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Jenna Sands March 30, 2018

Expression of the RNA binding protein, PABPN1, is regulated by insulin in muscle cells: Implication for oculopharyngeal muscular dystrophy

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Abstract

Expression of the RNA binding protein, PABPN1, is regulated by insulin in muscle cells: Implication for oculopharyngeal muscular dystrophy

By Jenna Sands

Oculopharyngeal muscular dystrophy (OPMD) is a late onset muscular dystrophy characterized by drooping eyelids, difficulty swallowing, and loss of mobility caused by weakness in eyelid, pharyngeal, and proximal limb muscles. The cause of OPMD is an autosomal dominant GCN expansion mutation in the *PABPN1* gene, which encodes the ubiquitously-expressed, RNA binding protein polyadenylate-binding nuclear protein 1 (PABPN1). The GCN triplet repeat expands an N-terminal alanine tract in the PABPN1 protein. In patients with OPMD, the alanine tract expands from an existing 10 alanine tract to 11 to 18 alanines. How this remarkably modest change in a single allele of *PABPN1*, a gene encoding a ubiquitously-expressed protein, causes tissue-specific disease is not known. A previous study revealed that PABPN1 protein levels are much lower in skeletal muscle than in other tissues, and PABPN1 protein levels were even lower in muscled affected in OPMD. These findings suggested a loss of function model in which low PABPN1 protein levels could predispose tissue to OPMD pathology. Few studies have defined the mechanisms that modulate *Pabpn1* expression. Here, we investigate three insulin regulated pathways. These pathways are protein degradation, the mechanistic target of rapamycin pathway (mTOR) pathway that is a major regulator of translation, and the insulin/Glucose transporter type 4 pathway (GLUT4). Our preliminary results suggest that PABPN1 protein turnover is not regulated by insulin, but we uncovered interesting results about both the mTOR pathway and the GLUT4 pathway. Our work suggests that these pathways merit further investigation for potential contributions to regulation of PABPN1 expression.

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Table of Contents

Introduction

OPMD

Oculopharyngeal muscular dystrophy (OPMD) is a late onset muscular dystrophy characterized by drooping eyelids, difficulty swallowing, and loss of mobility caused by weakness in eyelid, pharyngeal, and proximal limb muscles. Complications from pharyngeal muscle weakness include malnutrition, increased risk of choking, and aspiration pneumonia. Proximal limb muscle weakness may necessitate use of a walker or wheelchair in extreme cases. OPMD symptoms usually present at around age 50 and worsen with age (1). There are no drug treatments currently available for individuals living with OPMD; only surgical intervention is available in extreme cases. The incidence of OPMD within the United States, specifically, is not known, but the majority of cases are diagnosed in patients of French-Canadian, Ashkenazi Jewish, or Spanish American background (2). The autosomal dominant form of the disease is most common in a population of Israeli Bukhara Jews, affecting about 1 in 600 individuals. Estimations suggest that OPMD affects about 1 in 1000 French-Canadian ancestry individuals and about 1 in 100,000 French individuals (2).

The cause of OPMD is an autosomal dominant GCN expansion mutation in the *PABPN1* gene, which encodes the ubiquitously-expressed, RNA binding protein polyadenylate-binding nuclear protein 1 (PABPN1) (3). The GCN triplet repeat expands an N-terminal alanine tract in the PABPN1 protein (1). In patients with OPMD, the alanine tract expands from an existing 10 alanine tract to 11 to 18 alanines (4). While the autosomal dominant form of OPMD is the most prevalent, a small portion of OPMD patients display an autosomal recessive mutation that expands this alanine tract by a mere one alanine (1). How this remarkably modest change in a

single allele of *PABPN1*, a gene encoding a ubiquitously-expressed protein, causes tissue-specific disease is not known.

PABPN1, which is expressed in all tissues, plays multiple roles in RNA processing (1). One of the most well-characterized functions of PABPN1 is a role in modulating polyadenylation of nascent mRNAs. PABPN1 interacts with poly(A) polymerase to ensure proper poly(A) tail length. In addition, PABPN1 plays a vital role in export of poly(A) RNA from the nucleus. PABPN1 has also been implicated in alternative polyadenylation and poly(A) site selection through a mechanism that proposes masking of weak poly(A) site signals to ensure that poly(A) tails are added to the correct portion of the nascent mRNA (1). The ubiquitously-expressed PABPN1 protein plays an important role in all cells; why mutation in the *PABPN1* gene causes disease only in a specific subset of skeletal muscles is not known.

Muscle is comprised of large, multinucleated, post-mitotic cells termed myofibers (5). These myofibers are formed by the differentiation and fusion of muscle satellite cells—a pool of stem cells that reside within muscle tissue (5). Satellite cells are primarily quiescent in mature muscle tissue. In response to injury, satellite cells can proliferate, differentiate, and fuse to form myofibers. Satellite cells also self-renew to maintain a pool surrounding the fibers after injury (5). Skeletal muscle can be studied though multiple approaches including in vivo and in culture. In vivo studies of muscle typically involve use of mouse models. Such studies are expensive and labor-intensive particularly when defining molecular mechanism. For mechanistic studies, cell culture models allow for identification of molecular mechanisms that can then be tested in vivo (6). The present study utilized C2C12 muscle cells, an immortalized cell line. C2C12 cells are mouse myoblasts derived from satellite cells that originated from limb muscle, and due to their

wide-use, have been extensively characterized by other researchers (7, 8). In culture, C2C12 cells can be induced to differentiate into myotubes, which serve as a cell culture model of myofibers (9). Thus, C2C12 cells can be used to study both proliferating myoblasts and as a model for differentiated muscle cells.

Though the molecular mechanisms are not clear, recent studies suggest that loss of PABPN1 can contribute to pathology in OPMD. A previous study revealed that PABPN1 protein levels are much lower in skeletal muscle than in other tissues (Figure 1A) (10). This study also showed that levels of PABPN1 were particularly low in muscles affected in OPMD (Figure 1B) (10). These findings have led to a loss of function model for OPMD pathogenesis, in which low PABPN1 levels in muscle may predispose this tissue to OPMD pathology (10). As illustrated in Figure 2, patients with OPMD could have levels of PABPN1 that fall below a crucial threshold in muscle due to the presence of one mutant *PABPN1* allele. Understanding the mechanisms that modulate PABPN1 levels would be vital to better understanding OPMD disease pathology and could eventually lead to development of novel treatments.

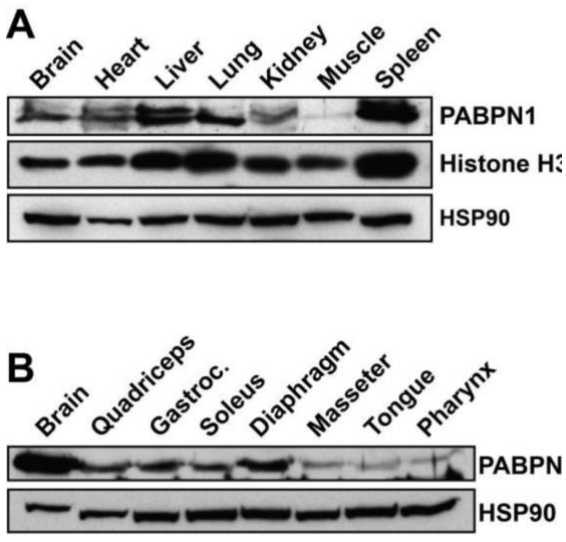

Image reproduced from Apponi, Corbett, Pavlath 2013.

Figure 1: Skeletal muscles have low levels of Nuclear poly(A) binding protein 1 (PABPN1). Immunoblots of lysates prepared from (A) different mouse tissues and (B) different mouse muscles, probed with anti-PABPN1 antibody. Loading controls used were histone H3 and heat shock protien 90 (HSP90).

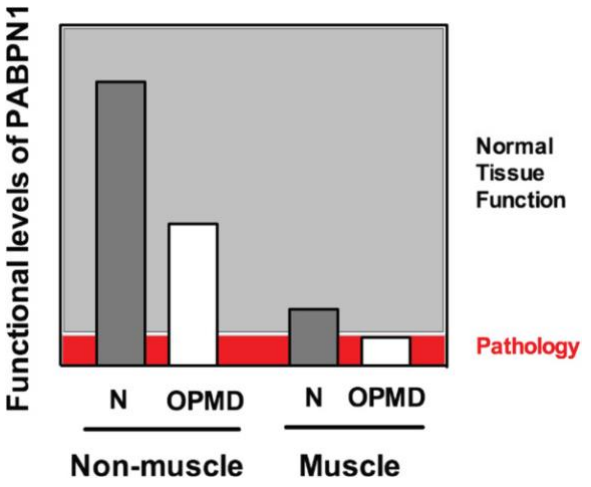

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Fig 2: Decreased levels of functional PABPN1 may lead to OPMD pathology. Unaffected muscle tissue has lower levels of PABPN1 than other tissues. However, this level of expression is sufficient for normal muscle function. In patients with OPMD, mutant PABPN1 could lead to a decrease in functional PABPN1 levels across all tissues. Muscle-specific pathology may occur if the functional level of PABPN1 falls below the necessary threshold to maintain normal tissue function only in muscle.

A previous study showed that PABPN1 is regulated through post-transcriptional mechanisms (10). Low levels of PABPN1 protein in muscle correlates with low levels of *Pabpn1* mRNA. Further analysis demonstrated that *Pabpn1* mRNA is unstable in skeletal muscle compared to other tissues not affected in OPMD, which suggests muscle-specific regulation of *Pabpn1* mRNA stability (10). Thus far, studies to define the mechanisms that regulate *Pabpn1* mRNA stability have identified the RNA binding protein HuR, an RNA binding protein and modulator of mRNA stability translational efficiency (11), as a negative regulator of *Pabpn1* expression, both in studies that employed cultured C2C12 cells and in a HuR knockout mouse

(6). These studies have begun to define mechanisms that regulate *Pabpn1* expression, but many questions remain about how PABPN1 expression is regulated in skeletal muscle.

Regulation of *Pabpn1* Expression by Insulin

While previous studies have explored mechanisms that regulate Pabpn1 mRNA stability (6), additional mechanisms likely contribute to tissue-specific expression of PABPN1. In fact, preliminary observations by the Corbett group suggest that PABPN1 expression is differentially regulated by the different media conditions employed to differentiate C2C12 myoblasts into myotubes.

Many C2C12 differentiation protocols include the use of insulin-transferrin-sodium selenite (ITS) (12). Differences in steady-state PABPN1 protein levels when C2C12 myoblasts were differentiated with and without ITS were observed by members of the Corbett group, which suggests that one of the components of ITS could regulate PABPN1 expression. Preliminary unpublished observations showed that when C2C12 myoblasts are differentiated into mature myotubes in the presence of ITS, steady-state PABPN1 protein levels are higher than in myotubes differentiated in the absence of ITS. Preliminary investigation compared the effects on steady-state PABPN1 protein levels when C2C12 myoblasts were differentiated in the presence of inulin, transferrin, and sodium-selenite individually. Neither transferrin nor sodium selenite affected PABPN1 levels (data not shown). These data suggested that insulin was most responsible for the increase in steady-state PABPN1 levels observed in the presence of ITS. This serendipitous observation provides another opportunity to study the mechanisms modulating PABPN1 levels. By investigating the mechanism by which insulin increases steady-state

PABPN1 protein levels, we can provide insight into the molecular mechanisms that regulate *Pabpn1* expression.

My goal for this project is to investigate which pathway(s) insulin modulates to increase steady-state PABPN1 levels. I hypothesized that insulin modulates PABPN1 protein levels by controlling PABPN1 protein turnover or that an insulin-dependent pathway modulates PABPN1 protein levels. While there are multiple mechanisms by which insulin can regulate various cellular processes (13), I investigated three insulin regulated pathways, which are summarized in figure 3. These pathways are protein degradation (14-19), the mechanistic target of rapamycin pathway (mTOR) pathway that is a major regulator of translation (20), and the insulin/Glucose transporter type 4 pathway (Figure 3) (21). My preliminary results suggest that PABPN1 protein turnover is not regulated by insulin, but I uncovered interesting results about both the mTOR pathway and the Glucose transporter type 4 pathway. My work suggests that these pathways merit further investigation for potential contributions to regulation of PABPN1 expression.

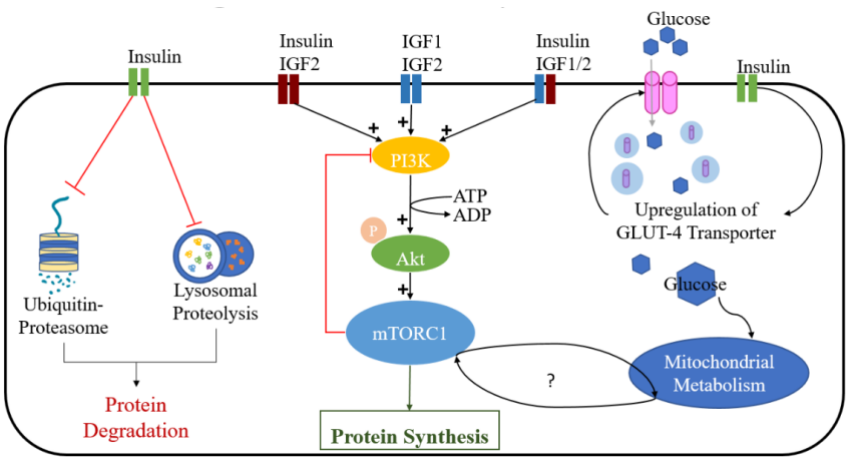

Figure 3: Schematic of investigated pathways. This paper investigates three insulin regulated pathways. From left to right: protein degradation, the mechanistic target of rapamycin pathway (mTOR), and the insulin/Glucose transporter type 4 pathway.

Materials and Methods

Cell culture

The mouse myoblast cell line C2C12 (ATCC CRL-1772) was used in this study. C2C12 myoblasts were maintained in a humidified incubator with 5% CO_2 at 37°C. Mouse C2C12 myoblasts were cultured in C2C12 growth media, also referred to as high glucose media (Dulbecco's Modified Eagle's Medium [DMEM] with 4.5 g/L glucose, 10% fetal bovine serum (FBS), 100 U/mL streptomycin, 100 U/mL penicillin). To induce differentiation, C2C12 myoblasts were plated on dishes coated with Entactin-Collagen IV-Laminin (ECL; Upstate Biotechnology) in C2C12 differentiation media that was changed every three days. A 6-day differentiation protocol was used in which cells were differentiated in DMEM with 4.5 g/L glucose, 1% horse serum, 100 U/ml streptomycin, and 100 U/ml penicillin. To condition with insulin, C2C12 myoblasts were cultured in the appropriate C2C12 media (high glucose or low glucose) with 10 µg/mL insulin (Sigma-Aldrich) for four days prior to use in experiments. This same procedure was used for Insulin-Transferrin-Sodium Selenite (ITS) conditioning, using 10 µg/mL insulin, 5.50 µg/mL transferrin, and 6.7 ng/mL sodium selenite (Gibco). To test the effect of glucose, C2C12 myoblasts were cultured in low glucose media (Dulbecco's Modified Eagle's Medium [DMEM] with 1.0 g/L glucose, 10% FBS, 100 U/mL streptomycin, 100 U/mL penicillin).

Immunoblotting

Cells lysis was performed using standard methods (22). After lysis/homogenization, samples were sonicated at 30% output for 10s to shear chromatin. A Bradford assay (BioRad) was used to determine protein concentrations, and equal amounts of protein were boiled in

reducing sample buffer (250 mM Tris-HCl pH 6.8, 500 mM DTT, 10% SDS, 0.5% Bromophenol Blue, 50% glycerol) and resolved using 4-20% Criterion TGX polyacrylamide gels (BioRad). Proteins were transferred to 0.2 µm nitrocellulose membranes, then the total protein loaded across samples was assessed by staining the membranes with Ponceau S Solution (Sigma-Aldrich). Next, membranes were blocked in 10% nonfat dry milk in Tris-buffered saline at pH 7.4 with 0.1% Tween-20 (TBS-T). Primary antibodies were diluted in 5% nonfat milk in TBS-T and incubated overnight at 4°C. Species-specific horseradish peroxidase conjugated secondary α-IgG antibodies (Jackson ImmunoResearch Laboratories) were used to detect primary antibodies, followed by enhanced chemiluminescence substrate (ECL, Sigma). Blots were exposed to autoradiography film to detect chemiluminescence. The following antibody concentrations were used: α-PABPN1 (1:4000) (22), α-eMyHC F1.652 (1:100, Developmental Studies Hybridoma Bank, Iowa City, Iowa) (23), and α-Heat Shock Protein 90 (HSP90) (1:5000, Santa Cruz). Image J software was used to quantify Ponceau staining and the immunoblots. To normalize protein loading, Ponceau stain was quantified across a region of the membrane with similar staining patterns for all lanes.

Protein stability assay

Translation was inhibited by treating cells with 50 µg/mL cycloheximide dissolved in sterile dimethyl sulfoxide (DMSO). For insulin conditioned cells, cycloheximide treatment and insulin were added to C2C12 myoblast cultures simultaneously. C2C12 cells were lysed after 15 minutes, 8 hours, 12 hours, and 24 hours following cycloheximide treatment. Protein lysates were prepared and immunoblot performed as described above. Changes in protein level were calculated relative to PABPN1 protein levels at the 15-minute time point for C2C12 myoblasts.

Each data point represents the mean of two technical replicates. HSP90 was used as a positive control for Cycloheximide treatment.

Rapamycin-mediated inhibition of mTOR pathway

Both insulin-conditioned and untreated C2C12 myoblasts were treated with 20 μM Rapamycin (Sigma-Aldrich) for 24 hours. Protein lysates were prepared and immunoblot performed as described above, using a digital imager to detect chemiluminescence. Image J software was used to quantify Ponceau staining and the immunoblots. Data for one technical replicate are displayed as arbitrary densitometry units normalized to ponceau.

RNA preparation, cDNA and qRT-PCR

Total RNA was isolated using TRIzol reagent (ThermoFisher Scientific) and treated with DNase I, Amplification Grade (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using the M-MLV reverse transcriptase kit (Invitrogen) and Rnasin (Promega). Approximately 10 ng of cDNA was mixed with appropriate primers and SYBR Select Master Mix (Applied Biosciences) for qRT-PCR analysis. Samples were analyzed using the comparative Ct method (24) on an Applied Biosciences Step One Real Time PCR System. Samples were normalized to actin as indicated.

Evaluation of normalizers for qRT-PCR

Standard laboratory methods utilized *Gapdh*, a gene encoding for a metabolic protein, as a normalizer for qRT-PCR analysis (6). However, preliminary data demonstrated that *Gapdh* mRNA fluctuated with experimental treatment. A systematic testing of three different normalizer options was performed (Figure 4), comparing Ct values of candidate primers. Samples from

untreated, insulin-conditioned, and ITS-conditioned C2C12 myoblasts were prepared for qRT-PCR analysis as described above, and utilized for this analysis. Based upon these findings, actin was used as the normalizer in all experiments.

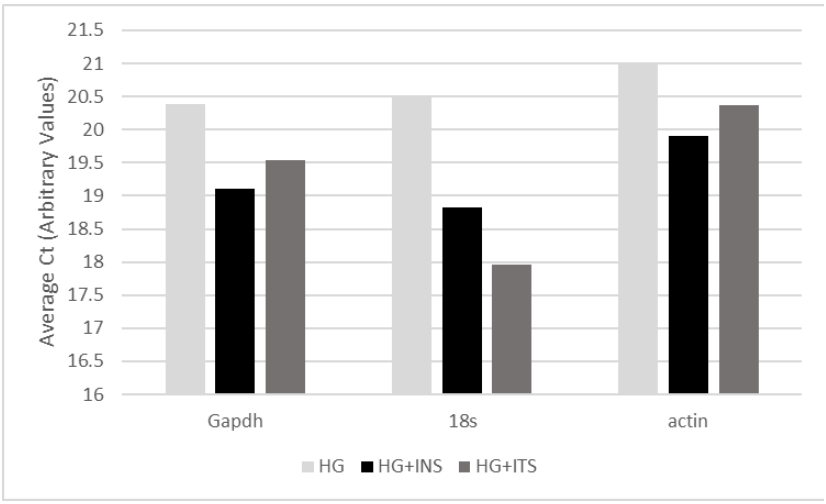

Figure 4: Comparison Ct values of candidate primers. Samples from untreated, insulin-conditioned, and ITS-conditioned C2C12 myoblasts were prepared for qRT-PCR analysis, and analyzed according to three cadidate primers: Gapdh, 18s, and actin. Bars represent the average C_T values for one technical replicate plated in triplicate to reduce pipetting error.

Data analysis

For all experiments, raw data are normalized to the appropriate values (Ponceau stain for immunoblots, Actin for qRT-PCR) as indicated. The average normalized values for control samples (C2C12 myoblasts, untreated C2C12 myoblasts, untreated C2C12 myoblasts cultured in C2C12 high glucose media) was set to 1, and all data are presented as values relative to this, unless indicated otherwise.

Statistical analysis

Non-preliminary data are presented as mean ± standard error (n), where n indicates the number of technical replicates. For all experiments, statistical analyses were performed using Microsoft Excel. Student's t-test was used for all experiments. In all cases, $P < 0.05$ is considered statistically significant.

Results

Insulin Increases Steady-State PABPN1 Protein Levels in C2C12 Cells

Steady-state PABPN1 protein levels were analyzed in both untreated control and insulin-conditioned C2C12 myoblasts. Results of this analysis (Figure 5) show a significant increase ($p = 0.007$) in steady-state PABPN1 protein when C2C12 myoblasts are treated with insulin.

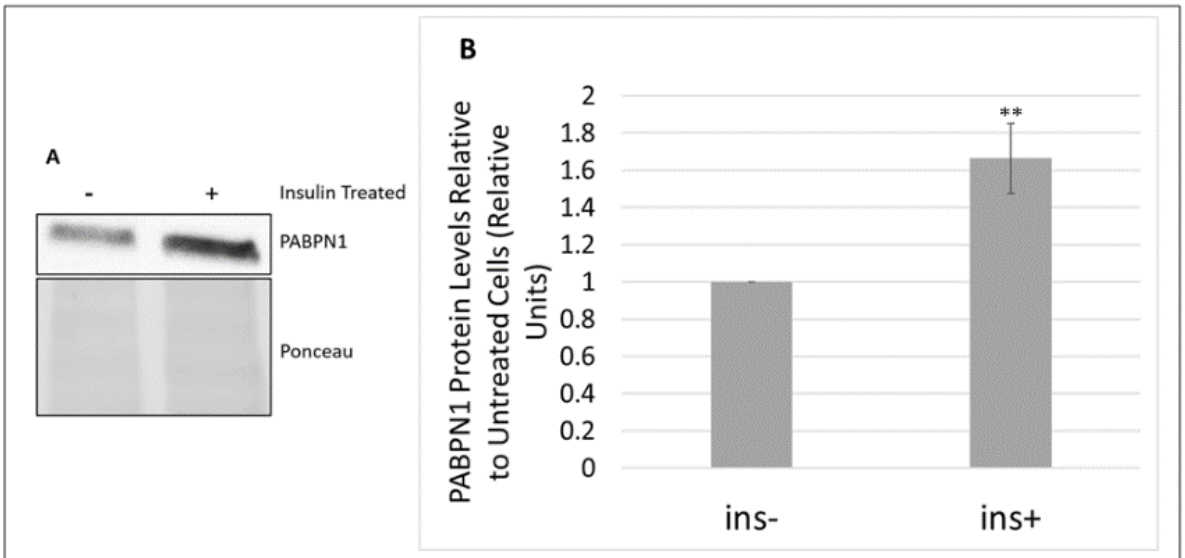

Figure 5: Insulin Increases Steady-State PABPN1 Protein Levels. C2C12 myoblasts (MB) were cultured for 4 days in high glucose DMEM in the presence (+) or absence (-) of 10 µg/mL insulin. Cells were collected and washed with phosphate buffered saline (PBS), and then lysed in RIPA-2+protease inhibitor. A) Immunoblot of C2C12 protein lysates showing C2C12 MB treated with insulin (+) have a higher steady-state level of PABPN1 protein than untreated (-) cells. The corresponding ponceau stain for total protein serves as a loading control. This blot is representative of five replicates. B) Quantification of five immunoblots as shown in (A). The level of PABPN1 in untreated (ins-) cells was set to 1.0 and compared to the insulin-treated (ins+) cells. Error bars represent the standard error of five experiments. Statistical significance was determined using Student's T-test, assuming equal variance (p-value = 0.007).

Insulin Modulates PABPN1 Protein Levels

Results shown in Figure 5 demonstrate that PABPN1 protein levels increase when myoblasts are treated with insulin. To assess how insulin regulates steady-state PABPN1 protein levels, I analyzed the steady-state *Pabpn1* mRNA levels. Control untreated and insulin-conditioned C2C12 myoblasts were collected, and the steady-state levels of *Pabpn1* mRNA was measured using qRT-PCR. As shown in Figure 6, no statistical difference was observed in the steady-state level of *Pabpn1* mRNA levels in untreated and insulin-conditioned C2C12 myoblasts ($p = 0.41$).

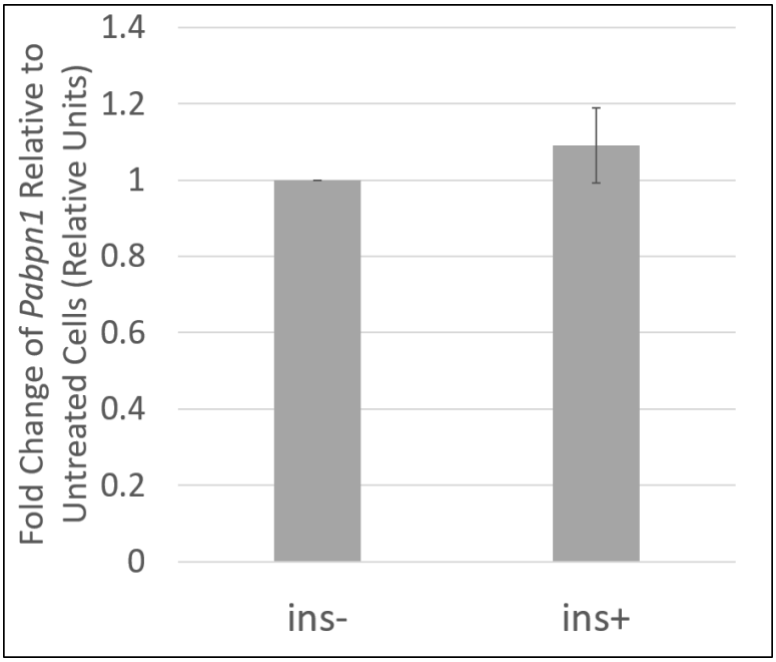

Figure 6: Insulin Does Not Significantly Change *Pabpn1* mRNA Levels. C2C12 myoblasts (MB) were cultured for 4 days in high glucose DMEM in the presence (ins+) or absence (ins-) of 10 µg/mL insulin. Cells were collected and washed with PBS, and then lysed in TRIzol reagent. mRNA was isolated and reverse transcribed into cDNA, which was used for RT-qPCR analysis. Samples were analyzed using comparative Ct method and normalized to actin mRNA. Fold change was calculated relative to untreated cells. Bars represent the average of three replicates, with error bars representing standard error. Student's t-test was used to determine statistical significance (p-value = 0.41)

Insulin Does Not Affect PABPN1 Protein Stability

Several studies have demonstrated that insulin decreases the activity of the two major eukaryotic protein degradation pathways, proteosome-dependent protein turnover and lysosomal turnover (14-19). To investigate whether the observed increase in steady-state PABPN1 is due to a decrease in the rate of protein degradation, the effect of insulin on PABPN1 protein stability was investigated. To exmaine the stability of the PABPN1 protein, cells were treated with cycloheximide to block translation, samples were collected over time and PABPN1 levels were analyzed by immunoblotting. This experimental approach allowed me to examine the rate of degradation of PABPN1 protein in the absence and presence of insulin. As shown in Figure 7, our preliminary data from two technical replicates show no statistical difference in PABPN1 stability in samples treated with insulin as compared to untreated samples. As a control, we examined heat shock protein 90 (HSP90), which shows a change in protein stability over the time course of the experiment. The HSP90 result confirms that cycloheximide inhibited translation in this experiment.

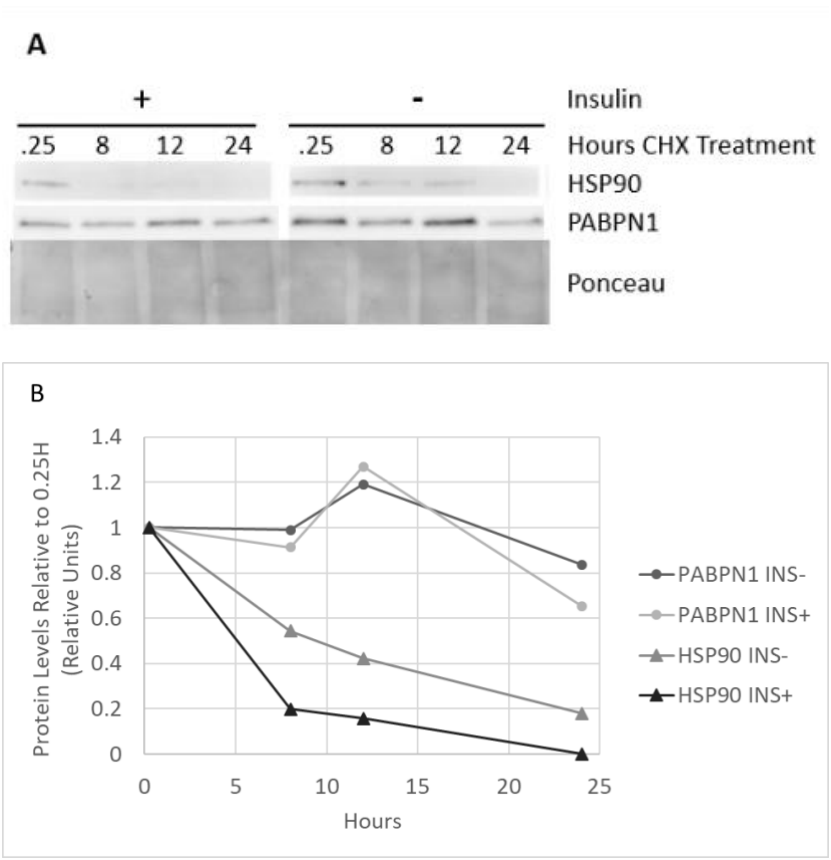

Figure 7: Insulin Does Not Affect PABPN1 Protein Stability. C2C12 myoblasts (MB) were cultured for 4 days in high glucose DMEM in the presence (+) or absence (-) of 10 µg/mL insulin. After 4 days of insulin conditioning, cells were treated with 50 µg/mL cycloheximide (CHX), with insulin conditioned cells being treated with insulin and CHX simultaneously. Cells were collected at 15 minute (0.25 hour), 8 hour, 12 hour, and 24 hour time points. Immunoblotting was used to quantify protein levels at the indicated timepoints. Heat shock protein 90 (HSP90) was used as a positive control for CHX treatment. A) Immunoblot and corresponding ponceau stain of C2C12 MB protein lysates after CHX treatment. B) Quantification of immunoblot. Each point represents the average protein level relative to the condition's (INS- / INS+) 15 minute time point.

Insulin May Regulate PABPN1 Steady-state Protein Levels through the PI3K/Akt/mTOR Pathway

The PI3K/Akt/mTOR pathway is a pathway downstream of insulin binding to the insulin receptor at the cell surface and can lead to an increase in protein synthesis (20). To investigate

whether insulin modulates PABPN1 protein levels through the mTOR pathway, I tested whether treatment of C2C12 myoblasts with rapamycin, an mTOR inhibitor, alters the insulin-dependent increase in the steady-state PABPN1 protein levels. As described in Materials and Methods, I treated insulin-conditioned and untreated cells with 20 µM rapamycin to inhibit the activity of the mTOR protein complex 1 (20). C2C12 myoblasts were cultured with rapamycin for 24 hours. Immunoblot was used to measure protein levels after rapamycin treatment. The preliminary data from one technical replicate suggest that rapamycin treatment decreases PABPN1 levels (Figure 8). However, PABPN1 protein levels in insulin-conditioned C2C12 myoblasts decreases by a comparable amount as in control cells. Further experiments will be required to determine whether mTOR signaling modulates PABPN1 protein levels.

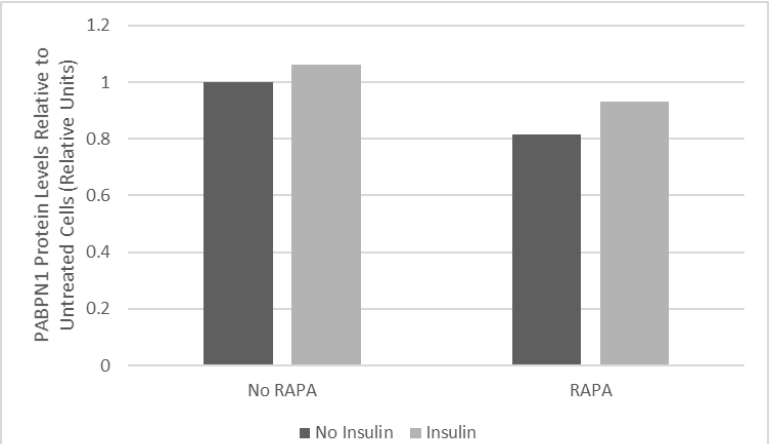

Figure 8: Rapamycin may decrease PABPN1 protein levels independent of insulin. C2C12 myoblasts (MB) were cultured for 4 days in high glucose DMEM in the presence or absence of 10 µg/mL insulin. After 4 days of insulin conditioning, cells were treated with 20 µM rapamycin. Cells were cultured for 24 hours in rapamycin media. Cells were lysed in RIPA-2 + protease inhibitor. Immunoblotting was used to quantify amount of protein in samples. Values represent the densitometry of PABPN1 after normalization to total protein as assessed by ponceau staining, bars represent the results of one technical replicate.

Glucose Decreases Steady-State PABPN1 Protein Levels in C2C12 Myoblasts

A well-characterized function of insulin is upregulation of the glucose transporter type 4 (GLUT4) protein, which then increases the rate at which cells import glucose (21). I investigated whether the increase in PABPN1 protein is a direct repsonse to insulin or a downsteam

consequence of changes in intracellular glucose concentration. To determine the role of glucose import in regulating PABPN1, I analyzed steady-state PABPN1 protein levels comparing C2C12 myoblasts cultured in high glucose media to C2C12 myoblasts cultured in low glucose media. As shown in Figure 9, steady-state PABPN1 protein levels increased when C2C12 cells were cultured in low glucose media, compared to high glucose media; however, more replicates are needed to determine whether this change is statistically significant.

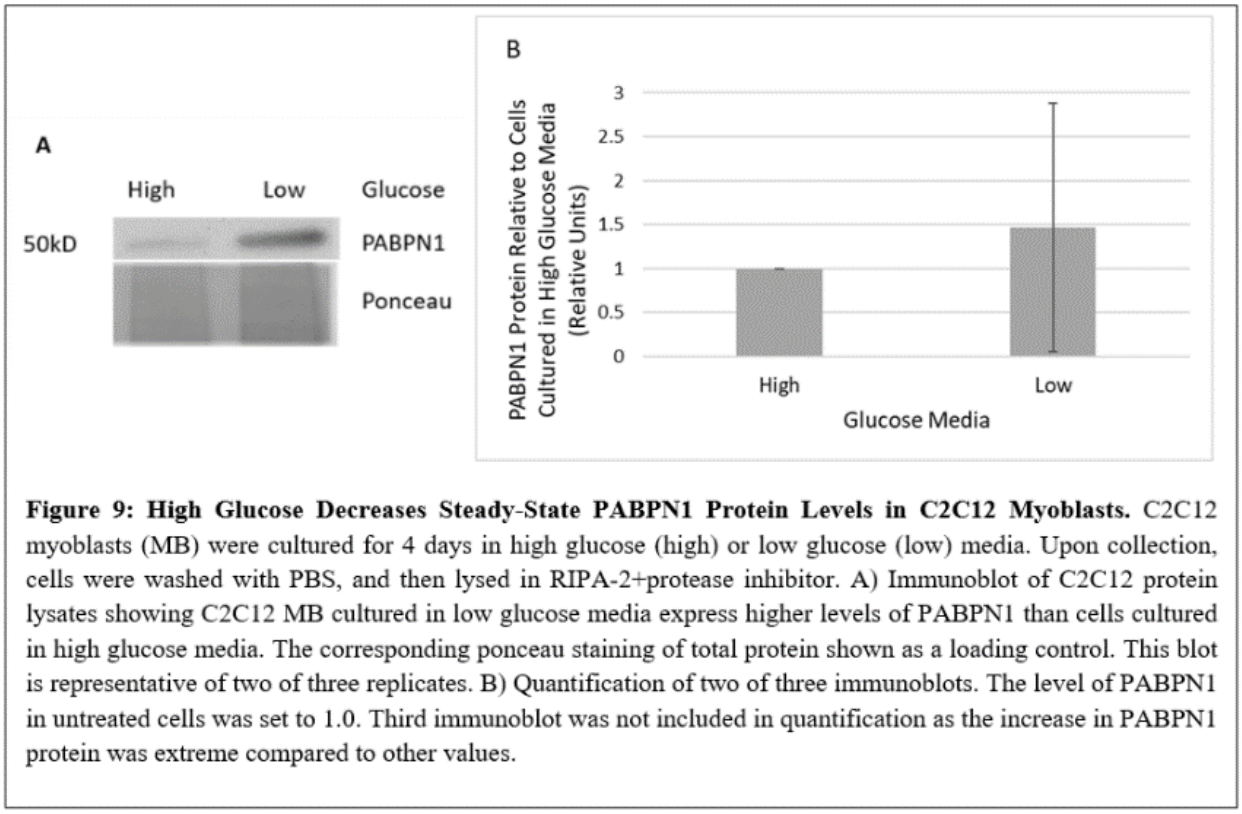

Figure 9: High Glucose Decreases Steady-State PABPN1 Protein Levels in C2C12 Myoblasts. C2C12 myoblasts (MB) were cultured for 4 days in high glucose (high) or low glucose (low) media. Upon collection, cells were washed with PBS, and then lysed in RIPA-2+protease inhibitor. A) Immunoblot of C2C12 protein lysates showing C2C12 MB cultured in low glucose media express higher levels of PABPN1 than cells cultured in high glucose media. The corresponding ponceau staining of total protein shown as a loading control. This blot is representative of two of three replicates. B) Quantification of two of three immunoblots. The level of PABPN1 in untreated cells was set to 1.0. Third immunoblot was not included in quantification as the increase in PABPN1 protein was extreme compared to other values.

To analyze the mechanism by which glucose regulates PABPN1 protein levels, I performed qRT-PCR to examine whether *Pabpn1* mRNA levels change in response to glucose. No significant difference was observed in the steady-state level of *Pabpn1* mRNA of C2C12 myoblasts cultured in high glucose and those cultured in low glucose media ($p = 0.77$) (Figure 10).

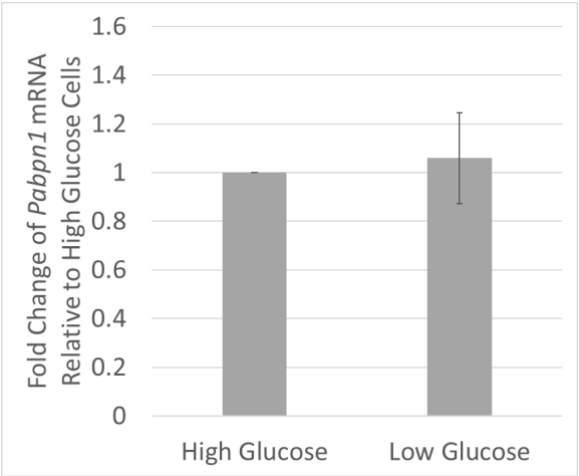

Figure 10: High Glucose Does Not Affect *Pabpn1* mRNA Levels. C2C12 myoblasts (MB) were cultured for 4 days in high glucose or low glucose media. Upon collection, cells were washed with phosphate buffered saline (PBS), and then lysed in TRIzol Reagent. mRNA was isolated and reverse transcribed into cDNA, which was used for RT-qPCR analysis. Samples were analyzed using comparative Ct method and normalized to actin. Fold change was calculated relative to cells cultured in high glucose media. Bars represent the average of three replicates, with error bars representing standard error. Student's t-test was used to determine statistical analysis (p-value = 0.77).

As steady-state PABPN1 protein levels change in response to glucose (Figure 9), I investigated whether glucose and insulin act independently to modulate PABPN1 protein levels. I compared steady-state PABPN1 protein levels of C2C12 myoblasts cultured in high glucose and low glucose media when insulin-conditioned and untreated. Steady-state PABPN1 protein levels increase when insulin is added in both high glucose and low glucose media; however, more replicates are needed to determine statistical significance of this result low glucose media samples (Figure 11).

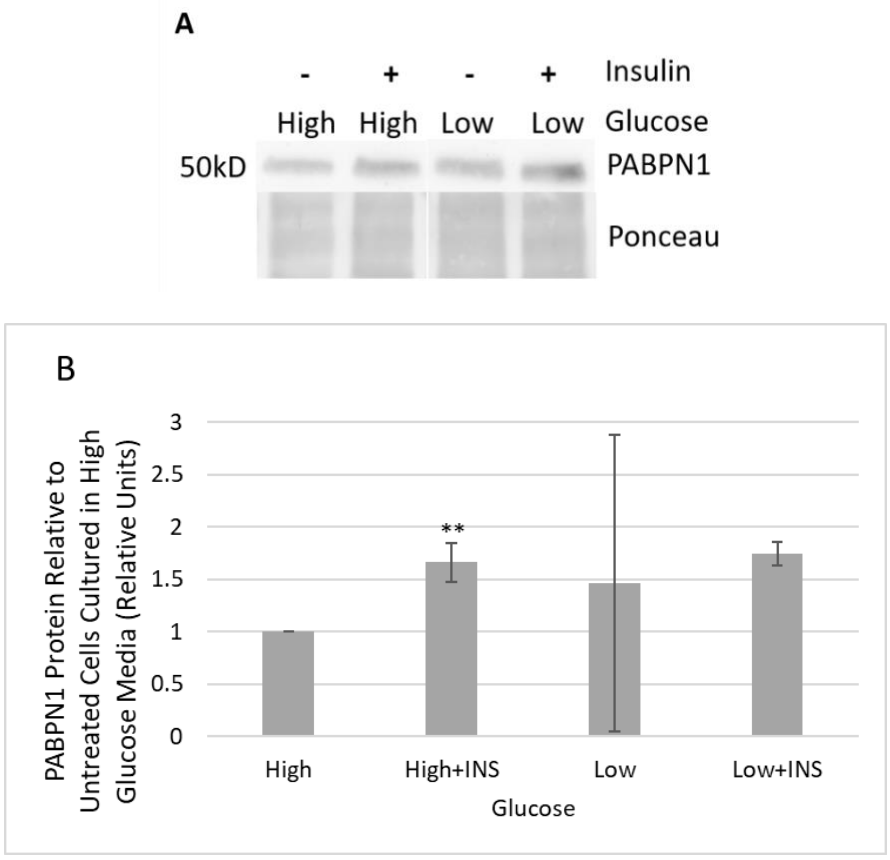

Figure 11: Insulin Increases Steady-State PABPN1 Protein Levels in C2C12 Myoblasts Independent of Glucose Levels. C2C12 myoblasts (MB) were cultured for 4 days in high glucose (HG) or low glucose (LG) media in the presence (+) or absence (-) of 10 µg/mL insulin. Cells were collected and washed with PBS and then lysed in RIPA-2+protease inhibitor. A) Immunoblot of C2C12 protein lysates showing C2C12 MB treated with insulin show an increase in steady-state PABPN1 levels as compared to untreated cells, independent of media glucose concentration (High or Low). The corresponding ponceau staining of total protein serves as a loading control. This blot is representative of three replicates. B) Quantification of three immunoblots as shown in (A). The level of PABPN1 in untreated cells was set to 1.0. Error bars represent the standard error of three experiments. Significance measured using Student's T-test, assuming equal variance.

Discussion

Over the course of this study, I investigated three well-characterized insulin regulatory pathways (Figure 12). Here I will discuss the results of my investigation of these three pathways: protein degradation, the mTOR pathway, and the insulin/GLUT4 pathway. These preliminary results suggest two pathways, the mTOR pathway and glucose regulation, that with further investigation may provide insight into how expression of PABPN1 is regulated.

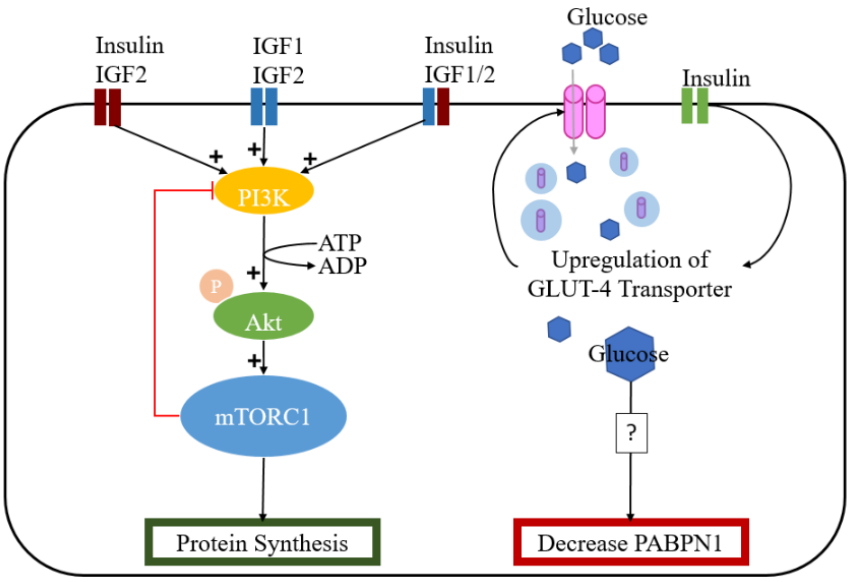

Figure 12: Involvement of investigated pathways in PABPN1 protein regulation. Preliminary results suggest two pathways, the mTOR pathway and glucose regulation, may provide insight into how expression of PABPN1 is regulated with further investigation.

Protein Degradation

Multiple studies have demonstrated that insulin decreases the activity of the two major eukaryotic degradation pathways: the ubiquitin-proteasome pathway and lysosomal proteolysis (14-19). In C2C12 myotubes, insulin treatment produced suppressed levels of ubiquitin conjugating enzymes essential for protein degradation (15). A separate study demonstrated that the activity of two lysosomal proteases, cathepsins B and L, has been shown to significantly

decrease in C2C12 myotubes after insulin treatment (18). I investigated whether the insulin-induced increase in steady-state PABPN1 protein was due to a decrease in protein turnover. To do this, I measured the stability of PABPN1 in both untreated and insulin-conditioned C2C12 myoblasts. The preliminary data suggest that insulin does not alter the stability of the PABPN1 protein in C2C12 myoblasts. Therefore, these findings suggest that the insulin-dependent increase in steady-state PABPN1 protein is not due to a decrease in PABPN1 protein degradation. This also suggests that insulin is regulating the expression of PABPN1 at a point prior to protein degradation.

Mechanistic Target of Rapamycin (mTOR) Pathway

The PI3K/Akt/mTOR pathway is a well-characterized downstream target of insulin signaling that positively regulates proliferation, cell growth, protein synthesis, lipid synthesis, and mitochondrial metabolism and biogenesis (20). I investigated whether the insulin-dependent increase in steady-state PABPN1 protein levels is due to upregulation of the mTOR pathway. To examine the mTOR pathway, I used rapamycin, which inhibits mTOR complex 1 (mTORC1) (20). The preliminary data suggest that rapamycin treatment decreases PABPN1 protein levels, but this decrease was approximately equal among untreated and insulin-conditioned C2C12 myoblast samples. Repetition of this experiment is necessary before any final conclusions can be made. However, these preliminary data suggest that insulin regulates PABPN1 protein independent of the PI3K/Akt/mTOR pathway. mTOR signaling is complex (20), and technical repeats with specific controls, such as measuring phosphorylated Akt and phosphorylated S6K1, are vital to investigating the role of this signaling pathway. Akt is a protein upstream of mTORC1, and its phosphorylation is inhibited after mTORC1 is activated. S6K1 is a protein

downstream of mTORC1, and is phosphorylated after mTORC1 activation. Repetitions of this experiment should suggest an increase in phosphorylated Akt and a decrease in phosphorylated S6K1 to confirm that rapamycin-mediated inhibition of the mTOR pathway was successful. The preliminary data suggest an effect of rapamycin on PABPN1 protein levels. Such controls would aid in determining the mechanisms causing this effect.

Glucose

One well-characterized function of insulin is increasing the amount of the glucose transporter type 4 (GLUT4) protein that is present at the cell surface (21). This increase in cell surface transporter allows faster glucose uptake and leads to an increase in intracellular glucose content. I investigated whether the insulin-dependent increase in steady-state PABPN1 protein levels is due to the insulin itself or due to the downstream increase in intracellular glucose. To perform this analysis, I compared the steady-state PABPN1 protein levels in C2C12 myoblasts cultured in high glucose and low glucose media. Steady-state PABPN1 protein levels were higher in samples cultured in low glucose media as compared to those samples cultured in high glucose. Thus, the data suggest that glucose decreases PABPN1 protein levels. I then measured the fold change of *Pabpn1* mRNA in C2C12 myoblasts cultured in both high glucose and low glucose media. No significant difference was observed in the steady-state levels of *Pabpn1* mRNA in C2C12 myoblasts cultured in high glucose and those cultured in low glucose media. This suggests that glucose regulates the PABPN1 protein independent of mRNA. These observations provide a novel method to study the mechanisms regulating PABPN1 protein in muscle.

Future Directions

My preliminary results suggest some very interesting future directions that can provide insight into how expression of PABPN1 is regulated. Some of the results shown need to be repeated to make definitive conclusions. For example, my initial results with rapamycin do not exclude or confirm regulation through the mTOR pathway. Due to the lack of sufficient controls in the experiment, it is impossible to determine if the mTOR pathway was inhibited by rapamycin treatment. There is variability in the susceptibility of mTOR to rapamycin (20), so the insulin-induced PABPN1 increase may be mediated through the rapamycin resistant activity of mTOR.

I would also investigate whether insulin alters *Pabpn1* translation, leading to the observed increase in steady-state PABPN1 protein levels. As my results show that PABPN1 protein stability is not altered, the most logical assumption is that translation could be more efficient. To examine translation, I would use polysome profiling. The polysome is the portion of the ribosome that is responsible for active translation of mRNA. Polysome profiling would be carried out using previously published methods for insulin-conditioned and untreated cells (25). The amount of *Pabpn1* transcript attached to the polysomes would be compared across samples. Additionally, such experiments could be used to investigate whether insulin regulates PABPN1 at the level of translation.

All the results described are from studies performed in C2C12 myoblasts. One goal is to repeat these experiments in C2C12 myotubes, which are a model more analogous to mature muscle tissue than C2C12 myoblasts. By differentiating C2C12 myoblasts into myotubes, I confirmed that PABPN1 protein levels are lower in myotubes than in myoblasts (Figure 13),

which is consistent with the finding that PABPN1 protein levels are low in skeletal muscle compared to tissues that are not affected in OPMD (10). A critical step future studies will be testing whether the preliminary results that I obtained studying myoblasts extend to myotubes and ultimately to muscle in vivo. These findings serve as proof of principle that within the C2C12 model, these experiments can be performed to begin to explore mechanisms that regulate the expression of the PABPN1 protein.

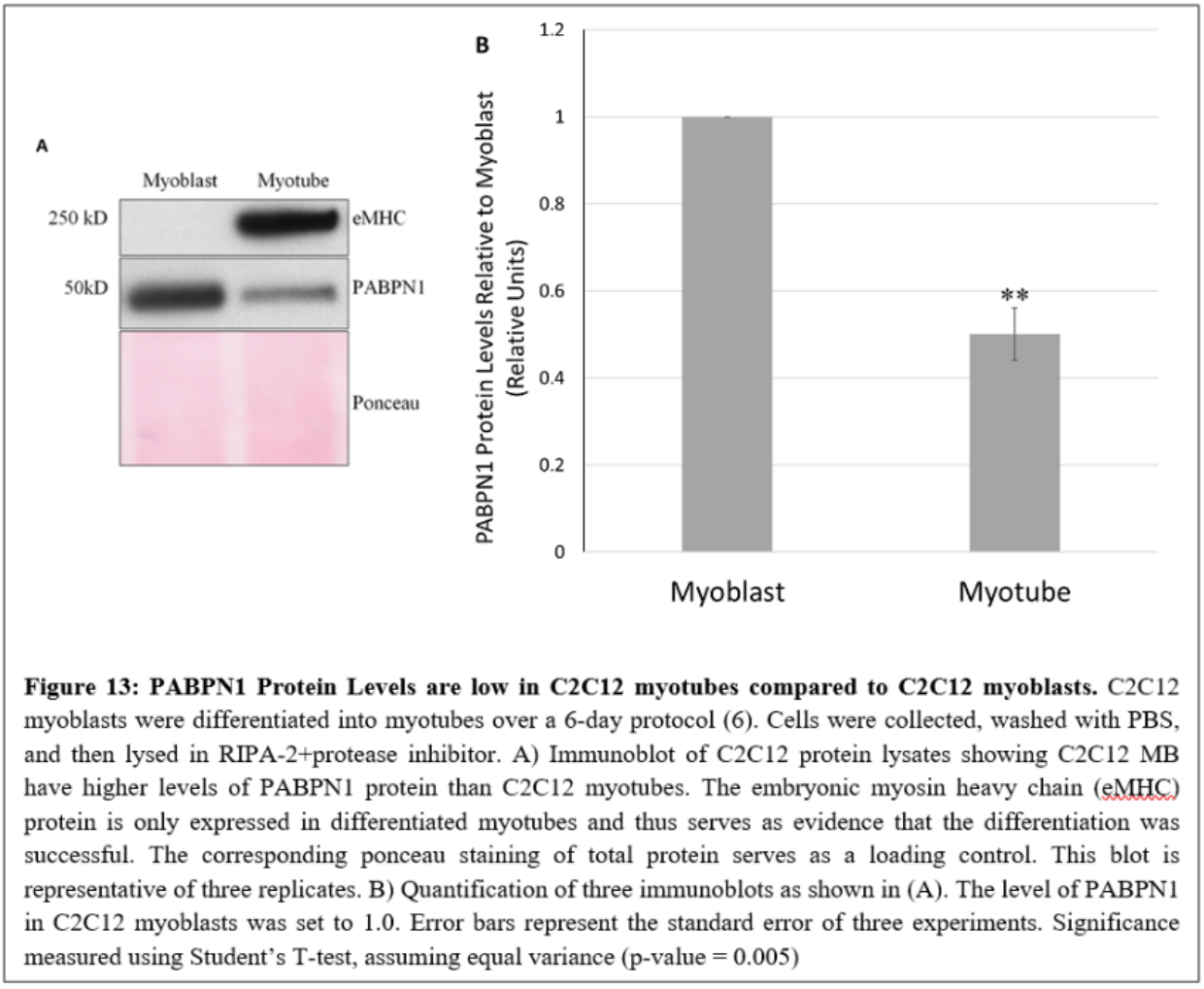

Figure 13: PABPN1 Protein Levels are low in C2C12 myotubes compared to C2C12 myoblasts. C2C12 myoblasts were differentiated into myotubes over a 6-day protocol (6). Cells were collected, washed with PBS, and then lysed in RIPA-2+protease inhibitor. A) Immunoblot of C2C12 protein lysates showing C2C12 MB have higher levels of PABPN1 protein than C2C12 myotubes. The embryonic myosin heavy chain (eMHC) protein is only expressed in differentiated myotubes and thus serves as evidence that the differentiation was successful. The corresponding ponceau staining of total protein serves as a loading control. This blot is representative of three replicates. B) Quantification of three immunoblots as shown in (A). The level of PABPN1 in C2C12 myoblasts was set to 1.0. Error bars represent the standard error of three experiments. Significance measured using Student's T-test, assuming equal variance (p-value = 0.005)

Perspective

Though these experiments did not definitively identify a pathway by which insulin increases the steady-state PABPN1 protein, they did eliminate one pathway and provide intriguing results that merit further investigation for two other pathways. Furthermore, this line of investigation identified a novel role of glucose as a regulator of steady-state PABPN1 protein levels. This work has set the stage for future investigation into the mechanistic relationship between metabolism and PABPN1. Low levels of PABPN1 protein in affected tissues in OPMD patients may explain why disease symptoms present only in specific muscles. Investigation into mechanisms of protein-level modulations of PABPN1 is vital for better understanding of the pathogenesis of OPMD and future therapeutic approaches for the disease.

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