An Investigation of the Non-Redundant Functions of PABP4 via Protein-Protein Interactions and PABP-Depletion Studies.

by

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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Molecular and Cellular Biology
Guelph, Ontario, Canada

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ABSTRACT

AN INVESTIGATION OF THE NON-REDUNDANT FUNCTIONS OF PABP4 VIA PROTEIN-PROTEIN INTERACTIONS AND PABP-DEPLETION STUDIES.

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Poly(A) binding proteins are a group of RNA binding proteins with specific affinities towards the poly(A) tract of eukaryotic mRNAs. Although the cellular function of PABP1 in mRNA metabolism is well characterized, the function of other cytoplasmic PABPs is less understood. The aim of this research was to elucidate the cellular function of PABP4. We observed a modest reduction in protein synthesis in PABP4 depleted cells. Additionally, we investigated protein-protein interactions, finding that PABP1 and PABP4 interact with one another. We also found that while PABP4 binds to Paip1, it failed to bind Paip2. Consistent with this observation, PABP1 depletion results in the degradation of Paip2. However, PABP4 depletion had no effect on Paip2 expression. Studies were also conducted to examine PABP1 and PABP4 expression during hypoxia and recovery. Our results did not show any detectable changes in cellular abundance of both PABP1, and PABP4 during hypoxia or recovery.
ACKNOWLEDGEMENTS

They say that no one ever accomplishes anything alone. I think that is doubly true in this case and I would like to profusely thank everyone who has supported me throughout this project. Firstly I would like to express my gratitude to my supervisor, Dr. Jnanankur Bag for his patient guidance and helpful critiques. His teachings have been invaluable throughout my project. Thank you also to Dr. Jim Uniacke for his advice and assistance regarding my thesis and defense preparation. His willingness to give his time so generously has been very much appreciated. I also wish to acknowledge the help and insight provided by Dr. Richard Mosser on my advisory committee. Thank you to the current and past members of the Bag and Yankulov lab for their help and much needed comradery. I would also like to express my appreciation to my friends, family and office mates for their support. I am particularly grateful for the constant love and guidance I have received from my mother. Without her support this would not have been possible. Thank you.
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INTRODUCTION

Regulation of Gene Expression

The multi-step process of gene expression involves the flow of information from DNA to mRNA, followed by translation of mRNA into protein. Correct protein expression is required for proper cellular function. The appropriate synthesis and localization of some proteins is vital to essential cellular processes. In eukaryotes the template strand of DNA carries the blueprint for mRNA and is transcribed by RNA polymerase II in the nucleus. The transcript is produced in the 5' to 3' direction, one nucleotide at a time. Once transcription is completed, the pre-mRNA undergoes further processing and the mature mRNA transcript travels through nuclear pores to the cytoplasm, where translation takes place (Mandel et al., 2008). When translation is initiated ribosomes bind the mRNA and recruit tRNA to translate the genetic code and bring the correct amino acid to the ribosome. Amino acids are joined by the ribosome to create a chain of amino acids. When the polypeptide is completed it is folded into a three-dimensional polypeptide and released from the ribosome (Livingstone et al., 2010). The released polypeptide often interacts with other polypeptides to form a functional protein. Before translation, the pre-mRNAs must undergo a number of post-transcriptional modifications. These modifications are essential for the pre-mRNAs, and are crucial for export to the cytoplasm, mRNA stability and initiation of translation. There are three main post-transcriptional modifications including, 5' capping, RNA splicing and 3' polyadenylation. The 5' capping process is carried out by a capping enzyme complex and occurs directly after transcription of the first 20-100 nucleotides. First the γ phosphate is removed from the 5' end, followed by the addition of GMP to the 5'-diphosphate end. Lastly a methyl group is added to the guanosine residue. The eukaryotic mRNA lacks a free phosphate group at the 5' end, hence this is referred to as the cap structure. The 5' cap is essential
for mRNA stability, protecting the transcript from 5’ exonucleases and is also crucial for mRNA translation.

Pre-mRNAs also undergo RNA splicing, carried out by a spliceosome complex. The spliceosome is a large RNA-protein complex composed of proteins and several small nuclear RNAs (SnRNAs). The spliceosome is able to recognize splice sites and remove introns. The remaining exons are joined together, resulting in a continuous mRNA transcript. Many pre-mRNAs can be spliced in a variety of ways, resulting in different mature mRNAs (Mandel et al., 2008). This allows the production of a number of unique mRNAs from a single DNA sequence.

Finally, eukaryotic mRNAs undergo 3’ polyadenylation, which is coupled with transcription termination. The RNA polymerase II continues transcription past the polyadenylation site. Thus, a cleavage step is required to produce a free hydroxyl group for polyadenylation. cis elements in untranslated regions help direct the 3’ cleavage. When a free hydroxyl group is available, a sequence of 200-250 adenosine monophosphates is added to the 3’ end of the transcript.

The 3’ poly(A) tail has integral functions in nuclear export, translation initiation and mRNA stability. For instance, mutations at the polyadenylation site have been found to reduce nuclear export of the mRNAs, thus diminishing protein expression. Additionally, polyadenylation directly assists in initiating the translation of mRNAs. Although the poly(A) tail is not essential for translation, polyadenylated mRNAs are translated more efficiently (Gorgoni and Gray 2004). Lastly, the binding of poly(A) binding proteins (PABP) to the poly(A) region plays a major role in protecting the mRNA from degradation in the cytoplasm.
Coller et al. (1998) conducted experiments to examine PABP function, independent of poly(A) by tethering PABP to a binding site in the 3’ UTR of a reporter mRNA, using MS2 coat protein. The decay of the MFA2 mRNA in yeast was investigated by inserting MS2 binding sites in the 3’ UTR of the mRNA. MFA2 is a relatively unstable mRNA, with a half-life of about 4 minutes. The PABP1/MS2 fusion protein was found to selectively increase the half-life of MFA2 mRNAs containing the MS2 binding sites from 4 to 21 minutes. Additionally, mRNAs with shortened poly(A) or even lacking poly(A) altogether were still stabilized by PABP1. The PABP1/MS2 fusion protein did not affect the decay of mRNAs lacking the MS2 binding sites, suggesting that PABP1 only acts in cis and is unable to stabilize all mRNAs. It was also found that ongoing translation was required for PABP to effectively stabilize the mRNA, suggesting that some part of the translational machinery is essential for PABP1’s function in stability. The findings suggest that PABP alone is sufficient to prevent mRNA decay, and poly(A) only passively stabilizes mRNA by recruiting PABP (Coller et al., 1998).

Proper cellular function depends on cellular localization and abundance of proteins. The regulation of gene expression is a delicate balance and there are many different strategies employed to regulate this multi-step process. Transcriptional control can regulate gene expression by altering cellular mRNA levels. Alternatively, translational regulation is utilized to generate a rapid change in expression and is often employed in response to environmental changes, stimuli or cell stress. Uncontrolled translation can be detrimental and is often related to disease (Livingstone et al., 2010). Translation is initiated when a 40S ribosome is recruited to the transcript, this is the rate-limiting step of translation and is often targeted for regulation (Martineau et al., 2008). Cytoplasmic PABP plays a crucial role in this process.
**Poly(A) Binding Proteins**

Poly(A) binding proteins (PABPs) are a family of eukaryotic RNA-binding proteins, characterized by their specific interactions with the poly(A) tract of mRNA and known to have prominent roles in mRNA metabolism. The repetitive structure of the poly(A) tail allows the multimerization of many PABPs which require at least 12 nucleotides to bind (Kuhn and Wahle, 2004; Gorgoni and Gray, 2004). The most extensively studied cytoplasmic poly(A) binding protein is PABP1. The molecular weight of PABP1 is approximately 70.2 kDa and the gene is located on chromosome 8, q arm, segment 22.2 and q arm, segment 23 (Kozlov et al., 2001). Structurally, PABP1 consists of an N-terminus containing four non-identical RRMs (RNA Recognition Motif) connected by a short linker sequence, which are conserved among most cytoplasmic PABPs. PABP's N-terminal domain is linked to a proline rich highly conserved C-terminus consisting of five α-helices (Gorgoni and Gray, 2004).

The 4 non-identical RNA Recognition Motifs (RRM) are each approximately 90-100 amino acids long (Kuhn and Wahle, 2004). The RRMs have a globular tertiary structure, folded in a β-α-β-α-β arrangement. These 4 antiparallel β sheets are backed by 2 α helices (Fig. 1). The two central β sheets contain 2 highly conserved sequence motifs, ribonucleoprotein (RNP) 1 and 2 (Deo et al. 1990). These highly conserved motifs facilitate the binding of the protein to the poly(A) tail. At least one RRM is needed for RNA binding and cell viability (Sachs et al., 1987). The RRMs facilitate PABP1's high affinity for poly(A), and a much lower affinity for poly(G) and poly(U), with no detectable affinity for poly(C). The 4 RRMs are strikingly dissimilar, indicating unique functionality. In fact, the equivalent RRMs of PABP1 found in different organisms seem more similar than those found within a single organism (Goss and Kleiman, 2013).
**Fig 1: The RRM Folded Structure.** The aromatic residues on the surface of the β-sheets interact with RNA by stacking with the bases (Kuhn and Wahle, 2004).

RRMs 1-2 are found in the N-terminal region of PABP1 and constitutes one functional unit together and are responsible for binding eIF4G, PABP interacting protein 1 (Paip1) and the poly(A) sequence. Paip1 is structurally similar to eIF4G and stimulates translation by stabilizing the closed loop structure of translating mRNA, which is necessary for optimum translation. RRM 1-2 in particular are responsible for PABP1 binding to the poly(A) tract of mRNA (Fig. 2) (Gorgoni and Gray, 2004; Mangus *et al.*, 2003). In fact, a protein fragment containing RRM 1-2 was able to bind eIF4G and had a stronger stimulatory effect on translation than the full length PABP1 protein (Gray *et al.*, 2000).
**Figure 2: PABP Domains.** The structure of PABP1 consists of RRMs connected to a C-terminal domain by a proline rich region. This structure is highly conserved among PABPs, excluding the nuclear PABP and embryonic PABPs (Gorgoni and Gray, 2004).

RRMs 3-4 make up the second functional unit. These RRMs are closer to the C-terminus and despite reduced poly(A) affinity are able to bind AU-rich RNA and facilitate protein-protein interactions (Brook *et al.*, 2012 & Neitfield *et al.*, 1990). Protein fragments containing only RRMs 3-4 were also able to stimulate translation (Gray *et al.*, 2000).

The N-terminal domain is linked to the C-terminus by a proline rich linker region, which plays a role in PABP-PABP interactions, facilitating the multimerization of PABP (Brook and Gray, 2012). The C-terminal domain (PABPC) is structurally composed of 4-5 α-helices folded into a globular cluster with a hydrophobic core (Kozlov *et al.*, 2001). This domain is 75 amino acids long and does not bind RNA, but instead binds proteins that contain the PABP interacting motif (PAM), such as Paip1, Paip2 and eRF3 (Kuhn & Wahle, 2004). Protein fragments containing only the carboxyl terminus domain were still able to stimulate translational activity (Gray *et al.*, 2000). PABPC is a highly conserved domain and is homologous to a domain found near the C-terminus of EDD. EDD is the mammalian ortholog of the *Drosophila* hyperplastic
disc protein (HYD) and is a member of the HECT family of E3 ubiquitin ligases. The structurally similar EDD domain can even bind PABPC-binding proteins such as Paip1 and Paip2. The EDD domain similar to PABPC likely specifies the targets of the ubiquitin ligase. Thus, EDD acts on proteins that are able to bind the PABPC domain as a means of regulating PABP (Deo et al., 2001).

**PABP Homologs**

The protein most closely related to PABP1 is PABP3 (tPABP), which is encoded by an intron-less gene (Kuhn and Wahle, 2004). The PABP3 gene is located on chromosome 13, q arm, segment 12 and q arm segment 13. PABP3 is very similar to PABP1 and is suspected to be the product of a retro transposition event. It is likely that PABP3 maintains very similar functions to PABP1, as a result of the conserved structure with possible additional roles in spermatogenesis, due to its specific expression in the mammalian testis, of both humans and mice. However, PABP3 has slightly less affinity for poly(A), compared to PABP1. This is likely due to various amino acid substitutions in the RRM regions and several small deletions in the proline linker region (Gorgoni and Gray, 2004). The unique expression pattern of PABP3 in the testis suggests a crucial role in mRNA stability during spermatogenesis. During meiosis mRNA transcripts are stored as messenger ribonucleoprotein particles (mRNPs). PABP3 mirrors these elevated levels of mRNPs, indicating that PABP3 may be responsible for the stability and translation of these stored mRNAs. The stability of mRNAs is crucial during the development of spermatozoa (Feral et al., 2001).

The third cytoplasmic PABP, PABP4 is often referred to as inducible PABP (iPABP). This gene is located on chromosome 1, p arm, segment 32 and segment 36. PABP4 has a number of amino acid substitutions in the RRM regions and has a striking dissimilarity in the proline rich
region, when compared to PABP1 (Gorgoni and Gray, 2004). As the name indicates PABP4 is rapidly induced in response to T cell activation and is found on the surface of activated platelets (Yang et al., 1995; Gorgoni and Gray, 2004). It is expressed at high levels in the heart and skeletal muscle tissues of mammals, however the function of this tissue specific expression is yet to be explained (Yang et al., 1995). In addition to its affinity for poly(A), PABP4 also binds eukaryotic Release Factor 3 (eRF3), which has a prominent role in translation termination. Interaction with eRF3 inhibits PABP-PABP interactions, preventing multimerization on the poly(A) tail (Cosson et al., 2002). However possible functions of PABP4 in mRNA stability or translation have not yet been determined.

Another unique cytoplasmic PABP, is PABP5 which is encoded by the X chromosome on the q arm, segment 21.3 and is expressed at high levels in the ovaries in humans and mice (Kuhn and Wahle, 2004; Gorgoni and Gray, 2004). The PABP5 gene lacks introns, indicating that it is likely the product of a retro transposition event (Mangus et al., 2003). PABP5 is structurally distinct from the aforementioned cytoplasmic PABPs, lacking the C-terminal domain and proline rich region (Kuhn and Wahle, 2004; Gorgoni and Gray, 2004). The function of PABP5 is as of yet unknown and an interaction with the poly(A) tail has not yet been proven. However, experiments in yeast show that the C-terminus is not required for functionality of PABP1, thus PABP5 may still be a viable PABP protein (Gorgoni and Gray, 2004).

PABPs are found to be highly conserved among species. PABP deletion and mutation experiments have been conducted on a number of different species. Sachs and Davis (1989) conducted a PABP depletion study in S. cerevisiae, using a repressible galactose promoter. When the carbon source was switched from galactose to glucose, the cells were depleted of PABP and an almost three-fold increase in poly(A) tail length was observed. Additionally, Sachs and Davis
(1989) used temperature-sensitive mutations to examine PABP depletion in yeast. In cells lacking a functional PABP protein, a decrease in polysomes was observed, coupled with an accumulation of monosomes. These findings suggest that PABP has a role in translation initiation and poly(A) shortening (Sachs and Davis 1989). A number of theories have been proposed to explain how PABP promotes translation initiation. Sachs and Davis (1990) observed that a mutation in the 60S ribosome or in the helicase required for 60S synthesis were able to partially rescue the phenotype of PABP depletion. Consistent with these findings, loss of poly(A) tails was shown to inhibit 60S joining (Munroe and Jacobsen 1990). These observations suggest that PABP stimulates recruitment of the 60S subunit. Additionally, studies on cell extracts depleted of PABP showed impaired recruitment of the 40S subunit (Tarun and Sachs, 1995). Kahvejian et al. (2005) showed that PABP acted as a translation initiation factor, aiding in recruitment of both the 40S and 60S subunits in an in vitro translation system.

PABP was also found to be necessary for proper development of Drosophila melanogaster. Insertion of P-elements into the Drosophila PABP gene (dPABP) has been shown to be embryonic lethal (Sigrist et al., 2000). A Drosophila homologue of the human Paip2 has also been characterized and is referred to as dPaip2. dPaip2 was shown to interact with dPABP in Drosophila cells, in a similar manner to the human homologues. When dPaip2 was overexpressed, growth of the larval fat body, eyes, wings, and wing-imaginal discs was reduced, without altering the development pattern. dPaip2 likely inhibits growth by blocking translation, much like human Paip2 (Roy et al., 2004). Drosophila also has an identified PABP2 (PABPN1 in mammals; previously called PABP2), which is mostly nuclear, expressed in all tissues and behaves much like its mammalian counterpart. It is able to stimulate poly(A) polymerase (PAP) and control poly(A) length. This protein contains a smaller N-terminal region and lacks an
alanine stretch in this region, found in the mammalian homologue. The PABP2 gene produces 4 different mRNAs. These are not a product of alternative splicing, but instead, alternative polyadenylation. There are 5 different poly(A) sites, all preceded by poly(A) signals. Two alternative poly(A) sites have also been identified in the mouse PABII gene (Benoit et al., 1999).

*C. elegans* contains 3 PABP homologues. PABP-1 is involved in gonad development as *C. elegans* depleted of PABP-1 showed abnormal germline development, resulting in small gonads in adults. PABP-1 was also found to interact with ataxin 2 (ATX-2), a protein associated with a neurodegenerative disorder, spinocerebellar ataxia type 2. Similar to PABP-1, ATX-2 is necessary for germline development, although to a lesser degree. PABP-2 is the most similar to the human PABP2, studies involving PAB-2 depletion resulted in defective somatic development. PAB-3 is the most similar to PABPN1 and does not seem to have a role in translation (Ciosk et al., 2004).

Novel embryonic PABPs (ePABP), like PABP1 were also discovered in *Xenopus*. The ePABP binds AU-rich regions and interacts with eRF3 and eIF4F (via the eIF4G subunit). The gene is located on chromosome 14, q arm, segment 11.2 and q arm segment 13 and is expressed at higher levels than PABP1 during oogenesis and early embryogenesis. ePABP has roles in regulating deadenylation but also has strong similarity to PABP1 in yeast. In fact, ePABP was shown to be a suitable substitution for PABP1 deletions in yeast, indicating that they share similar functionality (Voeltz et al., 2001). *Xenopus* oocytes contain a large pool of polyadenylated mRNAs, however, they're found to contain less than 1 PABP molecule for each poly(A) binding site. This is a stark contrast, compared to the approximate 3 fold excess (over poly(A) content) of PABP in HeLa cells. To investigate this disparity Wormington et al. (1996) examined the effects of PABP overexpression in the *Xenopus* oocytes. Results showed that the
high levels of PABP prevented deadenylases from accessing poly(A), thus inhibiting maturation-specific deadenylation and translational inactivation. These findings indicate that the low levels of PABP may facilitate maturation-specific deadenylation in maturing oocytes (Wormington et al., 1996). Due to the low levels of PABP in Xenopus oocytes, it has been suggested that PABP is not necessary for translation. However, additional studies examined an eIF4G mutant, unable to bind PABP. These oocytes showed a decrease in translation of polyadenylated mRNAs, which dramatically inhibited maturation (Wakiyama et al., 2000). Also Gallie et al. (2000) found that overexpression of PABP was only able to enhance translation of mRNAs with 5' caps and poly(A) tails. Thus, only transcripts able to bind PABP benefitted from the overexpression. Later, ePABP2 was found to be very similar to the Xenopus PABP2 protein (PABPN1 in humans), which has roles in poly(A) tail elongation. However, the precise cellular function of ePABP2 is still unknown. Although, it is only expressed during early stages of development, ePABP2 orthologs have also been found in humans and mice, and is expressed during early development and in the ovary, indicating its importance in mRNA metabolism during early vertebrate development (Good et al., 2004).

Most eukaryotes appear to only have 1-3 functional PABP genes. However, 8 PABP genes have been identified in Arabadopsis thaliana. The Arabadopsis PABPs show a higher sequence divergence and more differential expression than is commonly found in the highly conserved PABP proteins. It has been reported that only 2 of the 8 PABP genes contain 4 RRMs. The Arabadopsis PABs can be divided into 4 classes; those specifically expressed in reproductive tissue, PAB3 and PAB5; those that are broadly and strongly expressed, PAB2, PAB4 and PAB8; those that are weakly expressed in specific tissues, PAB6 and PAB7; and finally, PAB1 which has low tissue specificity (Belostotsky, 2003). The PABC portion of both
PAB2 and PAB5 are able to bind the PAM2 motif. However, the PABC domain of PAB7 contains a histidine instead of tyrosine, resulting in reduced PAM2 affinity. These varied protein interactions suggest unique functionalities (Kozlov et al., 2004). Cross complementation studies of yeast null PABP mutants show that expression of PAB3 from Arabidopsis in yeast was able to accelerate mRNA degradation and translation. Additionally, PAB3 was found in the nucleus of the complemented yeast strain and was able to restore the length of poly(A) tails by protecting mRNAs from poly(A) nucleases. These results are indicative of a conserved role for PABP in mRNA biogenesis and export (Chekanova and Belostotsky, 2003).

The miRNA regulatory pathways are able to control mRNA expression at a post-transcriptional level via translation repression or deadenylation and decay. miRNAs are partially complementary to their target sequences and together with Argonaute proteins form miRNA Silencing Complexes (RISCs). RISC represses translation of the target and triggers a pathway for mRNA deadenylation and decay. Several proteins are involved in this pathway, including GW182, a core component of RISC and the CCR4-NOT deadenylase. Interaction between GW182 and PABP1 is essential for silencing. GW182 competes with eIF4G to bind PABP1, therefore disrupting the PABP1-eIF4G complex and repressing translation, leaving the transcript susceptible to decay (Zekri et al., 2009). GW182 mutants that are unable to bind PABP show impaired miRNA-mediated silencing. Additionally, PABP is necessary for the miRNA-mediated deadenylation of mRNAs (Huntzinger et al., 2010). It has also been observed that polyadenylated mRNAs are silenced more efficiently than those lacking the poly(A) tail, however the cause of this trend is unclear. Moretti et al. (2012) found that the length of poly(A) tails, and the number of PABPs associated with it are positively correlated with miRNA-mediated repression. PABP and the poly(A) exert this stimulatory effect by facilitating miRISC
binding to mRNAs. miRISC displaces PABP and the mRNA is targeted for deadenylation. Although many studies show that PABP1 is not essential for miRNA-mediated silencing, it still contributes to the efficiency of this pathway. The precise mechanistic details of how PABP stimulates miRNA silencing are unclear and further studies need to be conducted (Zekri et al. 2009).

**Role of PABPs in Translation**

Although poly(A) tails are not necessary for translation to occur, polyadenylated mRNAs are translated preferentially and in a more efficient manner than non-adenylated mRNAs. Cells with PABP1 mutations exhibit defective translational processes, indicating that the involvement of the poly(A) track in mRNA translation is primarily mediated via PABP1. However, PABP1 has also been found to stimulate translation of non-adenylated mRNAs, indicating that PABPs role in translation is complex and may involve multiple pathways and additional PABP binding sites within the mRNA (Kahvejian et al., 2005). The translational process begins with the formation of the 43S preinitiation complex, which consists of the small 40S ribosomal subunit, eIF3, eIF2-GTP and Met-tRNA: A complex of eukaryotic initiation factors, collectively referred to as eIF4F helps the pre-initiation complex to bind the 5' cap and scans the mRNA for the initiation codon by unwinding the mRNA's secondary structure (Jackson et al., 2010). eIF4F associates with the 5' cap of the transcript. This complex consists of eIF4E, which is responsible for binding to the 5' cap; eIF4A which has ATP-dependent RNA helicase activity; eIF4B which stimulates the function of eIF4A; and lastly, eIF4G, a scaffolding protein that can interact with eIF4E, eIF4A, eIF3 and PABP1. eIF4G helps recruit the eIF3-40S ribosome complex by associating with eIF3 (Roy et al., 2002). While bound to eIF4E, tethering it to the 5' cap, eIF4G associates with PABP1 which is bound to the 3' poly(A) tail. This interaction brings the 5' and 3' ends together, forming a "closed loop" structure, which stabilizes and protects the mRNA from
degradation or decapping (Fig 3). The interaction of the active proteins also increases their affinity for the mRNA, locking this structure in place. The 40S ribosomal subunit is recruited to this complex to form the pre-initiation complex that scans along the transcript until it reaches a start codon. The initiation factors are released, allowing the 60S ribosomal subunit to join forming the 80S ribosome containing initiation complex, to begin mRNA translation (Gorgoni and Gray, 2004). The role of PABP in mRNA translation is conserved among many species (Kuhn and Wahle, 2004).

**Fig 3: Translation Initiation.** The eIF4F complex consisting of eIF4E, eIF4G, eIF4A and eIF3 interacts with the 5' cap via eIF4E. eIF4G interacts directly with PABP bound to the poly(A) tail. This interaction brings the 5' and 3' end of the transcript together, creating a close loop structure. The interaction is stabilized by Paip1, thereby stimulating translation. The 40S ribosome is recruited to eIF3 and will then scan along the transcript for a start site.

There have been a number of models proposed to explain the exact mechanisms of how PABP stimulates translation. The first of which suggests that a closed loop structure is mediated
by the interaction between PABP and eIF4G, by joining the two ends of the mRNA. This closed loop structure of translating mRNA is believed to promote ribosome recycling. This is supported by the interaction between PABP and eRF3 (Uchida et al., 2002). In addition, it has been proposed that PABP promotes 60S subunit binding to the mRNA. This is consistent with experiments showing that nonadenylated mRNAs had significantly less 60S subunit binding (Munroe and Jacobson 1990). However, other work suggests that PABP stimulates initiation by promoting 40S binding to mRNA (Tarun and Sachs, 1995).

Different mRNAs have differing intrinsic capabilities for translation which may depend on their specific secondary structures, codon usage and presence of additional regulatory sequences. In addition, translation of a specific mRNA may be controlled by fine tuning the interaction between PABP and translation initiation factors. Phosphorylation of initiation factors can significantly alter protein expression. Thus, altering the activity of initiation factors can differentially affect the translation of specific mRNAs. For example, interaction with eIF4G is enhanced by phosphorylation of PABP1, thus further stimulating translation. The MKK-2 pathway (mitogen-activated protein kinase) is also involved in the phosphorylation of eIF4E, PABP1 and eIF4G and in turn the MKK level is regulated by PABP1 levels (Ma et al., 2006).

**PABP and mRNA Stability**

PABP1 also has prominent roles in mRNA stability; this process has been extensively studied in yeast, and seems to be conserved in higher eukaryotes. Removal of the poly(A) tail is the rate-limiting step in mRNA decay pathways (Tourriere et al., 2002). After the poly(A) tail is significantly shortened by the poly(A)-nuclease (PAN) (Brook and Gray, 2012) the transcript is rapidly deadenylated followed by removal of the cap. The deadenylated and decapped transcript is now free to be degraded by exosomes. For some time, PABP1 has been thought to play a large
role in protecting mRNA deadenylation by preventing deadenylases from accessing the transcript. PABP1 interacts with the transcript and with the eukaryotic initiation factors, these proteins lock onto both ends of the transcript, simultaneously preventing the deadenylation and decapping processes, and thus maintaining mRNA stability (Tourriere et al., 2002). However, recently PABP has been found to interact with PAN and perhaps stimulate its activity, this seems counterintuitive due to the suggested role of PABP in protecting poly(A). It is possible that bound PABP protects poly(A) by blocking PAN, while free PABP recruits and stimulates the activity of PAN (Brook and Gray, 2012). It is notable that HeLa cells contain approximately 3 times more PABP1 than the poly(A) tracts available for PABP1 binding (Gorlach et al., 1994).

In addition to the global mRNA population, PABP regulates the stability of specific mRNAs such as c-fos, which encodes a transcription factor. The c-fos mRNA contains a major protein-coding determinant of instability (mCRD) cis element which stimulates deadenylation and then subsequent degradation of c-fos mRNA in resting cells (Shyu et al., 1991). The function of mCRD depends on its proximity to the poly(A) tail. The mCRD interacts with a complex consisting of PABP1, Paip1, heterogeneous nuclear ribonucleoprotein D (hnRNPD), NS1-associated protein 1 (NSAP1) and upstream of N-ras (Unr). Prior to translation, this complex brings the mCRD into close proximity with the poly(A) tail, preventing deadenylation by stabilizing the PABP-poly(A) interaction and blocking nuclease access to the poly(A) tail. But, during translation, the ribosome displaces this complex, exposing the poly(A) to nucleases (Grosset et al., 2000). A few other instances of this specific regulation have been studied, however it is likely that PABP functions as a widespread regulator of specific mRNAs (Gorgoni and Gray, 2004).
Regulation of PABP

PABP mRNAs have an unusually long 5'UTR that contains sequences responsible for regulating its own mRNA translation. PABP mRNA contains a TOP sequence (terminal oligopyrimidine) at the 5' terminus, which is a translational cis-regulatory element. Members of the TOP family of mRNAs are known for producing the machinery involved in protein synthesis (Bag and Bhattacharjee, 2010). The TOP sequence consists of a C residue at the 5' cap, followed by 4-14 pyrimidines and the activity of this sequence resides mainly with the first 30 nucleotides. The functionality of the TOP sequence is dependent on its proximity to the 5' cap; it is unable to function internally. The translation of TOP mRNAs is dependent on the rate of cellular growth. When cell growth is arrested, for instance when there is an amino acid shortage, TOP mRNAs will no longer be translated until more optimal growth conditions are achieved to prevent unnecessary energy waste. This is illustrated by the shift of TOP containing mRNAs from polysomes in growing cells to mRNA-protein complexes (mRNP) during growth arrest (Meyuhas, 2000). There is a correlation between the phosphorylation of the small ribosomal protein S6 (RPS6) and translation of TOP mRNAs. It is believed that when conditions are more suitable for cell growth mTOR activates S6 kinase (S6K), which phosphorylates RPS6, in turn increasing the affinity of ribosomes for TOP mRNAs leading to translation initiation. Despite the evidence of S6K involvement in TOP mRNA translation, more recent studies involving knock-in mice with mutated phosphorylation sites in RPS6 show that TOP mRNAs are still translated in the absence of S6K. These conflicting results suggest that another pathway may also be involved (Meyuhas, 2000).

Studies conducted in our lab investigated the response of TOP mRNAs to heat shock. During heat shock treatment PABP and eIF4G were found to co-localize in the nucleus with Heat Shock Protein 27 (HSP27). Translation of PABP decreased during heat shock, but was up
regulated 2.5 fold during recovery from heat shock. Studies in our laboratory showed that this response is facilitated by the TOP sequence (Ma et al., 2009; Datu and Bag, 2013). The TOP-mediated increase in translation may act as a signal to stimulate global mRNA translation to aid the cell in recovery (Ma et al., 2009). Similar enhanced translation during recovery from heat shock has been observed in other TOP mRNAs such as RPS6 and eEF1A. However, cells depleted of Zinc Finger Factor 9 (ZNF9) no longer showed increased levels of PABP1, RPS6 and eEF1A mRNA translation during recovery from heat shock. ZNF9 is known to stimulate both global and TOP mRNA translation, and these results suggest that it is necessary to regulate these 3 different TOP mRNAs simultaneously following recovery from heat stress. It is possible that different TOP mRNAs may be regulated by unique factors in response to various growth signals (Datu and Bag, 2013). In serum-deprived cells PABP displays a similar response to growth arrest. PABP mRNA accumulated in sub-polysomal fractions in serum deprived cells. Following addition of serum, a portion of PABP mRNA redistributed to large polysomes. Serum stimulation also increased the distribution of PABP mRNA in the polysomal fractions, showing that an increased serum level increases translation initiation of PABP mRNAs. These results indicate a demand for PABP under conditions of growth stimulation (Berger et al., 1992).

mTOR is another possible regulator of TOP mRNAs. mTOR integrates signals from upstream pathways involving insulin, growth factors, amino acids, nutrients and energy levels. mTOR acts on eIF4E Binding Protein (4E-BP) which binds and sequesters eIF4E. When 4E-BP is bound by mTOR, it is phosphorylated, causing it to release eIF4E. This allows eIF4E to interact with the eIF4F complex, initiating translation. This links mTOR growth signaling and eIF4F dependent translation initiation. Thus, it is possible that mTOR-dependent phosphorylation of 4E-BPs at least partially contributes to the regulation of TOP mRNAs.
In support of this, it has been observed that inhibition of the mTOR pathway represses the translation of TOP mRNAs (Patursky-Polischuk et al., 2009).

PABP contains an additional regulatory sequence in the 5' UTR, an (A)-rich region where PABP is able to bind to its own mRNA. This sequence acts as an auto-regulatory sequence (ARS) that inhibits PABP mRNA translation to avoid excess PABP synthesis. Unlike TOP, ARS is still effective when translocated within the 5' UTR (Bag, 2001). The ARS functions as a negative feedback mechanism. When PABP levels are high in the cell, it binds the ARS of the mRNA, repressing its own translation by stalling the 40S ribosome at the ARS. This repression can be relieved when intracellular polyadenylated mRNAs become abundant or when the length of poly(A) tails increases. Addition of exogenous poly(A) to the cell mimics this effect, stimulating PABP expression without increased mRNA synthesis. Similarly, overexpression of PABP leads to a repression of endogenous PABP (Melo et al., 2003). There is likely a level of PABP that is optimal for cellular function, since a high level of PABP has been observed in many cancer cells (Ozen et al., 2008; Verlaet et al., 2001). TOP and ARS function independently of one another, one stimulating PABP translation and the other repressing it, thus offering a precise control of the level of cellular PABP under different physiological conditions (Bag and Bhattacharjee, 2010).

In addition to regulating the PABP level by modulating the efficiency of its mRNA translation, the biological activity of the PABP polypeptide is regulated by various proteins. PABP1 has been found to interact with another group of proteins known as PABP-Interacting Proteins (Paip). Paips contain two conserved PABP-binding motifs (PAMs). PAM1 is able to bind the RRM region of PABP1 and PAM2 binds to the carboxy-terminus of PABP (Kozlov et al., 2004). Paip1 is structurally similar to eIF4G, and like eIF4G has been found to bind eIF4A
and eIF3. Cells which do not produce eIF3 or those that produce a mutated Paip1 that cannot bind eIF3 have shown a lack of Paip1 enhanced translation. This indicates that eIF3 is essential for the Paip1-mediated stimulation of translation. Paip1’s interaction with eIF3 and eIF4A help stimulate translation by stabilizing the closed loop structure formed by PABP1 and eIF4G. In contrast to Paip1, the Paip2 protein has an inhibitory role in mRNA translation (Martineau et al., 2008).

There are two Paip2 isoforms identified, Paip2a and Paip2b respectively. Any unique functionality between the two Paip2 isoforms is so far unclear. However, their expression does differ among various tissues. Both isoforms are highly expressed in the brain and testis; additionally, Paip2b is also expressed at high levels in the lung, heart and pancreas (Berlanga et al., 2006). In contrast to Paip1, the Paip2 isoforms act as negative regulators of PABP1 stimulated translation. Paip2 competes with Paip1 for binding to the RRM1-2 of PABP1, preventing interaction with Paip1 and the poly(A) tail. Interaction with Paip2 causes PABC1 to dissociate from the poly(A), repressing translation (Yoshida et al., 2006). Additionally, Paip2a binds PABP, preventing interaction with eIF4G, reducing mRNA circularization (Karim et al., 2006). Paip2 is conserved in Drosophila and also seems to reduce cell growth when over-expressed (Kuhn and Wahle, 2004). E3 ubiquitin ligase (EDD) contains a PABPC domain, enabling interactions with PABP1, Paip1 and Paip2. When PABP levels are low, Paip2 is free to interact with EDD, which ubiquitinates and degrades Paip2. This is thought to regulate PABP levels in a positive feedback mechanism, maintaining PABP homeostasis. (Yoshida et al., 2006). EDD was also shown to be up regulated in cancer cells. This overexpression may result in increased ubiquitination and degradation of Paip2, in turn raising PABP levels, enhancing translation and contributing to oncogenic transformation (Kahvejian et al., 2001). Although both
Paip1 and eRF3 contain the same PAM2 motif that facilitates the Paip2-EDD interaction, they are not found to be degraded by EDD (Yoshida et al., 2006).

PABP1 is also involved in the Nonsense Mediated Decay (NMD) pathway of mRNA. This pathway maintains mRNA quality by degrading mRNAs containing premature termination codons. This prevents the production of truncated proteins, which are often toxic to cells. In fact, one third of disease-associated mutations lead to the production of nonsense mRNAs (Muhlemann, 2008). Premature termination codons are recognized by mRNA surveillance machinery and the NMD pathway is initiated (Brook and Gray, 2012). The interaction of PABP1 with eRF3 is important for this "decision-making" step of NMD. eRF3 is important for the termination of translation and remains bound to the translating ribosome. For a termination to be deemed "correct" the termination codon needs to be close enough to the poly (A) tail so that eRF3 can interact with PABP1. When this interaction occurs the ribosome dissociates and the recruitment of decay factors is repressed. However, if a premature termination codon is present further away from the 3’ poly (A) it results in a much greater distance between the termination codon and the poly(A) tract, thus eRF3 fails to bind PABP1 allowing it to bind UPF1. When UPF1 has the opportunity to bind, the termination codon is deemed "incorrect" and UPF1 signals the start of the NMD pathway (Muhlemann, 2008).

**Nuclear Functions of PABP**

In addition to the four cytoplasmic PABPs, a nuclear PABP has also been identified in humans, referred to as PABPN1 or (PABP2). This nuclear protein is structurally unique from the cytoplasmic PABPs, containing an arginine rich C-terminal domain and only one RRM that is distinct from those found in the cytoplasmic PABPs (Kuhn and Wahle, 2004). PABPN1 is involved in the polyadenylation step of mRNA processing. Not only does PABPN1 stimulate
poly(A) polymerase (PAP) activity, it also protects the mRNA from degradation. Initially the Cleavage and Polyadenylation Specificity Factor (CPSF) binds a Polyadenylation Signal (PAS) on the 3’ end of the transcript and cleavage occurs. Following the cleavage step, Poly(A) Polymerase (PAP) is recruited to the 3’ end of the transcript and slowly begins poly(A) synthesis. PABPN1 binds this nascent poly(A) tail after the first 10-12 nucleotides of adenines have been added to the transcript. PABPN1 then stimulates PAP activity, leading to rapid addition of 200-250 adenylate nucleotides at the 3’ end, allowing more PABPN1 binding. PABPN1 isn’t necessarily required for polyadenylation but it does help regulate the correct length of the poly(A) tail and increases the efficiency of PAP 80-fold. (Mandel et al., 2008). After a length of 250 adenylate residues have been added to the mRNA the efficiency of this process slows and is terminated. There is a change in the poly(A)-PABPN1 complex that disrupts the CPSF-PAP complex, terminating polyadenylation (Kuhn et al., 2009).

Jenal et al. (2012) classified PABN1 as a repressor of alternative cleavage and polyadenylation (APA). In their studies PABPN1 was knocked down in U2OS osteosarcoma cells, using PABN1 siRNA. A deep sequencing technique, 3'Seq was then used to elucidate PABPN1’s role in cleavage site usage. Following knockdown, the use of the proximal cleavage sites was enhanced, thus shortening the 3’ UTR and changing the miRNA binding sites. This suggests that PABPN1 has a role in recognition of poly-adenylation sites and that PABPN1 interacts with the pre-mRNA prior to polyadenylation. PABPN1 also appears to have an important role in oculopharyngeal muscular dystrophy (OPMD). Patients with OPMD express a mutant PABPN1 (trePABPN1) that has polyalanine expansions from 10 to 12-17 alanines in the amino terminal end of the protein. This mutant protein appears to form inclusion bodies in the nuclei of OPMD patients. trePABPN1 was ectopically expressed in U2OS cells, using a GFP
tagged mutant PABPN1 and cells expressing trePABN1 showed the same trend in cleavage site usage as the PABN1 knockdown cells. Immunohistochemistry with the PABN1 antibody revealed that the mutant PABPN1 co-aggregated with the wild type PABN1, which would likely hinder the function of the wild-type protein in OPMD cells. These findings suggest that OPMD is associated with mis-regulation of APA. The symptoms may be a result of abnormal gene expression, causing irregular 3' end formation (Jenal et al., 2012). An OPMD model in mice showed that overexpression of wild type PABPN1 can improve OPMD symptoms and has anti-apoptotic effects. These effects are mediated through elevated levels of X-linked Inhibitor of Apoptosis (XIAP). Apoptosis has been proposed as a contributor to muscle fiber loss and some studies show that prevention of apoptosis improves muscle strength in some muscular dystrophy models (Davies et al., 2007).

Apponi et al. (2010) further investigated the role of PABPN1 in differentiation, by conducting PABPN1 knockdown studies in mouse myoblasts. In the PABPN1 depleted cells poly(A) tails were shortened and mRNA accumulated in the nucleus, possibly contributing to abnormal myogenesis, an essential part of muscle regeneration and maintenance (Apponi et al., 2010). Further studies in our lab were conducted on PABPN1 knockdown cells. This work showed no detectable effect of PABPN1 depletion on mRNA transport, mRNA abundance, mRNA translation, transcription or poly(A) tail length in HeLa cells. However, increased levels of PABP5 were observed, along with a small global increase in protein synthesis in PABPN1 depleted cells. It is possible that the enhanced levels of PABP5 work to stimulate global protein synthesis. Additionally, PABP4 was found to accumulate in the nucleus of PABPN1 depleted cells. PABP4 associated with pre-mRNA transcripts and CPSF in the nucleus, suggesting that it may be compensating for the function of PABPN1. Further work would be needed to determine
if PABP4 is able to stimulate PAP. However, despite these observations, 40% of the PABPN1 knockdown cells were apoptotic within 72 hours (Bhattacharjee and Bag, 2012). The differential effect of PABPN1 knockdown on mRNA metabolism in non-differentiated HeLa and differentiated myogenic cells could be due to additional function of PABPN1 in stimulating myogenesis (Apponi et al., 2010). In addition, PABPN1 also exhibited an anti-apoptotic function in mammalian cells. This was illustrated by increased levels of phosphorylated p53 and PUMA, common markers of apoptosis (Bhattacharjee and Bag, 2012).

**Shuttling PABPs Across the Nuclear Envelope**

PABPN1 has many essential functions for the mRNA in the nucleus. Due to the steady state localization of PABP1 and PABPN1 to the cytoplasm and nucleus respectively, it has been suggested that PABP1 "takes over" from PABPN1 once the mRNA exits the nucleus. It has been demonstrated that PABPN1 remains bound to the mRNA up until docking of the mRNA to the nuclear pore complex. However, once the mRNA is in the cytoplasm, little PABPN1 can be detected. This resulted in the development of a model in which PABPN1 is responsible for 3' end processing in the nucleus and transport but is removed when the mRNPs are remodelled as they travel through the nuclear pore complex. Once in the cytoplasm PABP1 is free to bind the poly(A) tail to carry out its translation related functions (Lemay et al., 2010).

Despite the demonstrated steady state localization of PABP1 and PABPN1, there is evidence to suggest that these proteins may also shuttle across the nuclear envelope. The cytoplasmic function of PABPN1 is also unclear, though evidence shows that PABPN1 is involved in the pioneer round of mRNA translation, a process used to identify and destroy mRNAs containing nonsense mutations (Chiu et al., 2004). The nuclear role of PABP1 has yet to be determined, however it has been found to associate with un-spliced pre-mRNAs and PAP in
the nucleus and evidence suggests it may be involved in a yet undetermined step in mRNA biogenesis (Lemay et al., 2010).

**PABP and Cell Stress**

Shuttling of cytoplasmic PABPs to the nucleus also occurs in response to cell stress. In response to heat shock HSP27 binds eIF4G, preventing PABP1 from binding and all three of these proteins are found to translocate to the nucleus. During recovery from heat shock PABP1 and eIF4G translocate back to the cytoplasm. Additionally, during UV treatment PABP1 and PABP4 are found to localize in the nucleus along with poly(A) RNA. This effect is accompanied by decreased protein synthesis (Burgess et al., 2011). However, studies conducted in our laboratory did not find any evidence of nuclear retention of mRNAs in PABPN1 depleted cells (Bhattacharjee and Bag, 2012). The abnormal localization of PABP along with other RNA-binding proteins to stress granules is also associated with neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) (Kim et al., 2014).

**Examining PABP Function via PABP Depletion**

Yoshida et al. (2006) conducted PABP depletion studies using siRNA and only observed a 10% decrease in global mRNA translation. They found that when PABP1 is depleted, Paip2 levels also decrease. In the absence of PABP, Paip2 is free to interact with EDD which results in ubiquitination and degradation, this may be a mechanism to maintain PABP homeostasis. In the absence of Paip2 the activity of the remaining PABP is enhanced due to relief from Paip2 mediated repression of PABP’s activity. Perhaps Paip2 levels are coordinately regulated with that of PABP to maintain an appropriate level of PABP activity (Yoshida et al., 2006). However, it seems paradoxical that following PABP depletion, the remaining 10% of PABP would be able to maintain translation, even in the absence of Paip2. Studies conducted in our lab showed that PABP depletion inhibited nearly 60% of mRNA translation and following 72 hours of PABP
depletion, 72% of the cells were apoptotic. In PABP depleted cells GAPDH localized in the nucleus, this signals a pro-apoptotic pathway by enhancing acetylation and serine 46 phosphorylation of p53 (Zannat et al., 2011). Studies in yeast show that depletion of PABP leads to lengthened poly(A) tails of mRNA and a shift of mRNA from polysomes to monosomes. These results support evidence of a role for PABP in poly(A) tail shortening and translation initiation (Sachs and Davis, 1989).

Additionally, Yanagiya et al. (2010) conducted an experiment using Paip2 knock-out mice. Loss of Paip2 resulted in sterile mice, due to spermatogenesis defects and expression of PABP1 increased. During this time of elevated PABP1, translation actually decreased and when Paip2 levels were restored, translation resumed at a normal rate. This suggests that PABP actually inhibits translation at high levels and that Paip2 regulates PABP levels as a means to enhance translation. Yanagiya et al. (2010) proposed a model to explain this phenomenon. It is possible that when excessive PABP1 is present, it competes with eIF4G for mRNA binding. This results in reduced interaction of eIF4E with the cap, inhibiting translation (Yanagiya et al., 2010).

Studies conducted in our lab found that PABPN1 depletion did not have a detectable effect on mRNA transport, poly(A) tail length, mRNA abundance or mRNA translation. Knockdown of PABPN1 did however lead to cell apoptosis, despite a compensatory response. The levels of PABP5 increased and PABP4 accumulated in the nucleus in both PABP1 and PABPN1 depleted cells. The enhanced levels of PABP5 may be an attempt to stimulate global protein synthesis. Additionally, PABP4 may be compensating for the loss of PABPN1 function in the nucleus. The relevance of PABP4 translocation into the nucleus remains unclear. While PABP4 accumulated in to the nucleus in PABPN1 depleted cells, it was found to be associated
with CPSF. Additionally, despite the well known function of PABPN1 in poly(A) tail extension, mRNAs in the PABPN1 depleted cells did not appear to have shortened poly(A) tails (Bhattacharjee and Bag, 2012). PABP4 has a similar affinity for poly(A) as PABPN1, thus PABP4 may be taking over the polyadenylation function of PABPN1 in the nucleus (Sladic et al., 2004). However, despite these compensatory effects, the PABPN1 depleted cells still underwent apoptosis. It was shown that PABPN1 depletion induced phosphorylation of p53, resulting in the expression of PUMA, a pro-apoptotic protein. However, PABPN1 depletion also induce glucose-regulated protein 78 (GRP78), a pro-survival protein and a marker of ER stress. These findings suggest that loss of PABN1 signals various complex pro-apoptotic and anti-apoptotic pathways. However the apoptotic response eventually overrides the compensatory effects (Bhattacharjee and Bag, 2012). It is also interesting to note that studies have revealed an anti-apoptotic role of PABPN1 in mammalian cells (Davies et al., 2007).

A considerable amount of research has been conducted in order to investigate the function and interactions of PABP1 and PABPN1. However, the function of the other PABPs remains largely unknown. The similar structure of these proteins indicates some shared functions. However, the inability of other PABPs to completely compensate for the loss of PABP1 or PABPN1 indicates additional non-redundant functions. Much remains unknown about the function of PABP4, particularly in PABP1 and PABPN1 depleted cells. Further work is needed to elucidate the significance of the movement of PABP4 to the nucleus in PABP1 depleted cells.

**Translational Control and Hypoxia**

PABP1 expression is regulated under a variety of cellular stresses including heat stress (Datu and Bag, 2013). However, the mechanism by which PABP1 and other PABPs are
regulated under hypoxic conditions is not known. A family of transcription factors called Hypoxia Inducible Factors (HIF) mediates the cell’s response to hypoxia. HIFs are able to trans-activate genes by binding to hypoxia response elements present in hypoxia responsive genes. HIFs contribute to the cell's hypoxia tolerance by stimulating glycolysis, and preventing oxidative phosphorylation (Semenza, 2003). Similar to PABP and other proteins involved in translational control, HIF-1α also contains a 5' TOP sequence (van den Beucken et al., 2006). In addition to HIF activity, cells also undergo biphasic repression of mRNA translation initiation in an attempt to aid in cell survival. If cells are unable to repress translation, they are more likely to undergo apoptosis (Bi et al., 2005). First, translation is inhibited by transient phosphorylation of eIF2α by PERK, which prevents the translation initiation complex formation (Koumenis et al., 2002). This response mediates translation repression under acute hypoxic conditions, within 30 minutes of hypoxia. mRNA translation shows a partial recovery around 4-6 hours of hypoxia and is fully reversible upon oxygenation. eIF2α is key in this initial translation inhibition. A study involving knock-in mice showed that cells with a mutation that prevented the phosphorylation of eIF2α were not able to repress translation until 16 hours of hypoxia had passed (van den Beucken et al., 2006). The second phase of translation repression occurs independently of eIF2α and involves disruption of the eIF4F complex. This alternative pathway maintains translational repression in prolonged hypoxia conditions (16 hours). eIF4F formation is limited by the availability of eIF4E. During periods of active translation, mammalian target of rapamycin (mTOR) phosphorylates eIF4E-binding protein (4E-BP). However, under hypoxic conditions, mTOR activity is reduced. This results in hypo-phosphorylation of 4E-BP, allowing it to interact with eIF4E and preventing eIF4F formation (Arsham et al., 2003).
Low oxygen conditions are known to lower global protein synthesis (Connoly et al., 2006). However, some proteins may be involved in an adaptive response and are in fact preferentially translated under hypoxic conditions. The mRNA of many proteins that are actively translated during hypoxic conditions contain the internal ribosome entry site (IRES), located in the 5' UTR. Most translation in the cell is cap-dependent, but 3-5% of mRNAs instead have internal ribosome entry sites (IRES), which can independently recruit the 40S ribosome to the mRNA near the initiation codon. Thus, the IRES was proposed as a possible mechanism for protein synthesis when cap-dependent translation is repressed (Young et al., 2008). Young et al. (2008) studied IRES activity, by analyzing capped and poly-adenylated RNAs in both in vitro and in vivo translation assays. IRES-mediated translation accounted for less than 1% of the level of cap-dependent translation indicating that another mechanism must also contribute to maintenance of translation levels during hypoxia conditions (Young et al., 2008).

Uniacke et al. (2012) identified a complex responsible for initiating cap-dependent translation of hypoxia-regulated proteins, independent of eIF4E. Low oxygen conditions stimulate the formation of a complex consisting of HIF2α, RNA-Binding Motif Protein 4 (RBM4) and eIF4E2, a homologue of eIF4E. This complex stimulates the cap-dependent translation of a select group of mRNAs under hypoxic conditions (Uniacke et al., 2012). RBM4 is responsible for binding to a specific cis element called RNA hypoxia response elements (rHRE) located in the 3' UTR of specific mRNAs and recruits HIF-2α to this site. The complex then interacts with eIF4E2 (a cap-binding protein) and eIF4A (the RNA helicase) to initiate cap-dependent translation in the absence of eIF4E. The majority of eIF4E is present in polysomes during normoxia but under hypoxic conditions, eIF4E shifts to monosomes and most of the cellular eIF4E2 associates with polysomes (Uniacke et al., 2012). However, in HIF2α-depleted
cells this switch of eIF4E2 distribution in polysomes was not observed indicating that HIF2α is essential for regulating the response to oxygen levels.

The mRNAs targeted by this eIF4E2-dependent mechanism code for various proteins regulated by hypoxia such as epidermal growth factor receptor (EGFR). EGFR expression is enhanced in tumor cells, suggesting a role for the eIF4E2 mechanism in the progression of cancer cells (Uniacke et al., 2012). rHREs are found in a number of other mRNAs that code for proteins involved in various processes characteristic of cancer cells. During the rapid growth of tumors, cancer cells often encounter hypoxic conditions and thus depend on eIF4E2 for translation initiation. When cells were depleted of eIF4E2, tumor growth and cell proliferation were reduced, and increased apoptosis was observed. Although cancer cells exploit eIF4E2-dependent translation, it was not sufficient to sustain hypoxic regions of the tumors (Uniacke et al., 2014).

SUMMARY AND RESEARCH GOALS

Poly (A) binding proteins are a group of RNA binding proteins with specific affinities towards the poly(A) tract of eukaryotic mRNAs. PABP1, PABP3, PABP4 and PABP5 are different cytoplasmic homologs present in different tissues. With the exception of PABP5 the other homologs bear considerable structural similarity with each other. An additional nuclear PABPN1, previously known as PABP2, is different in function and cellular localization than the cytoplasmic PABPs. PABP1 is a known regulator of protein translation and has a role in the cell's response to stresses such as heat shock and UV treatment (Burgess et al., 2011). Previous studies in our lab showed that protein synthesis is inhibited in PABP1 depleted cells (Zannat et al., 2011). Additionally, when the cell is depleted of PABPN1, PABP4 seems to attempt to compensate for the loss (Bhattacharjee and Bag, 2012). Although the cellular function of PABP1
in mRNA metabolism is well characterized, the function of other cytoplasmic PABPs is largely unknown.

**HYPOTHESES**

1. PABP4 and PABP1 share many structural similarities, and therefore may have common cellular functions and interact with similar proteins.

2. PABPs are involved in stress response and thus, may be differentially regulated during hypoxia and recovery.

**OBJECTIVES**

1. Examine the role of PABP4 in mRNA translation via depletion studies.

2. Isolate the binding partners of PABP4, using flag affinity chromatography and compare with the protein interacting with PABP1.

3. Determine if PABP1 or PABP4 are differentially expressed during hypoxic conditions.
MATERIALS AND METHODS

Co-Immunoprecipitation – Before co-immunoprecipitation, 100 µL of IgG-agarose beads (Sigma-Aldrich, Oakville, Canada) were washed three times with 1x PBS (125 mM NaCl, 1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$ and 2.5 mM KCl), followed by an additional wash with PLC lysis buffer (10% glycerol, 50 mM Hepes pH 7.5, 450 mM NaCl, 1.5 mM MgCl$_2$, 1mM EGTA, 100 mM NaPPi, 100 mM NaF, 1% Triton X-100). HeLa cells were grown to desired confluence at 37°C with 5% CO$_2$ in Dulbecco's Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were washed 3 times with 1x PBS before being lysed with 300 µL of PLC lysis buffer and incubated on ice for 10 minutes. A cell scraper was used to remove the cells from the plate and for complete lysis cells were then passed through a 27 1/2 gauge needle several times. Lysis of the cells was examined by placing a drop of the cell lysate on a coverslip and examined under an inverted microscope for the presence of lysed cells by monitoring the number of cytoplasmic tag free nuclei relative to intact cells. In all cases, greater than 90% cell lysis was achieved. Cell extract was centrifuged at 835 x g for 10 minutes at 4°C. To produce a pre-cleared extract, 300 µL of the cell extract was then incubated with 50 µL of the washed IgG-agarose beads for 2 hours with shaking in a nutator shaker at 4°C. Samples were centrifuged for 10 minutes at 835 x g at 4°C and the supernatant was transferred to a new tube. This pre-cleared extract was then combined with 400 µL of PLC buffer, 50 µL of the washed IgG-agarose beads and either PABP1 or PABP4 antibody. The samples were then incubated at 4°C overnight on a nutator shaker. Samples were then centrifuged at 835 x g for 1 minute and the supernatant was discarded. The beads were then washed three times with PLC lysis buffer. The pellet was resuspended in 50 µL of 2x SDS and boiled for 2 minutes. Samples were centrifuged to obtain the supernatant. Equal volumes of the samples were loaded and analyzed by 10% SDS
polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Silver Stain Plus kit (Bio-Rad, Mississauga, ON, Canada).

**Silver Nitrate Staining** - Protein samples were separated by 10% SDS-PAGE at 120V. The gel was then washed in a fixative solution (50% methanol, 10% acetic acid, 10% Fixative Enhancer (Silver Stain Plus kit, Bio-Rad, Mississauga, ON, Canada)) on a shaker overnight at room temperature. The gel was washed 3 times in water for 10 minutes. The Silver Stain Plus kit (Bio-Rad, Mississauga, ON, Canada) was then used, according the manufacturer's instructions to stain the gel. Once the gel was sufficiently stained the silver nitrate solution was removed and the gel was placed in 5% acetic acid. Lastly, the gel was rinsed with water and photographed for analysis.

**[^35S]-Methionine Labelling to Measure Protein Synthesis** - HeLa cells were incubated at 37°C with 5% CO₂ in methionine free DMEM (Invitrogen, Burlington, ON, Canada) with dialyzed 10% FBS (Invitrogen, Burlington, ON, Canada) for 2 hours to deplete the endogenous methionine. Cells were then incubated with 100 µCi[^35S]-methionine (259 MBq, MP Biomedicals, Santa Ana, California) in fresh methionine-free DMEM containing 10% dialyzed FBS for 2 hours. The media was then removed and cells were washed 3 times with 1x PBS prior to lysis with 200 µL of 1x SDS loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 5% 2-mercaptoethanol). The samples were boiled for 10 minutes and polypeptides were separated by 10% SDS-PAGE. The gel was fixed by boiling in 10% trichloroacetic acid (TCA) for 5 minutes to remove un-incorporated charged tRNAs from the gel. The gel was further washed twice at room temperature with 10% TCA and twice with 70% ethanol. The gel was then soaked in En3hance solution (Perkin Elmer, Waltham, MA, USA) for 30 minutes and after removing the En3hance, deionized water was added to the gel and
incubated for another 30 minutes at room temperature. In the presence of water, the En3hance precipitated within the gel. For autofluorography the gel was dried at 50°C for an hour and the dried gel was exposed to X-ray film overnight at -80°C and developed using an automated X-ray film developer (Thermo Scientific, Waltham, MA, USA) to analyze the $[^{35}\text{S}]$-methionine incorporation into newly synthesized polypeptides.

**Quantitative Measurement of $[^{35}\text{S}]$-Methionine Incorporation into Polypeptides** - Ten µL samples from $[^{35}\text{S}]$-methionine labeled cell extracts were spotted on a small square of Whatman chromatography paper (Fisher Scientific, Waltham, MA, USA), including one unspotted blank filter paper as a control. The filter papers were then boiled twice in 10% TCA for 10 minutes. The filter papers were then washed at room temperature with 10% TCA and twice with 95% ethanol. The filter papers were placed in scintillation vials with 5 mL of scintillation counting fluid. The count per minute (CPM) was measured using a liquid scintillation counter (Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter).

**Western Blotting** - Cells were washed 3 times with 1x PBS before being lysed with 1x SDS gel-loading buffer. Cells were removed from the plate using a cell scraper and heated in a boiling water bath for 10 minutes. Samples were separated by 10% SDS-PAGE at 120V. The separated proteins were electrophoretically transferred from the gel to a nitrocellulose membrane (Pall Life Sciences, St. Laurent, QC, Canada) for 1 hour at 4°C at 100V. The membrane was treated with a blocking solution containing skim milk (5% non-fat dry milk, 0.2% Tween-20 in PBS) for 30 minutes. The membrane was then incubated with the desired primary antibody (Table 1) in blocking buffer for 3 hours at room temperature followed by overnight incubation at 4°C. The membrane was then incubated with the appropriate HRP-conjugated secondary antibody in blocking buffer for 2 hours. For each antibody, the quantity of primary and secondary antibody
was optimized for western blotting. The images were developed using western lighting chemiluminescence reagent plus according to the manufacturer's instructions (Perkin Elmer, Waltham, MA, USA) and exposed to X-ray film for 5 seconds to 1 minute.

Table 1. Antibodies used for immunoblotting.

<table>
<thead>
<tr>
<th>Type</th>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Catalogue numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>GAPDH</td>
<td>Santa Cruz Biochemical, Santa Cruz, CA, USA</td>
<td>Sc-25778</td>
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<tr>
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<td>Sc-32318</td>
</tr>
<tr>
<td>Primary</td>
<td>PABP3</td>
<td>Abcam, Toronto, ON, Canada</td>
<td>Ab126178</td>
</tr>
<tr>
<td>Primary</td>
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<td>Ab66897</td>
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<td>Sc-168885</td>
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<tr>
<td>Primary</td>
<td>Paip1</td>
<td>Abcam, Toronto, ON, Canada</td>
<td>Ab92859</td>
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<tr>
<td>Primary</td>
<td>Paip2</td>
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<td>Sc-18617</td>
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<td>Primary</td>
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<td>Sigma-Aldrich, St Louis, MO, USA</td>
<td>F3165-2MG</td>
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<td>Anti-rabbit</td>
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<td>Sc-2004</td>
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<tr>
<td>Secondary</td>
<td>Anti-goat</td>
<td>Santa Cruz Biochemical, Santa Cruz, CA, USA</td>
<td>Sc-2020</td>
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**siRNA Transfection** - HeLa cells were grown to desired 30-50% confluence at 37°C with 5% CO₂ in DMEM supplemented by 10% FBS on 35 mm tissue culture plates. For each transfection
1.5 µL of siRNA duplex (20 µM annealed duplex from Invitrogen, Burlington, ON, Canada) was mixed with 250 µL of OPTI-MEM I (Invitrogen, Burlington, ON, Canada). In a separate tube 5 µL of Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) transfection agent was mixed with 250 µL of OPTI-MEM I. Both solutions were incubated for 5 minutes before being mixed together. The solution was added to the culture and incubated for 3 days at 37°C to achieve optimum results. Cells were transfected with a validated control, PABP1 or PABP4 siRNA (Table 2). Furthermore, non-transfected and mock-transfected cells were also used as additional controls.

**Table 2. siRNA sequences used for siRNA knockdown.**

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence</th>
<th>Manufacturer</th>
</tr>
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<tr>
<td>PABP1</td>
<td>Sense-AAGGUGGUUUGUGAUGAAAAUdTdT&lt;br&gt;Antisense- AUUUUCAUCACAAACCACCUCUdTdT</td>
<td>Thermo Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>PABP4</td>
<td>Sense-GGAAGGCCCUCCAUAUUAUATT&lt;br&gt;Anti-sense-UAUAAUAUGGAGGCCUCCCT</td>
<td>Qiagen, Toronto, ON, Canada</td>
</tr>
</tbody>
</table>

**Hypoxia Cell Treatment** - HEK cells were grown to 50% confluence at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS. Cells were incubated at 1% oxygen for either 6 or 24 hours. The cells were then returned to normal oxygen conditions to recover for the desired period of time. Cells were lysed with 1x SDS gel loading buffer. Samples were analyzed by SDS-PAGE and western blotting as described previously.

**Plasmid Transformation** - Two pReceiver-M12 Expression Clones (Fig. 4) were purchased from Genecopoeia (Rockville, Maryland, USA), expressing Flag (DYKDDDDK) tagged PABP1 and Flag tagged PABP4. The plasmids were transformed into competent *Escherichia coli* cells to
amplify the plasmid DNA. To begin the transformation, 50 µL of the competent cells were combined with 10 ng of plasmid DNA and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 45 seconds before being placed back on ice for 2 minutes. Afterwards, the cells were incubated at 37°C on a shaker for 1 hour with 1 mL of Luria Broth (LB). The cells were then plated on LB agar plates with the selective antibiotic ampicillin. The plates were then left to grow at 37°C overnight.

![Diagram](image.jpg)

**Figure 4. pReceiver-M12 Expression Clone**

**Flag Plasmid Transfection** - Following transformation, single colonies were taken from the LB agar plates and inoculated in LB with ampicillin. The plasmid DNA was then purified out of the bacteria cells, using the GeneJET Plasmid Miniprep Kit and following the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA). The purified plasmid DNA was tested for the presence of correct plasmid by agarose gel electrophoresis of circular and linearized restriction enzyme digested DNA. The plasmid DNA was then transfected into HeLa cells. HeLa cells were grown to desired 30-50% confluence at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS on 35 mm tissue culture plates. For each transfection sample, 1 µg of plasmid DNA was
mixed with 250 µL of OPTI-MEM I (Invitrogen, Burlington, ON, Canada). In a separate tube 5 µL of Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) was mixed with 250 µL of OPTI-MEM I. Both solutions were incubated for 5 minutes before combining. The DNA and the transfection mixture were incubated for 20 minutes prior to adding to the cell culture. Transfection was continued for 24 hours at 37°C for optimum results. For further analyses cells were lysed with 1x SDS gel loading buffer. Non-transfected cells were used as controls.

**Flag Tag Affinity Chromatography** - The anti-flag antibody coupled affinity gel (Biotool, Burlington, ON, Canada) was thoroughly mixed before transferring a 10 µL aliquot to a fresh tube. The beads were washed 4 times with 600 µL of 1x TBS (50 mM Tris-Cl pH7.5, 150 mM NaCl) before being used for chromatography. Cellular extracts for affinity chromatography were prepared from exponentially growing cells. Cells were washed 3 times with 1x PBS and lysed with 300 µL of PLC lysis buffer and left on ice for 10 minutes. A cell scraper was used to remove the cells from the plate, which were then passed through a 27 1/2-gauge needle. The cell extract was centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was then mixed with the anti-flag affinity gel. Samples were placed on a nutator shaker overnight at 4°C. In order to remove the unbound proteins, samples were centrifuged for 30 seconds at 5000 rpm in a microfuge and washed with 500 µL of TBS until the A280 of the supernatant liquid was less than 0.05. The proteins bound to the flag antibody beads were then eluted by adding 10 µL of 1x SDS gel loading buffer and boiled for 5 minutes.

**In Gel Digestion for Mass Spectrometry** - Protein samples were separated using 10% SDS-PAGE and stained with silver nitrate stain as described above. The band of interest, and a non-protein containing portion of the gel were excised and diced into small pieces. To oxidize the metallic silver attached to the protein, a solution of 30 mM potassium ferricyanide and 100 mM
sodium thiosulfate was added to the gel slices and incubated in the dark for 20 minutes. The solution was removed and the gel pieces were then washed with water until the gel pieces were colourless. Following washing 100% acetonitrile (ACN) was added to the gel slice until they were sufficiently white and shrunk. The gel pieces were then incubated at 50°C for 30 minutes in 10 mM dithiothreitol (DTT). The liquid was removed and the gel particles were shrunk again in 100% ACN. The gel pieces were then incubated in 55 mM iodoacetamide for 30 minutes to alkylate the particles. This solution was removed and the particles were washed with 50 mM ammonium bicarbonate for 15 minutes. The liquid was removed and the particles were treated again with 100% ACN and dried on the speedvac for 20 minutes. Sequencing grade trypsin (Sigma-Aldrich, St Louis, MO, USA) solution was prepared by adding 100 µL of 1 mM hydrochloric acid and 136 µL of 50 mM ammonium bicarbonate to 2 µg of trypsin. After the gel pieces were dry, 10 µL of the trypsin solution was added and the gel pieces rehydrated for 1 hour at room temperature. 136 µL of 50 mM ammonium bicarbonate solution was then added to the gel pieces that were incubated at 37°C overnight. The samples were centrifuged and washed with 50 µL of water before sonicating in a bath sonicator for 10 minutes. The supernatant, containing tryptic peptides was removed and kept in a fresh tube. A solution of 5% formic acid and 50% ACN was added to the gel pieces which were then centrifuged and sonicated for 5 minutes, the supernatant was removed and added to the fresh tube. The wash was repeated, again collecting the tryptic peptides in the supernatant. The volume was then reduced to 10-15 µL on the speedvac (modified from Shevchenko et al., 1996). Samples were then cleaned using a C18 ZipTip (Millipore, Etobicoke, ON, Canada).

**Liquid Chromatography-Mass Spectrometry Analysis** - Liquid chromatography-mass spectrometry was performed on an Agilent 1200 HPLC liquid chromatograph interfaced with an
Agilent UHD 6530 Q-TOF mass spectrometer at the Mass Spectrometry Facility of the Advanced Analysis Centre, University of Guelph. A C18 column (Agilent AdvanceBio Peptide Map, 100 mm x 2.1 mm 2.7 µm) was used for chromatographic separation with the following solvents, water with 0.1% formic acid for A and acetonitrile with 0.1 % formic acid for B. The mobile phase gradient was as follows: initial conditions, 2% B increasing to 45% B in 40 minutes and then to 55% B in 10 more minutes followed by column wash at 95% B and 10 minute re-equilibration. The first 2 and last 5 minutes of gradient were sent to waste and not the spectrometer. The flow rate was maintained at 0.2 mL/min. The mass spectrometer electrospray capillary voltage was maintained at 4.0 kV and the drying gas temperature at 350° C with a flow rate of 13 L/min. Nebulizer pressure was 40 psi and the fragmentor was set to 150. Nitrogen was used as both nebulizing and drying gas, and collision-induced gas. The mass-to-charge ratio was scanned across the m/z range of 300-2000 m/z in 4GHz (extended dynamic range positive-ion auto MS/MS mode. Three precursor ions per cycle were selected for fragmentation. The instrument was externally calibrated with the ESI TuneMix (Agilent Technologies, Santa Clara, CA, USA). The sample injection volume was 100 µl.

Raw data files were loaded directly into PEAKS 7.5 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada) where the data was refined and subjected to de novo sequencing and database searching. The following modifications were considered within the search parameters: methionine oxidation and carbamidomethylation of cysteine residues. The tolerance values used were 10 ppm for parent ions and 0.5 Da for fragment ions (Ma et al., 2003). The above procedure was performed by Dr. Armen Charchoglyan at the Advanced Analysis Centre, Mass Spectrometry Facility at the University of Guelph.
RESULTS
PABP Expression and Protein Synthesis

Previous studies have shown that PABP1 plays important roles in mRNA translation and stability (Sachs and Davis, 1989). Depletion of PABP1 in HeLa cells reduces protein synthesis and results in cellular apoptosis (Zannat et al., 2011). Whether PABP4 plays a similar role in mRNA translation was not known. Therefore, the role of PABP4 in global protein synthesis was examined by depleting PABP4 from HeLa cells. Previous studies in our lab found a 40-50% reduction of translation in response to PABP1 depletion (Zannat et al., 2011). The aim of this study was to investigate the effect of PABP4 depletion as well as a double knockdown (PABP1 and PABP4) on protein synthesis. HeLa cells were treated with either PABP1 siRNA, PABP4 siRNA or a combination of both for 72 hours and the effectiveness of siRNA in depleting cellular PABP1 and PABP4 was analyzed by measuring the abundance of PABP1 and PABP4 using SDS-PAGE and western blotting as described in materials and methods. The result of these analyses show that (Fig. 5A) compared to non-transfected cells both PABP1 and PABP4 siRNAs produced significant reduction of abundance of the target polypeptide. In three separate experiments approximately 45-50% and 60-80% reduction in the cellular level of PABP1 and PABP4 polypeptides were observed respectively (Fig. 5B). In experiments where both siRNAs were simultaneously used, a reduction in the effectiveness of both siRNAs was observed. However, both PABP1 and PABP4 abundance were still reduced by approximately 40-50%. The lower effectiveness of siRNA in double knockdown studies was likely due to reduced transfection of siRNA. In addition, transfection of cells with a validated control siRNA did not affect the cellular PABP1 and PABP4 levels. After achieving successful reduction of cellular PABP1 and PABP4 levels using siRNA mediated transfection, we measured the effect of reduced PABP levels on global protein synthesis. For these studies, newly synthesized cellular
proteins were radioactively labelled 72 hours after transfection by incubating with $[^{35}S]$-methionine for 2 hours as described in experimental procedures. The cells were lysed and the samples were analyzed after separating the polypeptides by 10% SDS-PAGE followed by autofluorography using X-ray film as described in materials and methods (Fig. 6A). Analysis of the newly synthesized polypeptides by SDS-PAGE followed by autofluorography suggested a reduction of protein synthesis when cells were treated with PABP1 siRNA but a smaller reduction in response to the double knockdown. This was expected since a reduction of cellular PABP4 level had a smaller effect on global protein synthesis. To obtain a quantitative measurement of the inhibition of protein synthesis the total $[^{35}S]$-methionine incorporation into TCA insoluble polypeptides was measured. Protein synthesis appeared to be reduced by approximately 40% in response to PABP1 siRNA treatment and only 10-20% reduction of protein synthesis by PABP4 knockdown. There only seemed to be a reduction of about 25% in protein synthesis in response to the double knockdown treatment (Fig. 6B).
Figure 5. PABP knockdown with siRNA. A) Cells were grown at 37°C until they reached 40% confluency. The cells were either kept as a control or transfected with PABP1, PABP4 siRNA or a combination of both for 72 hours. The cells were directly lysed on the plate and processed for SDS-PAGE and Western Blotting analysis. B) The abundance of specific proteins was determined by scanning the images and normalizing the values with the GAPDH levels as a loading control. The experiment was repeated 3 times and the mean values ± standard error are plotted above.
Figure 6. Effect of PABP depletion on global protein synthesis. HeLa cells were grown on 35 mm dishes and treated with either PABP1 siRNA, PABP4 siRNA or a combination of both for 72 hours. The cellular proteins were then labeled with radioactive $[^{35}\text{S}]$-methionine to measure global protein synthesis. A) The cells were lysed and the protein extract was analyzed by SDS-PAGE. The gel was autofluorographed with X-ray film overnight at -80°C and developed. B) To measure protein synthesis 10 µL of each $[^{35}\text{S}]$-methionine labeled sample was spotted on a square of filter paper and TCA insoluble radioactivity was measured using a liquid scintillation counter with a $[^{35}\text{S}]$ channel. The counts per minute (CPM) was measured and plotted.
PABP Depletion Studies

Previous work has shown that PABP1 depletion not only lowers the efficiency of global protein translation, the expression of specific proteins is also affected. (Zannat et al., 2011). Yoshida et al. (2006) showed that PABP1 knockdown caused a decrease in Paip2 expression. This may be a strategy to compensate for the loss of PABP1. Additionally, experiments conducted in our lab found that PABPN1 depletion resulted in enhanced PABP5 expression (Bhattacharjee and Bag, 2012). The aim of this study was to examine the effects of PABP1 and PABP4 knockdowns as well as a double knockdown on the expression of various proteins involved in translation initiation. HeLa cells were treated with PABP1 or PABP4 siRNA or a combination of both for 72 hours and the effectiveness of the siRNA in depleting the respective proteins was analyzed by measuring the abundance of PABP1 and PABP4 using SDS-PAGE and western blotting, as described in the materials and methods. The result of these analyses show that (Fig. 7A) compared to non-transfected cells both PABP1 and PABP4 siRNAs produced significant reduction of abundance of the target polypeptide. In three separate experiments approximately 45-50% and 60-80% reduction in the cellular level of PABP1 and PABP4 polypeptide were observed respectively (Fig. 7B). In experiments where both siRNAs were simultaneously used, a reduction in the effectiveness of both siRNAs was observed. However, both PABP1 and PABP4 abundance were still reduced by approximately 40-50%. The lower effectiveness of siRNA in double knockdown studies was likely due to reduced transfection of siRNA. The results of these studies are similar to those reported in Figure 1. After achieving sufficient depletion of PABP1 and PABP4, the abundance of other proteins known to interact with PABP1 was measured. As shown earlier, in our studies Paip2 abundance also showed a significant decrease in response to PABP1 depletion, however there was no detectable effect on Paip2 level in PABP4 depleted cells. In addition, results of our studies show that both PABP1
and PABP4 depletion, either independently or in combination did not appear to affect the cellular level of several proteins including Paip1, eIF4G, PABP 3 and PABP5. Thus homeostasis of PABP4 depletion was not observed as other cytoplasmic PABPs and PABP1 binding polypeptides eIF4G and Paip1 and 2 did not show any detectable effect on their cellular abundance. While these results could suggest different cellular roles, more experiments will be required to make these conclusions..
Figure 7. Effect of PABP1 and PABP4 knockdown on different cellular proteins.

A) Cells were grown at 37°C until they reached 40% confluency. The cells were either kept as a control or transfected with PABP1, PABP4 siRNA or a combination of both for 72 hours. The cells were directly lysed on the plate and processed for SDS-PAGE and Western Blotting analysis. B) The abundance of specific proteins was determined by scanning the images and normalizing the values with the GAPDH levels as a loading control. The experiment was repeated 3 times and the mean values ± standard error are plotted above.
Identifying Polypeptide Partners of PABP4

PABP1 has a number of identified binding partners. The aim of this study was to investigate whether PABP4 has similar or unique protein interactions. Initially, co-immunoprecipitation was used to identify proteins interacting with PABP4. To test the effectiveness, co-immunoprecipitation was carried out using cell extracts from control and PABP4 depleted cells. Analyses of eluted proteins from IgG-agarose beads by SDS-PAGE followed by staining the separated proteins with AgNO₃ showed the presence of several polypeptides in eluted fractions. We were expecting a distinct PABP4 band of 70kDa to be present in the eluted fraction of the control non-transfected cell extract which should be absent in the siRNA treated eluted fraction. However, there was no identifiable distinction between the two samples, indicating that the co-immunoprecipitation did not effectively purify PABP4 from the cell lysate and due to the presence of many polypeptides with similar molecular weights it was difficult to identify the PABP4 polypeptide band (Fig. 8A). To test whether the PABP4 antibody was working we analyzed the total cell extract and the eluted fractions by Western blotting. Results (Fig. 8B) show that the siRNA depleted PABP4 from the transfected cells and the majority of cellular PABP4 could be detected in the eluted fraction of non-transfected control cells. It is possible that the high polypeptide bands may be due to the presence of intact RNA bound to PABP4 rather than ineffective purification of PABP4.

Unsatisfied with the results of the co-immunoprecipitation we decided to use an alternative strategy using ectopic expression of a Flag tag-PABP4. Affinity chromatography using immobilized Flag antibody agarose beads were then used to purify Flag-PABP4 from cell extracts. HeLa cells were grown to 50% confluency and then transfected with a plasmid expressing either a flag-tagged PABP1 or flag-tagged PABP4 for 24 hours. The effectiveness of
the transfection was measured by analyses of the cell extract for the presence of ectopically expressed flag-PABPs using SDS-PAGE and western blotting with anti-flag antibody, as described in the materials and methods (Fig. 10A). Having established successful expression of flag-PABPs, anti-flag affinity beads were used to pull down the flag-tagged proteins from transfected cell extracts. Similar affinity purification was also carried out with non-transfected control cell extracts. The samples were subjected to SDS-PAGE and stained with AgNO₃. Results show (Fig. 9A) that one step affinity chromatography still did not eliminate many adventitiously bound proteins, however two additional bands of approximately 70 kDa were detectable in eluted proteins from the flag-PABP transfected cell extracts, which were not evident in the eluted proteins from the non-transfected cells. The bands marked in red were then excised from the gel and analyzed by liquid chromatography mass spectrometry to verify the presence of the flag-tagged proteins in each sample (Fig. 9A). Using Peaks 7.5, PABP1 was identified in the PABP1 band of the gel, using a false discovery rate parameter below 5% (probability > 95%). Other contaminating proteins were also observed and the potential low abundance of the PABP1 protein would account for only 9% sequence coverage observed. In the case of the PABP4 band, PABP4 was not adequately identified by mass spectrometry, which may be again due to its low abundance in comparison to other contaminating proteins. Since it is possible that the level of Flag PABP was below the sensitivity of our detection techniques we further tested the presence of PABPs in affinity-purified samples by using a more sensitive method than silver nitrate staining.

The samples eluted from the anti-flag affinity beads were analyzed by western blotting using antibodies for PABP as well as several known PABP interacting proteins. The results show that both flag-PABP1 and flag-PABP4 were detectable in the eluted fractions (Fig 10A). The
known PABP interacting protein Paip1 was present in the eluted fractions of both flag PABP transfected cells suggesting that Paip1 can bind to PABP4 almost as efficiently as with the PABP1. It is not clear however, why Paip1 was also present in the eluted fraction from the extracts of non-transfected cells. Perhaps the low level of endogenous PABPs that were detected in the flag affinity purified non-transfected cell sample may have interacted with Paip1. In contrast to Paip1, Paip2 was completely absent in the flag-affinity purified sample of flag-PABP4 transfected cell extract. As expected binding of Paip2 with PABP1 was detectable in our experiments. It is interesting to note that PABP4 is able to bind to the translational stimulator protein Paip1 but did not bind to the translational repressor Paip2. This observation suggests that PABP4 may participate in translating mRNA but Paip2 does not regulate its activity. Further work might be directed towards understanding the mechanistic details of the interaction between PABP4 and Paip1, and whether or not this interaction results in stimulation of PABP4 mediated mRNA translation.
Figure 8. Co-Immunoprecipitation of PABP4. A) Cells were grown at 37°C until they reached 40% confluency. The cells were then either transfected with PABP4 siRNA or kept as a control. Following transfection, a portion of the cytoplasmic extract was stored in a fresh tube and co-immunoprecipitation was carried out, as described in the materials and methods. Following overnight incubation with the IgG-agarose beads and PABP4 antibody, the supernatant containing unbound protein was removed and stored in a fresh tube. Finally, the protein was eluted from the beads. Equal volume of samples were analyzed with SDS-PAGE and silver nitrate staining. B) To determine the efficiency of the protein binding to the beads the total cell extract of the PABP4 siRNA transfected cells and non-transfected cells was compared to the unbound protein sample and the protein eluted from the flag affinity beads. The samples were analyzed by SDS-PAGE and western blotting with PABP4 antibody.
Figure 9. Plasmid Transfection. 

A) Cells were grown at 37°C until they reached 40% confluency. The cells were then either transfected with a plasmid to express flag-tagged PABP1 or flag-tagged PABP4 or they were maintained as non-transfected control cells. The flag affinity chromatography was carried out as described in the materials and methods and the samples were analyzed with SDS-PAGE and silver nitrate staining. The highlighted bands were sliced out of the gel and analyzed with liquid chromatography mass spectrometry to verify the presence of PABP1 and PABP4. 

B) To determine the efficiency of the protein binding to the beads the total cell extract of the plasmid transfected cells and non-transfected cells was compared to the protein eluted from the flag affinity beads. The samples were analyzed with SDS-PAGE and western blotting. PABP1 and PABP4 antibodies were used on the respective samples.
A) The flag affinity chromatography samples were also analyzed with SDS-PAGE and western blotting using different antibodies. B) The abundance of specific proteins was determined by scanning the images and normalizing the values with the control sample. The experiment was repeated 3 times and the mean values ± standard error are plotted above.
PABP Expression and Hypoxia

Previous studies from our lab showed PABP expression is regulated by heat stress resulting in preferential up-regulation of PABP mRNA translation during recovery from heat shock (Ma et al. 2009). In light of this I investigated PABP expression in response to hypoxia to further elucidate the role of PABP in cell survival. The cell's response to hypoxia is mediated by HIFs, the expression of which is up regulated in low oxygen conditions. Similarly to PABP1, HIF expression is controlled by a 5'TOP sequence and thus expression of both may be regulated by similar pathways (van den Beucken et al., 2006). Additionally, when cells are in low oxygen conditions they employ an alternative eIF4E2 mediated translation initiation (Uniacke et al., 2014), which does not require PABP and eIF4G for translation, therefore, it is likely that PABP expression may be fine tuned by down regulation during hypoxia and stimulated during recovery under normoxic conditions. However, it is unclear if PABP still plays a role in this alternative method of translation initiation. Hypoxia response was investigated by growing HEK cells to 50% confluency and incubating them at 1% oxygen for 6 or 24 hours followed by varying periods of recovery. Levels of PABP and Paip proteins were then measured by SDS-PAGE and Western blotting techniques, described in the materials and methods. In three separate experiments there was no detectable change in the cellular abundance of PABP1, PABP3, PABP4, PABP5, Paip1 and Paip2 (Fig. 11-12). Cellular levels of these polypeptides remained unchanged by hypoxia as well as during growth under normoxic conditions following hypoxia. Furthermore, the length of the hypoxic shock or the duration of the normoxic recovery had no detectable effect on the cellular abundance of all cytoplasmic PABPs and Paip 1 and 2. These results indicate that it is unlikely that any of the cytoplasmic PABPs are involved in eIF4E2-mediated translation.
Figure 11. PABP Expression and Acute Hypoxia. A) Cells were grown at 37°C until they reached 50% confluency. The cells were either kept as a control or incubated in 1% oxygen for 6 hours, followed by recovery in normal oxygen conditions for 0, 6, 12 or 24 hours. The cells were directly lysed on the plate and processed for SDS-PAGE and Western blotting analysis. B) The abundance of specific proteins was determined by scanning the images and normalizing the values with the GAPDH levels as a loading control. The mean values ± standard errors from three independent biological replicates are plotted above.
Figure 12. PABP Expression and Long Term Hypoxia

A) Cells were grown at 37°C until they reached 50% confluency. The cells were either kept as a control or incubated in hypoxic conditions for 24 hours, followed by recovery in normal oxygen conditions for 0, 6, 12 or 24 hours. The cells were directly lysed on the plate and processed for SDS-PAGE and Western blotting analysis.

B) The abundance of specific proteins was determined by scanning the images and normalizing the values with the GAPDH levels as a loading control. The results from three biological replicates were analyzed and mean values ± standard errors are shown above.
DISCUSSION

PABP Depletion and Global Protein Synthesis

PABP plays an important role in protein synthesis and when cells are depleted of PABP protein synthesis is hindered (Zannat et al., 2011). However, a role for PABP4 in protein synthesis is less clear. Studies conducted in our lab showed that PABPN1 depletion resulted in relocalization of PABP4 to the nucleus, where PABP4 was found to interact with poly(A) tracts and CPSF. These results are indicative of a compensatory function for the loss of other PABPs (Bhattacharjee and Bag, 2012). These studies were conducted to investigate a possible role for PABP4 in stimulating protein synthesis. Cells were treated with siRNA and translation was measured by $[^{35}\text{S}]$ methionine labeling and scintillation counting. A 40% reduction in protein synthesis was observed in response to PABP1 depletion (Fig. 6). However only a 10-20% reduction in protein synthesis was observed in response to the PABP4 depletion. Further studies would have to be conducted to determine the significance of these findings. This disparity between the effect of PABP1 depletion and PABP4 depletion on translation may be due to the relative abundance of these proteins in the cell. If PABP1 is more abundant in the cell than PABP4, this could explain its greater effect on protein synthesis. The relative abundance of these proteins could be determined by comparing them to purified protein samples of a known concentration. Despite the varied effects of knockdown on protein synthesis PABP1 and PABP4 may still share a common role in translation initiation. Additionally, these findings show a smaller decrease in protein synthesis in the double knockdown cells (treated with PABP1 and PABP4 siRNA) than the PABP1 knockdown. Translation was only decreased by 25% in these cells. This may be explained by the efficacy of the knockdown. Specifically, PABP4 depletion seems less effective in the double knockdown sample (Fig. 5). Thus, this difference is likely due to a lower efficiency of siRNA transfection in the double transfection.


**PABP Depletion and mRNA Translation**

The effect of PABP depletion on the translation of specific mRNAs was then studied. PABP1 and PABP4 were knocked down with siRNA and the abundance of various proteins were analyzed. Although sufficient PABP4 depletion was achieved (Fig. 7), there did not seem to be an effect on the abundance of PABP or PABP binding proteins. PABP1 depletion resulted in a 70% decrease in Paip2, this is consistent with previous studies (Yoshida *et al.*, 2006). It is hypothesized that Paip2 levels decrease as a means to maintain PABP homeostasis and promote the activity of the remaining PABP. However, PABP4 depletion did not show a similar effect on Paip2 abundance. Perhaps PABP4 homeostasis is mediated by a different mechanism. Our other findings also suggest that PABP4 may have a unique set of protein interactions.

**Investigating PABP Interactions by Flag Affinity Chromatography.**

Initially co-immunoprecipitation was used to isolate PABP4 and its binding partners. The results of this experiment showed a high number of bands present in the PABP4 pulldown. A number of these bands could be due to the presence of intact RNA bound to PABP4. When investigating the protein-protein interactions of an RNA binding protein, it is necessary to treat the sample with RNase to eliminate any proteins interacting with the RNA rather than the protein of interest. RNase treatment likely would have decreased the background associated with this immunoprecipitation. When the co-immunoprecipitation results seemed unsatisfactory, flag affinity chromatography was used as an alternative approach.

Flag affinity chromatography was used to study the proteins interacting with PABP1 and PABP4. Paip1 was found in both the PABP1 and PABP4 pull down samples, indicating that PABP1 and PABP4 both interact with Paip1 (Fig. 10). However, much lower levels of Paip2 were found in the PABP4 sample. This is consistent with results in the previous section and
suggests that PABP4 does not interact with Paip2, which interacts with PABP1. Further studies may be directed towards elucidating the significance of the PABP4 and Paip1 interaction.

The presence of PABP1 in the PABP1-Flag sample was identified by liquid chromatography mass spectrometry. However, PABP4 could not be adequately identified. This was due to the relatively high number of contaminants present in the sample. Further purification may be needed to properly analyze PABP4. Alternatively, tandem affinity chromatography may be a more effective approach to isolate PABP1 and PABP4. This procedure is often considered superior because it efficiently produces highly purified protein samples by utilizing two purification steps.

**Hypoxia and PABP Expression.**

The effect of hypoxia on PABP and Paips was also studied. Both acute (6 hours) and long term (24 hours) hypoxia were used with varying amounts of recovery time. There was no observable change in the expression of the various PABP or Paip proteins (Fig. 11-12). Previous studies have shown that PABP1 does not have a role in IRES-mediated translation (Thomas et al., 2004). These results could suggest that PABP proteins do not have a role in eIF4E2 mediated mRNA translation during hypoxia either. However, it's possible that proper hypoxic conditions were not achieved in the cells. To determine the success of the hypoxia treatment, the abundance of a known hypoxia marker such as hypoxia inducible factor 1α (HIF1α) would need to be measured.

Alternatively, even though PABP abundance may not change during hypoxia, the PABP proteins may undergo other changes in these conditions. For instance, studies that examined PABP during other kinds of cell stresses observed a relocalization of PABP to the nucleus (Burgess et al., 2011). In light of this, future studies might consider examining PABP
localization during hypoxia via fluorescent labeling and microscopy techniques. Additionally it would be important to investigate the involvement of PABP in translation under hypoxic conditions. It is unclear whether or not the PABP proteins are necessary for translation during hypoxia. Using polysome fractionation to examine the presence of PABP in polysomes and monosomes during hypoxia could provide a better understanding of PABP's involvement in translation under low oxygen conditions.

Gene expression is known to be affected by cell stress. For instance heat shock causes an overall inhibition of gene expression. This is facilitated by repression of RNA splicing, a highly conserved response to heat shock. A large number of genes contain introns, thus splicing repression can inhibit the expression of these genes. Interestingly genes encoding heat shock proteins tend to contain little or no introns, leaving them unaffected by splicing inhibition. This leads to the preferential expression of heat shock proteins during heat shock (Shi et al. 2011). Similarly, PABP3 and PABP5 also lack introns. Thus it may be worthwhile to further investigate PABP3 and PABP5 under heat shock or other types of cell stress.

Overall the results of these studies showed some differences between PABP1 and PABP4. However further experimentation would be needed to characterize the extent of these differences on the regulation of translation initiation.
REFERENCES


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