Investigating poly(A) binding protein dynamics in myoblast differentiation

Victoria Delisle

Experimental Medicine

McGill University, Montreal

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Contributions of Authors

The research carried out in this thesis was done under M.R. Fabian supervision, who helped conceptualize the experiments. Contributions by other researchers are outlined below.

Thesis Chapter 1: Introduction
M.R. Fabian helped edit this chapter.

I wrote and edited this chapter.

Thesis Chapter 2: Results
S. Kajjo helped perform and analyse the polysome profiles. He also graphed the polysome profiles displayed in Figure 4C, Figure 5A-B, and Figure 7C-D.

S. Sharma euthanized the mice and helped isolate the organs for the experiments outlined in Figure 2D and 8C-D. He also isolated the RNA and ran the qPCRs displayed in Figure 8C.

H. Benedeta generated the C2C12 PABPC4^{KO} cells used for experiments outlined in Figures 6 through 9.

M.R. Fabian helped edit this chapter.

I performed all other experiments and analysis in this chapter. I also wrote and edited this chapter.

Thesis Chapter 3: Discussion and conclusion
I wrote and edited this chapter.

Abstract

The regulation of gene expression is a tightly controlled phenomenon relying on transcription, translation, mRNA stability and protein stability. Translation can be stimulated by the poly (A) tail through PABPC1, which can bind simultaneously to the poly (A) tail and to eIF4G, which can allow for interactions between the 5' and 3' mRNA termini and has been proposed to facilitate translation initiation. PABPC1 also plays an important role in preventing the untimely decay of specific classes of mRNAs, notably those with constitutive functions, including ribosomal and mitochondrial encoding mRNAs. Overall, PABPC1 is thought to have a context-dependent role that fluctuates between mRNA translational efficiency and stability depending on a number of factors, including poly(A) tail status, PABPC1 availability and the availability of other RNA-binding factors. One of PABPC1's binding partners is the downstream target of mTORC1 LARP1. LARP1 is an RNA binding protein that can bind the 5'-cap and 5'terminal oligopyrimidine (TOP) motif of select mRNA populations, including mRNAs coding for cytoplasmic ribosomal proteins and select translation factors.

PABPC1 levels have recently been reported to decrease in C2C12 mouse myoblasts upon their differentiation into post-mitotic myotubes. How PABPC1 depletion levels affect mRNA translation and protein synthesis in terminally differentiated myotubes is not known. Moving forward, the overall aim of my research project have been to assess how PABPC1 and PABPC1-interacting protein expression changes over the course of myoblast differentiation, and to determine if PABPC1 plays a role in establishing protein synthesis rates in mature post-mitotic cells.

Using C2C12 cells and mouse organs, we established that PABPC1 and LARP1 protein levels are correlated in different terminally differentiated cells. Furthermore, we demonstrated that inhibition of mTORC1 in myotubes affects the translation of TOP mRNAs, irrespective of PABPC1 and LARP1 status. Additionally, we observed that ectopically expressing PABPC1 protein in myotubes leads to the rescue of LARP1 protein levels. However, this does not rescue global translation, global protein synthesis or mRNA steady state levels of certain ribosomal proteins known to be regulated by PABPC1 and LARP1 in cancer cell lines. Taken together, this suggests that there is a distinct gene regulation mechanism independent of PABPC1 and LARP1 proteins in myotubes.

Résumé

La régulation de l'expression des gènes est un phénomène étroitement contrôlé reposant sur la transcription, la traduction, la stabilité de l'ARN messager et la stabilité des protéines. La traduction peut être stimulée par la queue poly(A) grâce à PABPC1, qui peut se lier simultanément à la queue poly(A) et à eIF4G, ce qui permet des interactions entre les extrémités 5' et 3' de l'ARN messager et facilite l'initiation de la traduction. PABPC1 joue également un rôle important dans la prévention de la dégradation prématurée de certaines classes spécifiques d'ARN messagers, notamment ceux ayant des fonctions constitutives, tels que les ARN messagers codant pour les ribosomes et les mitochondries. Dans l'ensemble, on pense que PABPC1 a un rôle dépendant du contexte qui fluctue entre l'efficacité de la traduction de l'ARN messager et sa stabilité, en fonction de plusieurs facteurs, notamment l'état de la queue poly(A), la disponibilité de PABPC1 et la disponibilité d'autres facteurs de liaison à l'ARN. L'un des partenaires de liaison de PABPC1 est LARP1, une cible en aval de mTORC1. LARP1 est une protéine de liaison à l'ARN qui peut se lier au coiffeur 5'-cap et au motif oligopyrimidine terminal (TOP) des ARN messagers sélectionnés, y compris les ARN messagers codant pour les protéines ribosomiques cytoplasmiques et certains facteurs de traduction.

On a récemment signalé une diminution des niveaux de PABPC1 dans les myoblastes de souris C2C12 lors de leur différenciation en myotubes post-mitotiques. On ne sait pas comment la diminution des niveaux de PABPC1 affecte la traduction de l'ARN messager et la synthèse des protéines dans les myotubes terminalement différenciés. À l'avenir, l'objectif global de mon projet de recherche est d'évaluer comment l'expression de PABPC1 et des protéines interagissant avec PABPC1 évolue au cours de la différenciation des myoblastes et de déterminer si PABPC1 joue un rôle dans l'établissement des taux de synthèse des protéines dans les cellules post-mitotiques matures.

En utilisant des cellules C2C12 et des organes de souris, nous avons établi que les niveaux de protéines PABPC1 et LARP1 sont corrélés dans différentes cellules terminalement différenciées. De plus, nous avons démontré que l'inhibition de mTORC1 dans les myotubes affecte la traduction des ARNm TOP, indépendamment du statut de PABPC1 et LARP1. De plus, nous avons observé que l'expression ectopique de la protéine PABPC1 dans les myotubes permet de restaurer les niveaux de protéines LARP1. Cependant, cela n'entraîne pas la restauration de la

traduction globale, de la synthèse protéique globale ou des niveaux d'ARNm stables de certaines protéines ribosomales connues pour être régulées par PABPC1 et LARP1 dans les lignées cellulaires cancéreuses. Dans l'ensemble, cela suggère qu'il existe un mécanisme distinct de régulation génique indépendant des protéines PABPC1 et LARP1 dans les myotubes.

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Abbreviations

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
PABPC	Poly(A) binding protein cytoplasmic
LARP	La-related protein
UTR	Untranslated region
4EBP	4E binding-protein
eIF4	Eukaryotic initiation factor
PIC	Pre-initiation complex
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
S6K1	ribosomal protein S6 kinase 1
5’TOP	5’terminal oligopyrimidine
RRM	RNA recognition motif
PAM2	PABP-interacting Motif 2
PAIP2	poly(A) interacting protein 2
EDD	E3 ubiquitin ligase
eRF3	eukaryotic peptide release factor 3
PAN	Poly(A)-nuclease
BTG2	B-cell translocation gene 2
UV	Ultraviolet
DHFR	Dihydrofolate reductase
TMP	Trimethoprim
ECS	Embryonic stem cells
IL-4	Interleukin 4
ATM	Ataxia-telangiectasia mutated
PI3K	Phosphoinositide 3-kinases
Akt	Ak strain transforming
LaM	La-motif

DMEM	Dulbecco’s modified Eagle’s medium
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
PEI	Cationic polymer polyethylenimine
RT	Reverse-transcriptase
FL	Firefly luciferase
qPCR	Quantitative polymerase chain reaction
EDTA	Ethylenediaminetetraacetic acid
DTT	Dithiothreitol
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleoside triphosphates
cDNA	Copy DNA
SDS	Sodium dodecyl-sulfate
MRF	Myogenic regulatory factors
CHX	Cycloheximide
SUnSET	Surface sensing of translation
MW	Molecular weight
RPS14	Ribosomal protein S14
RPS29	Ribosomal protein S29
NPM	Nucleophosmin
KO	Knock-out
WT	Wild type
FSHD	Facioscapulohumeral muscular dystrophy
DUX	Double Homeobox
RBM24	RNA binding motif protein 24
DCM	Dilated cardiomyopathy
CNBP	CCHC-Type Zinc Finger Nucleic Acid Binding Protein
ZNF9	Zinc finger protein 9
DM2	Myotonic dystrophy 2
AMPK	AMP-activated protein kinase

Chapter 1:

General Introduction

General Introduction

1.1 Preface

The purpose of Chapter 1 is to provide an overview of gene expression regulation and the different mechanisms that influence mRNA translation. I will be focusing on the role of poly(A) binding protein cytoplasmic 1 (PABPC1) and it's interacting partner La-related protein 1 (LARP1), and the pathways through which these proteins act. Further, I will review how these mechanisms are context-dependent and how it is still unclear if/how PABPC regulates protein synthesis in terminally differentiated cells.

1.2 The regulation of gene expression

The central dogma of molecular biology is a theory explaining the flow of information resulting in protein synthesis (Figure 1). Specifically, how DNA is transcribed into RNA which is subsequently translated to generate protein. The coordination of this phenomenon is critical for our cells, as they need to be able to respond to rapid changes in environment. More recently, there has been an emphasis on the role of both mRNA stability and protein stability as methods for controlling gene expression (1). With translation being one of the most energy costly processes, its regulation is of highest importance (2).

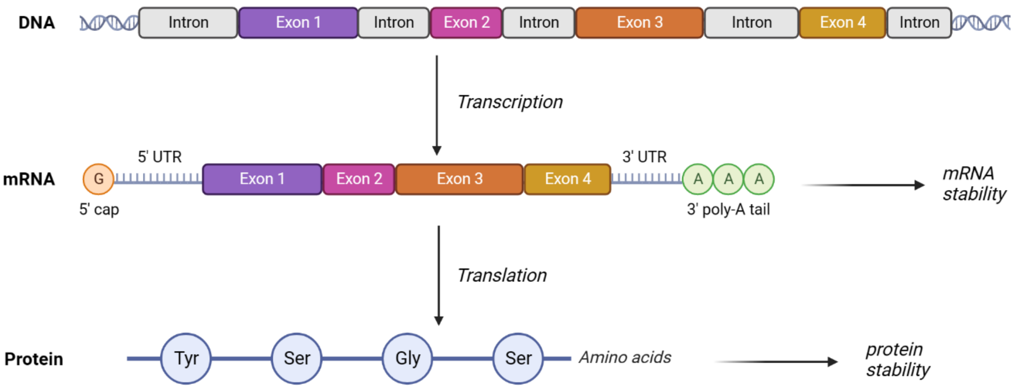

Figure 1. Eukaryotic gene expression regulation. Gene regulation is regulated at many different levels. DNA is transcribed to mRNA which is translated into proteins. mRNA stability and protein stability are two additional levels at which overall protein levels can be regulated.

1.2.1 Regulation of mRNA translation

All nuclear transcribed eukaryotic messenger (m)RNAs contain a 5' methylguanosine cap structure and, with the exception of certain histone mRNAs, a 3' polyadenylated [poly(A)] tail (3). For canonical translation to initiate, the 5'-cap must interact with the heterotrimeric eukaryotic initiation factor 4 (eIF4F) complex. It is composed of eIF4E, eIF4G, and eIF4A (Figure 2) (4). eIF4A has ATP-dependent helicase activity that plays a role in unwinding RNA secondary structures and eIF4G is the scaffolding protein that binds to both eIF4E and eIF4A, as well as several other translation factors. mRNA translation can also be stimulated by the cytoplasmic poly(A) binding protein (PABPC), which can simultaneously bind to the poly(A) tail and to eIF4G (5). This interaction has been proposed to result in mRNA circularization, which has been proposed to stimulate mRNA translation (6). eIF4E is also the rate-limiting factor to cap-dependent translation (7). While all mRNAs require eIF4E for their translation, certain classes of mRNAs are more sensitive to eIF4E and eIF4A availability. For example, mRNAs with extremely short 5' untranslated regions (UTRs) are sensitive to eIF4E levels, whereas mRNAs with long structured 5'UTRs are sensitive to both eIF4E and eIF4A activity (8,9). However, mRNAs that don't have long 5'UTRs can also be sensitive to eIF4E, suggesting other features of 5'UTRs could have a role in this sensitivity (10). PABPC has also been reported to stimulate mRNA translation, with PABPC binding to the poly(A) tail and to eIF4G promoting ribosome recruitment. This includes the recruitment of the 40S subunit through the 43S pre initiation complex and formation of the 80S complex (11). Additionally, PABPC depletion was shown to impairment of 80S ribosome initiation complex formation, suggesting it has a role in the 60S subunit recruitment (12).

In addition to its role in mRNA translation initiation, PABPC also plays a role in translation termination. This is done through its binding to eukaryotic peptide release factor 3 (eRF3) through its PAM2 motif (13). This eRF3 is then able to interact with eRF1, allowing it to load onto the ribosome and cause a conformational change which will result in translation termination (14). Due to the higher affinity of PABPC1 and eRF3, this inhibits the recruitment of the PAN2/3 and Ccr4-Not-Caf1 deadenylation complexes (15). However, this was shown to be true in a translation dependent matter, suggesting a model of translation termination-coupled mRNA decay (16).

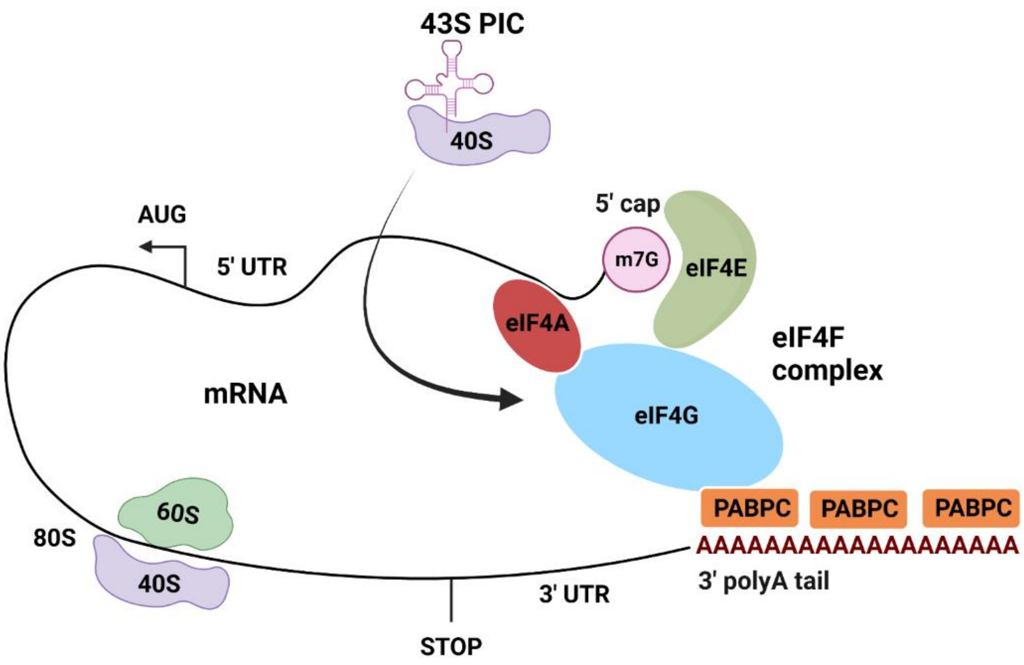

Figure 2. Model of cap-dependent translation. The binding of PABPC to the poly(A) tail and eIF4G promoted the circularization of the mRNA transcript and recruitment of the 43S pre-initiation complex (PIC) to the mRNA. Once the 43S PIC reaches the start codon, the 60S ribosomal subunit is recruited and with the 40S subunit forms the 80S functional ribosome complex which translates the mRNA to protein.

1.2.2 mTORC1

One of the pathways that regulates mRNA translation is the mammalian target of rapamycin (mTOR), a highly conserved serine/threonine kinase across eukaryotes. It acts through two complexes, mTORC1 and mTORC2, that control cell proliferation, metabolism, and cell growth. Though mTORC1 can modulate mTORC2 activation, each complex regulates their own set of functions. Select mRNA translation is tightly controlled by mTORC1 (17). It integrates signals from nutrients, energy, and growth factors to promote catabolism or cell growth (18). mTORC1 modulates protein synthesis via the phosphorylation of eukaryotic translation factor 4E-binding proteins (4EBPs), ribosomal protein S6 kinase 1 (S6K1), and La-related protein 1 (LARP1) (19,20). Binding of 4EBPs to eIF4E blocks the formation of the eIF4F complex, overall suppressing the translation of eIF4E-sensitive mRNAs in a mTOR dependent manner (21).

mRNAs containing a 5'terminal oligopyrimidine (5'TOP) motifs are a subset of mRNAs regulated by mTORC1 and code for ribosomal proteins and translation factors and are regulated by LARP1 and 4EBPs (20). The 5' TOP motif is characterized by an invariant cytosine after the 5'cap followed by a 4 to 14 pyrimidine tract (22).

Many different pharmacological agents have been used to inhibit mTORC1. Though rapamycin has been long used as an mTORC1 inhibitor, mTORC1 does have some rapamycin-resistant activity (23). Importantly, mTORC1 inhibition can lead to the hyperactivation of PI3K, which is regulates AKT, a protein regulated by mTORC2 upstream of mTORC1 (24). Torin1 is and ATP-competitive inhibitor that blocks both mTORC1 and mTORC2 activity. Relevantly, torin1 blocks 4EBP phosphorylation, an effect not observed with rapamycin (25). In actively dividing cells, torin1 treatment was shown to be more effective at repressing TOP mRNA translation in cells that have LARP1 compared to LARP1 knock-out (KO) cells (26). Another factor shown to influence TOP mRNA translation was the poly(A) tail length, shown to positively correlate with ribosome loading onto mRNA, though this length fluctuates in response to mTOR activity (27).

1.3 PABPC and PABPC-interacting proteins

1.3.1 PABPC structure

Cytoplasmic poly(A) binding proteins (PABPCs) are a family of proteins important for numerous metabolic pathways of the mRNA, including mRNA translation, mRNA degradation, and regulation of mRNA expression during development (28). PABPCs require a minimum of 12 adenosines to bind to the poly(A) tail, or 27 adenosines when it is in multimeric form (29). In humans, 6 different isoforms of PABPCs have been identified, with the most abundant and characterized isoform being PABPC1 (30). There are three other cytoplasmic isoforms (PABPC3, PABPC4, and PAPBC4L), an embryonic one (PABPC1L), and an X-linked isoform (PABPC5) (30). PABPC4, which has the same domain architecture as PABPC1, has been shown to compensate for the loss of PABPC1 in certain contexts (31).

PABPC1 is highly conserved in eukaryotes and contains four RNA recognition motifs (RRMs), a proline (p) rich linker region, and a C-terminal MLLE domain that interacts with a number of binding partners (Figure 3) (15,32). In addition to recognizing RNA, RRM2 was shown to be important for PABPC1 self-dimerization, interacting with the p-rich linker of another PABPC1 (33). This ability to dimerize was shown to be important for the ability of PABPC1 to stimulate cap-dependent mRNA translation *in vitro* (33).

The MLLE domain is a peptide-motif binding domain consisting of five α-helices with a conserved amino acid sequence of MLLE in the middle of the peptide recognition site (15). The conserved peptide sequence (LNxxAxEFxP) that binds to this domain is termed PAM2 for PABP-interacting Motif 2 (34). Some of the proteins that bind through this mechanism include poly(A) interacting protein 2 (PAIP2) and LARP1. Another protein that has an MLLE domain is EDD the E3 ubiquitin ligase (35). EDD has been shown to regulate PAIP2 protein levels in response to PABPC1 protein levels (36). Specifically, when PABPC1 is not bound to PAIP2, EDD can bind to it and transfer an activated ubiquitin to it, ultimately causing leading to its proteolysis (36).

Figure 3. PABPC1 domain architecture. PABPC1 is composed of 4 RRMs, a proline-rich linker and a C-terminal MLLE domain.

1.3.2 PABPC1 in mRNA turnover

mRNA decay is an important contributor to the regulation of gene expression. The most common degradation mechanism is eukaryotes is through deadenylation-dependent mRNA decay, though deadenylation-independent and endonuclease-mediated pathways also exist (37). Shortening of the poly(A) tail is the first step in deadenylation-dependent decay. The first nuclease to trim down the poly(A) tail is PAN2-PAN3 deadenylase complex and is dependent on PABPC1 (REF). Once the tail shortened, a second nuclease, CCR4-NOT deadenylase complex, can further trim the poly(A) tail (37). Though CCR4-NOT does not bind directly with PABPC1, it can interact with it through the PAM2 of the transducer of ERBB2 1 and 2 (TOB1 and TOB2) proteins (38). Once deadenylated, the mRNAs can then be decapped and rapidly decayed through 5' to 3' exonucleolytic degradation (39).

1.3.3 Controversy surrounding the role of PABPC in supporting protein synthesis

Previous *in vitro* data suggested that PABPC1 is critical for promoting mRNA translation, however recent published data from our lab and others suggest that this is not always the case (40,41). Our lab showed that actively dividing human HeLa cells need either PABPC1 or PABPC4 to be viable (Figure 4A). Depleting PABPC1 leads to an overall decrease in protein synthesis and decrease in ribosome association (Figure 4B-D). Though this shift in ribosome association usually indicates a decrease in translation, it can also be caused by mRNA abundance. Interestingly, the depletion of PABPC1 has negligible effects on mRNA translation (Figure 4E). In reality, PABPC1 plays an important role in preventing the untimely decay of specific classes of mRNAs, notably those with constitutive functions, including ribosomal and mitochondrial encoding mRNAs in actively dividing HeLa cells (41). Two important factors that were considered to determine whether an mRNAs stability and abundance would be affected due to PABPC1 depletion were length of UTR and poly(A) tail length (41). Having both short UTR's and poly(A) tails made the mRNAs being more sensitive to having a lower abundance in PABP-depleted cells (41). Interestingly, the poly(A) tail length was showed to have important role in mRNA stability with negligible effect on translational efficiency in most context (42).

Conversely, some mRNAs abundance went up in the absence of PABPC1 and PABPC4 (41). Notably, a considerable amount of these mRNAs can be linked to cell differentiation. Evidently, there are those that are linked to osteoblast differentiation, mesodermal commitment pathway, and the ECS pluripotency pathways. Additionally, the IL-4 signaling pathway regulates the differentiation of helper T cells, and the ATM dependent DNA damage response is linked to functional B cell differentiation, (43) (44). Finally. The focal adhesion-PI3K-Akt-mTOR signaling pathway, the ErbB signaling pathway, the nuclear receptors meta-pathways, and the glucocorticoid receptor pathway all have well documented roles in cell differentiation(45,46).

Interestingly, PABPC4 was shown to be crucial for erythroid differentiation (47). They showed that the depletion of PABPC4 in this context led to the destabilization of a subset of mRNAs, specifically those with short poly(A) tails and AU-rich sequences in their 3’UTR (47). Furthermore, colorectal cancer patients with higher PABPC4 protein expression were seen to have well/moderately differentiated colorectal tumors, which is correlated with better survival and prognosis(48). PABPC1 has also been shown to have diverse roles in different cancers. Though it’s been noted to be involved in esophageal cancer, breast cancer, prostate cancer, colorectal cancer, and hepatocellular cancer, its expression has been shown to be associated with both aggressive phenotypes in prostate cancer and better prognosis in gliomas(49). Taken together, this suggests that the role of PABPC1 and PABPC4 are context dependent.

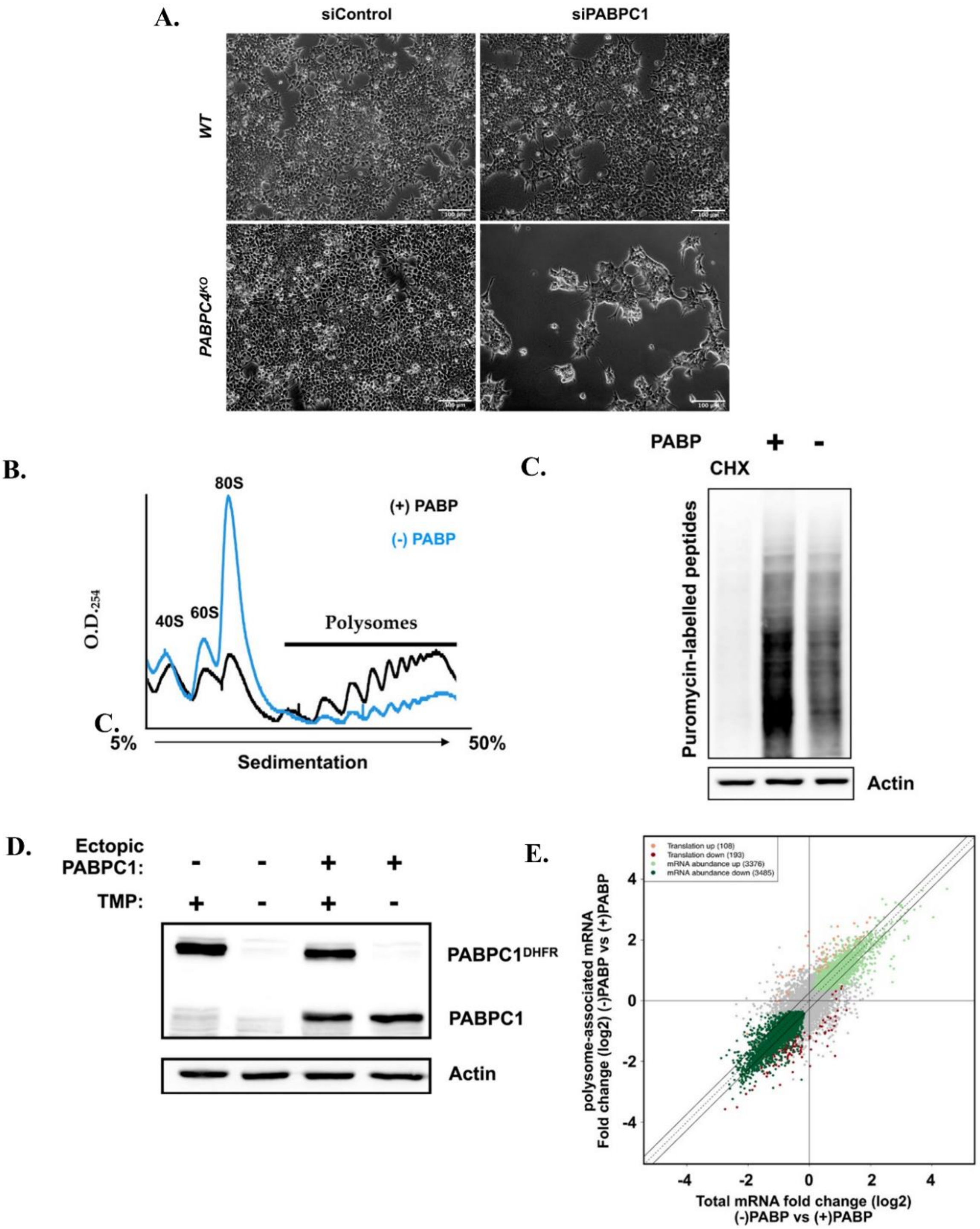

Figure 4. Depleting PABP alters transcriptome abundance with a minimal impact on mRNA translation. Adapted from (41). **(A)** Phase-contrast images of wild-type or PABPC4KO HeLa cells depleted of PABPC1 by siRNA-mediated knockdown. Scale bar, 100 µm. **(B)** Ribosome profiles from PABP^{DHFR} cells grown in the presence or absence of TMP for 12 h. Lysates were

subsequently resolved by ultracentrifugation on 5–50% sucrose gradients and fraction were collected during which time UV absorbance at 254 nm (Abs 254 nm) was monitored for tracing. 40S and 60S subunits, monosomes (80S) and polysomes positions are indicated above tracings. PABP-expressing and -depleted cells are denoted by a black and blue trace lines, respectively. **(C)** SUnSET assay of PABP^{DHFR} cells grown in the presence or absence of TMP for 12 h to maintain or deplete PABP. Cells were subsequently pulsed with either puromycin or puromycin and cycloheximide (control), lysed and equal protein amounts were resolved by SDS-PAGE. Western blot analysis was performed using a monoclonal antibody against puromycin or actin (loading control). **(D)** Western blot confirmation of homozygous PABPC1 tagging with DHFR and degron activity. Successful homozygous tagging of PABPC1 is indicated by the higher molecular weight migration (lane 2) as compared to untagged PABPC1 (lane 1). Degron activity was assessed by culturing PABPDHFR cells in the absence of TMP for 4 or 8 h and assessing PABPC1 levels by western blotting. **(E)** Scatter plot comparing log2 fold-changes [(−) PABP versus (+) PABP] in polysome-associated mRNA (y-axis) to corresponding changes in total cytoplasmic mRNA. Transcripts identified as regulated via altered translation efficiency (orange and red) or abundance (light and dark green) according to anota2seq are visualized together with non-regulated transcripts (grey).

In different systems, like oocytes and early embryos, gene expression is regulated through different mechanisms, probably due to their transcriptional status (42). In transcriptionally inactive embryos, a tail-length regulatory regime is utilised, in which case the length of the poly(A) tail would actually have a large effect on translational efficiency (42). This can be explained because regulation through mRNA stability when there is no transcription is not plausible. A model was recently proposed to explain the coupling of poly(A) tail length and translational efficiency, suggesting that it is context dependent (50). They showed that having a limiting amount of PABPC1 creates a coupled system that influences translational efficiency, whereas having PABPC1 in excess uncouples the system and influences mRNA stability (50). Moreso, they state that this coupling is dependent on having deadenylation coupled to decapping (50). This is congruent with our labs data on the role of PABPC1 for mRNA stability in actively dividing cells.

Other systems would be in cases were cells are not actively dividing. When looking at cardiomyocytes during mouse development, it was observed that PABPC1 protein levels are significantly downregulated during development, while no significant change in mRNA levels are seen (51). Interestingly, PABPC1 poly(A) tail length was seen to decrease during development, however, both during exercise and hypertrophy, PABPC1 protein levels and poly(A) tail length was seen to increase (51). This further suggests the role of coupling of poly(A) tail length and PABPC1 protein levels to determine whether PABPC1 has a role in mRNA translation or stability. It was also showed that in skeletal muscle, PABPC1 protein levels are down in adults compared to newly born mice (51). Whether PABPC1 can enhance mRNA translation in terminally differentiated mammalian cells has yet to be investigated, or if PABPC1 only enhances the translation of subsets of mRNAs under these contexts.

1.3.4 LARP1

One PABPC-interacting protein is the La-related protein 1 (LARP1). LARPs are family of seven proteins in humans characterized by having an La motif (LaM) conserved in eukaryotes (52). They are RNA-binding proteins that contain either an RRM or RRM-like motif (53). Two LARP isoforms, LARP1 and LARP4, have PAM2 motifs that they use to interact with PABPC1, which has been shown to protect mRNA poly(A) tails from decay (54). However, these proteins differ in their affinity for PABPC1 and the poly(A), their La-modules, and their regulatory mechanisms (54). Notably, LARP4 carries an additional PABP interacting motif on its C-terminal, while LARP1 has a highly conserved DM15 region (55). The DM15 of LARP1 allows it to bind to the 5' cap and invariant C of TOP mRNAs (56). Although both proteins bind to ribosomal encoding mRNAs, LARP1 is thought to be a negative regulator of their translation, while LARP4 functions as a constitutive positive regulator of their homeostasis (57).

LARP1 plays a role in translation, growth and proliferation, and is thought to be regulated through the mTORC1 signaling cascade (58). Its DM15 folds into two layers of alpha helices with a putative mTORC1-recognition motif within one of these flexible loops (59). In humans, LARP1 has been shown to be associated with actively translating ribosomes (60). One of the main functions of LARP1 is its involvement in TOP mRNA regulation; however, its exact function remains controversial (55).

Upon mTORC1 inhibition, either pharmacologically or by nutrient deprivation, the translational efficiencies of TOP mRNAs are suppressed (23). One model explaining this is that when mTORC1 is active, it phosphorylates both LARP1 and 4EBPs, rendering them unable to bind to the 5’ cap and eIF4E, respectively. This allows for enhanced translation of TOP mRNAs through eIF4E binding to the cap and the assembly of the eIF4F initiation complex. However, when mTORC1 is inactive, LARP1 can bind to the cap, preventing eIF4E binding and hence repressing translation (55). The affinity of its DM15 for TOP mRNAs allows it to selectively displace eIF4E from these transcripts, even at low concentrations (56). Interestingly, PABPC1 is a TOP mRNA regulated by LARP1 at the translational level (61).

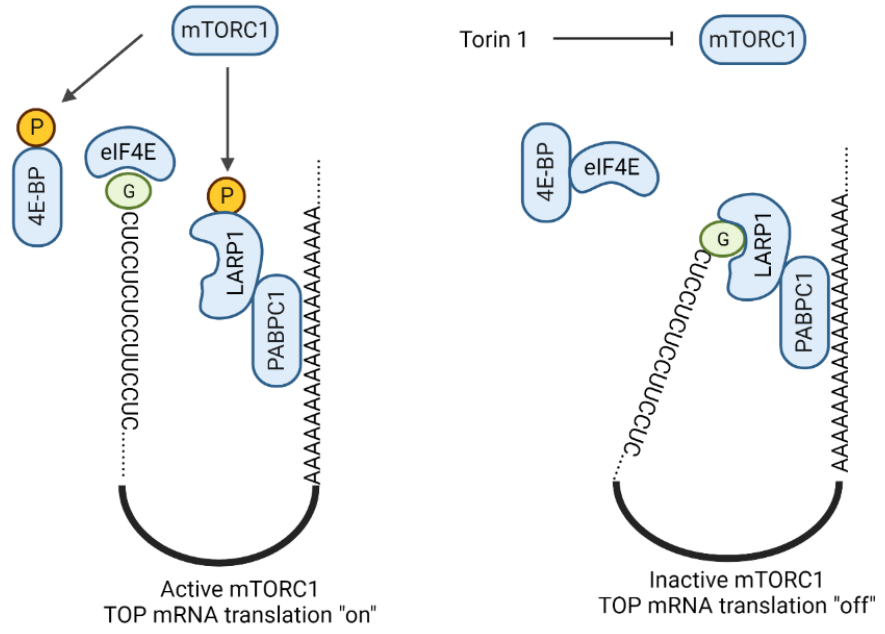

Figure 5. Model of mTORC1 translational regulation of TOP mRNAs. When active, mTORC1 phosphorylated 4E-BPs and LARP1, allowing for eIF4E to bind to the cap and initiate cap-dependent translation, resulting the TOP mRNA translation. When inactive, unphosphorylated 4E-BPs bind to eIF4E and LARP1 to the cap, shutting off TOP mRNA translation.

1.3.5 The roles of PABPC1 and LARP1 in mRNA regulation

Some mRNAs, notably those encoding for ribosomal proteins, are both TOP mRNAs regulated by LARP1 and part of the mRNAs whose stability was affected upon PABPC depletion in HeLa cells.

Although this overlap exists, PABPC1 and LARP1 don't have the same magnitude of regulation on these transcripts. Some groups have shown that the interaction between LARP1 and PABPC1 is necessary for the recruitment of LARP1 to specific mRNAs (62). A study looking at translational efficiency reported that transcripts bound by both LARP1 and PABPC1 were translated less efficiently than transcripts that were not bound or only bound by PABPC1 (63). They suggest that PABPC1 and LARP1 co-ordinately regulate mRNAs upon mTOR inhibition, and that inhibiting the binding of these proteins results in a large reduction in LARP1 mRNA binding. Interestingly, PABPC1 and LARP1 protein interaction has been shown to be independent of mTOR activity, despite their overlapping function in mTOR inhibition (64).

Another role for PABPC1 and LARP1 is for mRNA stability. It was recently showed that in hepatoblastoma, LARP1 competitively binds to PABPC1, blocking B-cell translocation gene 2 (BTG2) recruitment to the transcript (65). This in turns blocked PABPC1 and CCR4-NOT complex binding, inhibiting deadenylation and decay. Furthermore, the role of LARP1 complex in transcript protection could be dependent on whether it's a TOP mRNA or not (66). A model suggests that though the LARP1-PABPC1 complex has poly(A) tail length dependent activity in protecting against deadenylation, LARP1 could also protect TOP mRNA irrespective of poly(A) tail length.

1.4 Terminally differentiated cells

The cell cycle is composed of a series of events that results in the division of a cell dividing into two daughter cells. By the time humans are adults, most of our cells are not dividing anymore. These non-actively dividing cells can be divided into 3 main categories: quiescent, senescent, and terminally differentiated, with the majority falling into the later category (67). However, most cell models used are actively dividing cells. The process of terminal differentiation occurs for cells to perform a specialized role. This phenotypic and genotypic change results in irreversible proliferation loss, putting these cells in a postmitotic state (68). Importantly, these differences imply that much of what is learned from the study of actively dividing cancer cells lines cannot be directly translated to the majority of cells in our body. Some of these cells include skeletal muscles, or myotubes, and cardiac muscles, or cardiomyocytes.

1.4.1 Gene regulation in terminally differentiated cells

The process of adult stem cells activating, differentiating, and finally terminally differentiated requires the ability of cells to rapidly respond to stimuli and changes in the environment. Though early on a lot of emphasis has been placed on transcriptome, the discrepancy in transcription and protein synthesis in many contexts pushed for more importance to be put on post-transcription gene regulation. Most stem cells, including hematopoietic stem cells, neural stem cells, and hair follicle stem cells, show the same translational pattern; low translation in the stem cells followed by an increase in their differentiating progeny and finally back to the low translation in their terminally differentiated form (69). However, large-scale analysis of terminally differentiated tissues suggest that the importance of which cell-type may also have an important role in gene regulation. Notably, that different protein degradation rates, different RNA-binding proteins, specialized ribosomes and alternative UTRs account for the level of post-transcription regulation that is needed in different terminally differentiated cells (70).

1.4.2 Skeletal muscles

Skeletal muscles make up about 40% of adult human's bodyweight and contain 50 to 75% of all our bodies proteins, making them a key target for many studies, especially metabolism (71). Skeletal muscle development is controlled by the myogenic regulatory factors (MRFs). These transcription factors function together to control the four stages of myogenesis: myoblast construction, myocyte formation, fusion to multinucleated myotube, and myofiber formation. The tight regulation of their expression and activity is controlled through epigenetic processes (72). Another important factor to myogenesis is protein kinase activity. Different kinases have been linked to both the promotion of myogenesis and its regulation throughout the different stages (73). One study indicated that there might be certain classes of mRNAs who are translationally regulated during myogenesis (74). Specifically, those for mRNAs encoding for ribosomal proteins (74). Interestingly, PABPC1 protein, who has been showed to regulate this class of mRNA in cancer cell lines, has been observed to be downregulated in skeletal muscles (51). While numerous studies have looked at the early process of myogenesis, there has been relatively limited examination of translation and mRNA stability in terminally differentiated myotubes.

One of main signaling pathway through which skeletal muscles are thought to regulate protein levels is mTORC1. Studies suggest that in skeletal muscle the mTORC1 signalling pathway could account for 30 to 50% of basal rates of protein synthesis (75). In a study on the effect of branched-amino acids on muscle protein synthesis, it was shown that in food-deprived rats, leucine was the only one able to results in S6K and 4EBP hyperphosphorylation, plausibly through mTORC1 signaling (76). This overall lead to increased eIF4E availability and consequently increased protein synthesis in rat muscle. To further this point, rapamycin treatment blocked the observed effects on both translation initiation and protein synthesis (76).

Chapter 2:

Materials and Methods

Materials and Methods

DNA constructs and molecular cloning

The PABPC1 open reading frame was cloned into pBABE-puro plasmid (Addgene plasmid #1764) using conventional molecular cloning techniques using BamHI and SalI restriction enzyme sites. The PABPC1 ΔMLLE and LARP1 were cloned into pQCXIB (Addgene plasmid #22266) using conventional molecular cloning techniques using AgeI and MfeI, or AgeI and EcoRI restriction enzyme sites, respectively.

Cell lines

All cells were kept at 37°C and 5 CO_2. myoblast (C2C12) and rat myoblasts (H9C2) cell lines were used as models for differentiating cells. The human embryonic kidney 293T cells were used to make virus. Both cell types were cultured in of Dulbecco's modified Eagle's medium (DMEM). Both medias were supplemented with 50 U/mL of penicillin, and 50 µg/mL of streptomycin, but the DMEM for the C2C12 and HEK293T was with 10% fetal bovine serum (FBS) while the media for the H9C2 was with 20% FBS.

To obtain differentiated myotubes, C2C12 cells were plated at 40% on day 0. Once cells reached confluence on day 1, they were washed twice with PBS and media was changed to DMEM with 2% horse serum. Media was changed daily until myotube formation was seen (day 8 to 10), and cells harvested for subsequent experiments. To obtain differentiated cardiomyocytes, H9C2 cells were plates at 50% on day 0. Cells were washed twice with PBS and media was changed to DMEM with 0.1% FBS on day 1 and supplemented with 10 µM of all-trans retinoic acid. Media was changed every other day for 9 days, and cells harvested on day 10 for subsequent experiments.

Viral preparation and infection

Retroviruses, pBABE and pQCXIB were packaged in HEK. HEK293T cells were plated in 10 cm plates to reach 70% confluency the following day. Each of the plasmids (6.25 µg) were added with VSVG (2.5 µg) and pUMVC (3.75 µg) and transfected into the HEK293T cells. Transfections were performed using 2 mL of opti-mem and 50 µL of the cationic polymer polyethylenimine

(PEI). Media was changed 24-hours post transfection with 8 mL of DMEM, and virus was collected 48- and 72-hours post transfection using a 0.45-micron syringe filter.

Cells were plated in a 6-well plate to reach 30% confluency the next day. Cells were infected with 1 mL of the virus titer twice, 24 hours apart, and 24 hours later were selected using puromycin (2 ug/mL) for pBABE vector and blasticidin (10ug/mL) for pQCXIB 24- and 48- hours post-transfection. Cells recovered 24-hours post puromycin and 72-hours post blasticidin treatment in fresh media.

Western blotting

Adult BL6J mice (9-month-old) were euthanized as per animal handling protocols. Various tissues (brain, heart, muscle, kidney, testes, liver, bladder, spleen, and lung) were harvested and lysed using RIPA buffer (25mM Tris-HCl (pH 7.5); 150 mM NaCl; 2 mM EDTA; 1% NP-40; 0.1% Sodium Deoxycholate; 0.1% SDS; 1mM Dithiothreitol; 1x EDTA-free protease inhibitor cocktail and phenylmethylsulfonyl fluoride). Cells were harvested and lysed using a lysis buffer (50mM Tris-HCl, pH 7.5; 150 nN NaCl, 2 mM EDTA, 0.5% NP-40, 1x EDTA-free protease inhibitor cocktail and phenylmethylsulfonyl fluoride). Protein lysates were resolved by SDS-PAGE and western blotting was performed using various antibodies.

Polysome profiling

Sucrose gradients were prepared the morning of the polysome using 60% (w/v) sucrose solution made in ddH$_2$O. Solution was filtered using a 0.22 µm filter. 10x sucrose gradient buffer was prepared as 200 mM HEPES (pH 7.6), 1 M KCl, 50 mM MgCl2, 100 µg/ml CHX, 1x EDTA-free protease inhibitor cocktail and phenylmethylsulfonyl fluoride, 200 units/ml of murine RNase inhibitor, and 1mM Dithiothreitol. 5% and 50% sucrose buffer were made used the 60% sucrose solution, 10x sucrose gradient buffer and ddH$_2$O. Sucrose gradients were made by filling to the half-full point each polyallomer ultracentrifugation tube with the 5% sucrose solution. Next, starting from the bottom the 50% sucrose solution was added until it reached the half-way point once again. Tubes were sealed and put onto the gradient maker to make a linear 5% to 50% gradient. Gradients were kept at 4°C until use.

C2C12 cells were plated at 40% on day 0. Cells were washed twice with PBS and media was changed to DMEM with 2% horse serum on day 1. Media was changed daily for 7 days. On day 7, C2C12 cells were plated at 35%. On day 8, the media was changed on all of the cells, undifferentiated and differentiated with DMEM with 10% FBS and DMEM with 2% horse serum, respectively. One hour later, the medias were rechanged, and either DMSO or TORIN1 were added to the cells for 4 hours. Cells were then treated with 100 µg/mL of cycloheximide (CHX) for 10 minutes at 37°C. Cells were then harvested in PBS with every reagent having 100 µg/mL of CHX. Cells were lysed using 450 µL hypotonic lysis buffer (5 mM of Tris–HCl, pH 7.5; 2.5 mM of MgCl2, 1.5 mM of KCl; 100 µg/ml of CHX; 1x EDTA-free protease inhibitor cocktail and phenylmethylsulfonyl fluoride; 1X 2 mM of DTT; 0.5% Triton; 0.5% sodium deoxycholate) and vortexed. Next, 0.5% Triton X-100 and 0.5% Sodium deoxycholate were added to each sample and vortexed once again. Samples were left on ice for 15 min, then spun at 20,000 x g for 15 min 4°C. The OD at 260 nm was measured. Solutions were prepared to have a final OD at 260 nm reading of 24, and 10% of this solution for each sample was saved for inputs. 450 µL of the top of the sucrose gradients were removed and replaced with 450 µL of the sample with the OD reading of 24. Each gradient was weighed and balanced before being put to centrifuge. Samples were centrifuged at 39,000 rpm for 2 hr at 4 °C using SW41Ti rotor.

The polysome fractionator was set to pump at 1.5 mL/min with 30 sec on the fraction collector. The fractionator was started at the same time as the DAQ tracer, collecting the UV absorbance at 254 nm. Each condition was collected from fraction 5 to 17. 750 µL of TRIzol were added to each fraction and 500 µL for the inputs. Samples were put at -80°C overnight. Polysome graphs were generated using R.

RNA isolation

After staying at -80°C overnight, samples were left to thaw at room temperature. Once thawed, 100 µL of chloroform was added for the inputs and 200 µL for the fractions. Samples were vortexed twice and spun at 16,000 x g at 4°C for 15 min. The aqueous phase was then collected, and isopropanol was added in a 1:1 ratio to each sample. 3 µL of glycoblue was added to each sample and the samples were kept at -20°C overnight.

After staying at -20°C overnight, samples were spun at 16,000 x g at 4°C for 15 min. Isopropanol was discarded and 75% ethanol was added to the pellets. Samples were spun at 16,000 x g at 4°C for 15 min. All the ethanol was removed, and samples were left to air dry on ice. Once dry, 30 µL of sterile water were added to the pellets. The pellets were then resuspended, and 25 µL were put into a new tube. For each condition, the remaining 5 μL for fractions 5 to 11 were combined and labelled subpolysome (non-translating) and fraction 12 to 17 combined and labelled polysome (translating). Each fraction corresponds to a peak from the polysome profile. Isolated RNA was stored at -80°C.

RT-qPCR

RNA concentrations were measured using the nanodrop machine. Reverse-transcriptase (RT) reaction was done to get cDNA. 500 ng of each sample was combined with 0.5 μL of random primers, 0.5 μL of firefly luciferase (FL) and sterile water to make up to 6.25 μL. Using thermocycler, samples were heated to 65°C and dropped to 5°C. A master mix (2 μL RT buffer; 0.5 μL dNTPs; 0.5 μL maxima H minus enzyme; 0.25 μL murine RNAse inhibitor) was added to each sample and mixed. Samples were put back in thermocycler to finish the reaction.

Quantitative PCR (qPCR) was done. 40 μL of sterile water can added to each cDNA tube. 28 μL of master mix (9.1 μL of sterile water; 1X of the luna qPCR mix; 200 nM of forward and reverse primers) were added to new 1.7 mL tubes with 7 μL of diluted cDNA. Samples were run in triplicates with 10 μL in each well. qPCR plates were sealed and spun at 1400 g before being put into thermocycler. qPCR reactions were run in an Eppendorf Mastercycler realplex^2. Samples were first heated to 95°C for 2 minutes, followed by 40 cycle runs. Each cycle started at 95°C for 15 secs, followed by 15 secs at 55°C and 20 secs at 68°C. Mean Ct values for three technical replicates were used. Ct values were normalized to the FL spike-in. Graphs were generated using excel.

SUnSET assay

Both empty C2C12 and C2C12 ectopically expressing PABPC1 cells were used for these experiments. Myoblasts and myotubes were treated with 2 μg/mL of puromycin for 30 minutes at 37°C. As a control, myoblasts and myotubes were treated with 100 μg/mL of CHX for 5 minutes at 37°C prior to puromycin treatment. Cells were harvested and lysed using a lysis buffer (50mM Tris-HCl, pH 7.5; 150 nM NaCl, 2 mM EDTA, 0.5% NP-40, 1x EDTA-free protease inhibitor cocktail and phenylmethylsulfonyl fluoride). Protein lysates were resolved by SDS-PAGE and probed for puromycin.

Statistics

Statistical significance of qPCR data was calculated using two-tailed T-test. All data were normalized to an FL or RL spike-in. In experiments using C2C12 cells, inputs were set to 100 for polysome distributions, while myoblasts were set to 100 for mRNA abundance. In organs, mRNA abundance for the skeletal muscles were set to 100. Polysome distributions were done in 2 biological replicates, each represented by a dot. mRNA abundance was done in 3 biological replicates, and error bars represent the standard error of the mean.

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Cell line (*Mus Musculus*)	C2C12	Kostas Pantopolous' Lab		Cell line maintained in DMEM + 10% FBS, 50 U/mL of penicillin and 50 ug/mL of streptomycin
Cell line (*Rattus Norvegicus*)	H9C2	Kostas Pantopolous' Lab		Cell line maintained in DMEM + 20% FBS, 50 U/mL of penicillin and 50 ug/mL of streptomycin
Cell line (*Homo Sapiens*)	293T	ATCC	CRL-3216	Cell line maintained in DMEM + 10% FBS, 50 U/mL of penicillin and 50 ug/mL of streptomycin
Antibody	ACTIN	Cell signaling	4967S	WB (1:30,000)
Antibody	PABPC1	Cell signaling	4992	WB (1:1000)
Antibody	PABPC1	Abcam	ab21060	WB (1:1000)
Antibody	PABPC4	Bethyl	A301-467A-M	WB (1:1000)
Antibody	4EBP	Cell signaling	9644	WB (1:5000)
Antibody	4EBP-phospho	Cell signaling		WB (1:1000)
Antibody	MF 20	DSHB given by Dr. Vahab	AB_2147781	WB (1:60)

Antibody	M2-FLAG	Sigma	F1804-200UG	WB (1:1000)
Antibody	Puromycin			WB (1:1000)
Antibody	LARP1	ProteinTech	67810-1-Ig	WB (1:1500)
Antibiotic	Puromycin	Wisent	450-162-XL	
Antibiotic	Blasticidin			
Differentiation factor	All-trans retinoic acid	Sigma	R2625-100MG	Diluted to 10 mM using DMSO
Proteasome inhibitor	MG-132	EMD Millipore	80053-194	Diluted to 20 µM using DMSO
Protein synthesis inhibitor	Cyclohexamide	Sigma	C1988-1G	Diluted to 100 mg/mL using DMSO
mTOR inhibitor	Torin 1	TOCRIS	4247	Diluted to 1mM using DMSO
Control	DMSO	Fisher	BP231-1	
qPCR mix	Luna Universal qPCR Master Mix	NEB	M3003X	

Table 1. Reagents and Tools

Application	Name	Species	Sequence
qPCR	FL		F- CTCACTGAGACTACATCAGCTATTC R- CTCACTGAGACTACATCAGCTATTC
qPCR	GAPDH	Mouse	F- CCAATGTGTCCGTCGTGGATCT R- GTTGAAGTCGCAGGAGACAACC
qPCR	LARP1	Mouse	F- CCACAGGTACGGTTTGGAGTG R- ATGTCCAGCCGGAACTTTTTCT
qPCR	NPM	Mouse	F- CCGAGATCAAAGGGTCAAGA

			R-TCTTGAATAGCCTCCTGGTCA
qPCR	PABPC1	Mouse	F- GGCAAAGGAGTTCACCAATG R- TTAAGGCAGGCCCAAACT
qPCR	RPS6	Mouse	F- GATGATGTCCGCCAGTATGT R- CAGGACACGAGGAGTAACAAG
qPCR	RPS14	Mouse	F- GAAGGAAGAGCAGGTCATCAG R- CCAGAAAGATCGGTAACATGGA
qPCR	RPS29	Mouse	F- GCAGTACGCGAAGGACATAG R- GATCGGTTCCACTTGGTAGTAG
Cloning into pBABE	PABPC1	Human	F- cggcgccgcGGATCCAaccccagtgcccccagctacccc R- cggcgccgcGTCGACTtaaacagttggaacaccggtggc
Cloning into pQCXIB	PABPC1 ΔMLLE	Human	F-cacccgtgaccggtATGGGTGCTAGCtttatccagccctcac R-gaacaggccaattgttAACCTTGTACATGAACAGCAG
Cloning into pQCXIB	LARP1	Human	F- gAacaggcaccggtATGGGTCTTTGGAGGGTGCTTTTGTC R- gAacaggcgaattcCTTTGGAGGGTGCTTTTGTCAAAG

<u>Table 2.</u> Primers

Chapter 3:

Results

Results

3.1 Preface

This chapter contains data I collected using different cell lines and mouse organs to explore the status of PABPCs and their interacting partners in terminally differentiated cells, focusing on gene regulation in myotubes. Specifically, I looked at protein synthesis and mRNA translation, notably ribosomal encoding mRNAs. Additionally, I looked at how mTOR inhibition impacts protein synthesis in this system. Finally, I explored how re-introducing PABPC1 expression in myotubes impacts protein synthesis, mRNA translation, and mRNA levels.

3.2 Rationale and Hypothesis

Many studies have looked at the role of poly(A) binding proteins, notably PABPC1, in the contexts of mRNA translation and stability. However, these studies have focused on in vitro data and actively dividing cell models. PABPC1's role in terminally differentiated cells has yet to be investigated. Recently published data from our lab shows that in actively dividing cancer cells, PABPC plays a role in regulating the stability of a subset of mRNAs, notably those with constitutive functions. As previous works have suggested that PABPC levels are saturating in this system, we wanted to investigate whether this could be true in a system where PABPC is limiting (50). It has been reposted that when PABPC is limiting, it actually has a greater influence on translational efficiency compared to mRNA stability (50). C2C12 cells are a mouse myoblast cells line that has been widely used in the study of myogenesis (77). It had been shown that PABPC1 expression is downregulated in skeletal muscle and recapitulated in C2C12 differentiation (51). Additionally, most of the work done in PABPC-limiting systems have been in early embryos or oocytes. We wanted to test whether this holds true in post-mitotic cells. Furthermore, mTORC1 is known to be one of the main regulators on mRNA translation in skeletal muscles (75). We therefore wanted to know what role mTORC1 plays in this context. Overall, we hypothesized that PABPC1 may have a role in regulating the mRNA translation of certain classes of mRNA's in myotubes.

3.3 Results

3.3.1 PABPC levels dramatically drop during myoblast differentiation

We set out to assess PABPC levels over the course of myoblast differentiation. To this end, we cultured C2C12 myoblasts and differentiated them with horse serum into elongated myotubes (Figure 1A). Myotube formation was assessed by microscopy, as well as by assessing the expression of myosin heavy chain levels (Figure 1B). Myoblasts express PABPC1 and PABPC4 levels that are similar to their levels in cancer cell lines, including HeLa cells (Figure 1C). Interestingly, both PABPC1 and PABPC4 levels decreased dramatically over the course of myoblast differentiation, being barely detectable in differentiated myotubes (Figure 1D). These data therefore suggest that PABPC levels in general drop over the course of myoblast differentiation.

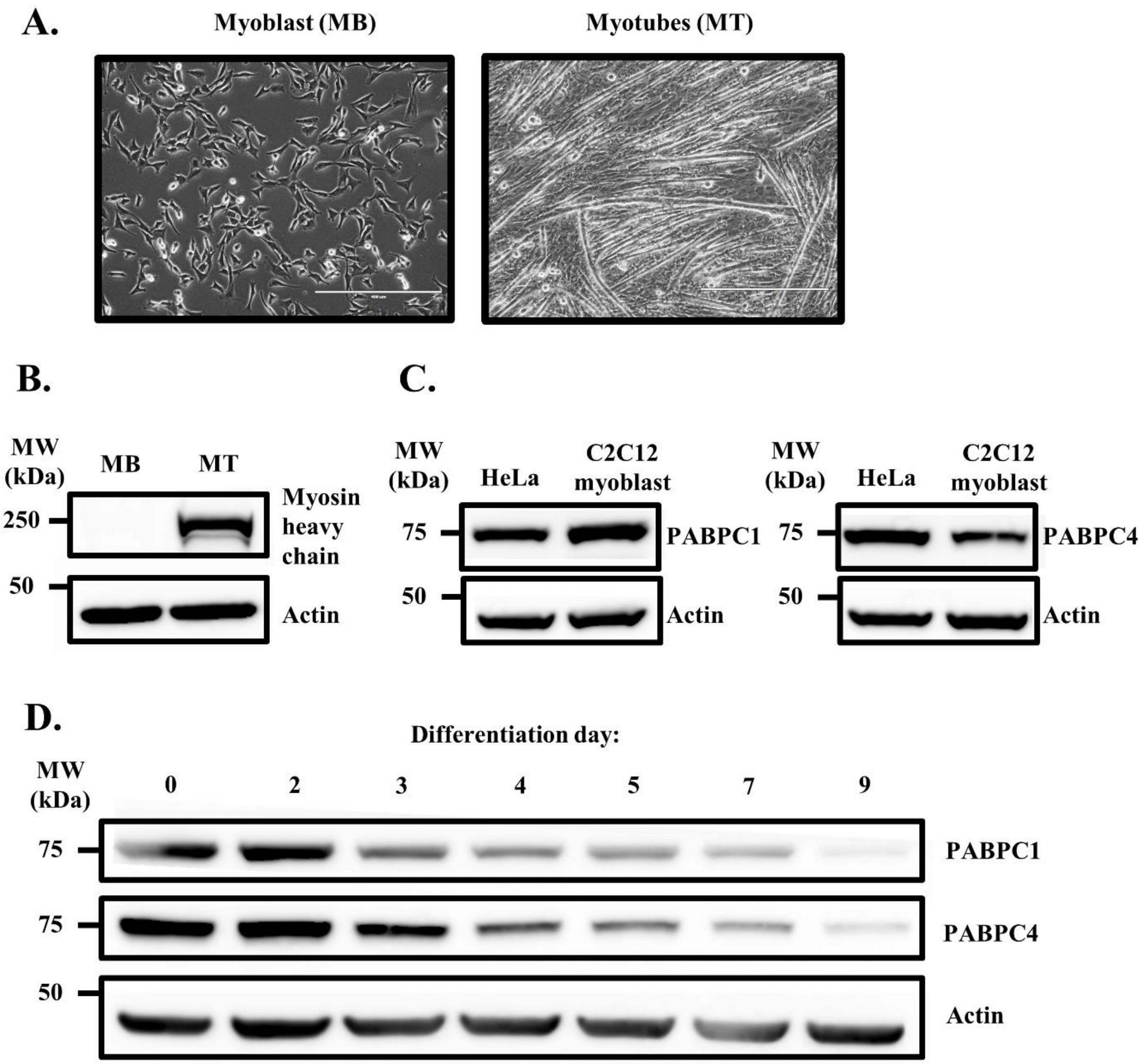

Figure 1. PABPC levels dramatically drop during myoblast differentiation (A) Microscope images of C2C12 myoblast and differentiated myotubes. **(B)** Western blot images of myosin heavy chain used as a differentiation marker. Actin was used as a loading control. **(C)** Western blot images of myoblast differentiation to myotubes showing a decrease in PABPC1 and PABPC4 protein levels. Actin was used as a loading control. **(D)** Western blot images comparing the protein levels of PABPC1 and PABPC4 in myoblast (C2C12) and HeLa cells. Actin was used as a loading control. PABPC4 was run on a different gel.

3.3.2 PABPC1 and LARP1 protein levels correlate during myoblast differentiation and in mouse tissues.

Next, we wanted to investigate the status of other known proteins that interact with PABPC1 and regulate mRNA translation. Interestingly, we observed that LARP1 protein levels decrease dramatically over the course of myoblast differentiation (Figure 2A). To verify whether this relationship could be seen in other terminally differentiated systems, we used the rat myoblast cell line H9C2, that can terminally differentiate to both myotubes and cardiomyocytes (Figure 2C). Once again, LARP1, PABPC1, and PABPC4 protein levels were downregulated in the terminally differentiated cells compared to the myoblasts (Figure 2B). Finally, we isolated various mouse tissues to determine their levels of PABPC1, PABPC4 and LARP1 (Figure 2D). We observed that PABPC1 and LARP1 protein levels correlate across mouse tissues. However, PABPC4 levels did not seem to be correlated to either LARP1 or PABPC1 (Figure 2D). PABPC4 levels were found to be significantly higher in cardiomyocytes, followed by skeletal muscles, while being either barely detectable or undetectable in other organs (Figure 2D). Taken together, these data terminally differentiated myotubes and heart and skeletal muscles before displaying low levels of PABPC1 and LARP1 proteins.

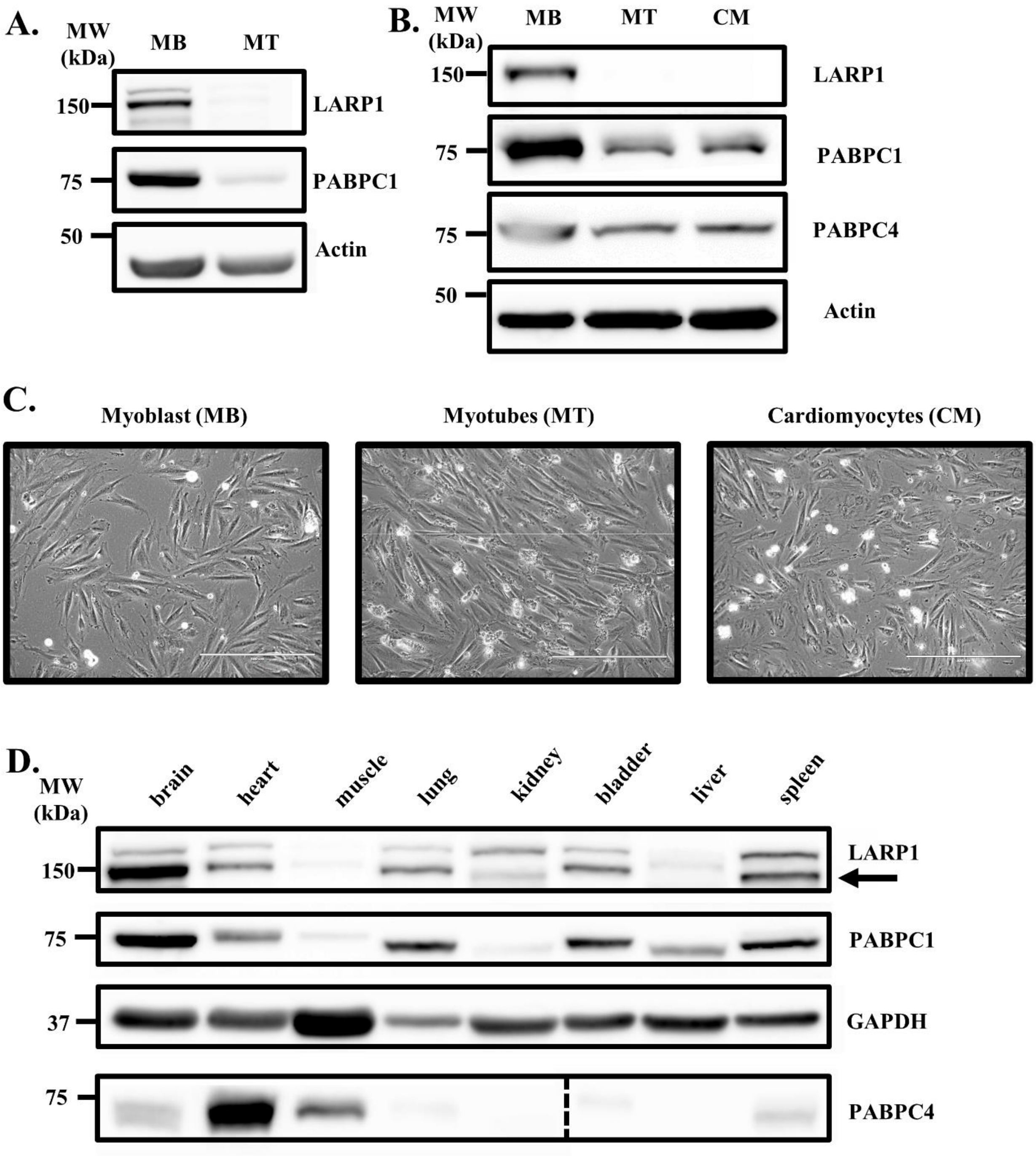

Figure 2. PABPC1 and LARP1 protein levels correlate during myoblast differentiation and in mouse tissues. (A) Western blot images of C2C12 myoblast differentiation to myotubes showing a decrease in PABPC1 and LARP1 protein levels. Actin was used as a loading control.**(B)** Western blot images of H9C2 myoblast differentiation to myotubes and cardiomyocytes showing

a decrease in LARP1, PABPC1, and PABPC4 protein levels. Actin was used as a loading control. PABPC4 was run on a different gel.**(C)** Microscope images of H9C2 myoblast, differentiated myotubes and differentiated cardiomyocytes. **(D)** Western blot images comparing the protein levels of LARP1, PABPC1, and PABPC4 in different mouse tissues, GAPDH was used as a loading control. Dotted line represents a cut in the membrane. PABPC4 was run on a different gel.

3.3.3 Global protein synthesis is down in terminally differentiated cells.

Our data suggest that myotubes display low levels of PABPCs as compared to myoblasts. As PABPCs can support protein synthesis, we set out to determine myoblast and myotube protein synthesis rates. This was accomplished using the surface sensing of translation (SUnSET) assay (Figure 1). As puromycin is a structural analogue of aminoacyl tRNAs, it gets incorporated into newly synthesized proteins (78). SUnSET assays utilize this property and use puromycin as a proxy for the amount of protein synthesis that occurred during puromycin incubation (79). These results can be visualized by western blotting using a primary antibody against puromycin. As a control, both myoblasts and myotubes were treated with the translation elongation inhibitor cycloheximide (CHX) (80). By blocking protein synthesis, CHX acts as a control to ensure that the puromycin detected was indeed only incorporated during the incubation period. Upon terminal myotube differentiation, a global decrease in global protein synthesis was observed (Figure 1B). This is consistent with the thought that once the cells have exited the cell cycle, less protein synthesis is required.

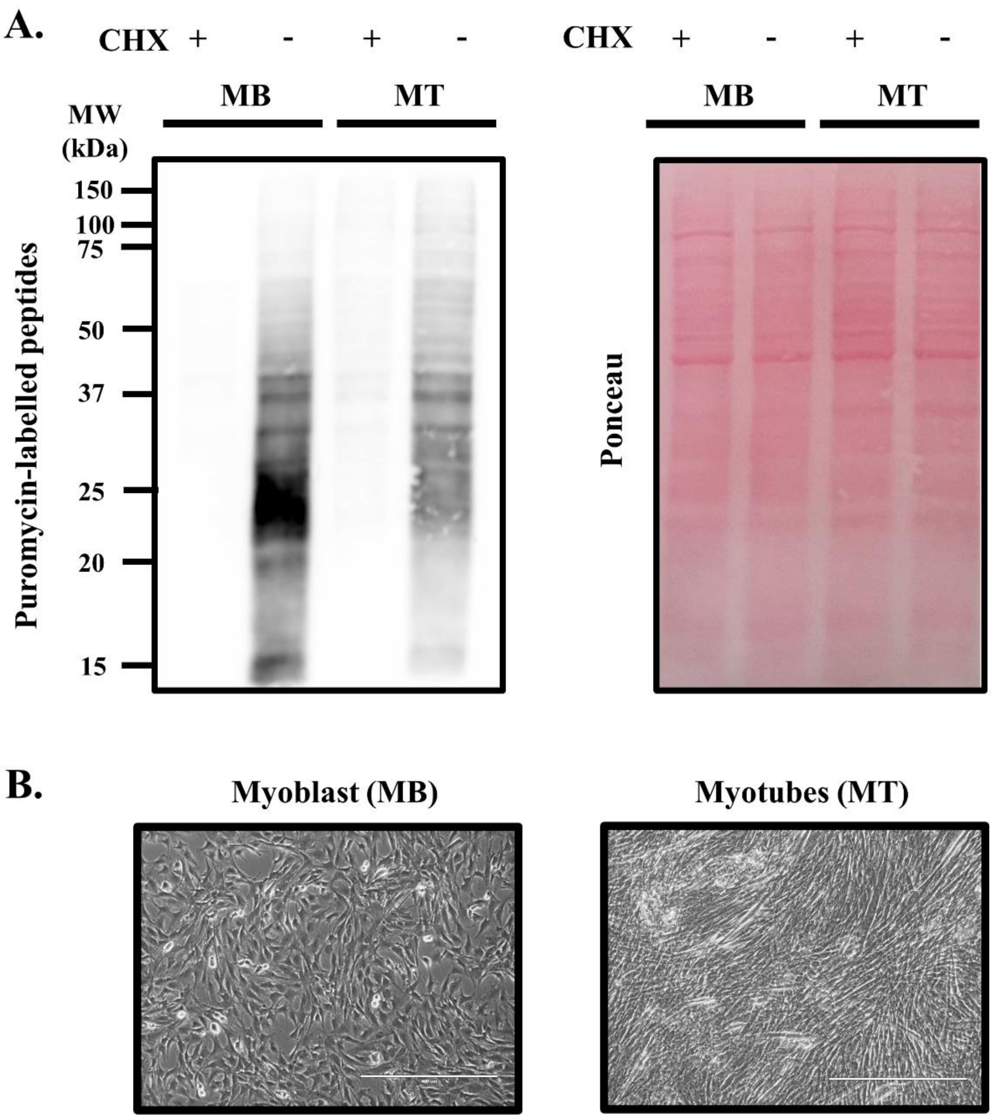

Figure 3. Global protein synthesis is down in terminally differentiated myotubes. (A) SUnSET assay showing a decrease in protein synthesis upon terminal myotube differentiation. Ponceau image shown for loading. **(B)** Microscope images of C2C12 myoblast and differentiated myotubes.

3.3.4 Global mRNA translation is down in terminally differentiated myotubes.

We then wanted to assess mRNA translation in myoblasts as compared to myotubes by carrying out polysome profiling (Figure 5). This technique allows for the separation of translated mRNAs based of the number of ribosomes associated to each mRNA, which allows us to infer its translational status (81). We observed that myotubes display a drop in polysomes and a concomitant increase in 40S and 60S peaks in myotubes as compared to myoblasts. These data are in line with observations made by SUnSET assays, suggesting that mRNA translation is downregulated in myotubes as compared to myoblasts. We next investigated the translational status of specific mRNAs by isolating RNA from individual ribosome profiling fractions and assessing their abundance by RT-qPCR. Surprisingly, PABPC1 mRNA shifted from subpolysome fractions (non-translating) to polysome fractions (translating) in myotubes as compared to myoblasts. This is despite PABPC1 protein levels being lower in myotubes as compared to myoblasts. RPS14 mRNA also shifted to polysome fraction in myotubes as compared to myoblasts. (Figure 4F). Contrarily, there was no significant differences in LARP1, RPS29, and NPM mRNA distributions between myoblasts and myotubes (Figure 4E-G-H). This suggests that the downregulation in PABPC1 and LARP1 protein levels observed upon terminal myotube differentiation is not a translational effect.

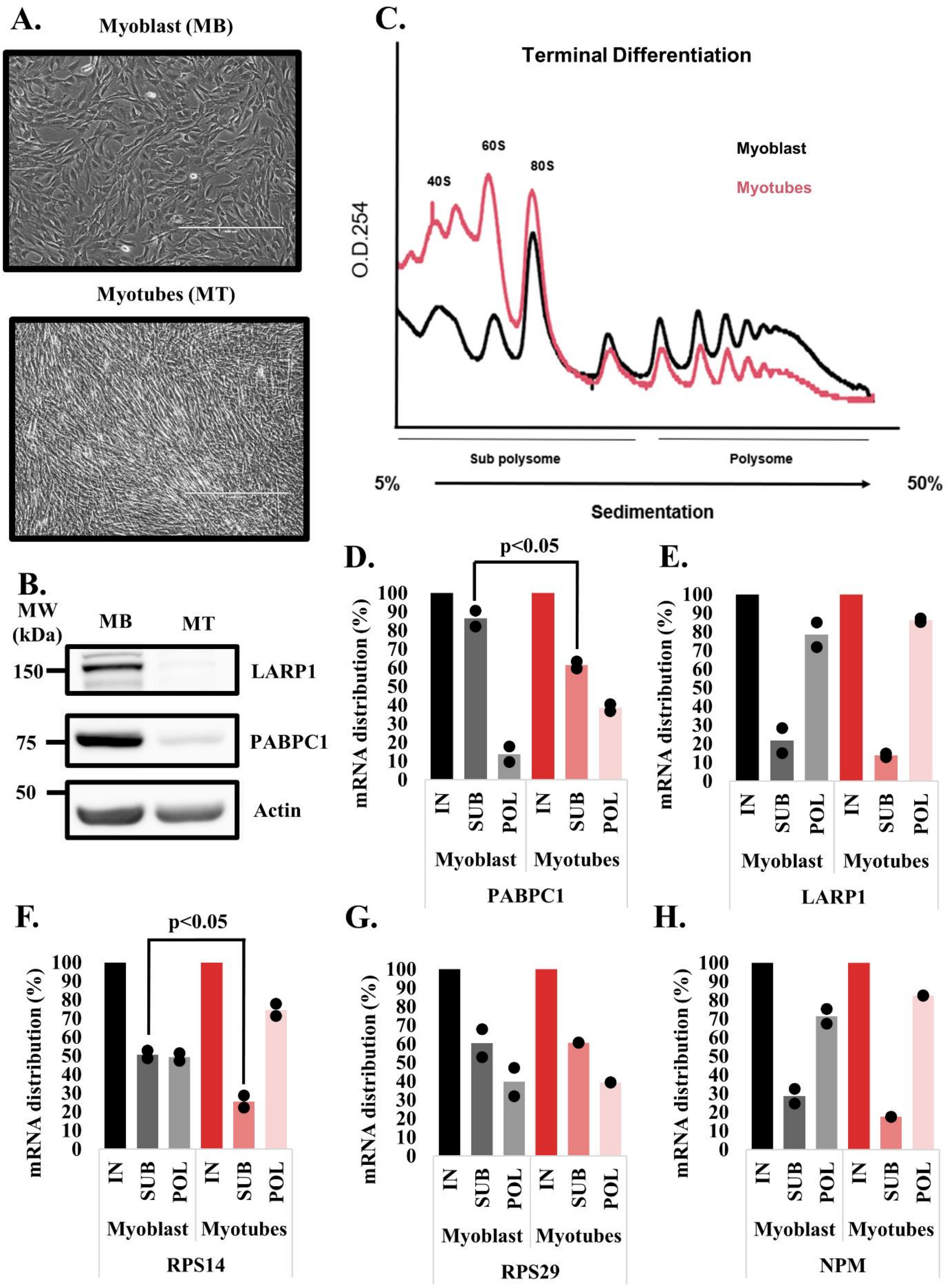

Figure 4. Global mRNA translation is down in terminally differentiated myotubes. (A) Microscope images of C2C12 myoblast and differentiated myotubes. **(B)** Western blot images of

myoblast differentiation to myotubes showing a decrease in PABPC1 and LARP1 protein levels. Actin was used as a loading control. **(C)** Polysome profiling of myoblast and myotubes. **(D-H)** qPCR data of polysome distribution of individual mRNAs in myoblasts and myotubes, based off of 2 biological experiments in technical triplicates.

3.3.5 mTOR inhibition causes a global shift in mRNA translation in myoblasts and myotubes.

mTOR is a key regulator of myogenesis (75), and signals through LARP1, we wanted to investigate its role in fully terminally differentiated myotubes (Figure 5). Since PABPC1 and LARP1 have overlapping roles in the regulation of ribosomal mRNAs, we also wanted to investigate the mechanism of regulation of these mRNAs in a system where both PABPC1 and LARP1 proteins are downregulated. As controls, ribosomal protein S29 (RPS29) and nucleophosmin (NPM) were used. RPS29 is a known TOP mRNA that was shown to sensitive in mTORC1 inhibition in actively dividing cells. Additionally, it was shown that LARP1 is the primary regulator of this protein through mTORC1 (82). NPM is a protein involved in both ribosomal biogenesis and is known to be downregulated upon mTORC1 inhibition (83). Unpublished data from our lab shows that upon PABPC depletion in actively dividing HeLa cells, mTOR inhibition through TORIN1 treatment does not cause a big shift in mRNAs to the subpolysome fraction. However, we see a similar shift to the subpolysome fraction upon TORIN1 treatment in myoblasts and in myotubes that have both PABPC1 and LARP1 protein downregulated (Figure 5A-B). Though no significant differences in mRNA distribution was found for PABPC1 and LARP1 upon mTOR inhibition, RPS29, RPS14, and NPM all shifted to the subpolysome fractions (Figure 5D-H). Overall, this suggests that mTORC1 may signal through different mechanisms in terminally differentiated myotubes compared to actively dividing HeLa cells.

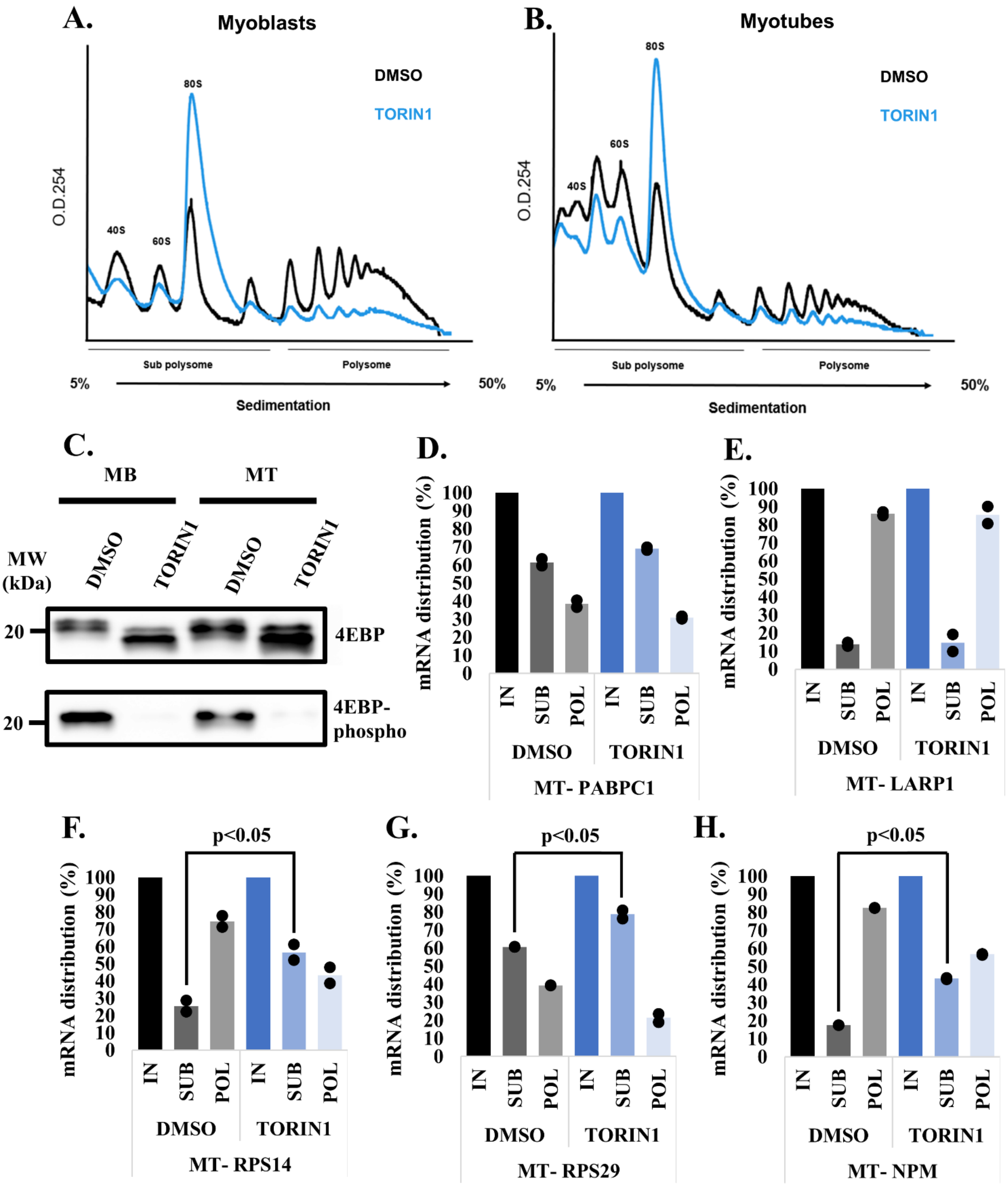

Figure 5. mTOR inhibition causes a global shift in mRNA translation in myoblasts and myotubes. (A-B) Polysome profile of C2C12 myoblasts and myotubes treated with DMSO or

TORIN1. **(C)** Western blot images of total 4EBP and 4EBP phosphorylation at thr37/46 upon TORIN1 treatment. **(D-H)** qPCR data of polysome distribution of individual mRNAs in myotubes treated with TORIN1 or DMSO, based off of 2 biological experiments in technical triplicates.

3.3.6 Ectopically expressed PABPC1 protein rescues LARP1 protein levels in myotubes.

Unpublished data from our lab has recently showed that adding back PABPC1 back after depleting it from PABPC4^{KO} HeLa cells rescues protein synthesis to the same level as before it was depleted (Kajjo S. Unpublished). This made us wonder whether re-expressing PABPC1 in myotubes could have a similar effect. Using PABPC4^{KO} C2C12 cells created by Benedeta Hasaj using CRISPR/Cas9 (Figure not shown), stable cells expressing flag tagged human PABPC1 were created (Figure 6). Cells were selected by antibiotic resistance. Upon terminal myotube differentiation, the ectopically expressed PABPC1 protein was not downregulated (Figure 6A). Morphologically, these PABPC4^{KO} +PABPC1 myoblasts were slightly elongated and the myotubes showed some cell death (Figure 5B). The flag-tag was put on PABPC1's C-terminal, allowing for it to be detected using a PABPC1 N-terminal antibody (Figure 6C). Surprisingly, expressing PABPC1 protein in myotubes seemed to rescue LARP1 protein levels (Figure 6C). Interestingly, unpublished data from our lab (Kajjo S. 2023) recently showed that LARP1 protein levels are not affected by PABPC1 depletion. However, protein synthesis was not rescued to the level of myoblasts in PABPC4^{KO} +PABPC1 myotubes (Figure 6D). Overall, this suggests that PABPC1 is not the main reason why protein synthesis is down in this context but may have a role is promoting the protein synthesis of certain proteins, like LARP1.

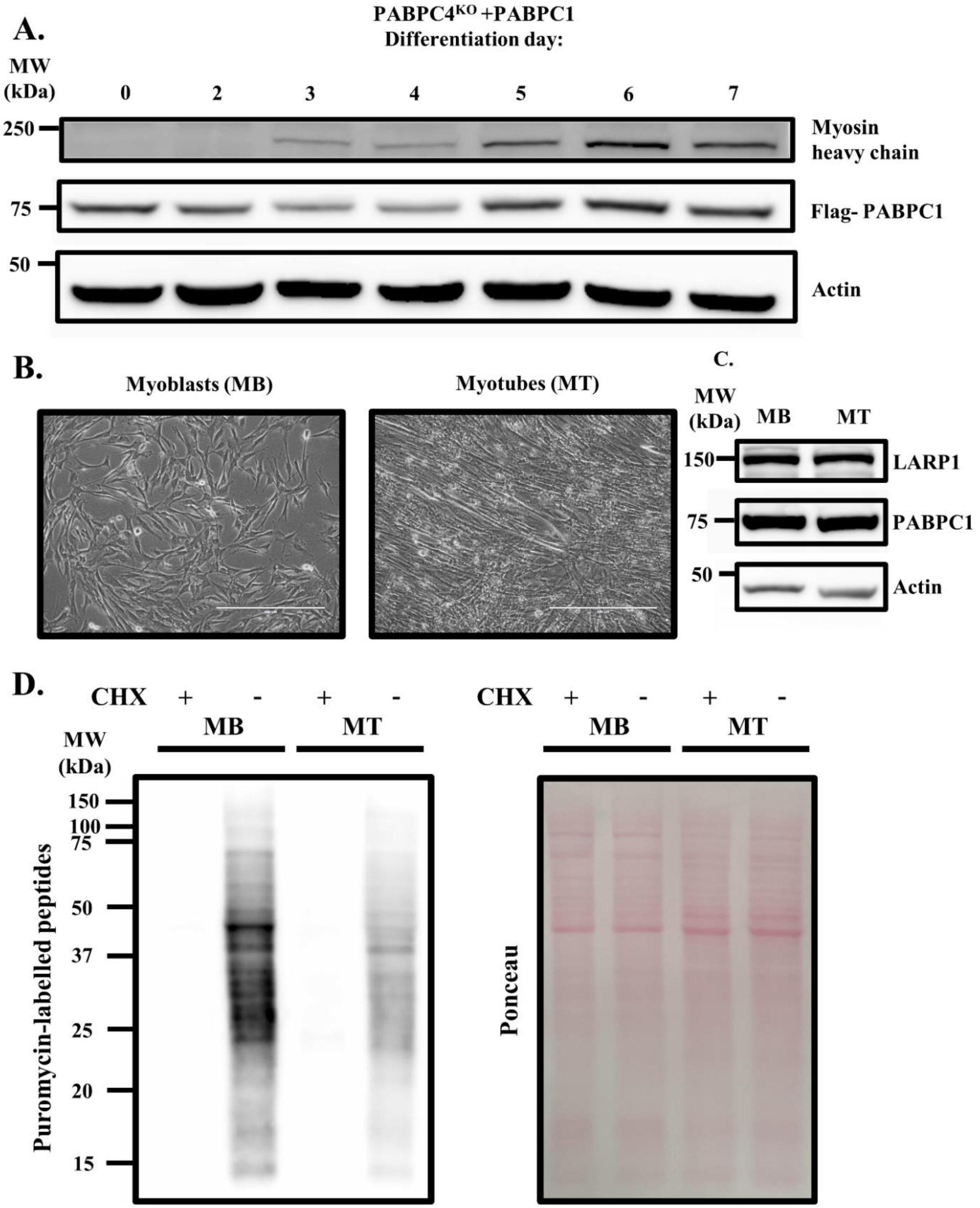

Figure 6. Ectopically expressed PABPC1 protein rescues LARP1 protein levels in myotubes. (A) Western blot images of myoblast differentiation to myotubes showing no significant change in Flag-PABPC1 protein levels during myotube differentiation. Myosin heavy chain as a differentiation marker and actin was used as a loading control. **(B)** Microscope images

of myoblast and differentiated myotubes. **(C)** Western blot images of myoblasts and myotubes showing no significant changes in LARP1 and PABPC1 protein levels. Actin was used as a loading control. **(D)** SUnSET assay showing a decrease in protein synthesis upon terminal myotube differentiation. Ponceau image shown for loading. All cells used in this figure where C2C12 PABPC4^{KO} +PABPC1.

3.3.7 PABPC1 protein expression in myotubes does not increase polysome association

Knowing that myotubes could be considered a PABPC-limiting system where PABPC1 is thought to play a bigger role in mRNA translation, we also wanted to look at whether expressing PABPC1 in myotubes had a global effect on mRNA translation (Figure 7). To do this, polysome profiling was done. A shift to the subpolysome fraction upon terminal myotube differentiation, as well as the two unidentified peaks before the 40S and 60S were observed (Figure 7B) This is similar to the polysome profile of the WT C2C12s (Figure 4C). When comparing the WT myotubes with PABPC1 downregulated to myotubes PABPC4^{KO} +PABPC1, no significant differences in the polysome peaks are observed (Figure 7C). However, a small increase in the 80S polysome accompanied by a decrease in the 40S and 60S is also observed (Figure 7C). All three mRNAs investigated by RT-qPCR show no significant differences in polysome distribution between WT and PABPC4^{KO} +PABPC1 myotubes (Figure 7 E-G). This suggests that PABPC1 is not influencing the translation of LARP1, RPS14, and RPS29 in myotubes.

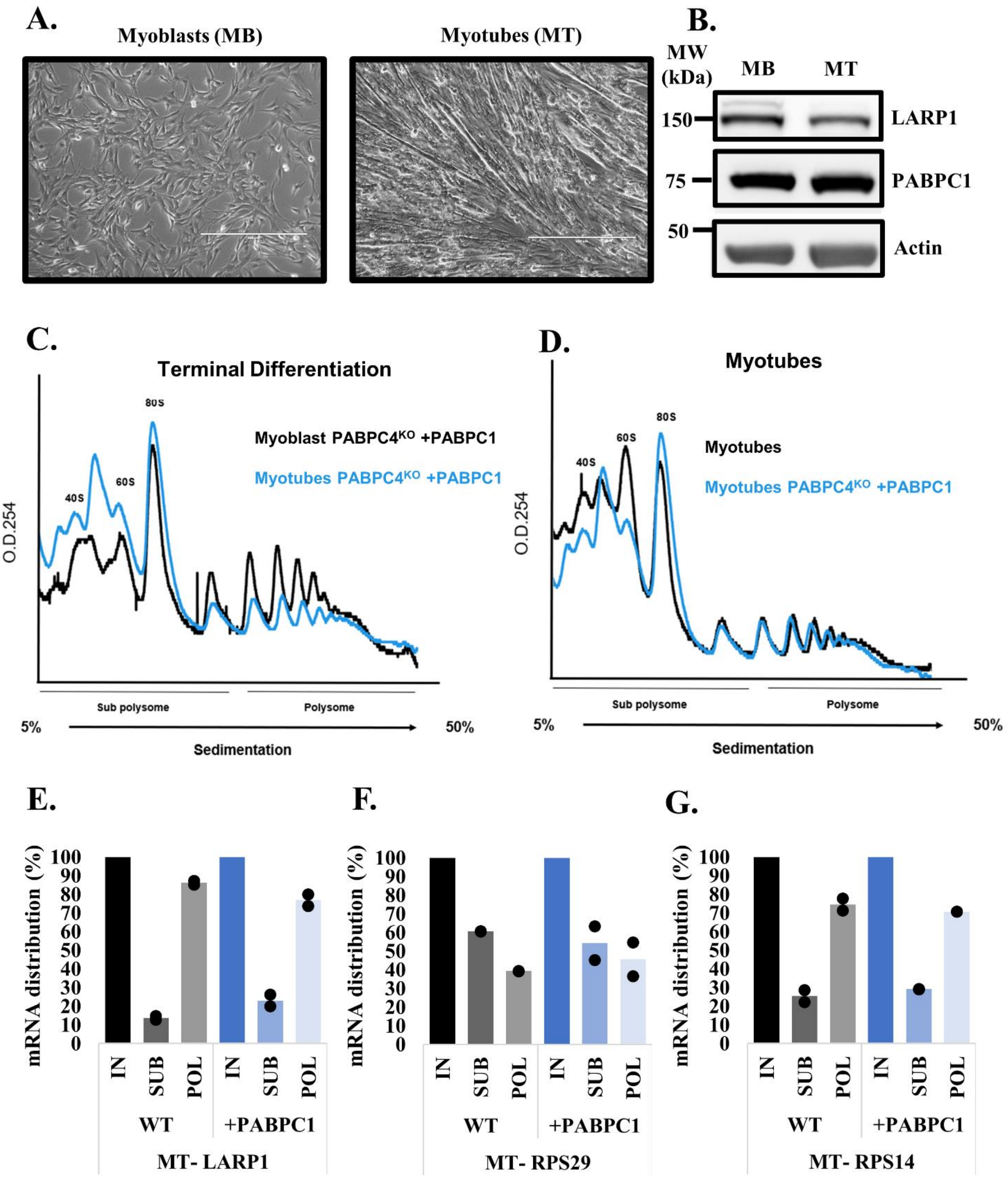

Figure 7. PABPC1 protein expression in myotubes does not increase polysome association

(A) Microscope images of C2C12 PABPC4^{KO} +PABPC1 myoblast and differentiated myotubes.

(B) Western blot images of C2C12 PABPC4^{KO} +PABPC1 myoblast differentiation to myotubes showing no significant changes in PABPC1 and LARP1 protein levels. Actin was used as a loading control. **(C)** Polysome profiling of C2C12 PABPC4^{KO} +PABPC1 myoblast and myotubes. **(D)** Polysome profiling of C2C12 WT and PABPC4^{KO} +PABPC1 myotubes. **(E-G)** qPCR data of polysome distribution of individual mRNAs in myoblasts and myotubes, based off of 2 biological experiments in technical triplicates. +PABPC1 identifies cells that are PABPC4^{KO} +PABPC1.

3.3.8 PABPC1 protein expression in myotubes does not increase the mRNA abundance of select mRNAs.

Recognizing that PABPC4 protein is downregulated in C2C12 myotubes, we created C2C12 +PABPC1 that had its endogenous PABPC4 (Figure 8A). Knowing that PABPC1 has a negligible effect on translation in HeLa cells, but an important role in certain mRNAs abundance, we wanted to see whether expressing PABPC1 in myotubes would affect mRNA abundance (Figure 8). As a control for differentiation NPM was used. NPM is a protein involved in both ribosomal biogenesis and cell cycle regulation (83). The destabilization of its mRNA was also shown to be required for muscle fiber formation (84). Both RPS14 and RPS29 which are known to be affected by both LARP1 and PABPC1 abundance did not significantly change with an increase in PABPC1, and consequently LARP1, protein levels in myotubes (Figure 8B). The PABPC1 we ectopically expressed was human and its mRNA was not detected in this experiment. However, endogenous mouse PABPC1 mRNA was not affected by it (Figure 8B). Interestingly, LARP1 mRNA abundance did not change, even though its protein expression was seen to be increased. We used mouse organs to look at the mRNA abundance association to protein levels in biological contexts (Figure 8C). Relative mRNA abundance was not seen to be correlated with protein abundance in the corresponding tissue. Overall, this suggests that PABPC1 and LARP1 may not play a role in regulating the mRNA abundance of ribosomal mRNAs in terminally differentiated myotubes. Additionally, this suggests that mRNA levels are not indicative of protein expression for LARP1 in myotubes.

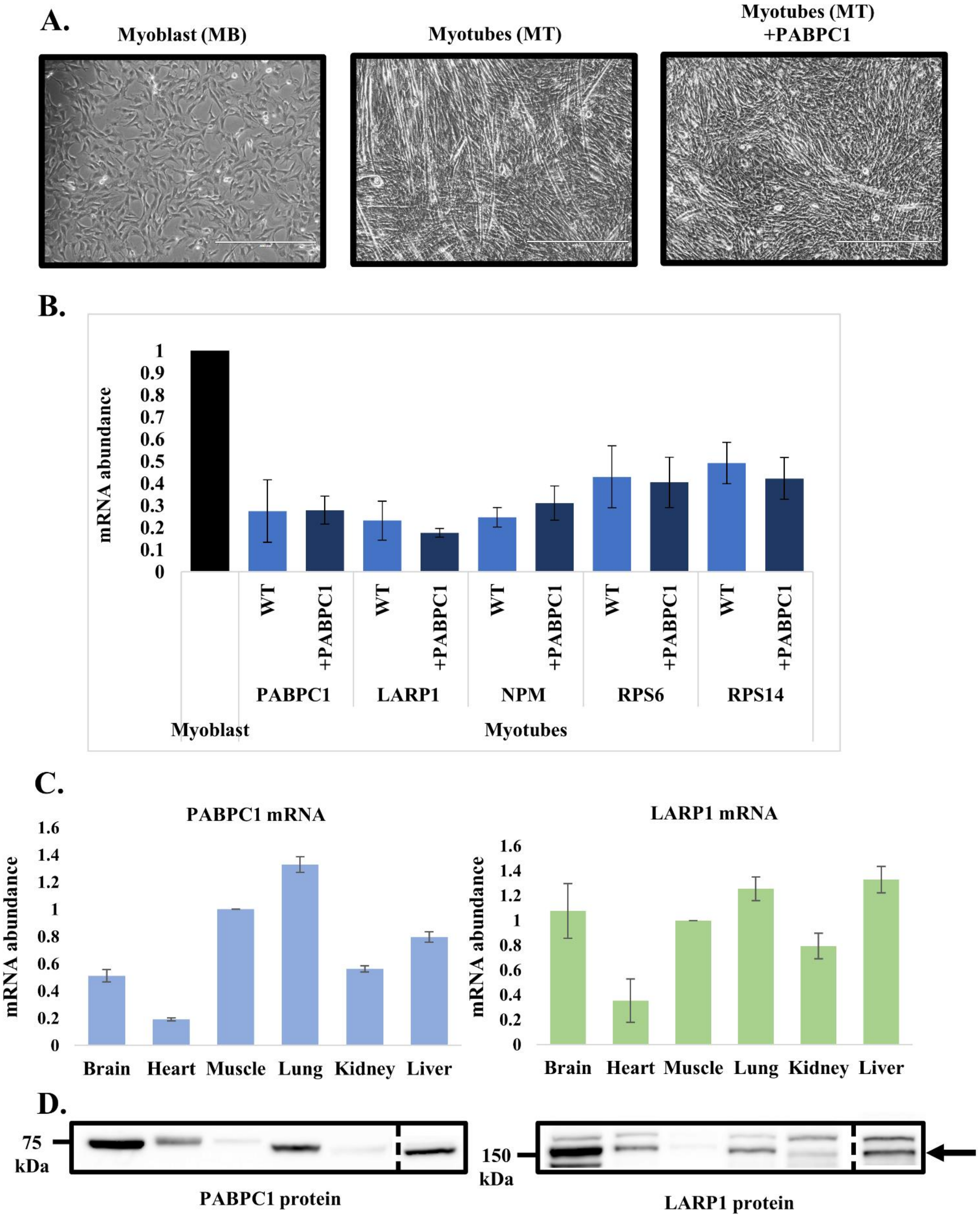

Figure 8. PABPC1 protein expression in myotubes does not increase the mRNA abundance of select mRNAs. (A) Microscope images of myoblasts, myotubes and myotubes +PABPC1.

Myotubes and Myotubes +PABPC1 were both differentiated for 8 days. **(B)** qPCR data of the mRNA abundance of different mRNAs in myoblasts, myotubes and myotubes +PABPC1, standardized with FL spike-in and normalized to myoblasts, based off of 3 biological experiments in technical triplicates.**(C)** mRNA abundance of PABPC1 and LARP1 in different mouse tissues, standardized with RL spike-in and normalized to skeletal muscle, based off of 3 biological experiments in technical triplicates. **(D)** Western blot PABPC1 and LARP1 protein levels from mouse organs for comparison.

3.3.9 PABPC1's MLLE domain is important to the rescue of LARP1 protein expression in myotubes.

Subsequently, we wanted to know whether the contact between PABPC1 and LARP1 was important for LARP1 protein levels to be rescued in myotubes. To investigate this, we created a PABPC1 mutant that lacks its MLLE domain necessary to bind to LARP1 and expressed it in C2C12 cells (Figure 9A). Expressing this PABPC1 ΔMLLE did not seem to rescue LARP1 protein levels in myotubes (Figure 9B). However, this protein also seemed to be slightly degraded upon terminal myotube differentiation (Figure 9B), suggesting it may not be as stable as the full-length PABPC1 in this context. Matching with the fact that LARP1 polysome association (Figure 7E) and mRNA steady state levels (Figure 8B) did not significantly change upon expression of PABPC1 in myotubes, this data suggests that PABPC1 protein contact with LARP1 could regulate its protein expression in terminally differentiated myotubes.

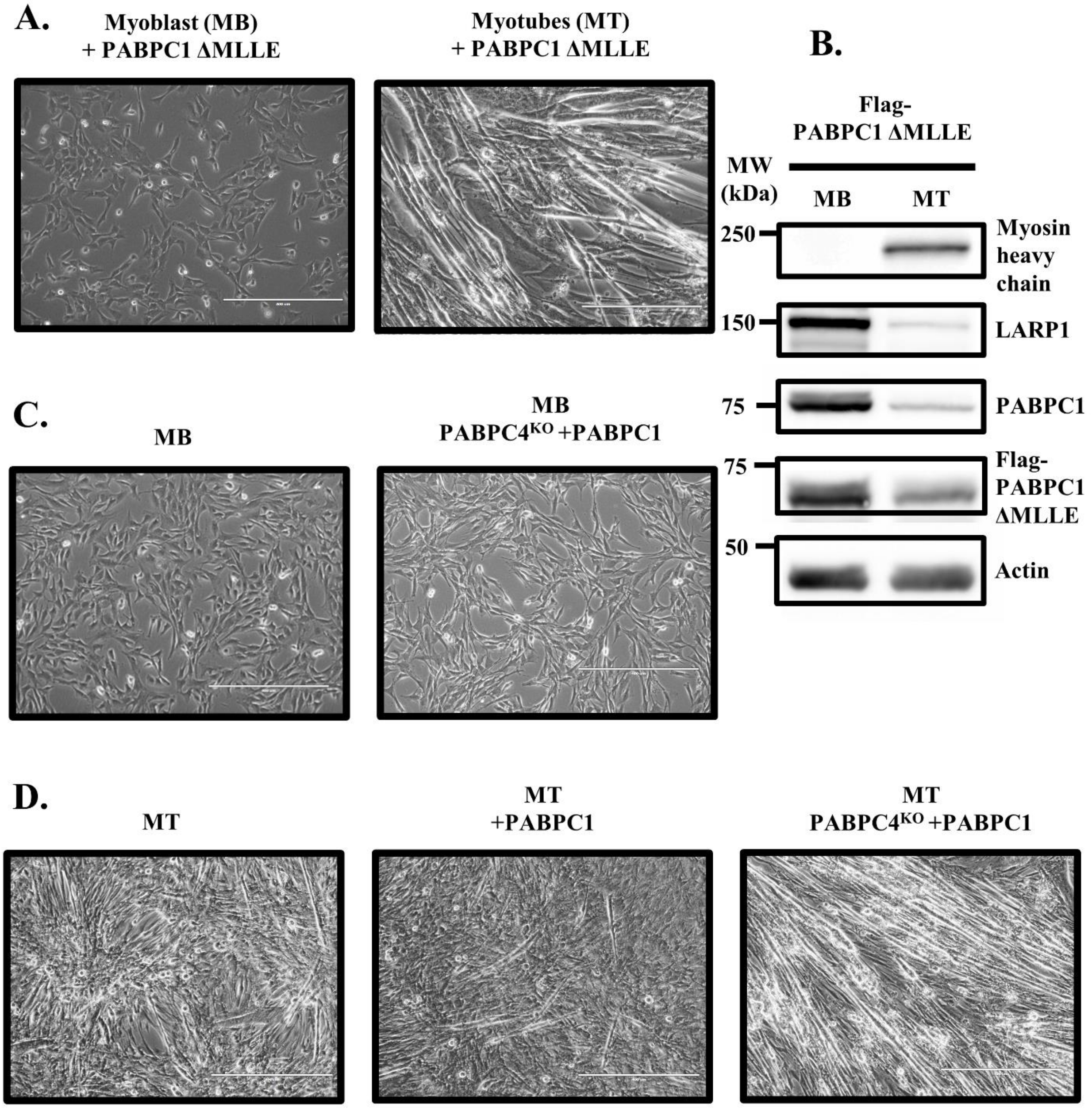

Figure 9. PABPC1 and PABPC4 play roles in myotube differentiation. (A) Microscope images of C2C12 + PABPC1 Δ MLLE myoblast and differentiated myotubes. **(B)** Western blot images of C2C12 + PABPC1 Δ MLLE myoblasts and myotubes. Myosin heavy chain used as a differentiation marker and actin as a loading control. **(C)** Microscope images of C2C12 myoblasts WT and PABPC4^{KO} +PABPC1.**(D)** Microscope images of C2C12 myotubes WT, +PABPC1 and PABPC4^{KO} +PABPC1. All myotubes in this figure were differentiated for 8 days.

3.3.10 PABPC1 and PABPC4 play roles in myotube differentiation.

Finally, we wanted to investigate whether PABPC1 and PABPC4 expression had effects on myoblast differentiation. Myotubes that were +PABPC1 were seen to differentiated at a slower speed than WT C2C12 (Figure 9D). However, this effect was not observed in the PABPC1 ΔMLLE cells (Figure 9A). This suggests that the downregulation of the PABPC1 protein may be important for the proper differentiation of myoblasts to myotubes, and more specifically that its MLLE domain could be important for this. Morphologically, C2C12 myoblasts that are PABPC4^{KO} are less round than their WT counterparts (Figure 9C). PABPC4^{KO} +PABPC1 C2C12 cells were seen to differentiate at a much greater speed than +PABPC1 C2C12, however, a greater amount of cell death could also be observed (Figure 9D). This suggests that PABPC4 may play a role in preventing myoblasts to differentiate before the right time.

Chapter 4:

Discussion and Conclusion

Discussion

4.1.1 PABPCs in terminally differentiated myotubes

The role of PABPC1 in the regulation of protein levels has been widely investigated, though a gap remains in its function in terminally differentiated cells. In an attempt to understand the role of PABPC1 in terminally differentiated myotubes, we investigated protein synthesis, mRNA translation, and mRNA stability in myoblasts and in myotubes. In agreement with previous literature, we saw that in the murine myoblasts system of C2C12 PABPC1 protein levels go significantly down upon terminal differentiation (51). We observed a similar drop in PABPC1 protein level in the rat myoblast H9C2 cell line upon differentiation to cardiomyocytes. A decrease in PABPC1 protein level from embryonic to adult mouse hearts has been previously reported (51).

A compelling model put forth for the role of PABPCs in different context is the "coupling between poly(A)-tail length and translational efficiency" (50). Succinctly, the coupled state is when PABPC is limiting, it strongly influences translational efficiency and mRNAs not bound to it are stable. Examples on an uncoupled state include HeLa cells, where PABPC is saturating and minimally influences translational efficiency. In HeLa cells, PABPs are found to be at a concentration of 4 μM (85). As we observed a comparable amount of PABPC1 and PABPC4 in C2C12 myoblasts as in HeLa cells, it is reasonable to assume that proliferating C2C12s have a similar concentration of PABPCs, and thus are not a PABPC-limiting system. Upon terminal differentiation, we see a vast decrease in both PABPC1 and PABPC4, suggesting that myotubes could be a PABPC-limiting system. However, to determine with certainty whether PABPCs are in excess or are limiting in this system, it would be necessary to quantify the overall concentration of both mRNAs and PABPCs.

In addition to the concentration of PABPCs, poly(A) tail length was also cited as a factor that differed in systems where PABPCs play a role in mRNA stability or translation efficiency (50). Nuclear PABP (PABPN1) has been shown to play critical roles in myoblasts proliferation and differentiation. Specifically, its depletion was shown to cause poly(A) tail shortening and nuclear poly(A) retention, leading to decreased myoblast proliferation and differentiation (86). However, in mature muscles, it is found at very low levels (87). Interestingly, a similar pattern of upregulation for both PABPC1 and PABPN1 was observed during muscle regeneration (87). In cardiac hypertrophy, PABPC1 poly(A) length was shown to be restored (51). Collectively, this suggests that, in addition to the amount of PABPC1, poly(A) tail status could influence whether PABPC1 plays a role in translational efficiency or mRNA stability. The involvement of PABPN1 in polyadenylation and myogenesis further supports the notion that poly(A) tail length could be important for gene regulation in this context.

Overall, this model states that PABPC saturation is not the only determining factor to dictate whether poly(A) tail length affects translation (50). Our lab came to a similar conclusion when showing that depleting PABPC in HeLa cells has minimal effects on translational efficiency (41). However, using a reporter system, they showed that PABPC overexpression in frog oocytes partially uncouples poly(A) tail length with translational efficiency. A similar reporter system could be used in myoblasts and myotubes. This would first allow us to know whether poly(A) tail length is influencing translational efficiency in myotubes compared to myoblasts. If this were the case, we could then investigate whether having +PABPC myotubes influences this effect.

Surprisingly, we also observed a significant reduction in PABPC4 protein levels in both the C2C12 and H9C2 systems, despite previous documentation of higher PABPC4 protein levels in both myotubes and cardiomyocytes compared to other terminally differentiated tissues (88).When examining various mouse tissues, we also found that PABPC4 protein was highest in hearts tissue and still present in skeletal muscle, which contradicted our observations in the cell systems. Interestingly, PABPC4 protein levels were observed to be downregulated in skeletal muscle in response to metabolic stresses, leading to an increase in mitochondrial function and biogenesis (89). This suggests that the reduction in PABPC4 protein levels we observed during C2C12 differentiation may be a result of increased mitochondrial function associated with cell differentiation. Consequently, using C2C12 differentiation as a model for terminally differentiated myotubes may not be ideal as some proteins may still be activated from the differentiation process. For instance, the enhanced mitochondrial function resulting from the differentiation process could contribute to lower PABPC4 protein levels compared to adult skeletal muscle. Moreover, though C2C12 myotubes are a useful and well studied model for myoblast differentiation, it remains that they have a different transcriptomic profile and metabolic profile compared to other models and human skeletal muscles (90).

Ectopically expressing PABPC1 in C2C12 cells resulted in a noticeable decrease in the rate of cell differentiation. The underlying reasons for this phenomenon require further investigation, but a plausible hypothesis is that the downregulation of PABPC1 is needed for the later stages of myogenesis. The comparison between C2C12 cells expressing endogenous PABPC4 or that were PABPC4^{KO} revealed observable differences in their appearance. The PABPC4^{KO} myoblasts appeared to be more elongated. Moreover, when PABPC1 was ectopically expressed in C2C12 cells, the impact on the differentiation speed seemed to be counteracted when PABPC4 was knocked out. These observations lead to three potential hypotheses explaining this phenomenon:

1- PABPC1 and PABPC4 have some overlapping functions in cell proliferation and differentiation. Co-expression of both proteins may interfere with the process of terminal myotube differentiation, leading to a reduction in differentiation speed.
2- PABPC1 plays a role in proliferation while PABPC4 plays a role in promoting differentiation. Though the ectopic expression of PABPC1 may influence the cells to continue proliferating, the effect of PABPC4^{KO} appears to be more significant, overriding PABPC1's influence.
3- The specific levels of PABPC1 and PABPC4 may have an impact on PABPC1's function. Knocking out PABPC4 or ectopically expressing PABPC1 could disrupt the balance required for a switch to a PABP-limiting system, which is necessary for proper myotube differentiation.

In cardiac hypertrophy, it was found that PABPC1 protein levels were restored (51). This finding aligns with the fact that cardiac hypertrophy is associated with an increase in translation initiation. Furthermore, it has been hypothesized that in PABP-limiting systems, PABPC1 plays a more significant role in mRNA translation (91). Exercise is one of the ways through which cardiac hypertrophy can be induced. In skeletal muscle, exercise can also lead to hypertrophy and metabolic stress. In a study using C2C12 cells, metabolic stress was linked with lower PABPC4 protein levels but not PABPC1 (89). Additionally, the study revealed that knocking down PABPC1, but not PABPC4, reduced protein synthesis. It's worth noting that this study was conducted on C2C12 cells that were differentiated for only 5 days, implying that these cells may not have reached full differentiation, as this typically occurs after 7 days. However, these difference in PABPC1 and

PABPC4 expression could indicate a distinct role for these proteins in terminally differentiated cells.

Facioscapulohumeral muscular dystrophy (FSHD) is a muscular disorder characterized by progressive weakness in face and limb muscles. In FSHD, myoblasts differentiate to two phenotypically different types of myotube: thin and atrophic (aFSHD) or large and disorganized with clusters of nuclei (dFSHD) (92). One of the causal proteins for FSHD is DUX4, which putatively binds to both PABPC1 and PABPC4. DUX4 is expressed during embryonic development, but is silenced in most adult tissues (93).

A homologue of DUX4 called DUX4c is also upregulated in FSHD and is another putative PABPC1 and PABPC4 binding partner. Although DUX4c is detected in healthy myotubes, its overexpression delays differentiation and promotes proliferation. Specifically, its overexpression induces the disorganized myotube phenotype, which appears hypertrophic (94). Interestingly, the most upregulated protein found in dFSHD is PABPC4, showing an 18-folds increase (92). This finding contradicts what was found in C2C12s, were PABPC4 was lower in metabolically stressed myotubes, a state linked to hypertrophy. These findings are more indicative of an overlapping role for PABPC1 and PABPC4 in hypertrophy. Another DUX4c putative binding partner is RBM24, a tissue-specific RNA binding protein associated with translational efficiency and polyadenylation (95). RMB24 has been shown to induce cell-cycle arrest and promote myoblast fusion in the development of myotubes and is a PABPC1-interacting partner (96).

Taken together, PABPC1 and PABPC4 probably have both distinct and overlapping roles in myoblasts proliferation and differentiation. A lot remains to be investigated for the roles of PABPC1 and PABPC4 in skeletal muscles, and in their interacting partners. The differential expression of PABPC1 and PABPC4 in skeletal and cardiac muscles further supports the idea that they have distinct roles. Future experiments to investigate this include verifying when differentiation markers (like myosin heavy chain) appear once differentiation is induced between C2C12's that are WT, +PABPC1, PABPC4^{KO} and PABPC1 PABPC4^{KO}. Additionally, stimulating the cells to induce metabolic stress once terminally differentiated and comparing the protein abundance of PABPC1 and PABPC4 could be useful to determine whether they play a role in protein synthesis regulation in this context. Finally, looking at the expression of DUX4c and

RMB24 in these different differentiation contexts could give insight on the mechanism by which PABPC1 and PABPC4 act to promote myoblast proliferation and differentiation.

4.1.2 PABPC1 alone does not regulate protein synthesis in terminally differentiated myotubes

Upon re-expression of PABPC1 in myotubes, overall protein synthesis was not rescued to the same levels as in myoblasts. This is in contrast to what was shown in cardiac muscle, where PABPC1 was sufficient to induce physiological hypertrophy (51). One explanation for this could be that in this study they expressed PABPC1 at 12-fold higher level than endogenous. To better verify whether PABPC1 expression leads to an increase in protein synthesis, a SUnSET assay comparing WT myotubes to +PABPC1 myotubes could be helpful, as reestablishing the protein synthesis rates seen in myoblasts may be unfeasible.

Ectopically expressed PABPC1 was not downregulated during differentiation, unlike the endogenous one. This provides valuable insight as to what may be regulating the decrease of PABPC1 in this system. Some of the main differences in these proteins are the following: human PABPC1 was expressed into mouse cells (Human and mouse PABPC1 share 95% identity (Blast)), the ectopic PABPC1 lacked UTRs, and the ectopic PABPC1 had a flag tag on it's C-terminal. A plausible explanation would be that the endogenous PABPC1 had elements in its UTR's that targeted it to not be translated in myotubes. HuR regulates PABPN1 by binding to cis-regulatory elements in its 3'UTR, which overall has a 50-fold decrease in steady state levels in muscle tissue compared to primary myoblasts (97). Though a study showed that only not having a 3'UTR lead to a decrease of less than 2 fold in mRNA steady stated upon myoblast differentiation, this study only differentiated the cells for 5 days and did not see a significant decrease in PABPC1 mRNA steady state upon differentiation (51). This is in contrast to what we observed and what has been reported by other groups (98). Overall, the 3'UTRs of PABPC1 could have a role in its regulation in skeletal muscles.

When analyzing the polysome profiles, a decrease in peaks before the 80s could be seen in +PABPC1 myotubes compared to WT myotubes. In certain contexts, like in neurons, some high-abundance proteins actually prefer to be associated with monosomes, even when actively translating (99). In *Saccharomyces cerevisiae*, they also observed that most 80S monosomes were in the elongation phase, not initiation, and that this was target specific (100). A way to verify whether these 80s peaks contain any mRNA would be to treat them with high KCl concentrations, which would break up ribosomes not bound to mRNA but keep those bound intact (101). The status of the mRNAs found in these 80S peak also needs to be investigated to determine whether PABPC1 expression is having an effect on monosomes in myotubes.

RNA stability was assessed in C2C12 myoblasts and myotubes to assess which genes were being differentially regulated at the mRNA stability level (98). This a biological system that naturally recapitulates the depletion of PABPCs protein that our lab previously looked at in HeLa cells (41). However, an important distinction between these two systems is that in HeLa cells PABPCs depletion was the triggering element for the changes observed, whereas is in myogenesis, PABPCs depletion may just be a byproduct of the differentiation process. Nonetheless, comparing which classes of mRNAs are being differentially regulated in two different cell lines that either have PABPCs, or don't, can help us understand its role. Interestingly, very little overlap in the classes of mRNAs whose stability is affected by either PABPC-depletion in Hela cells or myogenesis. Overall, most mRNAs showed no significant change in mRNA stability between myoblasts and myotubes. Curiously, one of the classes of mRNAs who were PABP-sensitive in HeLa cells, mitochondrial encoding mRNAs, were actually seen to be more stable in myotubes. Additionally, mRNAs who are more stable in both myotubes and PABPC-depleted cells were all related to cellular stress response. Future experiments to know whether the downregulation of PABPC1 protein in myogenesis played a direct role in altering the steady state levels of these transcripts after terminal differentiation will include checking these genes via RT-qPCR in WT myotubes compared to myotubes +PABPC1. Additionally, an Actinomycin D curve of these transcripts in WT myotubes versus myotubes +PABPC1 can give us more insight on what the decay dynamics of these transcripts look like, independent of transcription.

4.1.3 PABPC1 and LARP1 interactions in terminally differentiated myotubes

The response of skeletal muscle fibers to mechanical stimulus is closely linked to their ability to modulate protein synthesis rates (102). Both translational efficiency and capacity play critical roles in this adaptive response (103). Translational capacity, in particular, is associated with ribosome biogenesis, which can occur as a response to different mechanosensitive pathways, notably mTORC1 (103).

As mRNAs encoding for ribosomal proteins are regulated by both PABPC1 and LARP1, it was surprising that both these proteins are downregulated in terminally differentiated myotubes. Further, having their protein levels correlated across different tissues suggests their stability could be linked together. To further analyse this link, it would be interesting to verify the status of LARP1 in hypertrophic cardiac and skeletal muscles. Recently, a non-peer reviewed paper investigated the role of LARP1 in dilated cardiomyopathy (DCM). This is a disease characterized by myocyte hypertrophy (104). They saw that LARP1 phosphorylation was increased in a congenital mouse model of DCM. Further, they saw that this increase in phosphorylation led to increased binding of LARP1 to the TOP mRNAs RPS20 and RPL32 and an overall increase in ribosomal proteins in DCM (105).

As mTORC1 is thought to regulate ribosomal protein encoding mRNAs through LARP1, it was surprising to see that in myotubes where LARP1 protein is strongly downregulated, TORIN1 still had an effect on RPS14 and RSP29. Additionally, it had an effect on NPM. To our knowledge, no underlying mechanism has to how mTOR regulates TOP mRNAs in muscles has been reported. One protein, CNBP or ZNF9, has been proposed to play a role in TOP mRNA translation (106). This protein is dysregulated in myotonic dystrophy 2 (DM2), and was shown to bind between the TOP motif and start coding of mRNAs (107). It is also known to have roles in the regulation of both cap-dependent and cap-independent translation (108,109). Importantly, TOP mRNAs PABPC1 and RPS17 proteins were showed to be decreased in skeletal muscle of CNBP KO mice (110). Upon re-expressing CNBP in DM2 cell lines, TOP mRNA translation was restored.

AMP-activated protein kinase (AMPK) has been directly implicated in skeletal muscle metabolism, with its activation repressing global protein synthesis rates in this context (111). In liver, it was shown to specifically repress TOP mRNA translation though mTORC1 without acting on global protein synthesis (111). AMPK activation was also shown to phosphorylate and activate CNBP, resulting in the translation of ornithine decarboxylase (112). Importantly, MAPK and mTORC1 can signal through a double negative feedback loop. Under nutrient stress conditions, inhibition of mTORC1 leads to the inhibition of AMPK (113). It is plausible that in a system that lacks LARP1, instead of canonical TOP mRNA translation regulation, other pathways such as the AMPK pathway help regulate these transcripts. AS CNBD knockout was shown to not effect TOP mRNA translation in systems were cells are proliferating, this mechanisms may only be active in systems that are terminally differentiated and have lower PABPC1 and LARP1 protein (114). Future experiments could include knocking out CNBD in C2C12 cells and checking whether TORIN1 still affects translation of the TOP mRNAs in these myotubes.

A decrease in polysome peaks were observed upon differentiation. This is consistent with the thought that translation is lower in terminally differentiated cells (69). However, RPS29 did not show a significant change in polysome association in myotubes, despite being PABP-sensitive in HeLa cells and shown to be primarily regulated through LARP1 (41,82). This is in contrast to another TOP mRNA, RPS14, who was actually found to be more in the polysome fraction upon terminal differentiation. This further points to an alternate mechanism for TOP mRNA regulation in terminally differentiated myotubes, one that does not rely on PABPC1 or LARP1.

We observed that PABPC1 and LARP1 protein levels seemed to be correlated in different tissues, but that their corresponding mRNA abundance did not follow the same trend. Furthermore, PABPC1 actually shifted to the polysome fraction upon differentiation, even though its protein levels are down. LARP1 mRNA remained in the polysome fractions upon differentiation, despite its protein levels also going down. Overall, these findings suggest that these proteins are not regulated at the mRNA level in terminally differentiated myotubes.

PABPC1 expression was able to rescue LARP1 protein levels in myotubes. However, PABPC1 mutant lacking it's MLLE domain, and consequently who could not bind to LARP1, was unable to do so. Important to note is that this mutant seemed less stable during differentiation. An attractive hypothesis for this would be that in myotubes, PABPC1 and LARP1 have similar dynamic as PABPC1 and PAIP2, and when bound are protected from ubiquitination. To further study this, the use of proteasome inhibitors on myotubes can be used to verify whether these proteins are being regulated at the protein level. Additionally, adding back LARP1 and seeing if it can rescue PABPC1 levels will give us insight on whether they can both regulate each other. These experiments should also be repeated using a point mutant in the MLLE domain that disrupts PABPC1's contact with LARP1 without removing the whole domain. This will allow for a more precise assessment, as removing the whole MLLE domain may have more unintended consequences.

Conclusion

Skeletal muscle cells have unique regulatory mechanisms for protein expression in response to various stimuli. These mechanisms involve signaling pathways, such as mTORC1, which integrates these signals to regulate ribosome biogenesis and protein synthesis rates. The specific needs of these cells render them distinct from the models of translational efficiency and mRNA stability previously described in actively dividing cell systems. Nevertheless, these cells exhibit a high degree of plasticity, allowing them to undergo remodeling and hypertrophy in response to exercise or mechanical loading. Consequently, precise control over protein expression becomes essential. Understanding these unique regulatory mechanisms is crucial for unraveling the complexities of skeletal muscle protein expression and its adaptations to diverse physiological conditions.

The work outlined in this thesis suggests that PABPC1 is not a driving force of protein synthesis rates in myotubes. We outline several possibilities, including alternate signaling pathways like AMPK and time-sensitive roles, as to why this may be the case. Additionally, we show that mTORC1 inhibition alters mRNA translation of select TOP mRNAs in myotubes, irrespective of PABPC1 and LARP1's protein expression. This hints to alternate mechanisms for the regulation control of TOP mRNAs in this context. Furthermore, we show a correlation between PABPC1 and LARP1 protein expression, elucidating that their contact may be necessary for their protein expression in terminally differentiated myotubes. Overall, this work shows a discrepancy for the role of PABPC1 in protein expression regulation in terminally differentiated myotubes compared to both early embryos and actively dividing cells. Further experimentation and investigation are needed to validate the hypotheses outlined in this thesis and gain a better understanding of the intricate interactions between RNA binding proteins, notably PABPC1, PABPC4 and LARP1, in myogenesis.

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