Translational Regulation of Acetylcholinesterase by the RNA Binding Protein Pumilio-2 at the Neuromuscular Synapse

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TRANSLATIONAL REGULATION OF ACETYLCHOLINESTERASE BY THE RNA BINDING PROTEIN PUMILO-2 AT THE NEUROMUSCULAR SYNAPSE

By
Emilio Marrero

A DISSERTATION

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TRANSLATIONAL REGULATION OF ACETYLCHOLINESTERASE BY THE
RNA BINDING PROTEIN PUMILO-2 AT THE NEUROMUSCULAR SYNAPSE

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In skeletal muscle acetylcholinesterase AChE is highly expressed at sites of nerve-muscle contact where it is regulated at both the transcriptional and post-transcriptional levels. Scientists have elucidated many aspects of synaptic AChE structure, function, and localization during the past 80 years. However, our understanding of the molecular mechanisms underlying its regulation is incomplete, but it appears to involve both translational and post-translational events as well.

We found that Pumilio-2 (PUM2), an RNA binding translational repressor, is highly localized at the neuromuscular junction where AChE mRNA concentrates and that PUM2 binds to the AChE transcripts when immunoprecipitation studies were performed. A direct binding between a recombinant PUM2-HD and the Pumilio Binding Site (PBE) in a segment of the AChE 3'UTR was demonstrated by Gel shift assays. Transfecting skeletal muscle cells with shRNAs specific for PUM2 upregulated AChE expression, whereas overexpression of PUM2 decreased AChE activity. We conclude that PUM2 binds to AChE mRNA and regulates AChE expression translationally at the neuromuscular synapse. We found that PUM2 is regulated by the motor
nerve suggesting a trans-synaptic mechanism for locally regulating translation of specific synaptic proteins involved in modulating synaptic transmission, analogous to CNS synapses.

PUM2 expression is critically important in many cell types, virtually nothing is known about the regulation of PUM2 expression itself. Analyzing the PUM2 mRNA 3'UTR we found fifteen possible PBEs in the 3 Kb 3' UTR. We show that PUM2 binds in vivo to its own mRNA. Overexpression of PUM2 in several cell types transfected with a green fluorescent protein (GFP) reporter construct linked to the full length PUM2 3'UTR (GFP-PUM2-3'UTRFL) suppresses GFP expression suggesting that PUM2 downregulates its own expression by binding to its own 3'UTR. Mutations of the first five PBEs yield the expression of the reporter gene indicating that at least one PBE is functional in the autoregulation of PUM2. These observations suggest a novel model for the localized regulation of protein translation through a negative feedback loop.

Much is known about PUM2 as a translational regulative protein but little is known about PUM2 cell localization and possible mechanism of translational regulation. In this work we found PUM2 to be highly localized to the cell rough endoplasmic reticulum and that PUM2 is associated with ribosomal RNA. In addition, we found that the GFP protein itself, together with its mRNA and ribosomal RNA (rRNA), were localized in the PUM2 positive complexes when GFP-PUM2-3'UTRFL was transfected into muscle cells. These observations further suggest a mechanism of regulation where translation of the protein occurs but the protein remains associated with the ribonucleoprotein complex, possibly
to be transported together with its mRNA to specific domains inside the cell. Thus when needed, more protein is produced in those specific cell regions.
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LIST OF ABBREVIATIONS

A12: Asymmetric AChE form
A4: Collagen tailed AChE tetramer
A8: Collagen tailed AChE octamer
ACh: Acetylcholine
AChE: Acetylcholinesterase
AChE_H: AChE splice variant H
AChE_R: Read-through AChE
AChE_T: AChE splice variant T
BSA: Bovine serum albumin
C2/C12: Mouse skeletal muscle cell line
ColQ: Collagen tail of synaptic acetylcholinesterase in muscle
COS-7: African green monkey kidney fibroblast cell line
CTD: C-terminal domain
EGFP: Enhanced green fluorescent protein
ER: Endoplasmic reticulum
FBS: Fetal bovine serum
G1: AChE monomer
G2: AChE dimer
G4: AChE tetramer
GPI: Glycolipid
hAChE: Human AChE
HBSS: Hank’s balanced salt solution
HSBD: Heparin sulfate binding domain

IgG: Immunoglobulin

IP: Immunoprecipitation

mAChR: Muscarinic acetylcholine receptor

mRNA: Messenger ribonucleic acid

nAChR: Nicotinic acetylcholine receptor

NMJ: Neuromuscular junction

NREs: Nanos response elements

PBEs: Pumilio Binding elements

PBS: Phosphate buffered saline

PRAD: Proline rich attachment domain

PRiMA: Proline rich membrane anchor

PUM: PUMILIO

QMC: Primary quail muscle cultures

RFP: Red fluorescent protein

RNA: Ribonucleic acid

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

shRNA: Small hairpin ribonucleic acid

UTR: Untranslated region

WB: Western Blot
Chapter 1: Introduction

Discovery of AChE and its main features

Acetylcholinesterase (AChE) is localized at the neuromuscular junction (NMJ) and is the enzyme responsible for terminating neuromuscular transmission by rapidly hydrolyzing the neurotransmitter acetylcholine (ACh) released by the nerve terminal. AChE is one of the fastest enzymes known with one molecule of AChE capable of degrading about 25000 molecules of ACh per second. AChE was discovered in 1914 by Sir Henry Dale who made the observation that serum contained an “esteratic activity” (1). Thus, AChE has been studied for almost one century now.

More than twenty years after Dale’s observation, AChE was discovered to be highly concentrated at sites of nerve muscle contact (5) and shown to be regulated by the presence of the nerve (7). In 1946, it was shown that inhibition of AChE altered the muscle’s action potential, suggesting a possible role for the enzyme in neuromuscular transmission (8). These early studies were relevant in biology establishing the concept of protein localization at synapses to perform specific physiological functions.

After the discovery of AChE inhibitors, and many purification studies using muscles and nerves, many enzymatically active forms that differ in their physical properties and size have been isolated and characterized (reviewed by Massoulie et al.,1993). For example, these oligomeric forms have molecular masses between 75, 000 Daltons to 1.25 million Daltons. However, they are all the product of a single gene in vertebrates (9-11) where the diversity of forms
arises in part through alternative splicing (11-14) and the ability to assemble with non catalytic subunits (15;16).

**AChE structure and oligomeric forms**

The catalytic subunits of AChE are known as globular because of their

![3D structure of AChE](image)

**Figure 1.1: 3D structure of AChE.** (Sussman et al., 1991)

a) The unanticipated structure of this extremely rapid enzyme in which the active site was found to be buried at the bottom of a deep and narrow gorge (gray) b) The high aromatic content of the active-site gorge is a remarkable feature of this enzyme (2). c) Different oligomeric forms of AChE expressed in vertebrates (Rotundo 2003).
shape. The crystal structure of the protein was determined in the early nineties describing the active site at the bottom of a 15 nm gorge. There are 14 aromatic amino acids lined along the gorge of *Torpedo californica* AChE and the degree of flexibility of these 14 aromatic side chains is diverse (2) (Figure 1.1 A, B). Early purification studies described a “family” of AChE forms that differ in size and physical properties, but later it was clarified that AChE consisted of an organized family of oligomeric forms with the same catalytic subunit (17) (Figure 1.1 C). We now know that there are globular forms of the same enzyme referred to as monomers (G$_1$), dimers (G$_2$) and tetramers (G$_4$), and more complex and larger forms of the enzyme, the asymmetric or collagen-tailed forms consisting of one to three tetramers covalently linked to a three-stranded collagen tail (A$_4$, A$_8$, A$_{12}$) encoded by a separate gene, *ColQ* (16;18). ColQ is a triple helical collagen-like protein composed of three identical subunits of the same gene product. Each subunit contains a proline-rich attachment domain (PRAD) at the NH$_2$ terminus that binds an acetylcholinesterase tetramer, two heparan sulfate binding domains (HSBD) within the triple helical regions and a C-terminal domain (CTD) that is involved in and essential for anchoring AChE at the NMJ (19). There is also a membrane anchored tetramer G$_4$ linked to a transmembrane peptide named “p” peptide or PRiMA (15;18). The nature of PRiMA was unknown for many years and referred to as the “p” peptide until the primary structure of the protein was established (15) showing that contained a PRAD domain responsible for attachment of the AChE tetramers, a transmembrane domain and a small intracellular portion. This membrane anchor is the major form expressed in the
CNS of all mammals where it most likely associates with the neuronal nicotinic and muscarinic receptors at cholinergic synapses.

**AChE biogenesis**

In vertebrates, all AChE catalytic subunits are the product of one single gene, \(AChe\) (9-11) that can undergo alternative splicing producing two alternative splicing variants and one “readthrough” transcript (12;14;20-22). These variants share 95 % of the coding region, including the entire catalytic domain, and differ only in the carboxyl terminus. The two alternative splicing variants are referred as the H and T variants (Figure 1.2). The H variant encodes a C-terminal of 32 amino acids with a glycoposphoinositide (GPI) addition signal resulting in a hydrophobic membrane anchor; the T variant encodes a C-terminal of 40

![Diagram](image.png)

*Figure 1.2: Schematic representation of intron-exon organization of the AChE gene in Torpedo, mammals and birds.* (Modified from Cousin X, et al, 1997 with permission)

Homologous exons have the same color in different species. The first and second coding exons combine to encode the majority of the mature catalytic subunit (green); the remaining 3’ coding exons splice to encode the C-Terminal which determines the ability to form a GPI anchor (H exons in red) or to associate with the non-catalytic subunits PRiMA or ColQ (T exons in yellow).
amino acids that forms a specific binding domain to covalently link to the PRAD domain of ColQ or PRiMA. The “readtrough” transcript is rare and only expressed in the CNS of mice and rats under stress (23;24).

Early electron-micrographic studies using histochemical methods to reveal AChE activity showed the extensive intracellular distribution of AChE, including histochemical staining of the nuclear envelope, the rough endoplasmic reticulum (RER) and the Golgi apparatus (25). This began to make sense after the discovery of the secretory pathway by Palade (26). Several studies at that time suggested that AChE transited the secretory pathway (27;28;28). However this was conclusively demonstrated by Rotundo and Fambrough who showed co-transport with the nicotinic acetylcholine receptor (29). Subsequently, Rotundo (1984), analyzed the binding of newly synthesized AChE molecules to immobilized lectins that recognize specific sugars moeties, for instance AChE binding to concanavalin A (ConA), wheat germ agglutinin (WGA) or ricin agglutinin I (RCA), to provide direct evidence for the addition of mannose in the RER, GlcNAc in cis-Golgi or terminal galactose in trans-Golgi, respectively (30).

Results from this work indicated that the assembly of the dimers and tetramers of AChE probably occurs in the RER immediately following their translation and that the assembly of the asymmetric AChE (A_{12}) occurs in the trans-Golgi using tetramers previously processed in the RER.

Once the AChE molecules are translated and correctly folded as functional monomers they are assembled as disulfide bonded dimers. The dimers can be induced to tetramerize by either one of the non-catalytic subunits,
PRiMA in neurons or ColQ in skeletal muscle, that brings together three tetramers to form the $A_{12}$ synaptic form in skeletal muscle (15;31;32). These oligomeric forms are then transported through the cell and released into the synaptic cleft where they diffuse away into the circulation or become attached to the basal lamina. How these molecules are tagged for removal and degradation is still unknown (33).

**AChE expression in different tissues**

The oligomeric forms of AChE have been isolated from both nerve and muscle and there is a rare variant, glycolipid anchored AChE$_H$, that is expressed in hematopoietic cells of mice and rats that is not present in muscle or nerve (34). AChE is expressed in non-attached cells erythrocytes and lymphocytes of some mammals including humans (35). Globular forms of AChE are secreted in muscle and brain; a membrane anchored AChE tetramer G4 linked to a transmembrane peptide named “p” peptide or PRiMA is found in mammalian brain (15;18) and chicken (30). PRiMA was first described as a 20 kDa hydrophobic peptide that could anchor tetramers of catalytic subunits to the plasma membrane of nerve cells in the bovine caudate nucleus (36).

It is generally accepted that the $A_{12}$ asymmetric form consisting of tetramers of AChE linked to the collagen-like ColQ protein is attached to the extracellular matrix in the vicinity of the sites of nerve-muscle contact (37-41). Nevertheless, the ratio of its localization in the innervated compared to non-
innervated regions varies depending upon the fiber type and the activity state of the muscle (42-45).

Nyquist-Battie, in experiments using adult rat cardiac and non-cardiac muscle cells, showed that AChE globular forms were present in both, but the non-muscle cell fraction contained cholinergic nerve fragments, heart perfusion using collagenase and hyaluronidase was performed in this study extracting some AChE, suggesting that asymmetric AChE is bound to the extracellular matrix of heart (46). Two years later they described the distribution of the oligomeric forms in different regions of the heart, and they were able to detect that in all cardiac regions asymmetric A12 AChE comprised 8-10% of the total AChE pool (47). The expression of AChE and its mRNA have been reported in smooth muscle, for instance in the arterial and vein walls of the vascular pulmonary system (48-50).

Additionally, AChE is expressed in other cell types that are targets of cholinergic nerves, such as endocrine and exocrine glands, and sensory organs embedded in epithelia. For instance, Hiramatsu in 1988 and 1993 was able to determine the expression of AChE by electron microscopy in the pancreas of chickens and goats suggesting that the cholinergic system is involved in the regulation of both the exocrine and endocrine pancreas (51;52). Extending this work, Hiramatsu localized the expression of AChE to the chicken Hardenian gland (53).
Localized expression of AChE in skeletal muscle

In skeletal muscle the expression of many genes is highly "compartmentalized", with the nuclei that accumulate postsynaptically at the NMJ becoming under regulation by the nerve. Thus factors and stimuli from the nerve influence a differential pattern of gene expression in that region in which all the organelles of the secretory pathway are present and functional to export secretable and membrane proteins to the synaptic cleft, for instance AChE and acetylcholine receptor.

Using mosaic myotubes derived from quail myoblast homozygous for one of two AChE alleles, Rotundo showed that the AChE mRNA transcripts in multinucleated muscle cells remain localized near their nucleus of origin (54). These experiments indicated that although the majority of mRNAs are free to diffuse and be translated on free ribosomes in skeletal muscle cells, there is a population of mRNA localized and translated in “compartments” surrounding their nucleus of origin. Two years later, Rossi, using immunofluorescent localization of AChE in heterospecific mosaic myotubes, showed that the newly-synthesized AChE molecules are preferentially transported and localized to the muscle sarcolema directly above the nucleus of origin (55). One year later, Rotundo and collaborators were able to isolate single NMJs and perform quantitative reverse transcriptase PCR to show that AChE mRNA transcripts are concentrated at the NMJ, but little AChE mRNA was present in the vast extrajunctional regions (56). Results from other groups using different approaches, like in situ hybridization,
came to the same conclusion supporting the fact that AChE mRNA is highly localized at the NMJ (57-59).

Not only is the AChE transcript highly localized at the NMJ, there is a high concentration of collagen-tailed AChE in this region as well compared to extrajunctional or non-inervated regions of muscle. This was discovered by Zach Hall in the early 1970s (60). Much has been studied in this regard with the conclusion that collagen-tail forms of AChE are more highly expressed at the NMJ (6;61;62). Feng et. al. in 1999 showed that ColQ -/- mice completely lack asymmetric AChE in their skeletal muscles, establishing the role for the collagenic tail in AChE localization in the NMJ (63). At the same time Donger, Ohno and their colleagues discovered that patients with congenital myasthenia with a single mutation in the ColQ gene lack collagen-tailed AChE at their NMJ, supporting the role for ColQ in localizing AChE to sites of nerve muscle contact (64-66). In a subsequent study, using a heterologous transplantation technique developed in Rotundo’s Lab (41), it was determined that these mutations in the C-terminal domain of ColQ were not able to localize collagen-tailed AChE to the NMJ compared with the wild type (19). In this work, Kimbell and collaborators described the regions in ColQ essential for anchoring acetylcholinesterase at the synapse (C-terminal and heparin-binding domains). Previously, using perlecan null mice lacking AChE in their NMJ, Arikawa-Hirasawa et al., discovered the importance of the heparin sulfate proteoglycan perlecan in the localization of collagen-tailed AChE owing to the binding of the collagenic tail to perlecan (67). Perlecan has been shown to directly bind the trans-membrane protein alpha-
dystroglycan (αDG) (68;69), thus αDG indirectly interacts with the collagen-tailed AChE. Experiments using \textit{in vitro} solid phase binding assays carried out in our lab by Dr. Kimbell showed that collagen-tailed AChE exhibited high levels of binding to the proteoglycan biglycan, similar to the levels observed for perlecan. In contrast, globular AChE alone showed no binding to any of the protein tested, thus the presence of ColQ is needed for this interaction (Kimbell Ph.D., Dissertation).

Recently, it has been suggested that one role for the muscle-specific tyrosine kinase (MuSK), a synaptic protein, is localizing collagen-tailed AChE to the NMJ (70), but this work lacks strong experimental evidence and has not been replicated. Kimbell found that there is an interaction between MuSK and biglycan in the \textit{in vitro} solid phase binding assays, and thus put forth the hypothesis that the ternary complex described by Cartaud contained biglycan and that ColQ interacts with MuSK only indirectly via its association with biglycan (Kimbell Ph.D., Dissertation).

\textbf{Regulation of AChE expression}

Experiments performed as early as the 1930s have shown a decrease in AChE activity in the leg of guinea pigs after sciatic denervation and this activity persisted even after nerve death indicating that AChE is produced by the postsynaptic muscle and not the presynaptic nerve (7;71). This observation is supported by experiments in which frog muscles were crushed causing the disintegration and phagocytosis of the nerve terminals innervating the NMJ. The
basal lamina remained intact and when the AChE was irreversibly inhibited and the muscle fibers allowed to regenerate within this basal lamina but reinnervation of the muscle was prevented, newly synthesized AChE molecules accumulated where the NMJ was previously present, indicating that AChE is produced by the muscle fibers and that components of the basal lamina directs AChE accumulation at synaptic sites (72). Many denervation studies have been performed by many labs with the general result that denervation produces a large decrease in all AChE forms and almost the complete disappearance of the collagen-tail forms (60;73-75). Nevertheless, denervation studies in chicken resulted in an increase of AChE (76;77) although this was eventually all globular forms. While total cell associated levels of AChE tend to decrease after denervation, the collagenic-AChE persists attached to the ECM for weeks or months after denervation (74;78).

Transcriptional regulation of the AChE gene is complex and several transcription factors are involved in its expression. The promoter region has an N-Box similar to the AChR epsilon subunit gene (79) that is regulated through the Ets family of transcription factors (TFs) (80) following the activation of erbB receptors. An e-box has also been studied in the promoter region related to muscle specific gene expression, and other binding sites for TFs have been described including Sp1, AP2, NfkB, Egr-1, CCAAT, GATA-1, and CRE (the cyclic AMP response element) (81-85).

The role of muscle activity and membrane depolarization is important for the understanding of the regulation of AChE transcripts and the distribution of
AChE protein in muscle fibers. Rossi et al, in a study that used a two chamber culture system, showed that the number of cell surface cluster of AChE decreased in the presence of TTX (tetrodotoxin) (Na channel antagonist) whereas this number increased in the presence of Ver (veratridine) or ScVn (scorpion venom) (Na channel agonist) showing that individual muscle nuclei responded locally to membrane depolarization (86). Under either experimental conditions, chronically or blocked membrane depolarization, the changes in the AChE mRNA correlated positively with the change in total AChE activity, indicating transcriptional regulation of the AChE gene by membrane depolarization. However, the change in the mRNA levels do not correlate with the change in collagen-tailed AChE (ColQ-AChE) levels, suggesting posttranslational regulation (87). Initial studies on the co-expression of AChE and ColQ subunits in COS-7 cells and frog oocytes suggested that the presence of ColQ alone was sufficient to regulate the appearance of the ColQ-AChE forms (16;44). In a later study using skeletal muscle cells Ruiz and Rotundo showed that the abundance of the non-catalytic subunit ColQ could be limiting during assembly of the ColQ-AChE forms, raising the possibility that muscle activity regulates synaptic AChE levels by controlling ColQ and/or its assembly with catalytic subunits (87). The limiting role of endoplasmic reticulum molecular chaperones in the posttranslational regulation of ColQ-AChE forms was recently established as well (88). In this work, the levels of protein disulfide isomerase (PDI), endoplasmic reticulum protein 72 (ERp72) and Calnexin correlated positively with the levels of ColQ-AChE forms in skeletal muscle cells. PDI showed the largest effect and
was affected by muscle activity, which explains in part the increased number of ColQ-AChE forms after membrane depolarization even when transcription was inhibited for a 12 hours period with 4.9 μg/ml α-amanitin. The experimental evidence presented in this work unambiguously showed that the synaptic ColQ-AChE form is regulated by muscle activity at least in part through posttranslational controls and chaperones.

There is one report that AChE expression in rat superior cervical ganglion neurons is regulated via post-transcriptional mechanisms that involves the AU-rich element and the nerve-specific RNA binding protein HuD not present in muscle (89), suggesting the role of regulatory regions in the 3'UTR of AChE transcript in the translational regulation of the enzyme. Whether AChE is translationally regulated in muscle remain an open question.

**RNA-binding proteins, the PUF family of proteins**

RNA-binding proteins (RBPs) are important posttranscriptional regulators of gene expression (90). The processing, stability, localization and translation of many mRNAs depend upon these RBPs. Members of the PUF (Pumilio and FBF) family of RBPs function as translational repressors when bound to the 3’UTR of selected transcripts (91). Pumilio (PUM) from *Drosophila* and FBF (fem-3 binding factor) from *C. elegans* are founding members of this evolutionarily conserved family, present in all eukaryotic phyla (from yeast to mammals and plants) (92). There are six PUF genes in the budding yeast (*S. cerevisiae*), eleven in *C. elegans*, insects (*Drosophila* and *Anopheles sp.*) have only one. Among
vertebrates, zebrafish, *Xenopus*, mouse and humans each have two PUF genes (92-94). PUF proteins are characterized by a highly conserved C-terminal RNA-binding domain, composed of eight tandem repeats referred as PUM homology domain (PUM-HD) (95). This domain binds to a specific sequence motif in the 3' untranslated region (3'UTR) of target mRNAs known as the Nanos response element (NRE) (96). There are two members of this family in humans and mice, PUM1 and PUM2 (97). The consensus PUM RNA-binding site has been identified as 5'UGUANAUAA3', where each of the eight tandem repeats in the RNA-binding domain binds to one nucleotide from the RNA in an anti-parallel fashion (91).

**PUM-HD and its interaction with the NRE**

Alignment of the C-terminal region of *Drosophila* Pum, the Pum homology domain (PUM-HD), with the mammalian and *Xenopus* PUM demonstrates the typical architecture of PUM-HD (98). This domain is composed of eight imperfect repeats of 39 amino acids plus conserved flanking regions that resemble half repeats and called 1’ and 8’ repeats (99). The most conserved amino acids reside in the middle of each repeat and interact with the RNA bases in the NRE (Figure 1.3 A, B). The crystal structure of the *Drosophila* Pum and human PUM1 HD have revealed that the repeats are aligned in tandem to form an extended curved arc molecule (100;101). The RNA binds to the concave surface of the molecule and each of the repeats makes contact with a different RNA base via the conserved amino acids in the middle of the repeat (101) (Figure 1.3 C).
The first NRE discovered was in the 3’ UTR of *hunchback* mRNA, a transcription factor, and was thought to be bipartite and composed of Box A (GUUGU) and Box B (AUUGUA) (102). Nevertheless, the crystal structure of the PUM-HD was determined to be too small to fit both boxes together (99). We know now that PUF proteins bind only one box with the initial UGU which seem to be the core recognition sequence for them, however additional bases downstream are recognized and the NRE is composed of eight nucleotides (5’UGUANAUA3’) (91;99).

**Pumilio**

Studies carried out in Lehmann’s lab described *Drosophila Pumilio* (*pum*)

![Figure 1.3: PUM-HD interacts with NRE](Cheong and Hall 2006)

a) Anti-parallel amino acids residue-RNA base interaction. b) Three detailed examples of R groups interacting with RNA base. c) 3D structure of the human Pumilio-1 homology domain (blue) in complex with RNA (red). The 8 Pumilio repeats bind RNA using the concave surface of the protein.
as a large gene that encodes an mRNA present in ovaries and early embryos. The mRNA is localized in the posterior pole of embryos and encodes a 160 kDa protein that is of a great importance in the formation of the abdomen and the germline in *Drosophila* (103).

Only two Pumilio related genes are present in the human and mouse genome. The names PUM1 and PUM2 coincide with their chromosomal localization on in the human chromosomes 1 and 2, respectively (92). Mouse Pum1 localizes on chromosome 4 and mouse Pum2 on chromosome 12 (94). Human PUM1 and PUM2 share 75% homology, however their highly conserved PUM-HD is 91% identical, and PUM1 and PUM2 share 78% or 79% homology with the fly PUM-HD (92). Human PUM1 and mouse Pum1 genes consist of 22 exons, human PUM2 has 20 exons and mouse Pum2 has 21 exons. Most importantly, in all four genes exon size and exon/intron boundaries are conserved and the PUM-HD is encoded by the last 8 exons (92;94). This conserved gene structure differs from the gene structure of the fly *Pum* and argues for an early duplication event of a single *Pum* gene in vertebrate evolution (98).

PUM proteins may regulate up to 15% of the mammalian cells’ transcriptome determined by immunoprecipitation studies, however just a few mRNAs have been described as being regulated by them, and the protein composition of PUM complexes remains elusive (104). Future investigations will identify the molecular composition of Pum-containing ribonucleo-protein particles to unravel the molecular mechanisms governing translational control.
**PUM as a part of a repressor complex of protein translation**

*Drosophila* Pum is absolutely essential for polarity in the early embryo and abdomen formation in the *Drosophila* embryo (103). Seven *Drosophila* maternal genes are required for the development of this abdominal region (nanos, pumilio, oskar, valois, vasa, staufen and tudor). Pumilio acts downstream of nanos and is required for the distribution or stability of the nanos-dependent activity in the embryo, because Nanos is regulated by Pumilio. Staufen, oskar, vasa, valois and tudor act upstream of nanos. Embryos from females mutant for these genes lack the specialized posterior pole plasm and consequently fail to form germ-cell precursors (105). It was previously known that nanos specified abdominal development by repressing translation of maternally derived *hunchback* mRNA (106-108). Pumilio, like nanos, affects *hunchback* mRNA by translational repression in the posterior pole of the embryo (103;106), therefore it acts in conjunction with nanos repressing the expression of *hunchback* in that region of the embryo (103). Pum binds to the 3’UTR of *hunchback* mRNA, and causes translational arrest (109) by its association with the zinc finger protein nanos (110). Later it was found that another protein, Brain tumor (Brat), was needed to recruit nanos to form a quaternary complex consisting of Pum, *hunchback* mRNA, nanos and Brat (111). This complex promotes deadenylation of *hunchback* mRNA and inhibits its protein synthesis (112). Pum can also inhibit *hunchback* translation by another mechanism that does not require deadenylation, probably by interfering with the translational initiation factors (113).
Attenuated mutants and conditional knockouts have revealed that Pum supports mitotic proliferation and cell renewal of adult germline stem cells in the fly ovary (114-116). During migration of Drosophila primordial germ cells (PGCs) to the future gonad, Pum binds to cyclin B1 3'UTR and eventually arrest them in G2 phase by repressing Cyclin B1 translation (117). *Xenopus* PUM2 was shown also to bind to the NRE of *Xenopus* Cyclin B1 3'UTR and physically interacts with a nanos homolog (Xcat-2) and a cytoplasmic polyadenylation binding protein (CPEB) (118;119). Interestingly, nucleotide alignment of Cyclin B1 3'UTR from mouse, rat, human and *Xenopus* has revealed a highly conserved NRE, suggesting that mouse and human PUM2 protein may bind to Cyclin B1 and inhibit its translation (98). In fact, the finding that human PUM2 is highly expressed in human germline cells, neural and hematopoietic cells further strengthens this suggestion (94;120). Nevertheless, there is no direct evidence of PUM2 regulating Cyclin B1 in mammals.

The expression of PUF proteins in mammals and *Drosophila* is not restricted to stem cells, suggesting that these proteins are operating at different stages of development from early progenitor cells to the more differentiated and committed cell types. For instance, a recent study determined that PUM2 is expressed at all stages of neuronal differentiation in the rat brain, where PUM2 regulates dendrite morphogenesis, the formation of dendritic spines and synaptic function (121). In this work it was determined that the negative regulation of the initiation translation factor 4E (eIF4E) and a voltage-gated sodium channel (Scn1a), could account for the roles of PUM2 in dendritic spine morphogenesis.
and synaptic function, respectively. This study is supported by the finding that the miRNA, miR134, promotes dendritic outgrowth by inhibiting translation of PUM2 mRNA (122).

In a recent study it was determined that PUM1 and PUM2 are required for miR221 and miR222 mediated repression of the p27 tumor suppressor protein. The binding of PUMs induces a local conformational change in the p27 transcript that exposes the miRNA binding site (123). Furthermore p27 is a cyclin-dependent kinase (CDK) inhibitor that interferes with cell cycle progression by blocking CDK2 activity (124). The p27 is a well characterized tumor suppressor that is downregulated in many human cancers and upregulated in quiescent cells; however its mRNA is expressed at the same levels in both cell types suggesting a translational regulation mechanism. Kedde and colleagues revealed a model whereby p27 expression is upregulated in quiescent cells because the interaction of the miRNAs with its binding site is hindered. When cells re-enter the cell cycle, PUM1 is upregulated and phosphorylated leading to an increase in its RNA binding activity. Once bound to the mRNA PUM1 induces a conformational change that exposes the miRNA binding site to allow the RNA-induced silencing complex (RISC) recognition that leads to miRNA-mediated repression of p27 expression (123). I consider this a relevant example in vertebrates of the dual role of one RBP and two miRNAs in the translational control of an important regulator of the cell cycle and cancer cell progression. It will be interesting to study the cell signaling pathway(s) involved in the regulation and/or phosphorylation of PUMs and whether this finding is a more universal
mechanism of translational regulation by RBPs and miRNAs. There is another example that links *C. elegans* Pumilio homologue *puf-9* and the miRNA *let-7* repressing *hbl-1* expression (125). A genome-wide analysis has revealed NREs enrichment around predicted miRNA binding sites, suggesting evolutionarily conserved interactions between PUM proteins and the miRNA regulatory system (126). It will be important to study to what magnitude this interaction contributes to the control of gene expression.

**RBPs and AChE translational regulation**

Previous studies have shown the localization of the RNA-binding proteins Staufen1 and Staufen2 at the mammalian NMJ (127). However, no specific mRNA interaction with Staufen has been described at the motor end-plate. In these studies, it was found that Staufens were regulated by the motor nerve as well as by muscle contractility. When mice were denervated upregulation of both Staufens was observed. A recent study performed in hematopoietic cells revealed the role of a microRNA (miRNA-132) in the translational regulation of AChE by binding to a regulatory region in AChE 3'UTR. AChE was upregulated when an anti-miRNA-132 oligonucleotide was incorporated in the cells or when the miRNA binding region was mutated, and AChE expression was downregulated when the levels of miRNA-132 were increased using a lentiviral expression system (128). It will be interesting to determine whether there is a functional link between this miRNA and PUMs in regulating the expression of AChE in nerves and muscle cells.
It has been reported that AChE expression in rat superior cervical ganglion neurons is regulated via post-transcriptional mechanisms that involve the AU-rich element and HuD RNA binding protein (89). How AChE is translationally regulated at the NMJ is an open question. The RNA-binding protein (RBP) Pumilio from *Drosophila* is localized to the postsynaptic side of the NMJ in third instar larvae and is also expressed in larval neurons (129). In silico studies reported the E4-E6 splice variant of AChE as one of the possible targets of the RBP Pumilio-2 (PUM2) (130). We identified the presence of the canonic NRE octamer sequence in the 3’UTR of AChE transcripts which is the PUM2 RNA binding site. This led us to formulate the overall hypothesis of this project which is: “The RNA-binding protein PUM2 regulates the expression of AChE at the translational level in the NMJ”.
Chapter 2: Translational Regulation of Acetylcholinesterase by the RNA Binding Protein Pumilio-2 at the Neuromuscular Synapse

Summary

Our understanding of the molecular mechanisms underlying Acetylcholinesterase (AChE) regulation is incomplete, but they appear to involve both translational and post-translational events as well. Here we show that Pumilio-2 (PUM2), an RNA binding translational repressor, is highly localized at the neuromuscular junction where AChE mRNA concentrates. Immunoprecipitation of muscle cell extracts with a PUM2 specific antibody pulled down AChE mRNA, indicating that PUM2 binds to the AChE transcripts. Gel shift assays using a bacterially expressed PUM2 RNA binding domain showed a specific shift using a wild type AChE 3’UTR RNA fragment that was abrogated by mutation of the consensus recognition site. Transfecting skeletal muscle cells with shRNAs specific for PUM2 upregulated AChE expression, whereas overexpression of PUM2 decreased AChE activity. We conclude that PUM2 binds to AChE mRNA and regulates AChE expression translationally at the neuromuscular synapse. Finally, we found that PUM2 is regulated by the motor nerve suggesting a trans-synaptic mechanism for locally regulating translation of specific synaptic proteins involved in modulating synaptic transmission, analogous to CNS synapses.
Background

Acetylcholinesterase (AChE) and its transcripts are highly concentrated at the neuromuscular junction (NMJ) where they are regulated at both the transcriptional and post-transcriptional levels (reviewed in (33;40;61;131) ). The AChE mRNAs in multinucleated muscle cells remain localized near their nucleus of origin in tissue culture (54), and the newly-synthesized AChE molecules are preferentially transported and localized to the muscle plasma membrane directly above those nuclei (132). Moreover, regulation occurs in response to signals generated on the overlying regions of the plasma membrane (133). This high concentration of mRNAs encoding AChE at neuromuscular synapses (56;57;59;134) implies highly specific mechanisms for transcript localization and for insuring their translation at the appropriate times. The molecular mechanisms underlying this localization and how AChE is translationally regulated at the NMJ is not known.

The localization, stability and translation of many mRNAs depend upon ribonucleic acid binding proteins (RBPs). Members of the PUF (Pumilio and FBF) family of RBPs function as translational repressors when bound to the 3’ untranslated region (3’-UTR) of selected transcripts. Pumilio (PUM) from Drosophila is a founding member of this evolutionarily conserved family (95;103;114), and is present from yeast to humans (98). The PUF proteins are characterized by a highly conserved C-terminal RNA-binding domain, the homology domain (HD) (95;99;101), composed of eight tandem repeats. This domain binds to a specific eight nucleotide sequence in the 3’-UTR of target
mRNAs known as the Nanos response element (NRE) (91). There are two members of this family in mammals, PUM1 and PUM2 (135). They may regulate up to 15% of the mammalian cells’ transcriptome, however just a few mRNA have been described as being regulated by them, and the protein composition of PUM complexes remains elusive (136).

Pumilio was originally discovered in studies carried out in Lehmann’s lab. Drosophila Pumilio (pum) is a large gene that encodes an mRNA present in ovaries and early embryos. The mRNA is enriched in the posterior pole of embryos and encodes a 160 kDa protein that is of great importance in the formation of the abdomen and the germline in Drosophila (103;105;114;115). The first NRE discovered was in the 3’ UTR of hunchback mRNA, a transcription factor, and was thought to be bipartite and composed of Box A (GUUGU) and Box B (AUUGUA) (102). Nevertheless, the crystal structure of the PUM-HD was determined to be too small to fit both boxes together (99). We know now that PUF proteins bind only one box, with the initial UGU which seem to be the core recognition sequence for them. However, additional bases downstream are recognized and the NRE is composed by eight nucleotides (5’UGUANAUA3’) (91;99).

The expression of PUF proteins in mammals is not restricted to stem cells, suggesting that these proteins are operating at different stages of development from early progenitor cells to the more differentiated and committed cell types. A recent study determined that PUM2 is expressed at all stages of neuronal differentiation in the rat brain, where PUM2 regulates dendrite morphogenesis,
the formation of dendritic spines and synaptic function (121). Thus it was
determined that the negative regulation of the initiation translation factor 4E
(eIF4E) and a voltage-gated sodium channel (Scn1a), which account for the roles
of PUM2 in dendritic spine morphogenesis and synaptic function, respectively.
This study is supported by the finding that the miRNA, miR134, promotes
dendritic outgrowth by inhibiting translation of PUM2 mRNA (122).

In the present study we show that the AChE mRNA 3'UTR contains one
consensus NRE that it is highly conserved in mice, rats and humans, and that
PUM2 is highly localized at the mammalian NMJ. Moreover, Pum2 specifically
binds to the AChE transcript 3'-UTR where it regulates its translation in tissue
cultured skeletal muscle. These studies provide the first evidence for translational
regulation of a synaptic component (AChE) by an identified RNA binding protein
PUM2 at the neuromuscular synapse and provide a model system for studying
translational controls at both PNS and CNS synapses. Additional in vivo
evidence suggests that PUM2 itself is under regulation by the motor nerve.

Results

PUM2 is localized at the NMJ: Previous studies from our laboratory and
others have shown that AChE mRNA is highly concentrated in the NMJ (56-58).
Subsequent cloning and sequencing of the full length cDNAs by several
laboratories showed that the mammalian AChEs were highly conserved in both
their coding and non-coding sequences. We subsequently found that the mouse
AChE transcript contained a highly conserved NRE, the consensus Pumilio
binding site, in the 3'UTR. Our alignment of the C-terminal coding region and
proximal 3'UTR gene sequences from three mammalian species is presented in Figure 2.1, showing the high degree of conservation of the sequence surrounding the stop codon and the NRE over some 80 million years of evolution. We then proceeded to look for expression of Pum2 protein in both the C2/C12 mouse muscle cell line and mouse fast and slow muscle by Western blotting (Figure 2.2C). While the muscle cell line expressed predominantly the 120 kDa Pum2 band both the mouse fast EDL muscle (Ej) and slow soleus muscle (Sol) expressed more of the 96 kDa band. Higher levels of Pum2 expression were observed in the innervated junctional (J) regions of the fast fibers than the non-innervated extrajunctional (Ej) regions. Also, slow muscles consistently expressed higher levels of Pum2 than fast muscles (Figure 2.2C and unpublished observations). We then determined the subcellular distribution of PUM2 and PUM1 in skeletal muscle tissue by immunofluorescence. Anti-PUM2 antibodies selectively labeled the NMJ as well as the capillaries alongside the muscle fibers (Figure 2.2A, upper panel), whereas anti-PUM1 antibodies did not label the NMJ, but rather labeled the nerves and also throughout the muscle fibers (Figure 2.2A, lower panel). Thus the expression and localization of PUM2 in skeletal muscle is virtually identical to the expression of the AChE protein and mRNA.

PUM2 binds to the NRE in the AChE mRNA 3'UTR: To determine whether PUM2 interacts with AChE mRNA in muscle fibers, aliquots of the total C2C12 cell extracts were incubated with anti-PUM2 antibody coupled to protein-A Sepharose beads or anti-PUM1 antibody coupled to protein-G Sepharose beads.
After centrifugation and washes the RNA was extracted using TRizol reagent followed by RT-PCR using the mouse AChE specific primers as described in experimental procedures. From the anti-PUM2 antibody precipitate we amplified a band of approximately 1 Kb corresponding to the expected size of the AChE fragment (Figure 2.3) (P2) that was not precipitated by the anti-Pum1 antibody (P1). While usually negative, occasionally a small but barely detectable amount of AChE RNA was pulled down when anti-PUM1 antibody was used. This could occur if there was some cross-reactivity between this antibody and PUM2 or because some PUM1 in the tissue cultured cells was bound to AChE mRNA since PUM2 and PUM1 share the same NRE binding site. Similar results were obtained when quail muscle culture (QMC) extracts were used (data not shown). When a PCR to amplify alpha-actin was performed on the same samples we did not detect any DNA amplification (Figure 2B). These results suggest that PUM2 and AChE mRNA form complexes in muscle cells, possibly recruiting other factors as previously described (137).

To determine whether PUM2 and AChE mRNA interacted directly we performed a gel shift mobility assay. We transcribed a 95 nucleotide $^{32}$P-radiolabeled fragment corresponding to the mAChE 3'UTR RNA starting with the stop codon, or mAChE 3'UTR RNA with three nucleotides mutated in the NRE (G2/A; T3/A; T7/A) that does not bind PUM2 (Cheong and Hall, 2006). The labeled RNAs were incubated for 30 minutes with the recombinant purified PUM2-HD and the samples run in a 4% native polyacrylamide gel. The gel was dried and exposed to film and a phosphorImager. We found that the recombinant PUM2-
HD changed the mobility of the RNA fragment by more than 50 kDa, while the mobility of the NRE mutated RNA fragment remained unchanged (Figure 2.4). We detected two shifted bands when the RNA-protein complex was formed which could occur if two different secondary structures of the 95nt target sequence exist. While we do not have a certain explanation for this observation, RNA structure prediction programs predict a high degree of secondary structure in the 95nt target sequence (data not shown). Additionally, the protein could form two different secondary structures with the RNA complex that explain the different two forms in a native gel. We deliberately used the 60 kDa bacterially expressed and his tag-purified Pum2-HD and a mAChE 3’UTR produced in vitro to avoid contamination by other mammalian RNA binding proteins. In another set of direct in vitro solid state binding assays we observed a linear increase in mAChE 3’UTR binding when increased amounts of PUM2-HD was used (data not shown). Thus, we conclude that PUM2 binds specifically to the NRE of the mAChE 3’UTR.

The role of the NRE in AChE translational regulation: To test whether the NRE present in AChE 3’UTR could be responsible for PUM2 regulation the full length AChE 3’UTR was cloned after the EGFP coding region in the pEGFP-C1 vector (Clontech) to generate pEGFP-AChE-3’UTR. A three point mutation, described above, was introduced in the NRE region (pEGFP-AChE-3’UTRmut) as a control. QMCs cells were transfected separately with these constructs and GFP expression was determined by Western blot 48 hours later. The relative density of the bands was determined using ImageJ software. A similar reporter
assay has been used to determine the role of Huntingtin in dendritic RNA granules (138) and the localization of MAP2 and CaMKIIα mRNA into distinct ribonucleoprotein particles along dendrites of hippocampal neurons (139). Our results show that there is increased expression of GFP in the avian skeletal muscle cultures transfected with pEGFP-AChE-3′UTRmut compared to those transfected with pEGFP-AChE-3′UTR (Figures 2.5). In cells where the wild type 3′UTR was expressed there was repression of the reporter construct since the NRE is intact, while repression is absent when the NRE is mutated. To test that PUM2 is the factor involved in the regulation of the reporter gene we cotransfected QMC cells with pEGFP-AChE-3′UTR and PUM2-Cherry or pEGFP-AChE-3′UTRmut and PUM2-Cherry followed by Western blot to determine GFP expression. The results are shown in Figure 2.5 in which overexpression of PUM2 totally repressed the translation of GFP when the full length AChE 3′UTR was present after the GFP coding sequence, but did not affect translation of GFP when the NRE was mutated in a similar construct.

PUM2 regulates the translation of AChE in muscle cells: PUM2 has been shown to be a component of a translational repressor complex in several systems (118;119;140). To quantify the effects of PUM2 on AChE expression in skeletal muscle we overexpressed PUM2 in QMCs and assayed total cell-associated enzyme activity three days later. Overexpression of PUM2 in skeletal muscle cells downregulates the expression of AChE by about 40% (Figures 2.6). This partial reduction, however, could be due to an average 40% reduction in all cells or a near complete reduction in half the cells in the dish due to low efficiency
transfection. To distinguish between these two possibilities we transfected QMCs with a plasmid expressing PUM2-Cherry, three cultures per group. Three days after transfection the distribution of cell surface AChE was visualized by immunofluorescence. The number of cell surface AChE clusters was reduced specifically in those fibers that were transfected with the PUM2-Cherry encoding plasmid (Figure 2.7). Quantification of the numbers of AChE clusters per quadrant showed a dramatic decrease of about 90% in those fibers overexpressing PUM2 (Figure 2.7). This reduced number of cluster per transfected fiber is explained by the downregulation of AChE expression specifically in those fibers expressing Pum2-cherry. Conversely, reduction of PUM2 expression would be predicted to increase AChE levels. To determine the effects of PUM2 downregulation on AChE expression we used shRNAs to knock down PUM2. Downregulation of PUM2 promotes upregulation of AChE (Figure 2.8). When sucrose gradients were performed the globular forms (G1/G2), tetrameric forms of AChE (G4) and the collagen-tailed form (A12) were accumulated in the cells transfected with PUM2 shRNA (Figure 2.8). This increase was confirmed by Western blot of AChE extracted from tissue cultured muscle cells showing that when PUM2 was knocked down AChE protein increased (Figure 2.8). Together these results indicate that PUM2 acts as a translational repressor of AChE expression in muscle cells.

PUM2 expression is regulated by the presence of the motor nerve in skeletal muscle: Many proteins localized at the NMJ are regulated by the motor nerve such as AChE (75), AChR (141) (142), and the scaffolding proteins rapsyn
(143) and utrophin (144). To determine whether PUM2 is regulated by the motor nerve we unilaterally denervated the lower rear limb in mice (see methods for details). One, two or three days after denervation the animals were sacrificed and the EDL muscles dissected. Total protein extracts from EDL muscles were analyzed by Western blot to detect PUM2 (Figure 2.9). By three days after denervation we observed a large increase in PUM2 expression. In a second set of experiments we denervated five mice and found a tenfold increase in the expression of PUM2 three days after denervation in EDL muscle (Fig 2.9 B and C).

In a separate study we dissected and extracted the EDL and soleus muscles from the lower leg of a mouse followed by Western blot of total protein extract. We showed earlier that PUM2 levels in the mouse EDL and soleus muscles (Figure 2.2C) that slow twitch muscle like the soleus contained significantly more PUM2 than the typical fast twitch muscle like EDL. Also, we found PUM2 most highly expressed in the central region of the EDL muscle where the NMJs are located compared to the more distal non-innervated regions (Figure 2.2C). This is consistent with our finding that PUM2 is localized at the NMJ. We used C2C12 as positive control, where the size of PUM2 is around 120 kDa, while an additional smaller variant of approximately 96 kDa was found in skeletal muscle tissues. In these fibers both variants are present and further studies will clarify the basis of this observation. In conclusion, these results suggest a key role for the nerve in regulating the expression of PUM2.
Discussion

Because of their role in localized protein translation and their importance in memory and learning, research on RNA binding proteins in the nervous system has focused almost exclusively on CNS neurons such as those in the hippocampus. Mutations in the Fragile X Mental Retardation Protein (FMRP) gene are one of the major causes of inherited cognitive disorders in humans where it affects local translation of critical synaptic components in dendrites of hippocampal neurons (reviewed in (145;146)). Far less is known about RBPs in the peripheral nervous system and only two RNA binding proteins, Staufen1 and Staufen2, have been found to be localized at the vertebrate NMJ (127). Staufens however have not been found to regulate expression of any particular transcript at the NMJ. Since the only RNA binding proteins known to modulate RNA translation through this canonical PBE, previously known as NRE, are PUM1 and PUM2 (147), and PUM2 is the one localized at the NMJ where AChE mRNA is localized (Figure 2.2B), this suggests that PUM2 binding to the AChE 3’UTR has an important role in local translational regulation of AChE at the neuromuscular synapse.

Here we present the first evidence of a RNA binding protein acting as a translational regulator of the expression of a key component of the NMJ in mammalian skeletal muscle. In silico studies reported the E4-E6 splice variant of AChE as one of the possible targets of PUM2 (148) suggesting a role in electrically excitable cells since this splicing variant encoding the AChE isoform that is expressed primarily in nerves and muscle. Our data using tissue cultured
primary skeletal muscle cells (Figures 2.4 - 2.6) as well as in vivo indicate that PUM2 regulates the expression of AChE at the NMJ. This opens several important questions. What is the mechanism of PUM2 regulation of AChE? Two principal molecular mechanisms of PUM2 translational regulation have been described. PUM2 can target the mRNA for degradation (149) or PUM2 remains bound to the mRNA in a dormant conformation until that particular RNA is needed (150). Experiments are in progress to determine whether AChE mRNA is targeted for degradation after increasing PUM2 in muscle cells or whether it remains quiescent as a RNP particle. Another interesting question concerns the significance of translational regulation of AChE in electrically excitable cells such as skeletal muscle cells and neurons. Do the levels of AChE locally control the sensitivity of cholinergic circuits in the central and peripheral nervous systems? The ability to regulate translation of this enzyme on a short timescale would suggest that this is the case and thus might have important implications for modulating cholinergic sensitivity in the CNS as well.

The regulation of AChE expression in the central nervous system is still poorly understood. The possibility of PUM2 being involved in this capacity is not unlikely. It will be interesting to determine whether PUM2 binds to the AChE mRNA in CNS neurons since PUM2 is expressed by neurons and is involved in the formation of stress granules in dendrites (121) (151). AChE is highly expressed in cholinergic neurons and their targets in the CNS, and specifically in the hippocampus. In Drosophila, the Pumilio/Staufen pathway has been linked to synaptic plasticity and long term memory in the fly CNS (152). Thus PUM2
interacting with transcripts encoding proteins specific to subsets of hippocampal synapses could be related with learning and memory processes in mammals.

In conclusion, we find PUM2 highly localized at the NMJ as it was previously found in *Drosophila* (153). We unambiguously demonstrate the interaction of PUM2-HD with the consensus PBE that is present and highly conserved among mammal species. We present solid proof that PUM2 inversely regulates the expression of AChE in skeletal muscle cells and that the motor nerve is involved in the regulation of PUM2 which could affect in part the levels of AChE at the translational level after muscle denervation. The interaction of PUM2/AChE in the CNS requires special attention; hence PUM2 is involved in dendritic morphogenesis, learning and long term memory; and AChE plays an important role in brain disorders such as Alzheimer’s disease (154-156), in which long term memory is highly affected.

**Experimental procedures**

Cell cultures and transfection: Primary quail myoblasts (Quail Muscle Cells, QMCs) were obtained from pectoral muscles of 10-day-old embryos and plated at 5×10^5 cells/ml in Eagle’s Minimum Essential Medium (Gibco) supplemented with 2% chicken embryo extract and 10% horse serum (GemCell) and 0.1% gentamicin (Invitrogen). The avian cultures were maintained at 39°C in a water-saturated incubator with 5% CO₂ and fed with fresh medium on days three and five post-platting. On day three the medium was supplemented with 10^-6 M cytosine-arabinoside (Sigma) to reduce fibroblast proliferation.
The C2/C12 mouse muscle cell line (ATCC) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals). Cultures were maintained at 37°C in a water-saturated incubator with 5% CO₂. The cells were induced to differentiate by changing the medium to DMEM supplemented with 2% horse serum when the cells approached confluency.

QMCs were transfected with plasmids encoding mouse PUM2 (pCMV-Sport6 mPUM2, Open Biosystems) or PUM2-Cherry fusion protein (chicken PUM2 fused with Cherry/GFP cloned in the NotI sites of pIRES vector). pEGFP was used to clone AChE3'UTR after the GFP coding sequence in the EcoRI site. Plasmids were transfected using Exgen-500 (Fermentas) following the manufacturer's suggested protocol. Control groups were transfected in parallel with plasmids encoding GFP or an unrelated secreted protein (pTarget-MuSK ectodomain). A mixture of shRNAs (OriGene; product #TR302189) was used to suppress PUM2 expression and transfected into QMC using the same protocol described above. Control groups consisted of cells transfected with a mixture of shRNA (OriGene; product #TR30003) targeting an unrelated protein, GFP.

Protein extraction and Western blotting: Cultured cells were washed in PBS with 0.1 mM calcium and 2 mM magnesium and extracted in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1 % SDS). The relative levels of AChE and PUM2 expression were determined by Western blot (WB). After centrifugation 10% aliquots of the supernatants were run in a 10% SDS-PAGE. Proteins from the gel were electrophoretically
transferred to nitrocellulose membranes (Whatman). After blocking the membrane with skim milk (Carnation) rabbit polyclonal anti-PUM2 (Abcam) (1:5000) and a mouse monoclonal anti-AChE antibody 1A2 (1.6 µg/ml) (30) were used as primary and peroxidase conjugated antibodies (Cell Signaling) (1:1000) were used for detection. The membranes were washed three times between incubations with 0.025% NP-40 in phosphate-buffered saline (PBS). The membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and recorded on Kodak film. Mouse extensor digitorum longus (EDL) and soleus muscles were isolated and homogenized using 1% SDS extraction buffer (100mM Tris-HCL, 5 mM EDTA, 1% SDS, pH 7.2) 10X weight/volume, and sonicated. The Western Blots of the muscle homogenates were carried out using the same procedure as for cultured cell extracts. The relative density of the bands was estimated using ImageJ software (NIH).

Assay of AChE activity and analysis of oligomeric forms: AChE activity was measured using Ellman's method (157). To determine the distribution of the AChE oligomeric forms cells were extracted with RIPA buffer, microfuged, and the supernatants pooled from triplicate cultures for analysis by velocity sedimentation in a Beckman SW41ti rotor at 36,000 rpm for 16 hours on 5-20% sucrose gradients. Fractions were collected and AChE activity was measured using the Ellman’s method.

Immunoprecipitation: QMCs or C2/C12 cells were extracted with a lysis buffer containing 50 mM Tris-HCL, 150 mM NaCl, 1% NP-40 and protease inhibitor cocktail (Roche Diagnostics; Complete Mini, EDTA-free). Cell extracts
were immunoprecipitated using protein-A sepharose beads (Amersham Pharmacia Biotech) pre-incubated with 3 µg of rabbit anti-PUM2 or protein-G sepharose (Amersham Pharmacia Biotech) pre-incubated with 3 µg of goat anti-PUM1. Protein A or G conjugated sepharose beads without antibodies were used as negative controls. After immunoprecipitation the beads were extracted with TRIzol reagent (Invitrogen) to isolate RNA, followed by RT-PCR using oligo dT (Promega) and amplification using AChe specific primers (forward: TTTCTGGGCATCCCCTTTGC, reverse: ATCCCAAGCTTTAAATAAATAG) giving a 1000 nucleotide fragment. The PCR products were run in an agarose gel. An additional PCR to amplify alpha actin was performed as negative control using ATGGAGCCACCGATCC as forward and AACTGGGACGACATGG as reverse primers (800 nucleotides).

In vitro binding assay: The mouse amino acid sequence of PUM2 RNA binding homologous domain (PUM2-HD)(158) with a poly-His tag at the N-terminus was cloned into the bacterial expression vector pQE30-HA and transformed into E. coli M15 cells. The bacteria were grown in LB culture medium containing both ampicillin (100 µg/ml) and kanamycin (25 µg/ml) until the OD600 was 0.5. PUM2-HD expression was induced for 4 hours with 1mM IPTG at which time the cells were collected and resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0). After centrifugation, the supernatant was loaded onto a nickel column (Ni Sepharose 6 fast flow; Amersham). After three washes with wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0) the column was eluted with elution buffer (50 mM NaH2PO4,
300 mM NaCl, 200 mM imidazole, pH 8.0). The eluate was washed and concentrated using centicon centrifugal filter devices (Amicon) and PBS buffer, pH 7.4. The final product with a protein concentration of 1 mg/ml was stored in PBS at -80°C until used one week after the purification procedure.

A 95 base pair fragment of the AChE 3'UTR starting at the stop codon was cloned into pCRII-TOPO plasmid and verified by sequencing. Three point mutations (G2/A; T3/A; T7/A) were introduced in the PBE of the AChE 3'UTR using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) to be used a negative control since Hall and colleagues have shown that these nucleotides are essential for PUM2 binding (3). For transcription of probes each plasmids were digested with BamHI for 3 hours and gel purified. The linearized plasmids were extracted using a gel extraction kit (Invitrogen) and in vitro transcriptions performed using 1µg DNA, 4 µl rNTP mix (2.5 mM each) and P32rUTP 50 µCi for 1h at 30 °C with T7 polymerase (Promega) and 20 units of the RNase inhibitor RNasin (Promega). The plasmid DNA was digested with RQ1 DNase (Promega) for 30 min at 37°C and the RNA denatured for 5 min at 65°C and 2 min on ice. One ng of the probe was incubated with 20 ng of native PUM2-HD in binding buffer (20 mM HEPES pH 7.5, 10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, 200 ng of yeast tRNA and 200 ng of BSA). After 20 min at room temperature the samples were loaded directly onto Tris-Glycine 4% polyacrylamide gel and run for 2 hours at 120 V in Tris-glycine buffer (12 mM Tris-HCl, 120 mM glycine, pH 7.4). The gel was dried at 80°C for 1 hour and exposed to both an X-ray film and a PhosphorImager.
Immunofluorescence: Cryostat cross-sections of mouse gastrocnemius and EDL muscles were mounted on Superfrost microscope slides (VWR) and blocked for 20 minutes with 1% bovine serum albumin in PBS. PUM2 immunoreactivity was detected using a rabbit anti-PUM2 polyclonal antibody (Abcam, 1:200). A goat polyclonal anti-PUM1 antibody (Abcam, 1:100) was used to localize the PUM1 protein. Alexa-488 goat anti-rabbit or rabbit anti-goat antibodies (Invitrogen) were used as secondary antibodies, and Alexa-555 α-Bungarotoxin (Invitrogen) was used to label the nicotinic acetylcholine receptors at the NMJs. The sections were fixed with 4% paraformaldehyde in PBS, pH 7.0, for 20 minutes. Between incubations the sections were washed 4 times with PBS. All images were acquired with identical exposure parameters using a Leica DMR-A fluorescence microscope operated by 3i Slidebook 4.0 software. The cells transfected with the PUM2-Cherry fusion protein were used directly for fluorescence microscopy after fixation. Three days after transfection the cells were immunostained with mouse anti-AChE antibody (mAb 1A2) (30). Conjugated rabbit anti-mouse FITC (Jackson, 1:100) was used as secondary antibody. The number of AChE clusters was count in 100 nuclei per coverslip in untransfected and transfected fibers. Three coverslips were counted per group and the results expressed as the mean ± SEM.

Animal care and surgery: BALB/C mice were housed in the University of Miami Animal Care Facility and surgical procedures were performed in accordance with the guidelines established by the University of Miami Animal Care & Use Committee under an institutional ACUC approved protocol. The mice were
anesthetized with ketamine and xilazine and denervated through a small incision in the mid-thigh region by cutting and removing a 5 mm segment of the exposed sciatic nerve followed by ligation of the nerve stumps and suturing of the incision. Three and seven days after surgery the animals were sacrificed using CO₂ and the EDL and soleus muscles excised and rapidly frozen in liquid nitrogen to be analyzed by Western Blot.
Figure 2.1: Alignment of AChE mRNA 3’UTRs from rat, mouse and human showing the location of the PBE in the 3’UTR.

The PBE consensus octamer sequence for PUM2 binding is highlighted in bold. The stop codon is the TGA beneath the word stop. The position of the PBE as well as the flanking sequences have been very highly conserved.
Figure 2.2: PUM2 is localized at the mouse NMJ. Immunofluorescence localization of PUM1 and PUM2 on cross-sections of mouse gastrocnemious muscle.

A. Upper panel: immunofluorescence on adjacent sections labeled with goat anti-PUM1 antibody as primary and rabbit anti-goat Alexa 488 as the secondary antibody, together with Alexa 555 α-BTX to stain the NMJ (arrows), PUM1 is highly localized in the nerve (arrowhead).

B. Lower panel: a rabbit anti-PUM2 antibody was used as the primary antibody to localize PUM2 with rabbit Alexa 488 as the second. Alexa 555 α-BTX α-bungarotoxin was used to label the NMJ (arrows).

C. Western Blot analysis of differentiated mouse C2/C12 cells (C2), and mouse EDL junctional (J), EDL extrajunctional (EJ) and soleus (Sol) muscles, showing expression of both the 120 and 96 kDa variants of PUM2 in skeletal muscle. The Western blot for tubulin (55 kDa) is shown as a loading control in the lower panel.
Figure 2.3: PUM2 associates with the AChE transcript in skeletal muscle cells.

A. Agarose gel showing the RT-PCR products after immunoprecipitation of C2C12 cell extracts using either anti-PUM2 Ab or anti-PUM1 antibodies, followed by RNA purification and RT-PCR. B. 40 Cycles of PCR using primers for muscle actin. I: Total Input RNA; PA, Protein-A Sepharose without Abs.; PG, Protein-G Sepharose without Abs.; P2, Protein-A Sepharose + Rabbit anti-PUM2; P1, Protein-G Sepharose + Goat anti-PUM1. The polynucleotide ladder is shown in kilobases (Kb).
Figure 2.4: The PUM2 homology domain binds specifically to the PBE in the AChE 3'UTR.

Gel mobility shift assay of a radiolabeled 95 nucleotide fragment of AChE 3'UTR RNA after 20 minutes incubation with PUM2-HD. AChE is the wild type 3UTR of AChE with the intact PBE. AChEm is 3'UTR of AChE with the PBE mutated. The apparent molecular weight is shown in kilo Daltons (kDa).
Figure 2.5: The PBE in AChE3'UTR plays an important role in the translational regulation of the gene by PUM2.

A. Anti-GFP Western blot of QMCs transfected by triplicates with pEGFP-AChE-3'UTR or pEGFP-AChE-3'UTRmut. Anti-tubulin antibody (lower panel) was used as loading control. B. Quantitation of the experiment shown in A using ImageJ software. C. Anti-GFP Western blot of QMCs co-transfected by triplicates with pEGFP-AChE-3'UTR and PUM2-Cherry or pEGFP-AChE-3'UTRmut and PUM2-Cherry.
Figure 2.6: PUM2 regulates the expression of AChE in skeletal muscle cells.

A. QMCs were transfected with pTarget coding a secreted protein as control plasmid (Control) or mouse PUM2 (PUM2). Three days later the cells were extracted in 20mM borate extraction buffer and total AChE activity measured. *p<0.05 (t test).

B. Western Blot of the cell extracts used in panel A, probed with anti-PUM2 antibody showing the overexpression of PUM2 (upper panel) or anti-tubulin antibody (lower panel) as loading controls. The molecular weight is represented in kilo Dalton (Kda).
Figure 2.7: Expression of cell surface AChE is downregulated specifically in PUM2 transfected fibers.

A. Localization of cell surface AChE using anti-AChE mAb 1A2 (green) on myotubes, some of which are expressing PUM2-Cherry (red). Myotubes overexpressing PUM2 do not express cell surface AChE. B. Quantitation of the experiment shown in a. The number of AChE clusters per 100 nuclei was counted in non-transfected and PUM2 transfected fibers. * p<0.001 (t-test).
Figure 2.8: Increased AChE expression following PUM2 knockdown.

A. AChE activity in total cell extracts of 7 day old QMCs was measured using the Ellman’s assay from triplicate culture dishes transfected with a mixture of an unrelated shRNA for GFP (shControl) or a mixture of PUM2 shRNA (shPUM2) as described in Methods. *p<0.005 (t test).

B. Velocity sedimentation analysis of a pool of the samples used in panel “a”. (A12) asymmetric collagen-tailed form of the enzyme, (G4) globular tetramers and (G2/G1) globular dimers and monomers of the enzyme, respectively.

C. Western Blot of samples from panel “a” incubated with anti-AChE antibody mAb 1A2, anti-PUM2 antibody or anti-tubulin antibody. The apparent molecular weight is given in kilo Daltons (kDa).
Figure 2.9: Neural regulation of PUM2 in mouse skeletal muscle.

A. Western blot analysis of mouse fast medial EDL showing that PUM2 is highly upregulated three days after denervation. WB for actin is shown as a loading control in the lower panel. (S) Sham operated animal, (1) one day denervated and (3) three days denervated animals. B. Western blot analysis of mouse fast medial EDL showing that PUM2 is highly upregulated in five mice three days after denervation. Tubulin used as loading control is shown in the lower panel. C. Graph showing the quantitation of the Western Blot bands of PUM2 in sham and denervated (Denv) mice from “B”. The apparent molecular weights are given in kilo Daltons (kDa).
Chapter 3: The RNA Binding Protein Pumilio-2 Regulates its Own Translation

Summary

Pumilio-2 (PUM2) is highly localized at the neuromuscular junction in skeletal muscle tissue as well as the central nervous system. Although PUM2 expression is critically important in many cell types, virtually nothing is known about the regulation of PUM2 expression itself. Analyzing the PUM2 mRNA 3'UTR we found fifteen possible PUM2 binding sites (PBEs or NREs) in the 3 Kb 3' UTR. We therefore hypothesized that PUM2 regulates its own translation. Using immunoprecipitation assays we show that PUM2 binds to its own mRNA. Overexpression of PUM2 in Cos7 cells, C2C12 mouse muscle cells and primary cultured skeletal muscle cells transfected with a green fluorescent protein (GFP) reporter construct linked to the full length PUM2 3'UTR (GFP-PUM2-3'UTRFL) suppresses GFP expression suggesting that PUM2 downregulates its own expression by binding to its own 3'UTR. We found that at least one PBE is active in the first fragment of the PUM2 3'UTR containing the first five PBEs. These observations suggest a novel model for the localized regulation of protein translation through a negative feedback loop.
Background

The PUF family is an evolutionary conserved family of RNA binding proteins that act as translational repressors. A typical feature of this family of proteins is a C-Terminal RNA binding domain, composed of eight imperfect tandem repeats, known as the Pumilio homology domain (PUM-HD)\(^{(98)}\). The crystal structures of PUM-HD from \textit{Drosophila} \(^{(100)}\) and human Pumilio-1 (PUM1) \(^{(101)}\) have revealed that the repeats are aligned in tandem to form an extended curved arc-like molecule. The RNA binds to the concave surface of the domain, where each of the eight repeats makes contact with a different RNA base via three conserved amino acid residues positioned in the middle of the repeat \(^{(99)}\). PUF proteins recognize specific RNA sequences, known as nanos response elements (NREs) or Pumilio binding elements (PBEs) present in the 3’UTR of target mRNAs. The first PBE discovered to interact with Pumilio was located in Drosophila hunchback (hb) 3’UTR \(^{(102)}\). It was recently determined that human PUM1 \(^{(99)}\) and mouse Pumilio-2 (PUM2) \(^{(91)}\) bind to a sequence composed of eight nucleotides (5’UGUANAUA3’) and by consensus was named Pumilio Binding element (PBE).

The role of Pum is well documented in the proper formation of the embryo in Drosophila \(^{(109;110;112)}\), and in the proliferation and self-renewal of adult germline stem cells in the fly ovary \(^{(114-116)}\). This protein also plays several roles in the fly nervous system. First, Pum controls dendrite morphogenesis in peripheral neurons \(^{(159)}\). Second, it regulates synaptic growth and function by controlling the expression of eIF4E mRNA at the fly NMJ \(^{(129;153)}\). Third, it
inhibits neuronal excitability by repressing the translation of a voltage-gated sodium channel (160;161). Finally, loss of fly Pum impairs long-term memory (152). A homolog of Pum, mammalian PUM2, is expressed in hippocampal neurons and is found in ribonucleoparticles (RNPs) in the somatodendritic compartment (151). Recently, a role for PUM2 in regulating dendrite morphogenesis and synaptic function in rat neurons has been established (121).

While much is known about the importance of PUM2 in development and in the adult nervous system, little is known about the regulation of expression of this important protein. In the present studies, we found two consensus PBEs and thirteen non-consensus PBEs in the PUM2 3’UTR (Figure 3.1), and we hypothesis that PUM2 regulates its own expression. We found PUM2 interacting with its own mRNA and repressing the expression of a reporter gene under the regulation of its 3’UTR. Moreover, we found that at least one of the first five PBEs is necessary and sufficient for PUM2 translational regulation, thus the position of the PBEs with respect to the stop codon could be relevant regarding PUM2 translational regulation.

**Results**

PUM1 and PUM2 bind to the PUM2 transcript: To determine whether PUM2 interacts with its mRNA in muscle myotubes aliquots of the C2C12 cell extracts were incubated with the anti-PUM2 antibody coupled to protein-A Sepharose beads or anti-PUM1 antibody coupled to protein-G Sepharose beads. After centrifugation and washes the RNA was extracted using TRIzol reagent,
followed by RT-PCR using the mouse PUM2 specific primers as described in Methods. From the anti-PUM2 antibody precipitate we amplified a band of approximately 0.6 Kb corresponding to the expected size of the PUM2 fragment (Figure 3.2 A), that was precipitated by the anti-Pum1 antibody also. Therefore, PUM1 and PUM2 in the skeletal tissue cultured cells bind to the PUM2 mRNA and confirming that PUM1 and PUM2 share the same PBE binding site (98;162) and suggesting that both proteins can regulate the expression of PUM2. When a PCR reaction to amplify alpha-actin was performed on the same samples, we did not detect any DNA amplification (Figure 3.2 B). These results suggest that PUM2 and its transcript form complexes in muscle cells, and that PUM2 could be translationally autoregulated.

PUM2 represses expression of a transcript under the control of a fragment of its own 3’UTR containing the first five PBEs: To test whether PUM2 can regulate its own expression, we first cloned a 620 base pair fragment of the PUM2 3’UTR containing the first five PBEs downstream of the GFP sequence in the pEGFP expression vector. This reporter construct was called pEGF-PUM2-3’UTR620 (Figure 3.3 A). As a control we mutated 3 nucleotides in each of the five PBEs to produce the plasmid pEGFP-PUM2-3’UTR620mut1-5. Two cell lines and primary QMCs were co-transfected with this construct and the PUM2-Cherry expressing plasmid. The results were visualized by fluorescence microscopy (Figure 3.3 B). Overexpression of PUM2 decreased expression of GFP when the wild type 3’UTR construct was used, but did not affect the expression of pEGFP-PUM2-3’UTR620mut1-5 plasmid.
To study which of the first five PBEs is functional we introduced sequential mutations in three positions of each PBE as shown in Figure 3.4 A. The first PBE mutated was the canonical one as indicated by the number one. We found that PUM2 auto-represses the reporter under control of a fragment of its 3'UTR only when all of the first five PBEs are mutated the pEGFP3'UTR629 was expressed. This indicates that at least one of these PBEs is functional with respect to PUM2 autorepression (Figure 3.4 B).

The full length PUM2 3'UTR can repress expression of a reporter under its regulation: To determine whether transcripts under the regulation of the wild type PUM2 3'UTR are translationally repressed by PUM2, we cloned the full length wild type PUM2 3'UTR downstream of the GFP sequence in the pEGFP expression vector. This reporter construct was called pGFP-PUM2-3'UTRFL. Additionally, we substituted the fragment containing the first five PBEs with another in which the first five PBEs were mutated (pEGF-PUM2-3'UTRFLmut5) to study the role of these PBEs in PUM2 translational autoregulation. We cotransfected QMCs with either pEGF-PUM2-3'UTRFL or pEGF-PUM2-3'UTRFLmut5 together with PUM2-Cherry, and 48 hours latter the cells fixed, stained with DAPI and the results visualized by fluorescence microscopy. Overexpression of PUM2 suppressed expression of GFP when the wild type PUM2 3'UTR construct was present, but did not affect expression of the mutated plasmid (Figure 3.5 A). Quantification of the transfected QMCs is shown in Figure 3.5 B. One hundred transfected myotubes were counted per coverslip and three transfected coverslips were counted per each group. PUM2 overexpression
resulted in approximately 80% reduction of GFP expression from the wild type PUM2 3’UTR compared to pGFP-PUM2-3’UTRmut. This study shows that PUM2 regulates its own expression and that at least one PBE is active in PUM2 3’UTR.

In a parallel experiment we co-transfected triplicate QMC cultures with the same constructs as the previous experiment. Three days after transfection the cells were extracted and Western blot was performed to detect GFP expression (Figure 3.6). As expected, there was a large downregulation of GFP expression with the wild type construct compared to the mutated one. These results indicate that PUM2 represses in some way translation of a transcript under the regulation of the full length wild type PUM2 3’UTR, and that this function of PUM2 is specific to the first five PBEs located after the stop codon of PUM2 transcript. The function of the following PBEs, if any, remains to be studied. The fact that only the PBEs that are close to the stop codon are functional suggest a function for PUM2 in the final step of protein translation may also involved.

Discussion

In silico studies of transcript sequences from HELA cells have shown that PUM2 itself is one of its own possible targets (163). We performed alignment studies and showed that the PUM2 3’UTR contains two consensus PBEs that are highly conserved in mice, rats and humans. Additionally, we found thirteen other non-consensus PBEs in the mouse PUM2 3’UTR. A non-consensus PBE differs from the consensus one in one or two nucleotides located in positions 5 and/or 8 of the octamer and can still functionally bind the PUM-HD (3). Based on these
observations we performed Immunoprecipitation studies and found that PUM1 and PUM2 bind PUM2 mRNA in skeletal muscle cells (Figure 3.2). Previous studies in which a similar experimental approach was carried out indentified PUM2 mRNA after PUM2 immunoprecipitation in cancerous cells and these authors mentioned the possibility of a negative feedback mechanism of PUM2 translational regulation (126).

Our findings demonstrate that PUM2 can repress translation of a GFP reporter under control of its own 3'UTR (Figures 3.3 and 3.4). Thus, PUM2 can repress its own expression in skeletal muscle cells and in other cells thereby describing a possible negative feedback mechanism of PUM2 translational regulation. Drosophila PUM regulates the translation of Nanos a protein that has an important role in PUM translational regulative mechanism (164). This suggests a possible mechanism of autoregulation in which if too much Nos is produced, it acts on its mRNA repressing its own expression. On the other hand if there is little amount of Nanos, the mRNA is not repressed and Nanos is produced. Our findings suggest a similar mechanism can occur in the mouse model in which PUM2 regulates its own translation.

The PBEs near to the stop codon are the ones involved in PUM2 binding to its 3'UTR and they are responsible for PUM2 translational regulation (Figure 3.5). Our data therefore suggest that the binding of PUM2 must be close to the stop codon in order to be functional with respect to translational repression. This observation suggests the interesting hypothesis that repression of translation by PUM2 in some cases may reside in the termination step of protein synthesis.
Recently, a similar finding has been described in studies using a cell free translational system obtained from yeast lysates (165). Their results indicated that PUF-mediated repression was sensitive to the distance between the ORF and the regulatory elements in the 3'UTR; excessive distance decreased repression activity.

A role for Pumilio has been determined for the establishment of long-term memory in Drosophila (152), and PUM2 is expressed at all stages of neuronal differentiation in the rat brain where it regulates dendrite morphogenesis, the formation of dendritic spines and synaptic function (121). In that work it was determined that PUM2 is involved in the negative regulation of the eukaryotic translational initiation factor 4E (eIF4E) and a voltage-gated sodium channel (Scn1a), which can account for the role of PUM2 in dendritic spine morphogenesis and synaptic function, respectively. Thus, how PUM2 is regulated in dendrites is an important and an open question. Another study found that the miRNA, miR134, promotes dendritic outgrowth by inhibiting translation of PUM2 mRNA (122), thus we can not rule out a possible role for miRNAs and the RISC complex in the translational regulation of PUM2. It is known that PUM1 can interact with the p27-3’ UTR producing changes in the secondary structure of the mRNA that favors miR-221 and miR-222 binding to with their respective sites in the p27 3’UTR, efficient suppression of p27 expression, and rapid entry to the cell cycle (123). In our study we used different cells lines and found that PUM2 can regulate its own translation in each case. It would be relevant to determine whether this autoregulation can also occur in nerve cells.
In conclusion, we demonstrated an interaction of PUM2-HD with the PBEs present on its own 3’UTR and highly conserved among mammal species, and we presented strong evidence to support the hypothesis that PUM2 inversely regulates its own expression in skeletal muscle.

**Experimental procedures**

Cell cultures and transfection: Primary quail myoblasts (Quail Muscle Cells, QMCs) were obtained from the pectoral muscles of 10-day-old embryos and plated at 5x10⁵ cells/ml in Eagle’s Minimum Essential Medium (Gibco) supplemented with 2% chicken embryo extract, 10% horse serum (GemCell) and 0.1% gentamicin (Invitrogen). The avian cultures were maintained at 39°C in a water-saturated incubator with 5% CO₂ and fed with fresh medium on days three and five post-plating. On day three the medium was supplemented with 10⁻⁶ M cytosine-arabinoside (Sigma) to reduce fibroblast proliferation. The mouse muscle cell line C2/C12 (ATCC), the human embryo kidney HEK cell line and the Cos-7 green monkey kidney cell line were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) supplemented with 10% of fetal bovine serum (Atlanta Biologicals). The mammalian cultures were maintained at 37°C in a water-saturated incubator with 5% of CO₂. The C2/C12 cells were induced to differentiate by changing the medium to DMEM supplemented with 2% horse serum when the cells approached confluency.

QMCs were transfected with plasmids encoding PUM2-Cherry fusion protein (chicken PUM2 fused with Cherry/GFP cloned in the NotI sites of pIRES
vector (generous gift of Drs. Pantelis Tsoufas and Andrew Darr). The pEGFP plasmid was used to clone a PUM2 3'UTR portion of 620 bps containing the first five PBEs (pEGFP-PUM2-3'UTR 620) or the full length PUM2 3'UTR after the GFP coding sequence in the EcoRI site (pEGFP-PUM2-3'UTRFL). Plasmids were transfected using Exgen-500 (Fermentas) following the manufacturer’s suggested protocol. Control groups were transfected in parallel with plasmids encoding cytoplasmic GFP alone (pEGFP) or pEGFP-PUM2-3'UTR 620 in which the 5 PBEs were each mutated in three positions (G2/A; T3/A; T7/A) (pEGFP-PUM2-3'UTR 620mut). This mutated fragment was cloned in the pEGFP-PUM2-3'UTRFL in the AccI site to exchange for the wild type 620 portion in the PUM2 full length 3'UTR (pEGFP-PUM2-3'UTRFL5mut).

Protein extraction and Western blotting: Cultured cells were washed in PBS with 0.1 mM calcium and 2 mM magnesium and extracted in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS). The relative levels of GFP expression were determined by Western blot (WB). After centrifugation 10 μl aliquots of the supernatants were run by 10% SDS-PAGE. Proteins from the gel were electrophoretically transferred to nitrocellulose membranes (Whatman). After blocking the membrane with skim milk (Carnation) rabbit polyclonal anti-GFP (Abcam) (1:3000) was used as primary and peroxidase conjugated antibodies (Cell Signaling) (1:1000) were used for detection. The membranes were washed three times between incubations with 0.025% NP-40 in 20 mM phosphate-buffered saline pH 7.4
(PBS). The membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and recorded on Kodak film.

Immunoprecipitation: Three days after transfection the cells were extracted in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail (Roche Diagnostics; Complete Mini, EDTA-free) and (RNAsin from Promega) and centrifuged at 20,000 rpm in a microfuge at 4°C. The supernatants were processed immediately or stored at -80 °C until assayed. Cell extracts were immunoprecipitated using protein-A or protein-G Sepharose beads (Amersham Pharmacia Biotech) pre-incubated with 3 µg of rabbit anti-PUM2. Protein A or Protein-G conjugated sepharose beads without antibodies was used as negative control. Beads were washed with lysis buffer four times for ten minutes, followed by with RNA extraction or western blot.

RNA extraction and RT-PCR: After immunoprecipitation the beads were extracted with TRIzol reagent (Invitrogen) to isolate RNA, followed by RT-PCR amplification using Oligo dT (Promega) and PUM2 specific primers (forward: GCAGTCTGGTAGGTCTACAAC) and (reverse TGACAGAGAGATATAGAGGG) (600 nucleotides). The PCR products were run in an agarose gel for detection. An additional PCR amplification of the same sample to detect α-actin was performed as a negative control using ATGGAGCCACCGATTC as forward and AACTGGGACGACATGG as reverse primers (800 nucleotides). The input in each experiment is the amplification of the product after direct RNA extraction from a separate dish of cells.
Microscopy: For visualization, cultured cells transfected with constructs expressing one or both fluorescent proteins were grown on glass coverslips, and washed 3 times with HBSS. After the last wash the cells were fixed with 4% paraformaldehyde in 20 mM PBS, pH 7, for 20 minutes. All images were acquired with identical exposure parameters using a Leica DMR-A or DMI 6000 fluorescence microscopes equipped with a Princenton Instruments MicroMax CCD camera and using 3i Slidebook 4.0 software.
Figure 3.1: Location of PBEs in the 3'UTR of PUM2 mRNA.
There are two canonical PBEs (blue) present in the PUM2 mRNA 3'UTR and 13 non-canonical PBEs (orange). The scale bar shows the Pumilio-2 transcript size in kilo bases.
Figure 3.2: PUM2 binds to the 3'UTR of its own mRNA in skeletal muscle cells. Agarose gels showing the RT-PCR products obtained after immunoprecipitation of mouse muscle C2/C12 cell extracts using anti-PUM2 or anti-PUM1 antibodies followed by RT-PCR. **A.** PCR for PUM2. **Panel B.** PCR for Actin. *IP: Input, PA Protein-A Sepharose without Abs. PG: Protein-G Sepharose without Abs. P2: Protein-A Sepharose + Rabbit anti-PUM2. P1: Protein-G Sepharose + Goat anti-PUM1.*
Figure 3.3: PUM2 represses the expression of pEGFP-PUM2-3'UTR fragment of 620 bp in several different cell types.
A. Schematic representation of the construct used. The 620 nucleotide region of the PUM2 3'UTR immediately after the stop codon was cloned after GFP in the pEGFP plasmid. B Fluorescence expression of PUM2-Cherry and pEGFP in different cell types, pEGFP was used as control.
Figure 3.4: PUM2 represses expression of pEGFP-PUM2-3’UTR 620 nucleotide fragment and at least one PBE is active
A. Schematic representation of the construct used and the position of the canonical (dark blue) and noncanonical (orange) PBEs. B. Fluorescence expression of PUM2-Cherry (red) and pEGFP (green) in QMC. pEGFP-PUM2-3’UTRmut1 is the mutation of the canonical PBE only. (pEGFP-PUM2-3’UTRmut1-n) the PBEs 1 to 5 are mutated.
Figure 3.5: PUM2 represses expression of GFP-PUM2-3’UTR full length (FL).
A. QMC co-transfected with either pEGFP-PUM2-3’UTR-FL plasmid or pEGFP-PUM2-3’UTRmut with the first 5 PBEs mutated (pEGFP-PUM2-3’UTR-FLmut1-5) and pIRES-PUM2-Cherry plasmid (PUM2-Cherry). Fluorescence expression of PUM2-Cherry and GFP. B. Quantification of the experiment illustrated in A, show the percent of PUM2 positive cells that were also expressing GFP.
Figure 3.6: PUM2 represses the expression of GFP-PUM2- full length 3’UTR. Quail muscle cells were co-transfected either with pEGFP-PUM2-3’UTR with the first 5 PBEs mutated plasmid (pEGFP-PUM2-3’UTR-FL5mut) or with pEGFP with the 3’UTR of PUM2 linked plasmid (pEGFP-PUM2-3’UTR-FL) and pIRES-PUM2-Cherry plasmid. A. Schematic representation of reporter construct used in these experiments, GFP (light blue) followed by PUM2 3’UTR (yellow), canonical PBEs (dark blue) non-canonic PBEs (orange). B. Western Blot of extracted quail muscle cells. In the upper panel GFP was detected. In the lower panel tubulin was detected as loading control.
Chapter 4: The RNA Binding Protein Pumilio-2 localizes to the Endoplasmic Reticulum and Interacts with the Ribosomes

Summary

Pumilio-2 (PUM2) is an RNA binding protein that recruits a translational control complex to the Pumilio Binding Element (PBE) in the 3'UTR of cognate mRNAs. It has been shown that PUM2 mRNA is expressed during neuronal development and plays a key role after differentiation where it is involved in the formation of dendritically localized ribonucleoprotein particles and stress granules. While it is well established that PUM2 is an important regulator of protein translation little is known about its cellular localization or its mechanism of translational regulation. In these studies we found that PUM2 was highly localized on the cell endoplasmic reticulum where it may interact with target transcripts. In addition, when a reporter plasmid containing the GFP sequence and PUM2 3'UTR was transfected into muscle cells we found that the GFP protein itself was localized in the PUM2 positive complexes. We precipitated GFP in immunoprecipitation studies using anti-PUM2 antibodies and cells transfected with the construct described above. We immunoprecipitated ribosomal RNA (rRNA) and GFP transcript using anti-PUM2 antibodies, suggesting that the ribosome and the mRNA are present in PUM2 complexes as well. These observations suggest a novel mechanism of localization and regulation where translation of the protein occurs and the protein remains associated with the ribonucleoprotein complex possibly to be transported together with its mRNA to specific domains inside the cell.
**Background**

PUM2 is an RNA-binding translational repressor protein that plays a key role both during development and in the nervous system. For example, it has been shown that PUM2 mRNA is expressed during neuronal development and that it is involved in the formation of dendritically localized ribonucleoprotein particles and stress granules (151). *Drosophila Pumilio* is a large gene that encodes an mRNA expressed in ovaries and early embryos. The mRNA is enriched in the posterior pole of embryos and encodes for a protein that is of a great importance in the formation of the abdomen and the germline (103).

The classical mechanism of PUM translational regulation was first described in oocytes where PUM is involved in deadenylation of maternal mRNA thereby reducing their translation. The maternal mRNAs that are stored in the oocyte in an untranslated state have short poly(A) tails, but normal polyadenylation occurs in the nucleus of frog and mouse oocytes (166;167). Pumilio binds directly to cytoplasmic polyadenylation binding protein (CPEB), and therefore could contribute to the deadenylation of all cytoplasmic polyadenylation element (CPE) containing mRNAs (119;168). Many CPE containing mRNAs also contain Pumilio binding sites, including cyclin B1 and Gld-2 (119;168-170).

Human PUM1 and yeast Puf5 interact with the conserved deadenylase complex CCR4–Pop2–Not, by binding to Pop2/Caf1(171;172). This complex is involved in deadenylation of the mRNA thereby repressing protein translation. In addition, Pumilio recruits the translational repressor Nanos in Drosophila and Xenopus
Nanos can recruit CCR4–Pop2–Not complex by binding to Not4 and contribute to the repression of Cyclin B (173).

In addition to the recruitment of CCR4–Pop2–Not other alternative PUF-dependent repression mechanisms have been proposed. They include the inhibition of translation initiation factors and interaction with the 5' mRNA cap, changes to the ribonucleoprotein structure and effects on translation elongation and termination (174). For instance, Drosophila Pumilio can recruit the translational inhibitor d4EHP, via Brain tumor (Brat) (175). The translational inhibitor competes with the translation initiation factor eIF4E for binding to the cap (176). Changes to the ribonucleoprotein structure have been suggested for the yeast Puf3. Puf3 affects the structure of the mRNA and the poly(A) tail binding protein Pab1, thereby exposing the poly(A) tail to deadenylation (177). Finally, Puf5 could use an alternative mechanism of repression. Puf5 could recruit a partner protein via the AUU element in the HO mRNA, the two repressors could then form a complex that would inhibit translation elongation and/or termination (165).

Even though expression of PUM2 seems to be critically important in development and differentiation, virtually nothing is known about PUM2 localization and the mechanism regulating PUM2 expression itself. We have now found that PUM2 localizes to the rough endoplasmic reticulum where it colocalizes with GFP in cells transfected with a green fluorescent protein coding region linked to the PUM2 3'UTR (construct GFP-PUM2-3'UTRFL), suggesting that PUM2 is forming a complex that induces the newly translated GFP protein
along with ribosomal RNA (rRNA) when GFP-PUM2-3'UTRFL was transfected in muscle cells. This suggests a mechanism of translational regulation in which translation of the protein may occur, but the protein remains associated with the ribonucleoprotein particle to be transported along with its mRNA. This provides a mechanism for localizing RNAs and protein when they are needed in specific cell regions.

**Results**

PUM2 localizes to the rough endoplasmic reticulum: While labeling skeletal muscle fibers and culture cells for the experiments described in chapter 2 we observed fragmented and intense perinuclear labeling and/or reticulated pattern in the cytoplasm that resembles ER labeling. To determine whether PUM2 localizes to the ER we used the C2C12 mouse muscle cell line and QMCs and transfected them with plasmids encoding the fusion protein GFP-Sec61, the ER translocon, and Cherry-PUM2 (Figure 4.1 A). As expected, GFP-Sec61 shows an ER distribution. Similarly, PUM2-Cherry is distributed co-localizing with Sec61 inside the cell. In a separate experiment we transfected the same cell line with the fusion protein Cherry-PUM2. We then performed immunofluorescence of the transfected cells using the Golgi marker antibody anti-Giantin (Abcam) to determine whether PUM2 localizes to the Golgi as well. The antibody marker labeled the Golgi as expected but the distribution of PUM2 did not correlate with Golgi (Figure 4.1 B). We then performed a similar experiment using QMC and observed a co-localization between GFP-Sec61 and PUM2-Cherry in myoblasts.
and myotubes surrounding the nuclei (Figure 4.2 A) where the ER forms and where AChE mRNA is localized, since it is known that AChE mRNA interacts with PUM2 (Marrero et al. submitted for publication) and is localized to the ER.

As an alternative test of whether PUM2 localizes to the rough endoplasmic reticulum (RER), we performed subcellular fractionation experiments using C2/C12 cells. The cells were extracted in an isotonic buffer and gently homogenized. The cell extracts were then fractionated using different velocities of sedimentation to obtain the corresponding fractions. We found that PUM2 is predominantly in the microsomal fraction (Figure 4.3). This suggests that PUM2 is localized to the RER, but does not rule out the possibility that PUM2 is present in the other fractions in concentrations that are below the sensitivity of the Western blot. Two additional separate experiments gave a similar result, thereby we can conclude that PUM2 localizes to the external membrane of the RER possibly bound to mRNAs.

A protein under PUM2 translational regulation is bound to PUM2 complexes. When investigating the PBEs present in the PUM2 3'UTR and their role in translational autoregulation we observed that the reporter GFP protein translated from the GFP-PUM2-3'UTR formed particles in some cells that co-localize with PUM2. There are cells in which GFP is being translated but somehow it remains associated with the PUM2 complex since PUM2 is present in the same particle. This could be a translational mechanism in which PUM2 is stalled with the rRNA in the mRNA containing particle and the newly synthesized protein is not released from the complex. In vitro cell free translational studies using cell
free yeast extracts suggest that protein translation occurs even when PUM is present and that PUM2 translational regulation may occur during the elongation or termination steps of translation. We are observing that protein translation may occur in PUM2 positive complex in skeletal muscle cells.

GFP-PUM2-3’UTR is present in PUM2 containing complexes: To determine whether GFP was present in PUM2 complexes we carried out immunoprecipitations using anti-PUM2 antibodies followed by western blots using anti-GFP antibodies. As a control, we expressed GFP from a plasmid that did not have a PUM2 3’UTR. The expression of GFP was highly repressed when the 3’UTR was present, however the small amount of GFP expressed was almost entirely present in the immunoprecipitate (Figure 4.4 B). Thus, we conclude that the newly synthesized GFP protein remains bound to the PUM2 complexes when the PUM2 3’UTR is present after the GFP sequence.

If a complex is formed in which the newly synthesized protein remains bound to the PUM2 complex the linker must be the mRNA. To test whether the GFP mRNA was present in the PUM2 complexes we again performed immunoprecipitations using anti-PUM2 antibodies followed by RT-PCR (Figure 4.5). We used as control a construct in which the PUM2 3’UTR was mutated in the PBEs abrogating the possible binding of PUM2 to this RNA. We found that the GFP mRNA was present in the immunoprecipitates for the GFP-PUM2-3’UTR while the mutated control did not yield GFP mRNA. We conclude that GFP mRNA remains bound to the PUM2 complex when its 3’UTR is present.
The small rRNA subunit is present in PUM2 Complexes: To determine whether rRNA was present in the PUM2 particles we performed an immunoprecipitation of QMC extracts with anti-PUM2 antibodies followed RNA isolation and RT-PCRs using specific primers to amplify the 18S small rRNA. We were able to detect a fragment of 1.2 Kbs only when PUM2 antibodies were used in the immunoprecipitation (Figure 4.6). This amplification was not observed when the specific anti-PUM2 antibodies were not coupled to the protein-A sepharose beads. With the same RNA samples RT-PCRs using oligo dT and actin primers were performed (Figure 4.6, lower panels). We were not able to amplify any actin fragments as would be expected since actin mRNA does not have an PBE in its 3’UTR. This set of experiments suggests that at least the small subunit of the rRNA is bound to PUM2 complexes and that translation of protein may occur in those complexes.

Discussion

Defects in local mRNA regulation have been linked to neurological disorders like intellectual disabilities. One typical example is the loss of RNA binding protein FMRP that causes fragile X syndrome which is characterized by intellectual and behavioral abnormalities. The FMRP regulates dendritic mRNA transport and local translation through metabotropic glutamate receptor (mGlu) which is the major regulator of FMRP-mediated local translation (146). The role of FMRP and other RNA binding protein like Pumilio and Staufen in local
translation is widely studied but the mechanism of mRNA localization is not that well understood.

Previous studies have shown the localization of the RNA-binding protein Staufen in the rough endoplasmic reticulum (RER), suggesting that Staufen is responsible for directing the mRNA to the RER to be translated into proteins (127). A specific interaction between Staufen/Pumilio is known to occur in the Drosophila CNS where it is involved in long term memory (Dubnau et al 2003). Dubnau and colleagues found that disruption of four genes, *staufen, pumilio, oskar and eIF-5C* yielded mutants defective in memory. The finding of a translational gene affecting long term memory is indicative that protein translation is involved in memory and learning in *Drosophila*. Based on these observations we propose that PUM2 localizes to the external leaflet of the ER. To study PUM2 localization we first determined the colocalization of PUM2 with the translocon member-protein Sec61 and found that PUM2 was highly colocalized with Sec61 (Figure 4.1 and 4.2). Next, we performed subcellular fractionation studies and found that PUM2 fractionates in the microsomal fraction together with the RER membranes (Figure 4.3). These studies are the first direct evidence that a large fraction of PUM2 localizes to the RER external leaflet membranes.

During the course of our studies we observed that PUM2 colocalized with GFP in cells cotransfected with PUM2-Cherry and GFP-PUM2-3’UTR (Figure 4.4 A). To study this interaction we carried out Immunoprecipitation (IP) studies to test whether GFP could be detected in immunoprecipitates using anti-PUM2 antibodies. Although only a small amount of GFP was expressed since PUM2 is
translationally repressing GFP-PUM2-3'UTR, the amount of GFP in the immunoprecipitates was much higher than the negative control that expressed more GFP since it does not have any 3'UTR (Figure 4.4 B). This is the first direct evidence that protein translation occurs in PUM2 complexes and that the newly synthesized protein remains bound to those complexes. Moreover, we found that the mRNA encoding for that protein was also bound to the PUM2 complexes (Figure 4.5).

Further probing of the anti-PUM2 immunoprecipitates complexes showed that the ribosome was associated with PUM2 complexes (Figure 4.6). This finding, together with the other data leads us to propose the model that is presented in figure 4.7. In this model we postulate that once the PUM2 mRNA is transcribed, matured and exported to the cytoplasm, PUM2 protein binds to the PBEs that are close to the stop codon and recruits other components of the repressor complex. The translational machinery, including the ribosomes, starts protein translation until the ribosomes reach a region close to the stop codon that is in some way under PUM2 influence. The newly synthesized protein is almost completely translated but remains bound to the complex because the translational release factor cannot reach the stop codon. This way the mRNA, the newly synthesized protein, PUM2 and the rest of the complex can move in the cell to a region which under the appropriate stimuli, that could be phosphorylation of PUM2, PUM2 relives its inhibition of the mRNA and the ribosomes finish the proteins. The mRNAs and the ribosomes can then start translating new proteins in that region of the cell. Once the stimuli stop, PUM2 again represses the mRNA
and the complex can be reassemble protecting protein and RNA from degradation. A similar alternative mechanism was found in vitro for the yeast RNA binding protein Puf 5 (165). These studies suggest that in some cases protein translation may occur, but the repression is in the termination step after the protein is made.

**Experimental procedures**

Cell cultures and transfection: Primary quail myoblasts (Quail Muscle Cells, QMCs) were obtained from pectoral muscles of 10-day-old embryos and plated at 5x10^5 cells/ml in Eagle’s Minimum Essential Medium (Gibco) supplemented with 2% chicken embryo extract and 10% horse serum (GemCell) and 0.1% gentamicin (Invitrogen). The avian cultures were maintained at 39°C in a water-saturated incubator with 5% CO₂ and fed with fresh medium on days three and five post-platting. On day three the medium was supplemented with 10^-6 M cytosine-arabinoside (Sigma) to reduce fibroblast proliferation. The mouse muscle cell line C2/C12 (ATCC) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) supplemented with 10% of fetal bovine serum (Atlanta Biologicals). Cultures were maintained at 37°C in a water-saturated incubator with 5% of CO₂. The C2/C12 cells were induced to differentiate by changing the medium to DMEM supplemented with 2% horse serum when the cells approached confluency.

Cell transfection: Cultured cells were transfected with plasmids encoding PUM2-Cherry fusion protein (chicken PUM2 fused with Cherry/GFP cloned in the
NotI sites of pIRES vector). The pEGFP plasmid was used to clone the full length PUM2 3’UTR after the GFP gene in the EcoRI site (pEGFP-PUM2-3’UTRFL), pIRES-GFP-Sec61 plasmid was used to label the ER. Plasmids were transfected using Exgen-500 (Fermentas) following the manufacturer’s suggested protocol. Control groups were transfected in parallel with plasmids encoding GFP (pEGFP) or pEGFP-PUM2-3’UTR in which the 5 first PBEs were mutated in three positions (G2/A; T3/A; T7/A) (pEGFP-PUM2-3’UTR mut5). This mutated fragment was cloned in the pEGFP-PUM2-3’UTRFL in the AccI site to substitute the wild type 620 portion in the PUM2 full length 3’UTR (pEGFP-PUM2-3’UTRFL5mut).

Subcellular fractionation: After proliferation the C2C12 cells were grown in differentiation medium (DMEM and 2% horse serum) for 7 days; the cells were scraped off the dish and homogenized in sucrose buffer (250 mM sucrose, 100 mM Tris-HCl, pH 7.4) using 10 strokes of a Dounce homogenizer on ice. The nuclei were pelleted at 1,000 x g using the Sorvall rotor SS 34 for 10 minutes to give pellet 1 (P1), the supernatant from this was spun at 15,000 x g using a Sorvall rotor SS34 for 20 minutes to separate mitochondria, lysosomes, peroxisomes and Golgi membranes (P2). The microsomes were pelleted at 100,000 x g using a Beckman rotor SW 50.1 for 45 minutes (P3). After resuspension in SDS-Tris Buffer aliquots of the 3 pellets and the last supernatant (S3) were run in SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with a rabbit anti-PUM2 primary antibody followed by
goat anti-rabbit HR peroxidase conjugated secondary (Jackson). After washes with PBS/NP40 the membrane was developed with SuperSignal West Pico Chemiluminescent Substrate from Pierce.

Protein extraction, Immunoprecipitation and Western blotting: Cultured cells were washed in PBS with 0.1 mM calcium and 2 mM magnesium and extracted in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1 % SDS). The relative levels of GFP expression were determined by Western blot (WB). After centrifugation 10 µl aliquots of the supernatants were run in a 10% SDS-PAGE. Proteins from the gel were electrophoretically transferred to nitrocellulose membranes (Whatman). After blocking the membrane with skim milk (Carnation) rabbit polyclonal anti-GFP (Abcam) (1:3000) was used as primary antibody and goat anti-rabbit peroxidase conjugated antibodies (Cell Signaling) (1:1000) were used for detection. The membranes were washed three times between incubations with 0.025% NP-40 in phosphate-buffered saline (PBS). The membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and recorded on Kodak film. Three days after transfection the cells were extracted in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail (Roche Diagnostics; Complete Mini, EDTA-free) and RNAsin (from Promega) and then centrifuged to keep the supernatants. The cell extract supernatants were either used immediately or stored at -80°C until procedure. Cell extracts were immunoprecipitated using protein-A sepharose beads (Amersham Pharmacia Biotech) pre-incubated with 3 µg of rabbit anti-PUM2. Protein A conjugated
sepharose beads without antibodies was used as negative control. Beads were washed with lysis buffer four times for ten minutes to proceed with RNA extraction or western blot.

RNA extraction and RT-PCR: After immunoprecipitation the beads were extracted with TRIzol reagent (Invitrogen) to isolate RNA, followed by RT-PCR using Oligo dT (Promega) as the reverse primer. To reverse transcribe the 18S rRNA subunit the reverse primer TCAATTCCTTTAAAGTCAG was used and to amplify that subunit the forward primer ATTAAGCCATGCATGTCTAAG and reverse primer CTCCTGGGTGGTCGCTTCCG were used (around 1000 bps). The PCR products were run in an agarose gel. An additional PCR to amplify α-actin was performed as negative control using ATGGAGCCACCGATCC as forward and AACTGGGACGACATGG as reverse primers (800 nucleotides). The input for the experiments is the RT-PCR product after direct RNA extraction from a separate dish of cells. It is important to explain that the input used for 18S rRNA and α-actin was diluted 1:20.

Microscopy: Cultured cells were grown on glass coverslips, washed 3 times with HBSS and fixed with 4% paraformaldehyde in 20 mM PBS, pH 7, for 20 minutes. All images were acquired with identical exposure parameters using a Leica DMR-A or DMI 6000 fluorescence microscopes equipped with a Princenton Instruments MicroMax or Hamamatsu CCD camera using 3i Slidebook 4.0 software.
Figure 4.1: PUM2 localizes to the ER in C2C12 myoblasts but not Golgi.

A. Fluorescence Microscopy of C2C12 myoblasts transfected with the ER resident protein Sec61-GFP (green) and ChPUM2-Cherry (red). B. C2C12 muscle cells were transfected with a plasmid encoding for a fusion protein (ChPUM2-Cherry) and immunostained for the Golgi marker Giantin (Green). DAPI was used to stain the nuclei. Golgi (arrow). Scale bars 10 μm
Figure 4.2: PUM2 localizes in the ER in QMC myoblasts and myotubes.

Fluorescence Microscopy of a QMC myoblast (upper panel) and myotubes (lower panel) transfected with the ER resident protein Sec61-GFP (green) and ChPUM2-Cherry (red). Scale bars 10 μm
Figure 4.3: PUM2 is localized in the microsomal fraction with the RER in muscle cells

The figure represents a Western blot of different cell fractions using anti-PUM2 antibody. MM: molecular markers, P1: nuclear fraction, P2: mitochondrial fraction, P3: microsomal fraction and C: cytoplasm. PUM2 is found in the P3 microsomal fraction where the RER membranes pellet down. Equal amount of protein loaded in each lane.
Figure 4.4: A protein under PUM2 translational regulation is bound to PUM2 complexes.

A. Fluorescent microscopy of QMC myotubes co-transfected with two plasmid encoding for the fusion protein PUM2-Cherry and pEGFP linked to PUM2 3’UTR (pEGFP-PUM2-3’UTR). The arrow points to a PUM2 complex showing the co-localization of PUM2 and GFP-PUM2-3’UTR. Scale bar 10 μm. B. QMCs were co-transfected with either pEGFP plasmid (GFP) or pEGFP with the PUM2 3’UTR (3’) and pIRES-PUM2-Cherry, three days after transfection the cells were extracted and immunoprecipitated with anti-PUM2 antibodies followed by Western blot. In the upper panel GFP was detected. In the lower panel immunoglobulin was used as loading control. Immunoprecipitation (Immunop). We loaded 20 μl aliquots of the run through in the Western Blot to detect the GFP that was not bound to the column (Run through).
Figure 4.5: GFP-PUM2-3’UTR is in PUM2 complexes.

Agarose gels showing the RT-PCR products after Immunoprecipitation (with anti-PUM2 antibodies) of HEK cells transfected with pEGFP-PUM2-3’UTR-FL green fluorescent protein coding region linked to PUM2 3’UTR construct (G3’) or with pEGFP-PUM2-3’UTR-FL 5mut (G3’m) green fluorescent protein coding region linked to PUM2 3’UTR construct in which the first five PBEs have been mutated. In the upper panel is the RT for GFP, actin was used as negative control (lower panel). T: RT-PCR using 1/20 total RNA from the same amount of cells.
Figure 4.6: rRNA is present in PUM2 complexes.

Agarose gel showing that the 18S rRNA is present in PUM2 complexes suggesting protein translation may occur in the complex. Triplicates of QMCs were transfected with pIRES-PUM2-Cherry and immunoprecipitated with anti-PUM2 antibody (PUM2immunop.); protein-A without antibody was used as negative control of the immunoprecipitation (ProteinA), after immunoprecipitation total RNA was isolated and RT-PCR carried out. Upper panel is the RT-PCR for rRNA small subunit. Lower panel is RT-PCR for actin. T: RT-PCR using 1/20 total RNA from the same amount of cells.
Figure 4.7: Possible model of PUM2 translational autoregulation.

A. PUM2 binds to the PBE/PBEs closer to the stop codon in PUM2 3' UTR and recruits a translational repressor complex, while the NH-terminal of the protein starts translating.

B. As the ribosome reaches the region close to the stop codon and the protein is almost completely translated, it pauses, and remains attached to the complex together with the mRNA, the ribosome and PUM2 translational repressor complex.
The neuromuscular junction remains an important system for studying cholinergic synapses. Synapses, like the NMJ, are responsible for initiating and terminating neurotransmission. This event is regulated in part by the number and density of key molecules present in the synapse itself. The molecular mechanisms underlying the regulation of these molecules are important questions in cell biology and molecular neuroscience. The enzyme AChE is a key component and marker of cholinergic synapses where it breaks down the neurotransmitter acetylcholine to terminate neurotransmission. Studies of this enzyme and how it is regulated provide important information that can be applied to virtually all synapses in the organism. The postsynaptic domain of the NMJ is a highly specialized region of the muscle fiber. Specific genes encoding synaptic components are expressed in the postsynaptic region that are not expressed in the non-innervated regions. Furthermore, it is known that protein translation occurs in the postsynaptic side where all components of the cell’s translational machinery are present. Ribosomes, ER, Golgi, mRNAs and translational regulators are all components of the subsynaptic sarcoplasm. Thus, how the moment to moment translation of key molecules, like AChE, is regulated is important for understanding the fine tuning of synapses and synaptic plasticity.
Regulation of synaptic AChE by PUM2

In eukaryotic cells many transcripts are controlled through specific elements comprised of nucleotide sequences located in their 3’UTRs. Proteins that bind to those elements are important players in controlling mRNA stability, translation and localization. One of these proteins is Pumilio, which as part of a ribonucleoprotein complex represses protein translation and may also control RNA localization. An initial observation that PUM2 was highly concentrated at the NMJ, and that the AChE had a single PBE located approximately fifty nucleotides downstream from the stop codon, led us to the hypothesis that PUM2 played an important role in regulating AChE at the NMJ. That this hypothesis proved to be correct has contributed significantly to our understanding of the regulation of AChE translation at the NMJ. In chapter II we showed that PUM2 is highly localized at the mammalian NMJ. Previously, and published independently after this project began, it was found that Drosophila Pumilio was highly localized at the fly NMJ where it regulated expression of the glutamate receptor GluRIIA (129;153). We presented experimental evidence that PUM2 regulates AChE expression at the translational level using different cellular and molecular approaches including immunoprecipitation, RT-PCR, immunofluorescence, gel shift mobility assay, cell transfection and gene knockdown using shRNAs. We concluded that mammalian PUM2, like its homolog Drosophila Pumilio, plays an important role in the translational regulation of this key component of the mammalian NMJ, AChE. We have not determined whether the translational regulation of AChE by PUM2 can occur at cholinergic synapses in the CNS,
however PUM2 and AChE are both localized at those synapses and therefore an interaction between PUM2 and AChE transcript in CNS neurons is clearly possible. Thus, PUM2 may be playing a role in the fine tuning of AChE expression not only at the NMJ but also at cholinergic synapses in the CNS as well. One possible study for the future would be to create a knock-in mouse in which the AChE 3’UTR PBE is mutated, thereby preventing the interaction between PUM2 and the AChE transcript. One prediction is that this could create changes in the NMJ and in CNS synapses of these animals with potential effects on memory and learning. Soreq et al., 2009, (Meeting in Brazil) presented a similar model where she created a knock-in mouse expressing AChE without its 3’-UTR. These mice had severe behavioral changes and their memory and learning abilities were deeply impaired. The authors later claimed that miRNAs were involved in the regulation of the AChE 3’UTR (presented in the XIIIth International Meeting on Cholinesterases, Croatia). However, these authors did not perform any additional studies to determine whether those miRNAs were affecting AChE expression directly, as we did for PUM2 in our studies, or even take into consideration the possibility that elements other than the miRNA site might be involved.

Another potentially interesting line of investigation would be to test in vivo the effects of PUM2 repression of AChE by creating a muscle PUM2 conditional knock-out mouse with a Tet-on promoter system that can be turned on to express PUM2 at the NMJ, then determine whether there is a change in AChE levels and what the effect of this overexpression is on the animal’s motor activity
and coordination. We also found that PUM2 itself is regulated by the motor nerve. Three days after denervation PUM2 was overexpressed, thereby affecting AChE expression. However, we cannot conclude that only the overexpression of PUM2 accounts for the downregulation of AChE because other factors are known to be involved in this regulation including several muscle-specific transcription factors (83). The fact that we did not determine AChE mRNA levels after PUM2 overexpression is an important limitation of our study. One possible solution might be to study changes in AChE transcript levels after denervation and/or PUM2 overexpression. Studies are in progress in our laboratory to determine whether AChE transcripts are affected after changes in PUM2 levels, since two different mechanisms for PUM2 translational regulation have been proposed, one involves transcript degradation (178) while the other the RNA remains stable but untranslated (119).

**Regulation of PUM2 by negative feedback**

Feedback mechanisms involving translational repression have been described by several laboratories during early development of the embryo. One well established example is the regulation of Nanos expression by the RNA binding protein Pumilio in Drosophila oocytes (115). Nanos is required in this system for Pumilio inhibition of protein translation. If Nanos is overproduced it binds Pumilio in a complex that represses Nanos translation. On the other hand if Nanos is underexpressed Pumilio cannot repress translation of Nanos and more
Nanos is produced. In that way Nanos regulates its own expression through its interaction with Pumilio.

While doing sequence alignments of several mammalian PUM2 3'UTRs we found two consensus PBEs and thirteen non-consensus PBEs with only one nucleotide substitution each from the canonical. These findings led us to propose the hypothesis of a feedback mechanism of translational regulation by PUM2 of its own translation. We then performed Immunoprecipitation studies and found that PUM2 was bound to its own mRNA in tissue cultured cells, suggesting an autoregulatory feedback of translational regulation. We also found that PUM1 interacts with the PUM2 transcript. It is known that PUM1 and PUM2 can bind to the same consensus PBE present in a 3'UTR (147). However, PUM1 is not localized at the mammalian NMJ so the interaction of PUM1 with the PUM2 transcript is unlikely to occur \textit{in vivo} at this synapse.

To determine whether PUM2 regulates its own translation we first created a reporter construct with only a small initial portion of the PUM2 3'UTR starting with the stop codon and containing the first five possible PBEs, cloned after the GFP coding sequence. When co-expressed in different cell types with PUM2 this construct was not able to express GFP. When the five PBEs were mutated by changing three critical nucleotides in each necessary for PUM2 binding, the construct was able to express GFP. This suggests that PUM2 regulates its own translation, that the PBEs in the 3'UTR play an important role in this regulation and that at least one PBE is active. We then studied the expression of a reporter construct in which the full length PUM2 3'UTR was cloned after the GFP coding sequence.
sequence and we obtained the same results. PUM2 regulates its own translation and only the first five PBEs are involved in this regulation. However, it appears that the PBEs nearest the stop codon are necessary. When they were mutated we observed GFP expression even when the PBEs located farther away from the stop codon were not altered. We presented experimental evidence that PUM2 is translationally regulated by its own product in vertebrates in chapter III.

In our experiments we determined the autoregulation of PUM2 in several different cell types such as: Cos cells, HEK and muscle myotubes, but not in stem cells. The role of PUM2 in the maintenance and self-renewal of stem cells is widely accepted (98). It would be relevant to study whether the same feedback mechanism affects these types of cells and what would be the significance of such regulation. Another relevant future study would be to create a knock-in mouse in which the ability of PUM2 to autoregulate is abrogated by mutating the first five PBEs in the PUM2-3’UTR. One would then determine whether stem cells can be maintained and whether the NMJ is affected in these same mice. Another set of experiments could involve behavioral studies to determine whether brain function of these animals is normal since PUM2 is involved in neurogenesis and synaptic function, as well as synaptic plasticity, memory and learning (129;159;160).

Subcellular localization and function of PUM2

While conducting preliminary experiments using anti-PUM2 antibodies on the localization of PUM2 in QMCs and C2C12 we found that PUM2 was highly
localized in one cellular compartment that looked like ER-Golgi. In the same studies, using a Golgi marker, we did not find any co-localization. PUM2 is assumed to be localized in the cell’s cytoplasm since it is a soluble protein and does not have a signal sequence or any known nuclear or mitochondrial localization sequence. In chapter IV we showed that a large fraction of PUM2 is localized on the ER, co-localizing with the translocon component protein Sec 61, and in a subcellular fractionation study we found PUM2 highly localized in the microsomal fraction together with rough ER membranes. These results lead us to conclude that a major fraction of PUM2 is localized on the cytoplasmic face of the RER membranes, possibly interacting with the translational machinery of the cell and with its mRNA targets. In addition, we found that PUM2 was present together with ribosomes where the newly synthesized protein was still bound to the complex. Not only was the RNA present, we found that its protein was present in the complex as well. Thus protein synthesis can possibly occur in PUM2 complexes but the termination step of protein translation is regulated by the RNA binding protein PUM2. A similar mechanism of translational regulation has been described for Oskar that is regulated by the RISK complex. There the regulation is on the elongation step of protein translation (179). We propose a model in which PUM2 binds to its own mRNA and recruits other components of the repressor complex. The translation initiation complex can recognize the start codon and begin protein translation until the ribosome approaches the stop codon. However PUM2 does not allow translation termination and then the newly synthesized protein remains bound to the PUM2 complex including its mRNA
and the ribosome. One possible function for this is that the complex could be transported to different regions of the cell and, with the appropriate stimuli, the newly synthesized protein could be released from the complex and the ribosome could then translate the mRNA again to produce more protein.

It is important to point out that we did not observe complexes of PUM2/GFP when the AChE 3’UTR was cloned after the GFP coding sequence. This suggests a different mechanism of translational regulation of AChE compared to PUM2, possibly because there are at least 5 active PBEs in the PUM2 3’UTR and only one in the AChE 3’UTR. In fact, when one single PBE was mutated in the PUM2 3’UTR the PUM2/GFP complex was not formed compared to the wild type PUM2 3’UTR. It appears that the more PBEs in a 3’UTR the stronger is the regulation of PUM2 (180), thus we speculate that the regulation by PUM2 of its own transcript should be stronger than the regulation of the AChE transcript. We can only say that when there is too much PUM2 at the NMJ, AChE can still be regulated. If too much AChE transcript is produced under stimulation, PUM2 releases its autoregulation to bind to the AChE mRNA, and then more PUM2 can be produced to further regulate AChE and other targets including itself. This would allow fine tuning the regulation of target transcripts at the NMJ and also help the localization of those transcripts at synapses.

To summarize, the overall conclusions from these studies are that we found that PUM2 is highly localized to the NMJ; PUM2 regulates the translation of AChE at the NMJ; the PBE at AChE 3’UTR is active and responsible to PUM2 translational regulation; we found a role for the PUM2 3’UTR in the
autoregulation of PUM2 translation in which at least one PBE is active, and that the PBEs located near the stop codon are active for repression of translation, but not the ones located farther away from the stop codon. We also found that a major fraction of PUM2 localizes to the ER and we described the interaction of PUM2 with the ribosome. Surprisingly, we also found that a newly synthesized GFP protein under PUM2 3’UTR regulation remained bound to the PUM2 complex, and, based on this observation, we proposed a possible mechanism for translational autoregulation in which PUM2 mRNA is translated but cannot reach the stop codon and protein translation is stalled just before the termination step leaving the newly synthesized protein attached. These observations need to be pursued in greater detail, and clearly there is much work left to be done.
Bibliography


