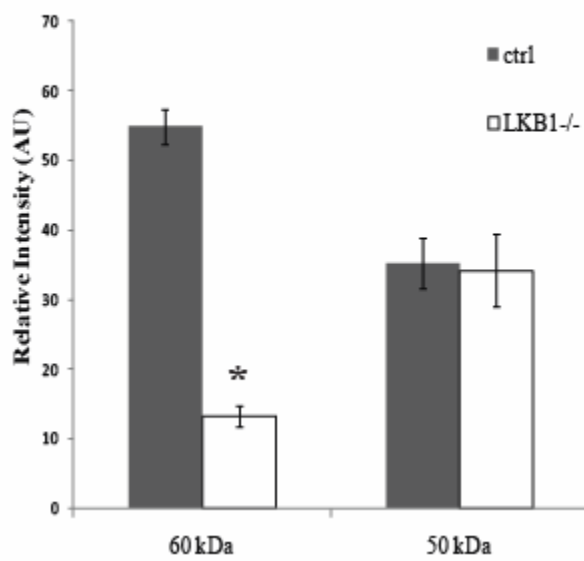
Gene	F/R	Primer	bp start	PL
LKB1	F	TTGGGCCTTTTCTCCGAGG	107	138
	R	CAGGTCCCCATCAGGTACT	244	
MO25α	F	CCGTTCCCATTTGGCAAGTCT	422	160
	R	ACAGAATTTCTTTCATGGCGACC	581	
MO25β	F	CAAAACGCAGCCTATCGTGGA	1140	94
	R	CTCGTCGTCTGTCCTTCTTTC	1233	
STRADα	F	TTCCCTCTGTTCCCATGTCC	1687	118
	R	AGTCCAGCCTTAACTGACC	1804	
STRADβ	F	CCTTACGCTGTGGGATTATGC	701	111
	R	TGGACAGCTCAACATACTTGAAG	811	
CamKKβ	F	GCCATGGGTGTGACGCTGTA	1173	116
	R	GGCTGGTCGGGAACTCCAG	1288	
18S	F	GTGCATGGCCGTCTTAGTTG	1328	133
	R	GCCACTTGTCCTCTAAGAAGTTG	1460	

Figure 1

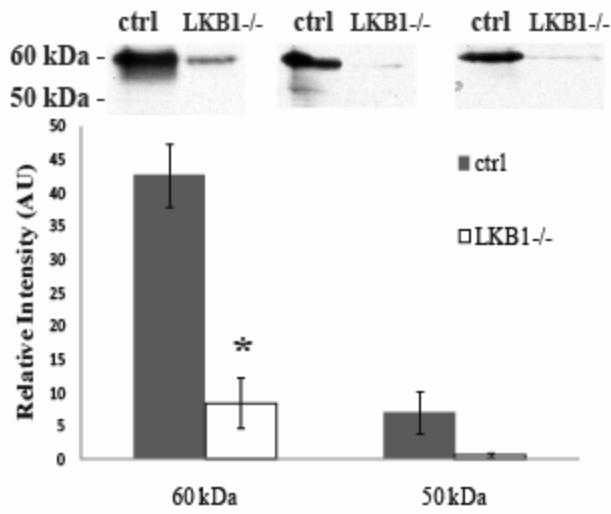
LKB1 protein is detected at a molecular weight of approximately 60 kDa, and not 50 kDa. (A) LKB1 protein detection in untreated muscle lysates using three different anti-LKB1 antibodies (Anti-LKB1 #1: Upstate, cat#: 07-694, kinase domain epitope; Anti-LKB1 #2: Santa Cruz Biotech, cat#: sc-5640, C-terminal epitope; Anti-LKB1 #3: Santa Cruz Biotech, cat #: sc-5638, N-terminal epitope) and two different protein molecular weight standards (BioRad, cat#: 161-0373; Fermentas, cat#: SM0441, identified in parentheses). Untreated muscle lysates were prepared by homogenizing mouse gastroc muscles in enough lysate buffer to make a 5% homogenate using a rotating pestle. Enough sample volume was mixed with the appropriate amount of loading buffer and ddH₂O to allow approximately 90µg of protein to be loaded into each gel well. (B) Quantification by densitometric analysis of LKB1 detection using the Anti-LKB1 #1 antibody. Western blot images were scanned and loaded into AlphaEase software which was used to quantify relative band intensities. (C) LKB1 protein detection in PEG lysates using the Upstate anti-LKB1 antibody. PEG-lysates were obtained by adding enough 25% PEG (m/v) to achieve a 10% PEG concentration in muscle lysates. Centrifugation caused precipitation of PEG and the AMPKK complex. The precipitate was re-suspended in lysate buffer to reconstitute the AMPKK complex. (D) LKB1 detection in FPLC fractions using the Upstate anti-LKB1 antibody. FPLC fractions were collected as PEG lysates were applied to a DEAE FF Sepharose ion-exchange chromatography column, eluted with 200 mM NaCl Buffer B. Protein bands at 50 kDa were undetectable in FPLC fractions. In all charts, * = p<0.01 compared to ctrl 60 kDa protein band intensity.



B) LKB1 Protein in Untreated Muscle Lysates



C) LKB1 Protein in Re-Suspended PEG Lysates



D) LKB1 Protein: PEG Lysates and FPLC Fractions

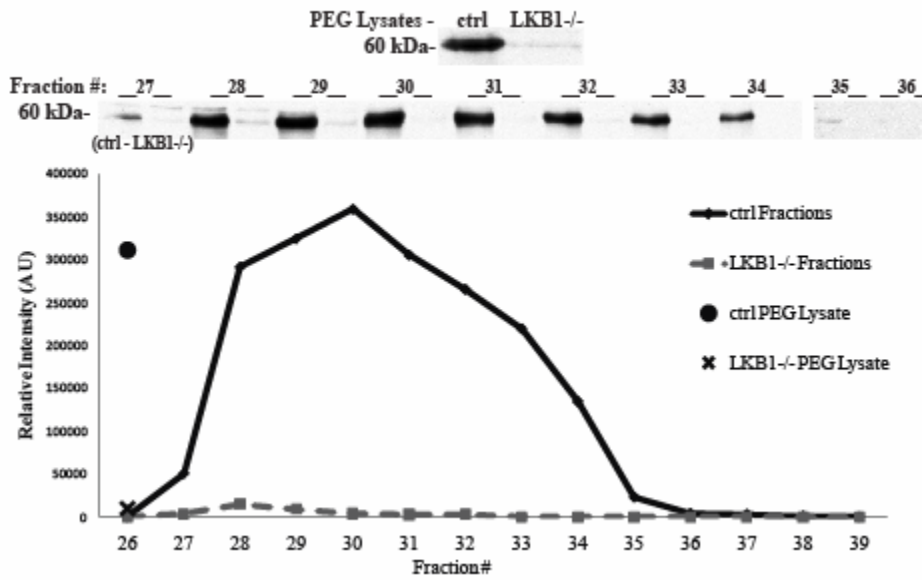


Figure 2

Tryptic-digest mass spectrometric analysis of the 60 kDa protein band detected the following peptide sequences from the LKB1 amino-acid sequence. Twelve separate peptide fragments were detected in all. Prior to analysis, ctrl PEG-lysates were immunoprecipitated using anti-LKB1 antibody (Santa Cruz Biotech: (M-18), cat#: sc-5640). After gel electrophoresis, a Coomassie blue stain was applied to the gel and allowed to incubate over-night. Gel bands at the putative molecular weights were excised from the gel and analyzed by mass spectrometry.

12 Peptide Fragments Detected by Mass Spectrometry Align with the LKB1 Amino-acid Sequence

MDVADPEPLG LFSEGELMSV GMDTFIHRID STEVIYQPRR KRAKLIGKYL MGDLLGEGSY
GKVKEVLDSE TLCRRAVKIL KKKKLRRI PN GEANVKKEIQ LLRRLRHRNV IQLVDVLYNE
EKQKMYMVME YCVCGMQEML DSVPEKRFPV CQAHGYFRQL IDGLEYLHSQ GIVHKDIKPG
NLLLTTNGTL KISDLGVAEA LHPFAVDDTC RTSQGSPAFQ PPEIANGLDT FSGFKVDIWS
AGVTLYNITT GLYPFEGDNI YKLFENIGRG DFTIPDCGP PLSDLLRGML EYEPAKRFSI
RQIRQHSWFR KKHPLAEALV PIPSPDTKD RWRSM TVVPY LEDLHGRAEE EEEEDLFDIE
DGIIYTQDFT VPGQVLEEEV GQNGQSHSLP KAVCVNGTEP QLSSKVKPEG RPGTANPARK
VCSSNKIRRL SACKQQ

**Note: No peptide fragments detected in the 50 kDa protein band aligned with the LKB1 amino acid sequence.

Figure 3

FPLC fractions containing LKB1 protein show positive LKB1-MO25-STRAD associations, suggesting complete complex formation. Co-immunoprecipitation followed by Western blot assays demonstrated positive associations between all combinations of LKB1, MO25, and STRAD proteins. Representative blots were chosen based on the ctrl FPLC fraction exhibiting the highest protein detection. Ctrl and LKB1^{-/-} fractions were assayed side-by-side.

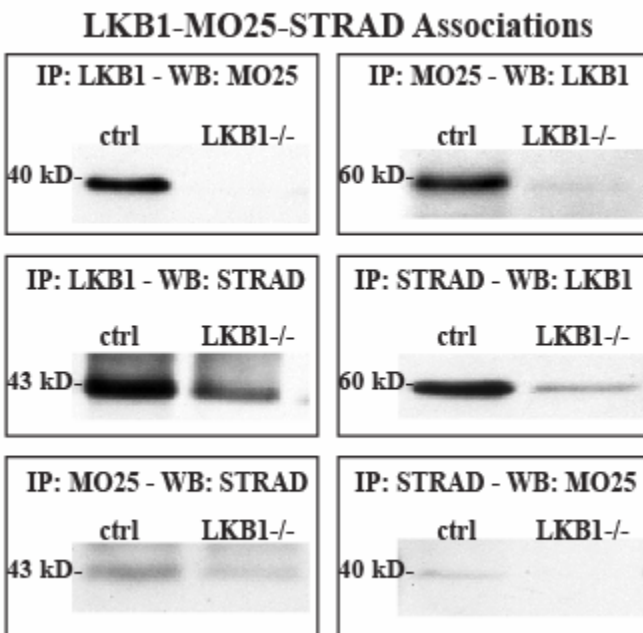


Figure 4

(A) CaMKK α and (B) CaMKK β protein was detected in untreated muscle lysates, demonstrating the presence of other AMPKK protein expression in skeletal muscle. Untreated muscle lysates were prepared by homogenizing mouse gastroc muscles in enough lysate buffer to make a 5% homogenate using a rotating pestle. * = $p < 0.05$ compared to ctrl.

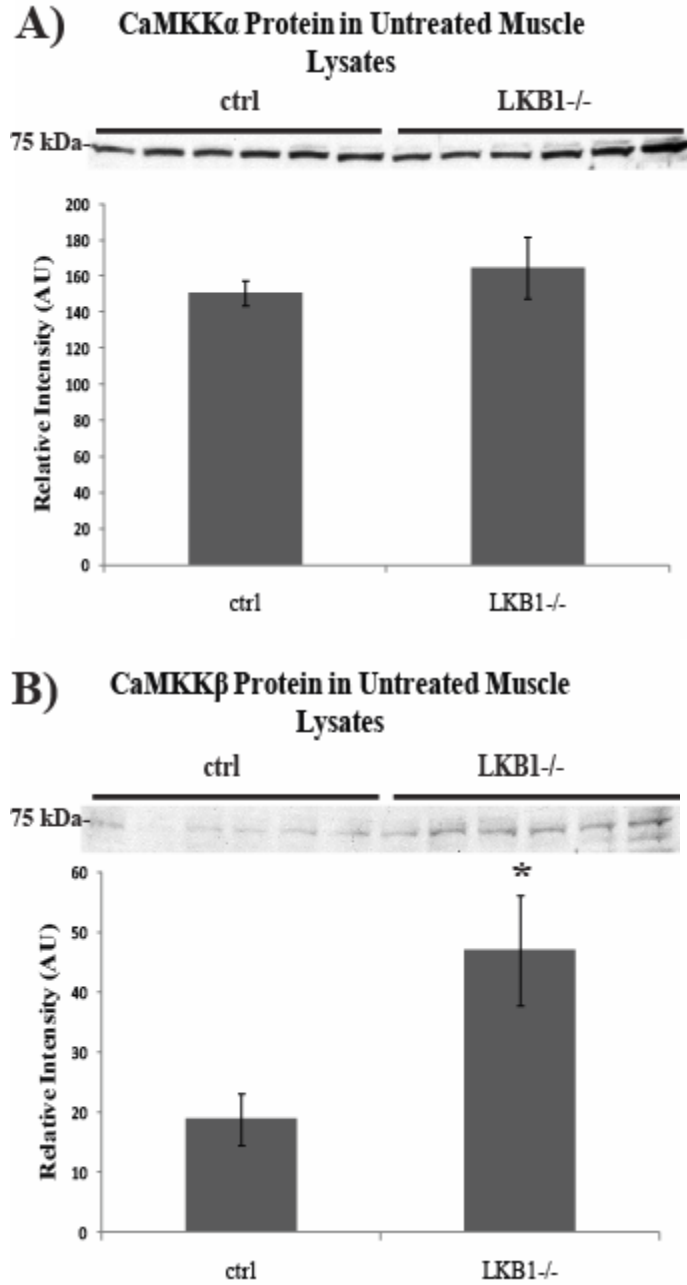
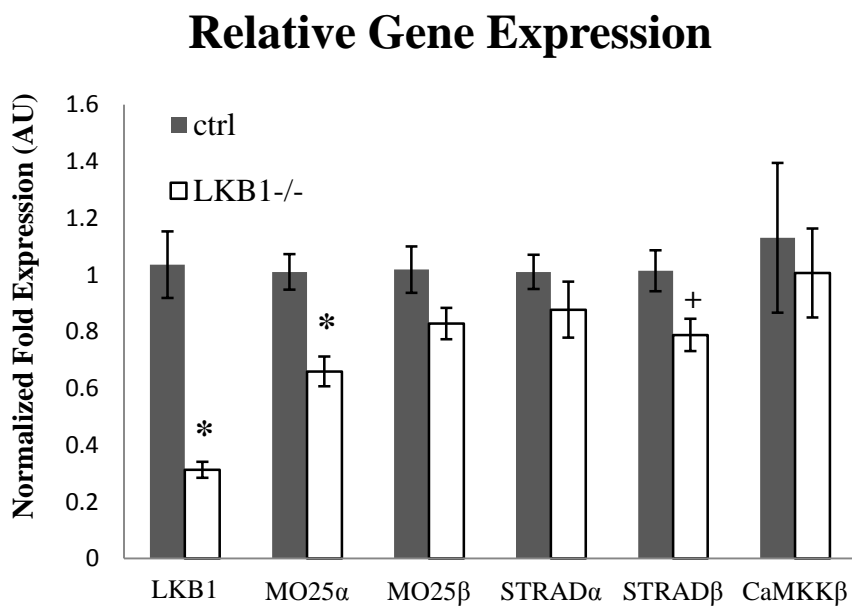


Figure 5

The relative gene expression profile in ctrl and LKB1^{-/-} skeletal muscle was determined using quantitative-PCR. Custom, gene-specific primers, yielding average amplicons of 100-200 bp in length were used to determine the relative mRNA expression levels of LKB1, MO25 α / β , STRAD α / β , and CaMKK β genes; 18S amplification was used as the reference for normalization calculations. Proper optimization procedures were performed prior to analysis as described in the experimental procedures section. * = p<0.01 compared to ctrl; + = p<0.05 compared to ctrl.



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Curriculum Vitae

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United States Citizen
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Education

MS, Physiology and Developmental Biology

December 2010

Brigham Young University, Provo, UT

Committee Members: Dr. William Winder (Chair), Dr. David Thomson, Dr. Barry Willardson

Thesis: Characterization of the LKB1-MO25-STRAD AMPKK complex in Adult Mouse Skeletal Muscle

Course work: Cellular Signal Transduction, Cell and Molecular Physiology, Renal/Gastrointestinal Physiology, and Mass Spectrometry.

BS, Physiology and Developmental Biology – Magna Cum Laude

April 2008

Brigham Young University, Provo, UT

Related course work: Advanced Physiology with Lab, Endocrinology, Histology, Pathophysiology, Pharmacology and drug mechanisms, Biochemistry, Developmental Biology, Advanced Cell Biology, and Biochemical methodology.

Work Experience

Research Assistant—Exercise Endocrinology/Metabolism

June 2008—Present

BYU Department of Physiology and Developmental Biology

Dr. William Winder

General focus: Characterization of the LKB1-MO25-STRAD AMPKK complex in adult mouse skeletal muscle, entailing: Identification of the protein components, confirmation of mRNA expression and identification of any possible splice variants, confirmation of protein-protein associations, and determination of the relative mRNA expression levels of the LKB1, MO25, and STRAD genes.

Methods used: Polyethylene glycol protein purification, Ion chromatography using fast protein liquid chromatography (FPLC), Immunoprecipitation followed by Western blot analysis, Mass spectrometry, Radiolabeled-ATP activity assays, RT-PCR, Gene sequencing, and Quantitative PCR.

Research Assistant – Inorganic Chemistry - Ion Chromatography

August 2007-June 2008

BYU Department of Chemistry and Biochemistry

Dr. John Lamb

General Focus: To study the inclusion properties of different macrocyclic molecules through ion chromatography. These macrocyclic structures are incorporated into ion exchange chromatography columns and are used to detect harmful electrolytes based on their retention properties within the macrocyclic compound. These macrocyclic structures are also used in drug microencapsulation.

Methods used: Routine ion chromatography and Acid-base titrations.

Journal Secretary –

August 2007-April 2008

BYU Department of Chemistry and Biochemistry
The Journal of Inclusion Phenomena and Macrocyclic Chemistry
Dr. John Lamb, Editor

Description: Performed many of the editorial duties under Dr. Lamb's direction: organized incoming manuscripts, invited reviewers, sent editorial decisions, and reviewed manuscripts.

Research Assistant – BAC Library of Llama Genome

January 2007-April 2007

BYU Department of Physiology and Developmental Biology
Dr. David Kooyman

General Focus: To create a bacterial artificial chromosome (BAC) library of the llama genome.

Methods Used: PCR, Ligations, Transfections, and PAGE.

College Chemistry Teaching Assistant –

August 2005-December 2005 & August 2006-May 2007

BYU Department of Chemistry and Biochemistry
Drs. John Lamb and Paul Savage

Description: Regularly taught college students principles of general inorganic and organic chemistry and assisted the professor during lectures and with grading students' work.

Publications

Regretfully I have not authored/co-authored any publications despite my research experience. However, I do expect to publish my research on the AMPKK complex by the end of this year.

Conferences Attended

American Diabetes Association 70th Scientific Session

Poster presentation of current AMPKK research

June 2010

Regional Meeting of the American Chemical Society

June 2008

Volunteer Work

Emergency Room Volunteer

June 2008-December 2009

Orem Community Hospital, Orem, UT

Volunteer Tutor – Inorganic and Organic Chemistry

January 2005-December 2009

BYU Center for Service and Learning, Provo, UT

Church Mission – Richmond, Virginia

September 2002-August 2004

The Church of Jesus Christ of Latter-day Saints

Awards

NIH Grant Research Stipends (7)

August 2008 – December 2010

Garth L. Lee Undergraduate Teaching Award

December 2006 & 2007

Eagle Scout Award

November 2001

Interests and Hobbies

Reading, Sports (Wakeboarding, Football, Basketball, Soccer, Jogging, etc.), Cooking, Camping, White-water rafting, and Traveling.