# Identification and Characterisation of a Novel Posttranslational Modification of Translation Repressor 4E-BP2

Michael Bidinosti

Department of Biochemistry
McGill University, Montreal
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## Abstract

In eukaryotes, control of protein synthesis or translation is critical for maintenance of cellular function and adaptation to environmental stimuli or stresses. The entire process of producing a functional protein molecule from an mRNA template is elaborately controlled and involves several integrated phases. It is the initiation phase of recruiting the protein synthesis machinery to an mRNA that is rate-limiting for translation. Importantly, a major regulatory mechanism of translation initiation is performed by the eIF4E-binding proteins (4E-BPs). These small proteins interact with the mRNA 5' cap-binding protein, eIF4E, and inhibit translation by preventing it from forming a complex that promotes translation. This is accomplished by the competition of the 4E-BPs with eIF4G for association to eIF4E. This competition is determined by the degree of stimulus-induced 4E-BP phosphorylation: whereas hypophophorylated 4E-BPs bind tightly to eIF4E, hyperphosphorylation causes their dissociation from eIF4E and permits translation. In the nervous system, translational control is obligatory for learning and memory. 4E-BP2, the predominant mammalian 4E-BP in the brain, is required to ensure normal functioning of translation-dependent memory processes. This thesis describes the identification of asparagine deamidation as a novel posttranslational modification of 4E-BP2 in the brain. Deamidation is the spontaneous conversion of asparagines to aspartates. Deamidated 4E-BP2 exhibits increased binding to the mammalian Target of Rapamycin (mTOR)-binding protein, Raptor. Furthemore, 4E-BP2 deamidation, which occurs during postnatal development, alters neuronal activity. It is conceivable that this modification of 4E-BP2 compensates for its attenuated phosphorylation in the brain. 4E-BP2 is also identified here, by virtue of its propensity to deamidate, as a novel substrate for the enzyme Protein L-Isoaspartyl Methyltransferase (PIMT). As a whole, this thesis describes a posttranslational modification, previously unknown in the study of protein synthesis, and its involvement in the regulation of translation control by 4E-BP2 in the mammalian nervous system.

## Résumé

Le contrôle de la synthèse protéique ou traduction chez les eucaryotes est d'une importance capitale dans le maintien de l'homéostasie cellulaire et dans l'adaptation aux stimuli et stress environnementaux. La production de protéines fonctionnelles à partir d'ARN messagers est soumise à un fin contrôle et consiste en une succession d'étapes intégrées. C'est au cours de la première étape de la traduction, l'initiation, que la machinerie traductionnelle est recrutée au niveau de l'ARN messager. L'initiation est l'étape limitante du processus de synthèse. Au cœur du processus de régulation de l'initiation de la traduction se trouve les facteurs liant eIF4E, les 4E-BP. Ces protéines de faible poids moléculaire interagissent avec le facteur eIF4E qui lie le 5'-cap des ARN messagers et inhibe ainsi la traduction en empêchant eIF4E de participer à la formation du complexe d'initiation. Cette inhibition est causée par la compétition entre les 4E-BP et eIF4G pour lier eIF4E. Le niveau de compétition est déterminé par le degré de phosphorylation de 4E-BP induit par les stimuli extracellulaire. Les formes hypophosphorylées de 4E-BP lient eIF4E avec une forte affinité, tandis que les formes hyperphosphorylées relâchent eIF4E, ce qui stimule la traduction. Dans le système nerveux, le contrôle de la traduction est nécessaire pour l'apprentissage et la mémoire. 4E-BP2, le facteur liant eIF4E dont l'abondance est prédominante dans le système nerveux des mammifères, est requis afin d'assurer un fonctionnement normal des processus de mémoire qui dépendent de la traduction. Cette thèse décrit la déamidation d'asparagine en tant que nouvelle modification post-traductionnelle du facteur 4E-BP2 dans le cerveau. Cet événement de déamidation consiste en la conversion spontanée de résidu asparagine en résidu acide aspartique. La forme déamidée de 4E-BP2 présente une affinité accrue pour la protéine Raptor, un facteur associé au complexe protéique « cible mammifère de la rapamycine » (mTOR). De plus,la déamidation de 4E-BP2, qui survient lors du développement postnatal, altère l'activité neuronale. Il est postulé que cette modification de 4E-BP2 compenserait son hypophosphorylation dans le cerveau. 4E-BP2 est aussi identifié, par sa capacité à être déamidé, comme un nouveau substrat de l'enzyme PIMT (L-isoaspartylmethyltransférase). Dans son ensemble, cette thèse identifie le rôle d'une modification post-traductionnelle, précédemment inconnue dans le contrôle de la traduction, comme ayant un rôle à jouer dans la régulation de la traduction effectué par 4E-BP2 au niveau du système nerveux.

## Preface

This thesis is compiled from two manuscripts: one that is currently under consideration for publication and another that will be submitted upon acceptance of the first. Several people who are included as co-authors contributed to the preparation of these manuscripts in many important ways, as detailed below. I am grateful to all of them for their expertise and dedication during our collaboration.

**Chapter 2**

**Bidinosti, M**., Ran, I., Sanchez-Carbente, M., Martineau, Y., Gingras, A.-C., Gkogkas, C., Raught, B., Bramham, C., Sossin, W., Costa-Mattioli, M., DesGroseillers, L., Lacaille, J.-C., and Sonenberg, N. (2009). Postnatal deamidation of 4E-BP2 in brain enhances its association with Raptor and alters kinetics of excitatory synaptic transmission. Molecular Cell (*Submitted*).

Israeli Ran performed electrophysiology experiments and contributed to writing and editing of the manuscript. Maria Sanchez-Carbente prepared cultured neurons and performed experiments in this system that are not included in this revised version of the manuscript. Yvan Martineau constructed many of the expression vectors used in this study, in particular all of the numerous 4E-BP2 deamidation mutants. Anne-Claude Gingras and Brian Raught performed mass spectrometry analysis of recombinant 4E-BP2. Christos Gkogkas.performed an experiment that was omitted from the revised manuscript and contributed numerous ideas. Clive Bramham, Wayne Sossin, Mauro Costa-Mattioli, and Luc DesGroseillers contributed, in part, to supervision of this work and helped conceive and design experiments. Jean-Claude Lacaille supervised the electrophysiology experiments and contributed to writing and editing of the manuscript. Nahum Sonenberg supervised this project, helped conceive experiments, and edited the manuscript. I performed the majority of experiments, including the original purification of endogenous 4E-BP2 described in the supplemental data section, and wrote the manuscript in near entirety.

**Chapter 3**

**Bidinosti, M**., Martineau, Y., Frank, F., and Sonenberg, N. (2009) Translational Repressor 4E-BP2 is a Substrate for Protein L-Isoaspartyl Methyltransferase in the Brain.

Yvan Martineau performed interaction studies, conceived experiments, and contributed to editing the manuscript. Filipp Frank helped with AdoMet labelling experiments. Nahum Sonenberg supervised this project, helped conceive experiments, and edited the manuscript. I performed all experiments presented in this chapter and wrote the entire manuscript.

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To the lovely Andréane, thank you for being my new roommate and for somehow managing to tolerate me during the writing of this thesis. Thanks also for being the wonderful person that you are.

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# Chapter 1

# Introduction

## 1.1 Prologue

The sequencing of the human genome within the past decade has revealed that human cells possess 20,000-25,000 protein-encoding genes. Remarkably, it is estimated that the total number of cellular protein isoforms is as much as 1 to 2 orders of magnitude greater than this. Processing of RNA transcripts, by splicing in particular, accounts for a large proportion of this diversity. However, posttranslational modifications (PTM) to fully translated proteins likely expand the cellular repertoire of protein variants considerably more. There are approximately 200 known PTMs, most of which involve the chemical modification of amino acid side-chains by the covalent attachment of other molecules or by the rearrangement of side-chain groups (Walsh, 2006). Aside from their multiformity, the known occurrence of specific PTMs in proteins is largely biased. For instance, phosphorylation at serines, threonines and tyrosines is widespread and modifies proteins involved in nearly every aspect of cellular function; at the other extreme, hypusination of lysine has only been found in eIF5A. Posttranslational protein modification can dramatically alter protein function and structure. PTMs allow rapid and energy-efficient modulation of cellular outputs in response to environmental conditions. In this way cells coordinate and regulate the myriad of functions required for life that is based on a limited set of genes. It is this fascinating means to alter protein activity that prompted the work for this thesis in which a novel and tissue specific posttranslational modification, asparagine deamidation, of the translation inhibitor 4E-BP2 was identified and characterized.

## 1.2 Translational Control

Control of gene expression in eurkaryotes is regulated at multiple levels of varying complexity. Two of the major forms of control, which involve the biosynthesis of macromolecules and require enormous cellular energy investments, are transcription and

translation. Translation, the synthesis of new proteins, assumes a central position in the flow of information from the genome to the final assembly and translocation of active proteins within cells. Translational control refers to the efficiency of cellular protein synthesis (proteins produced per mRNA) per unit time. Thus, it follows that a principal aim of this process is to regulate the copy number of cellular proteins produced under a given cellular condition. This may apply globally to most mRNAs or instead to a specific mRNA or mRNA subset. Well-defined translational control mechanisms are rare in prokaryotes owing to the lack of physical separation between transcription (nucleus) and translation sites and to the rapid rate of synthesis of macromolecues in these organisms. However, in eukaryotes translational control is widespread; its study arose from observations such as the rapid, transcription-independent changes in translation rates of preexisting mRNAs in fertilized sea urchin eggs and of ion-dependent synthesis of proteins in enucleated reticulocytes (Mathews et al., 2007).

The regulation of gene expression at the translational level is critical because it allows rapid and reversible responses to environmental cues; this obviates the need to invoke time and energy expensive nuclear pathways of transcription and mRNA processing and transport. Moreover, translational control can allow for spatially limited or localized protein synthesis at distinct cellular sites where the new proteins are required in processes such as developmental patterning (Thompson et al., 2007) and synaptic plasticity (Klann and Richter, 2007; Sutton and Schuman, 2006). The importance of translational control is underscored by the numerous and varied pathologies or physiological perturbations that are attributed to its deregulation, which include but are not limited to: cellular transformation and cancer (Meric and Hunt, 2002; Pandolfi, 2004; Schneider and Sonenberg, 2007), metabolic disorders (Harding and Ron, 2002; Kozma et al., 2007; Shi et al., 2003), learning and memory alterations and nervous system degeneration (Abbott

and Proud, 2004; Costa-Mattioli et al., 2009; Klann and Richter, 2007), susceptibility to viral infection (Buchkovich et al., 2008; Costa-Mattioli and Sonenberg, 2008; Mohr et al., 2007), developmental impairments (Thompson et al., 2007) and changes in organism longevity (Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007).

### 1.3 Overview of Translation

In eukaryotes, mRNA translation is classified into four phases: initiation, elongation, termination, and ribosome recycling. During initiation the two ribosome subunits are separately recruited (40S followed by 60S) to the mRNA 5' untranslated region (UTR) and are assembled at the initiation codon such that the AUG is located in the ribosome P (peptidyl) site, base-paired with the initiator methionyl tRNA (Met-$tRNA_i^{Met}$) anticodon. Initiation is, in the vast majority of cases, the rate-limiting step of translation, thus guarding against the wasteful energy expenditure of arresting the entire process at a later stage. Eukaryotes employ both general (many mRNAs) and mRNA-specific mechanisms of initiation. As this thesis is intimately concerned with the initiation phase of translation, this topic is addressed in greater detail below (section 1.5).

Elongation follows; here the mRNA is decoded by the ribosome and the nascent peptide chain is formed. Aminoacyl (aa)-tRNAs are recruited to the ribosome A (aminoacyl) site by the GTP-bound eukaryotic elongation factor 1A (eEF1A) such that the anticodon stemloops are postioned in the small ribosomal subunit to establish base-pairing with cognate codons. The large ribosome subunit binds the aa-tRNA acceptor arms and catalyzes peptide bond formation by transferring the peptide from the P site peptidyl-tRNA to the A site aa-tRNA (peptidyl transferase activity). The uncharged tRNA of the previous peptide bond, located in the E (exit) site, is concomitantly expelled from the ribosome. Translocation of the mRNA and tRNAs frees the A site for subsequent aa-

tRNA entry and places the newly formed peptidyl-tRNA in the P site and the deacylated tRNA in the E site (Noller, 2007; Taylor et al., 2007). Termination of translation commences when a stop codon enters the A site. This codon is bound by release factors, rather than tRNA, which begets hydrolysis of the completely synthesized protein in the P site peptidyl-tRNA and its release from the ribosome. Finally, the ribosome is recycled for new translation by removal from the mRNA and its dissociation into subunits. In prokaryotes this is accomplished by the concerted action of a ribosome recycling factor and an elongation factor. The initiation factor 3 (IF3), which is involved in originally recruiting the ribosome to the mRNA, is believed to prevent association of the large and small subunits prior to initiation. Ribosome recycling is poorly understood in eukaryotes (Ehrenberg et al., 2007).

## 1.4 The Message and the Machinery

The components required for the initiation of protein synthesis in higher eukaryotes are mRNA, the 80S ribosome (assembled from the 40S and 60S subunits), the eukaryotic initiation factors (eIFs), aminoacyl-tRNAs, and the hydrolysis of ATP and GTP.

### 1.4.1 mRNA

Eukaryotic mRNAs possess several unique characteristics, aside from the protein-encoding open reading frame (ORF). The 5' termini of all nuclear-transcribed mRNAs contain a 7-methylguanosine cap-structure, $m^7$GpppN (where N is any nucleotide). The cap is bound by the initiation factor 4E (eIF4E) and consequently, through a series of events, is involved in 40S ribosome recruitment. At the other extreme is a 3' poly(A) tail of 50-300 adenylates. These two mRNA elements synergistically enhance translation initiation via the interaction of their respective binding proteins, the eIF4F cap-binding complex and the poly(A)-binding protein (PABP) (Pestova et al., 2007b). The start codon

is typically the first AUG triplet encountered in the mRNA 5'UTR by the ribosome and should occupy a favourable sequence context as given by, GCC(**A/G**)CCAUG**G** (most important positions in bold) (Kozak, 1983, 1991). Small upstream open-reading frames (uORF) in the same mRNA can affect the translation at canonical protein-coding ORFs (Hinnebusch et al., 2007). Extensive secondary structure in the 5'UTR dampens initiation efficiency by acting as an encumbrance to ribosome scanning toward the start codon (Pestova and Kolupaeva, 2002). Other 5'UTR secondary features termed IRESs (internal ribosome entry site), frequently found in viral mRNAs, internally recruit specific eIFs and the 40S ribosome independently of the 5' cap (Doudna and Sarnow, 2007; Elroy-Stein and Merrick, 2007). The 3'UTRs of specific mRNAs often harbour *cis*-acting elements for protein interactions that impact translation. Importantly, recent breakthroughs in the understanding of posttranscriptional regulation of gene expression indicate that many mRNA 3'UTRs (and possibly some 5'UTRs) contain miRNA binding sites. These sequences generally elicit translational silencing or mRNA degradation via the RNA-induced silencing complex (RISC) (Chekulaeva and Filipowicz, 2009).

### 1.4.2 Ribosomes

The ribosome is the engine of protein synthesis, performing the functions of mRNA decoding, peptidyl transfer and translocation (the latter of which is catalyzed by an elongation factor), and is conserved throughout all forms of life. These macromolecular complexes are assembled from two subunits, each comprising ribosomal RNA (rRNA) and proteins. Eukaryotic ribosomes (> 3 MDa) are larger than those of prokaryotes (2.5MD) as is reflected by their sedimentation values: 80S (40S and 60S subunits) for eukaryotes compared to 70S (30S and 50S subunits) in prokaryotes. The 40S subunit contains 32 ribosomal proteins and the 18S rRNA, while the 60S subunit is comprised of 46 proteins and the 25S, 5.8S, and the 5S rRNAs (Taylor et al., 2007). Peptidyl transfer

from the P site peptidyl-tRNA to the A site aa-tRNA is catalyzed solely by the 60S rRNA (Nissen et al., 2000; Noller et al., 1992), effectively demonstrating that the ribosome functions as a ribozyme. Actively translating mRNAs may be engaged concurrently by several ribosomes in the elongation phase that have initiated consecutively. As such, the rate of translation is proportional to the number of initiation events.

### 1.4.3 Eukaryotic Initiation Factors

Eukaryotes require at least 12 initiation factors for translation. These factors are often formed from stable complexes of many subunits, which themselves may have several isoforms. In total, more than 30 proteins or polypeptides, some of which are not fully characterized, comprise the canonical eIFs (Dever, 2002; Pestova et al., 2007b). The roles of most of these factors will be outline briefly in section 1.5. eIF4E is examined in greater detail owing to its importance to this thesis.

#### 1.4.3.1 eIF4E

The 24 kDa cap-binding protein, eIF4E, was initially purified by its specific interaction with the 5' cap of an mRNA or a resin containing the cap-analog, $m^7$GDP (Sonenberg et al., 1978; Sonenberg et al., 1979). Structural analyses of this highly conserved protein indicated that the associated cap-structure is stacked between two conserved tryptophans within a narrow groove on the concave surface of the folded protein, which resembles a cupped hand (Marcotrigiano et al., 1997; Matsuo et al., 1997). The addition of purified eIF4E alone or together with other factors specifically increases translation of capped-mRNAs (Sonenberg et al., 1979; Sonenberg et al., 1980) in cell-free extracts, while its depletion has the opposite affect (Svitkin et al., 1996). The ability of eIF4E to stimulate recruitment of the 40S subunit, and therefore cap-dependent translation, relies on its inclusion into the eIF4F complex, along with eIF4G and eIF4A. eIF4G bridges eIF4E and

eIF4A (Richter and Sonenberg, 2005; Sonenberg and Hinnebusch, 2009) and also enhances the association of eIF4E with the cap (Haghighat et al., 1995; Ptushkina et al., 1998; von Der Haar et al., 2000; Yanagiya et al., 2009) eIF4E is modified by phosphorylation at Ser209, an event that is catalyzed by the kinases Mnk1 and Mnk2 (Fukunaga and Hunter, 1997; Pyronnet et al., 1999; Wang et al., 1998; Waskiewicz et al., 1999; Whalen et al., 1996). It is thought that eIF4E phosphorylation weakens its affinity for the mRNA cap, although the function of this in translational regulation is unclear (Scheper and Proud, 2002; Scheper et al., 2002; Zuberek et al., 2003). Notably, mutation of the homologous eIF4E phosphorylation site in *Drosophila* impaired organismal development and viability (Lachance et al., 2002). Perhaps, the most striking characteristic of eIF4E is its oncogenic capacity. Overexpression of this factor causes transformation of cell lines and several forms of carcinomas in transgenic mice (Lazaris-Karatzas et al., 1990; Ruggero et al., 2004; Wendel et al., 2004). Moreover, eIF4E appears to be overexpressed in various human cancers (Mamane et al., 2004; Schneider and Sonenberg, 2007). The function of eIF4E in tumour development is thought to result from the enhanced translation of several mRNAs encoding proteins involved in cell growth and division. These mRNAs exhibit exquisite sensitivity to eIF4E levels by virtue of their long and structured 5'UTRs which are normally inhibitory to translation (see section 1.5.3) (De Benedetti and Graff, 2004; Koromilas et al., 1992; Schneider and Sonenberg, 2007). The concentration of eIF4E is among the lowest of initiation factors in many cell types and thus its recruitment to the mRNA cap-structure is rate-limiting for translation (Duncan et al., 1987; Hiremath et al., 1985). Considering this, it is not surprising that increases to its expression can dramatically upregulate the translation of secondary-structure inhibited mRNAs.

### 1.4.4 Aminoacyl-tRNAs

Aminoacyl-tRNAs are tRNAs that are rendered competent for translation by the addition of amino acids to their acceptor stem regions. This aminoacylation reaction is catalyzed by the enzyme aminoacyl tRNA synthetase. A second salient tRNA feature, located at the other extreme of the molecule, is the anticodon loop necessary for binding to mRNA codons. Met-$tRNA_i^{Met}$ is the sole aa-tRNA not utilized in translation elongation. Rather, it is critical in the initiation phase via its incorporation into the ternary complex along with eIF2 and GTP. Met-$tRNA_i^{Met}$ is distinguished from its elongation-specific counterpart, Met-$tRNA_m^{Met}$, by the presence of unique bases in certain regions and by specific base-pairing interactions in both of the acceptor and anticodon stems. These features specify interaction with eIF2-GTP and exclude association with eEF1A, thereby ensuring its destination to the ribosomal P site during initiation as opposed to the A site for elongation (Astrom et al., 1993; Basavappa and Sigler, 1991; Farruggio et al., 1996; von Pawel-Rammingen et al., 1992).

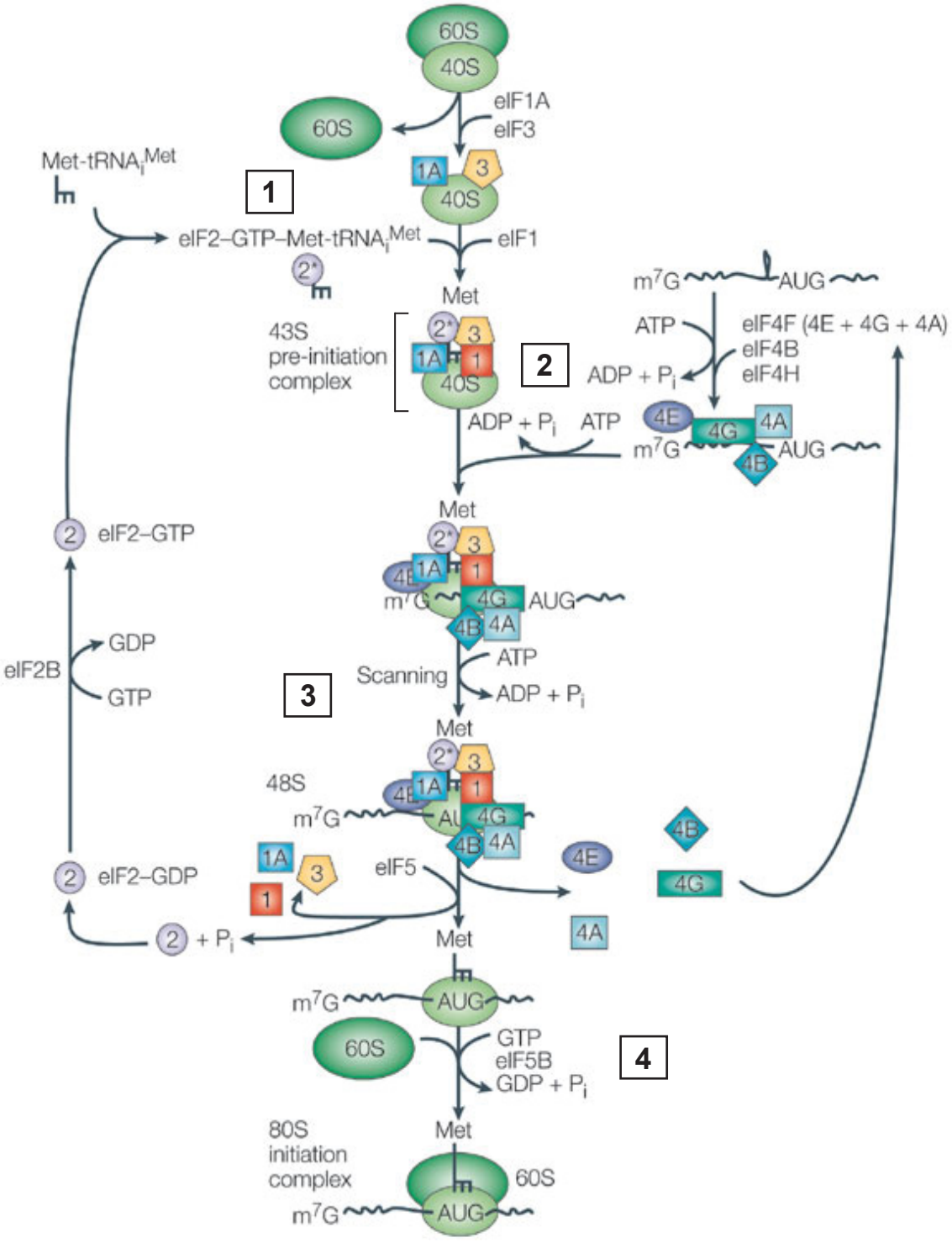
## 1.5 The Mechanism of Eukaryotic Translation Initiation

The initiation phase of translation, which culminates with the initiation codon positioned in the P site of an assembled 80S ribosome, involves several complex and energy-demanding steps mediated by the initiation factors. These steps may be conceptually divided, as follows: 1) 43S preinitiation complex formation, 2) PIC recruitment to mRNA, 3) Start Codon Selection, and 4) 60S Subunit Joining (Figure 1.1).

### 1.5.1 43S Preinitiation Complex Formation

eIFs 1, 1A, 3 and 5 assemble with the 40S subunit and the eIF2 ternary complex (eIF2-GTP-tRNAMet-$tRNA_i^{Met}$) to form the 43S Preinitiation Complex (PIC). Ternary complex formation itself is regulated by GTP loading of eIF2. GTP-bound eIF2 has a much greater affinity for Met-$tRNA_i^{Met}$ than does eIF2-GDP, the form in which eIF2 is found

**Figure 1.1 Eukaryotic Translation Initiation.**
Control of translation at the initiation phase involves the recognition of the mRNA 5' end by the eIF4F complex and its subsequent recruitment of the 43S preinitiation complex (43S PIC). This complex scans the linearized 5'UTR until the start codon is recognized and located in the 40S ribosome P site. Dissociation of several iniation factors is coupled with 60S subunit joining to form the 80S ribosome that is competant for peptide elongation. Numbering indicates four major steps of the initiation process: (1) 43S PIC formation, (2) Recruitment of 43S PIC to mRNA, (3) Start Codon Selection, (4) 60S Subunit Joining. See text for details. (Figure Adapted from Holcik and Sonenberg, 2005).


60S
40S
eIF1A
eIF3
60S
Met-tRNA$_i^{Met}$
1A
3
40S
1
eIF2–GTP–Met-tRNA$_i^{Met}$
eIF1
2*
Met
43S
pre-initiation
complex
2*
3
1A
1
40S
2
m$^7$G
AUG
ATP
eIF4F (4E + 4G + 4A)
eIF4B
eIF4H
ADP + P$_i$
4E
4G
4A
4B
m$^7$G
AUG
ADP + P$_i$
ATP
Met
2*
3
1A
1
4B
m$^7$G
4G
AUG
4B
4A
2
eIF2–GTP
ATP
Scanning
ADP + P$_i$
3
eIF2B
GDP
GTP
Met
2*
3
48S
1A
4B
m$^7$G
AU
4G
4B
4A
1A
3
1
eIF5
4E
4B
4G
2
eIF2–GDP
2 + P$_i$
Met
4A
m$^7$G
AUG
60S
GTP
eIF5B
GDP + P$_i$
4
80S
initiation
complex
Met
60S
m$^7$G
AUG

following a previous initiation event. eIF2B, a guanine nucleotide exchange factor (GEF), accelerates the replacement of GDP with GTP. A key regulatory mechanism in this process occurs via phosphorylation of eIF2, which consists of three subunits (α,β, and γ), on the α subunit at Ser51. Phosphorylated eIF2α is a competitive inhibitor of eIF2B. As cellular eIF2α levels are greater than those of eIF2B, only a substoichiometric amount of phosphorylation is required to inhibit nucleotide change on eIF2 (γ subunit) and thus impair translation by occluding ternary complex formation (Dever, 2002; Hinnebusch et al., 2007). Following ternary complex assembly eIF1, eIF1A, and eIF3 promote its recruitment to the 40S ribosome. Evidence from yeast suggests that these three initiation factors form a multifactor complex with the ternary complex prior to 43S PIC formation (Asano et al., 2000; Hinnebusch et al., 2007). Interestingly, 43S PIC formation is also directly linked to 80S ribosome dissociation as eIF3 and other factors together with mRNA or the ternary complex can dissociate 80S ribosomes and prevent their reformation (Chaudhuri et al., 1999; Kolupaeva et al., 2005; Pestova et al., 2007b).

### 1.5.2 Recruitment of 43S Preinitiation Complex to mRNA

Association of the 43S PIC with an mRNA is mediated by the eIF4F complex. As mentioned above, eIF4F is a tripartite complex of the factors eIF4E, eIF4G, and eIF4A, of which eIF4E selectively binds the mRNA 5' cap. eIF4G is a large mRNA-binding protein that bridges the mRNA to the incoming 43S PIC. eIF4A is an ATP-dependent RNA helicase. The interaction of eIF3, which contains13 subunits, with eIF4G connects the 43S PIC to the mRNA 5' end. The eIF4F complex is believed to be assembled prior to mRNA-binding. The eIF4E-binding proteins (4E-BPs), which are translation inhibitors, disrupt eIF4F formation by preventing eIF4G from binding eIF4E (Altmann et al., 1997; Gingras et al., 1999b; Mader et al., 1995). The poly(A)-binding protein, PABP, also interacts with eIF4G, thereby bridging the 5' and 3' mRNA termini in a 'closed loop'.

This is thought to stimulate eIF4F affinity for the cap and to promote reinitiation of post-termination ribosomes on the same mRNA (Hinnebusch et al., 2007).

### 1.5.3 Start Codon Selection: The Scanning Mechanism

The most complete model to explain ribosomal selection of the initiation codon is that of scanning. Here the recruited 43S complex migrates through the mRNA 5'UTR until it encounters the start codon. The eIF4A helicase of eIF4F acts with eIF4B and eIF4H to unwind secondary structure in the 5'UTR that otherwise would impede ribosomal scanning and impair translation. Indeed, the requirement of both eIF4E and eIF4A for ribosome recruitment is proportional to the degree of secondary structure in the mRNA 5'UTR (Jackson, 1991; Svitkin et al., 2001), thus explaining the sensitivity of mRNAs with structured leader sequences to eIF4E levels. Evidence suggests that eIF4F retains eIF3/43S at the cap structure during scanning. This implies that the mRNA is actually pulled through and loops out of the ribosome (Gross et al., 2003; Pestova and Kolupaeva, 2002; Poyry et al., 2004). eIF1 and eIF1A promote scanning of the PIC through the 5'UTR and its fidelity of start-codon selection. This is accomplished by favouring an open conformation of the 40S mRNA-binding cleft until the initiation codon, located in the correct sequence context (section 1.4.1), is positioned in the ribosome P site (Sonenberg and Hinnebusch, 2009). eIF1 also prevents premature hyrdrolysis of eIF2-GTP, which is catalyzed by the GTPase-activating protein (GAP) activity of eIF5, by preventing interaction of eIF2 and eIF5 (Conte et al., 2006). Base-pairing of the start codon to Met-tRNA$_i^{Met}$, thereby establishing the 48S complex, effects conformational changes that promote eIF1 dissociation and hydrolysis of eIF2-GTP (Maag et al., 2005; Unbehaun et al., 2004; Valasek et al., 2004).

### 1.5.4 80S Ribosome Formation: Subunit Joining

Following eIF2-GTP hydrolysis to eIF2-GDP, the 60S ribosome subunit is recruited to the 48S complex. eIF2-GDP has a weakened affinity for Met-tRNA$_i^{Met}$ and is released from the complex. However, eIFs 1, 1A, and 3 remain and prevent 60S joining by occupying its binding surfaces on the 40S subunit. GTP-bound eIF5B, together with the 60S subunit, displaces eIFs 1 and 3 (Pestova et al., 2000). This is aided by the interaction of the eIF5B-GTP with eIF1A and putatively with Met-tRNA$_i^{Met}$ (Acker et al., 2006; Guillon et al., 2005; Marintchev et al., 2003; Myasnikov et al., 2005). Correct 80S formation then occurs by the joining of the 60S subunit and its completion yields hydrolysis of eIF5B-GTP and release of eIF5B-GDP and eIF1A (Acker and Lorsch, 2008).

### 1.6 Regulation of Translation Initiation

As the rate of protein synthesis is principally controlled at the initiation phase, it is intuitive that several regulatory mechanisms are in place at this step. Three major mechanisms of initiation regulation are known in higher eukaryotes.

One involves the inhibition of ternary complex (eIF2-GTP-Met-tRNA$_i^{Met}$) formation by eIF2$\alpha$ phosphorylation (section 1.5.1). Phosphorylated eIF2$\alpha$ is a competitive inhibitor of eIF2B's GEF activity and blocks translation by preventing formation of active eIF2-GTP. Regulated translation inhibition by eIF2$\alpha$ phosphorylation is catalyzed by four kinases (HRI, PERK, PKR, and GCN2) which respond to diverse cellular stresses, in particular: heme deprivation, endoplasmic reticulum stress, double-stranded RNA (viral infection), and amino acid deficiency (Dever, 2002; Dever et al., 2007).

Another mechanism for translational control at the initiation phase is via disruption of PABP:eIF4G-mediated mRNA circularization (section 1.5.2). Normally, this form of

mRNA circularization synergistically enhances translation and is further activated by the PABP-interacting protein 1 (Paip1) (Craig et al., 1998). Two small, homologous proteins Paip2 (Paip2A) and Paip2B inhibit translation by abrogating PABP association with the mRNA 3' poly(A)-tail and by competing with Paip1 for PABP binding (Berlanga et al., 2006; Khaleghpour et al., 2001; Roy et al., 2004); consequently, the association of the mRNA 5' and 3' termini is lost (Kahvejian et al., 2001). A more recent report indicates that Paip2 can also inhibit translation by disrupting the association of PABP with eIF4G (Karim et al., 2006).

The third regulatory mechanism of translation initiation, which is integral to work of this thesis, is mediated by the 4E-BPs. These small polypeptides prevent eIF4F formation by competing with eIF4G for the same binding site on eIF4E. A common eIF4E-interacting motif, YXXXXLΦ (where X may be any amino acid, and Φ is a hydrophobic residue), harboured in both eIF4G and 4E-BPs directs this interplay (section 1.5.2). The competition for eIF4E-association is regulated by the phosphorylation status of the 4E-BPs; hypophosphorylated 4E-BPs bind eIF4E and preclude eIF4F formation, whereas 4E-BPs phosphorylated on several sites (hyperphosphorylated) dissociate from eIF4E thereby permitting interaction with eIF4G and association with the mRNA 5' cap (Gingras et al., 1999b; Richter and Sonenberg, 2005). In this way, translation is regulated in a dynamic manner by signaling pathways sensing cellular conditions favourable or prohibitive to *de novo* protein production that impinge on 4E-BP kinases. This will be discussed in the following sections.

It is noteworthy that transcript-specific mechanisms of translational control exist. To date, most have been described in developmental systems (*Drosophila* embryogenesis and *Xenopus* oocyte maturation) and the vertebrate central nervous system where spatial and

temporal restriction of specific protein expression is often critical. These mechanisms frequently rely on sequences in the mRNA 3'UTR that are targeted by various *trans*-acting factors (Hentze et al., 2007; Thompson et al., 2007). Moreover, miRNAs, target specific mRNAs containing complementary 3'UTR sequences for translational control (Chekulaeva and Filipowicz, 2009). Gene-specific translation repression will not be examined further in this thesis.

### 1.7 The eIF4E-Binding Proteins (4E-BPs)

The 4E-BP translation inhibitors are a family of three mammalian paralogous phosphoproteins (named 4E-BP1, -2, and -3). 4E-BP1 and 4E-BP2 were first identified (Lin et al., 1994; Pause et al., 1994). The capacity of these proteins to inhibit translation of reporter mRNAs was therein demonstrated *in vitro* or upon 4E-BP overexpression in cells (Pause et al., 1994). 4E-BP3 was later identified based on homology to the other 4E-BPs (Poulin et al., 1998). Overall, the human 4E-BPs are 56% identical at the amino acid level (Figure 1.2), yet, the middle portions containing the eIF4E-binding sequence and regulatory phosphorylation sites are strikingly similar. 4E-BP1, as the best characterised, is the prototype 4E-BP.

The inhibitory role of the 4E-BPs towards translation relies on their competition with eIF4G for binding to eIF4E by way of the common eIF4E-recognition motif (section 1.6) (Altmann et al., 1997; Haghighat et al., 1995; Mader et al., 1995; Marcotrigiano et al., 1999). Structural analyses have shown that the 4E-BP/eIF4G binding site on eIF4E is located on the dorsal convex surface, opposite the concave mRNA 5' cap-binding site (Figure 1.3) (Marcotrigiano et al., 1999; Matsuo et al., 1997). Moreover, the 4E-BPs, which are small polypeptides (100-120 amino acids), are inherently unstructured in solution but gain alpha-helical structure in the eIF4E-binding region upon association

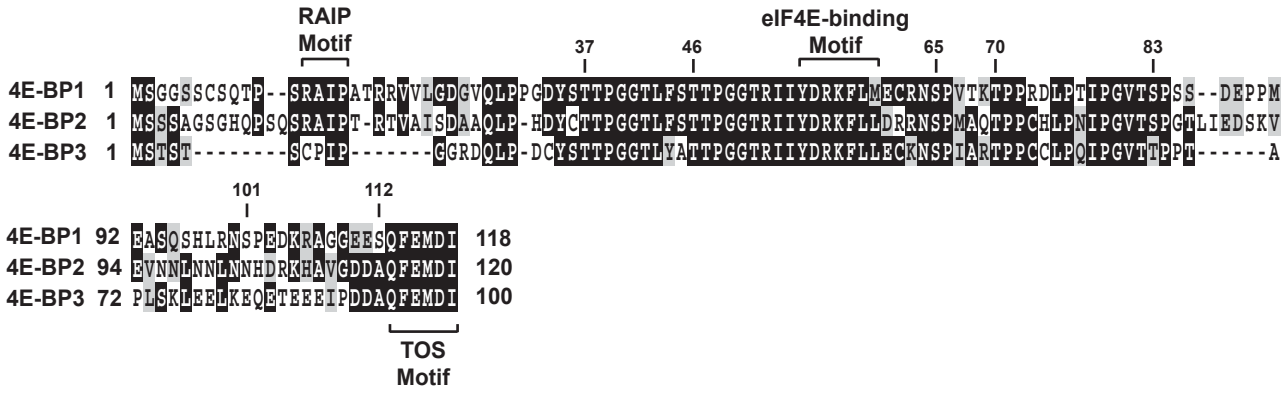



**Figure 1.2 The 4E-BPs: regulatory sequence motifs and phosphorylation sites.** Amino acid sequence alignment of the three human 4E-BPs. Regulatory motifs are labeled. Phosphorylation sites, with respect to 4E-BP1, are denoted by residue number.

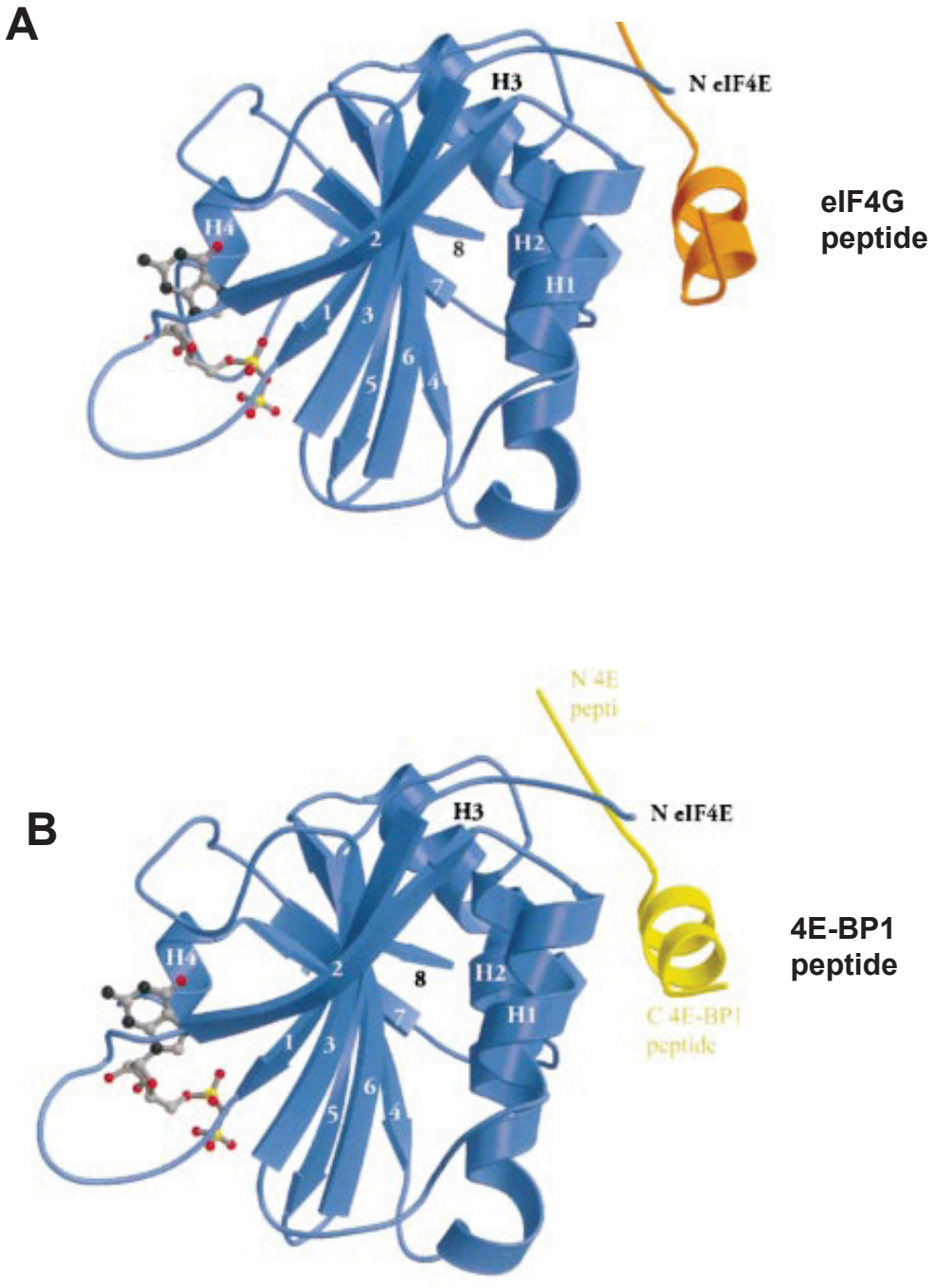



**Figure 1.3 4E-BPs and eIF4G compete for binding to eIF4E.**
Ribbon diagrams derived from crystal structures of the ternary complexes of $m^7$GDP-eIF4E-eIF4G peptide (A) or $m^7$GDP-eIF4E-4E-BP1 peptide (B). The peptides (orange or yellow) recognize the same region on the convex dorsal surface of eIFE (blue) thus solidifying the competition model for translation repression by 4E-BPs. The $m^7$GDP cap-structure is represented as an atomic ball and stick figure. (Adapted from Marcotrigiano et al., 1999).

with eIF4E (Fletcher and Wagner, 1998; Marcotrigiano et al., 1999; Tomoo et al., 2005). Perhaps not surprisingly then, the 4E-BPs exhibit remarkable stability to heat or acid which was emphasized in their original nomenclature as PHAS proteins (phosphorylated heat- and acid-stable protein regulated by insulin) (Blackshear et al., 1982; Ramakrishna and Benjamin, 1983).

The 4E-BPs exhibit disparate tissue expression in mammals. Notably, 4E-BP1 is particularly abundant in adipose tissue and muscle, while 4E-BP2 is predominant in brain (Tsukiyama-Kohara et al., 2001). 4E-BP knock-out mice and other transgenic models have revealed several important physiological functions for these proteins. Mice lacking 4E-BP1 or both 4E-BP1 and 4E-BP2 (double knock-out) display altered metabolic function and fat regulation (Le Bacquer et al., 2007; Tsukiyama-Kohara et al., 2001). Double knock-out mice were also found to be dramatically resistant to some forms of viral infection, owing to translational upregulation of the type-I interferon pathway (Colina et al., 2008). In contrast, ablation of the sole *Drosophila* 4E-BP homologue, Thor, rendered flies more susceptible to bacterial infections as Thor was upregulated during immune induction (Bernal and Kimbrell, 2000). In separate studies, Thor mutant flies had reduced longevity relative to controls (Tettweiler et al., 2005), while overexpression of a hyperactive mutant for eIF4E-binding reduced wing size owing to decreases in cell number and size (Miron et al., 2001)**.** The oncogenic potential of eIF4E (sections 1.4.3.1 and 1.5.3) also suggests the possibility that the 4E-BPs might act as tumour suppressors. Considerable evidence for this exists in that overexpression of 4E-BP1 and 4E-BP2 can at least partially revert eIF4E-transformed cells (Li et al., 2002; Polunovsky et al., 2000; Rousseau et al., 1996). Together, these latter findings suggest that a function of the 4E-BPs is to provide a counterbalance to translation-mediated cell growth and proliferation. On the other hand, increased expression of 4E-BP1 has been noted in some tumour types

(Kremer et al., 2006; Martin et al., 2000; Nathan et al., 2004) and, in at least one instance, contributed to hypoxia-induced cancer progression by favouring cap-independent (IRES-mediated; section 1.5.3) translation of mRNAs with oncogenic properties (Braunstein et al., 2007). In the nervous system, 4E-BP2-/- mice displayed alterations in certain forms of learning and memory (discussed in section 1.9) (Banko et al., 2006; Banko et al., 2007; Banko et al., 2005).

### 1.7.1 Phosphorylation of the 4E-BPs

The responsiveness of the protein synthesis machinery to external stimuli governing intracellular conditions is mediated, at least in part, by 4E-BP phosphorylation. Early studies revealed that 4E-BP1 (then designated phosphoprotein m) was amongst the most highly phosphorylated proteins following insulin-stimulation of rat adipocytes (Blackshear et al., 1982; Ramakrishna and Benjamin, 1983). Although, the significance of this was unknown until the function of eIF4E-binding and translational control was later described (Lin et al., 1994; Pause et al., 1994). 4E-BP hyperphosphorylation effects their dissociation from eIF4E thereby permitting the joining of eIF4G (Gingras et al., 1999b, 2001b). Four principal phosphorylation events are required for the dissociation of 4E-BPs from eIF4E. These phosphorylation sites are conserved in the three mammalian 4E-BPs, as well as those of lower eukaryotes, and flank the eIF4E-binding site. Considerable work has led to a model in which the phosphorylation on these four residues occurs in an inducible, hierarchical manner. The residues N-terminal to the eIF4E-binding site, Thr37/Thr46, appear to be readily phosphorylated, even in conditions of growth factor depletion (for example, serum-starvation), and together act as priming sites for further phosphorylation. Serum-stimulation elicits phosphorylation of the residues C-terminal to the eIF4E-binding site: Thr70 precedes Ser65 and subsequent dissociation from eIF4E (Figure 1.4) (Gingras et al., 1999a; Gingras et al., 2001a; Mothe-Satney et al., 2000).

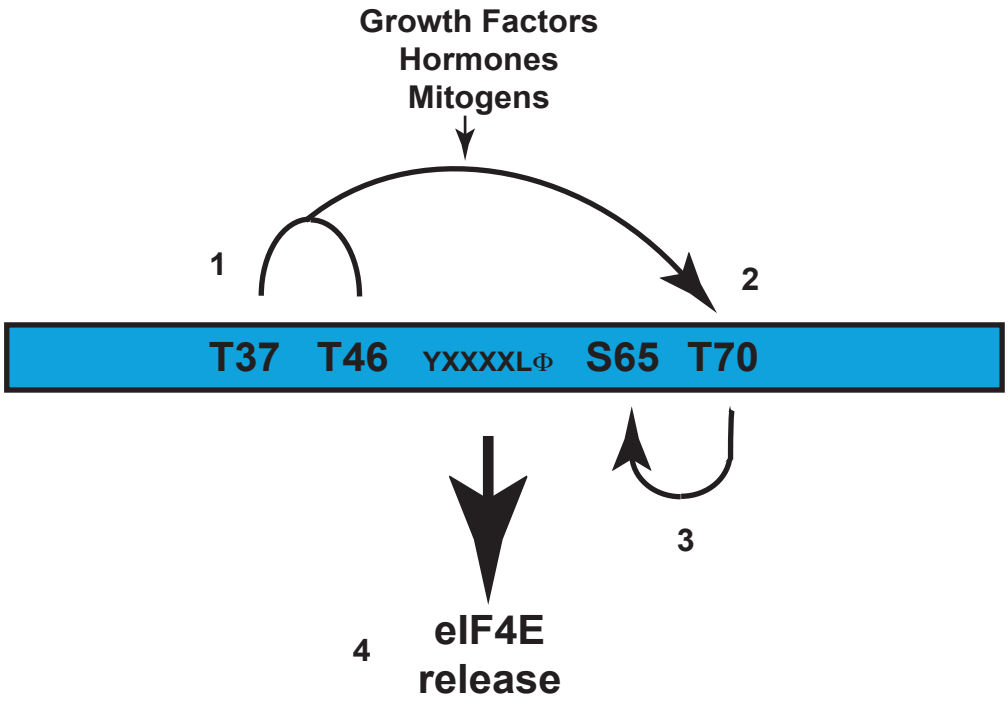



**Figure 1.4 Schematic diagram of hierarchical 4E-BP phosphorylation.** Phosphorylation at T37/T46 is insensitve to serum-starvation or rapamycin. Together these sites are required for further phosphorylation (1). Upon stimulation, T70 phosphorylation (2) preceeds that at Ser65 (3). The culmination of these phosphorylation events effects 4E-BP dissociation from eIF4E (4). (Figure adapted from A.C. Gingras, 2001).

Cumulative phosphorylation of the four residues is critical for dissociation from eIF4E. Interaction and biophysical studies have shown that phosphorylation of Ser65 alone or together with Thr70 can not abolish the interaction with eIF4E (Gingras et al., 2001a; Tomoo et al., 2006; Tomoo et al., 2005). Phosphorylation appears to increase 4E-BP peptide structural rigidity, thus providing a possible explanation for the loss of eIF4E-association (Tomoo et al., 2006; Tomoo et al., 2005). Other sites of 4E-BP phoshphorylation have been described at Ser83, which is conserved in all mammalian 4E-BPs (Thr in 4E-BP3), and at Ser101 and Ser112 that are present only in 4E-BP1 (Figure 1.2). Although the function of phosphorylation at these sites is poorly defined, work suggests that Ser83 may assist in dissociation from eIF4E (Eguchi et al., 2006; Mothe-Satney et al., 2000), while Ser101 and Ser112 may be required for phosphorylation at the serum-responsive sites (Ser65/Thr70) and release from eIF4E (Heesom et al., 1998; Wang et al., 2003; Yang and Kastan, 2000).

MAP kinase was initially implicated in 4E-BP phosphorylation based on its proclivity to phosphorylate PHAS I (rat 4E-BP1) *in vitro* (Haystead et al., 1994; Lin et al., 1994). However failure to inhibit insulin-stimulated phosphorylation with a MAPK inhibitor, among other observations, rendered this untenable in cells (Lin et al., 1995). It is now widely believed that the mammalian Target of Rapamycin (mTOR) is the *in vivo* kinase of the 4E-BPs. The lipophilic macrolide rapamycin, a potent inhibitor of mTOR, was instrumental in the seminal works delineating the signaling pathways to 4E-BP phosphorylation (Beretta et al., 1996; Diggle et al., 1996; von Manteuffel et al., 1996). Surprisingly, mTOR preferentially phosphorylates Thr37/Thr46 of 4E-BP1 *in vitro*, yet *in vivo* it is the serum-responsive sites, Ser65/Thr70, whose phosphorylation is inhibited by rapamycin (Gingras et al., 1999a; Gingras et al., 2001a). Thus, rapamycin treatment of cells effectively mimics growth factor deprivation in that it is permissive to

phosphorylation at Thr37/Thr46 but inhibits that at Ser65/Thr70. While the reasons for this discrepancy are not clear, it is possible that another kinase might directly phosphorylate the 4E-BPs in an mTOR-dependent manner. Alternatively, there is some evidence that mTOR may promote 4E-BP hyperphosphorylation by targeting and inhibiting 4E-BP phosphatases. One report identified a phosphatase with activity toward 4E-BP1 in mTOR immunoprecipitates (Peterson et al., 1999). Moreover, in yeast mTOR signals to PP2A-type phosphatases (Di Como and Arndt, 1996; Jacinto et al., 2001), although it is unclear whether this mechanism impacts the 4E-BPs in mammalian cells.

The 4E-BPs contain two regulatory motifs necessary for efficient phosphorylation. The C-terminal TOR signalling motif (FEMDI; Figure 1.2) mediates 4E-BP interaction with Raptor, an mTOR-associated protein necessary for the rapamycin-sensitive outputs. Mutation of this sequence in 4E-BP1 impairs its dissociation from eIF4E by preventing Ser65/Thr70 phosphorylation (and to a lesser extent that at Thr37/Thr46) (Choi et al., 2003; Nojima et al., 2003; Schalm et al., 2003). A second sequence near the 4E-BP N-terminus, known as the RAIP motif (based on the amino acid sequence; Figure 1.2**)**, is required for phosphorylation at Thr37/Thr46 (Choi et al., 2003; Tee and Proud, 2002). It may also be involved in Raptor-binding (Choi et al., 2003; Lee et al., 2008), although this was not observed in another study (Beugnet et al., 2003). Interestingly, 4E-BP3 exhibits impaired insulin-induced phosphorylation and dissociation from eIF4E, perhaps owing to a sub-optimal RAIP sequence (CPIP in 4E-BP3) (Tee and Proud, 2002). However, other work suggests that the isoleucine, which is conserved in 4E-BP3, is the critical residue in this motif (Lee et al., 2008).

### 1.8 mTOR

TOR proteins are central regulators of cell growth (Wullschleger et al., 2006). These proteins are large, evolutionarily conserved Ser/Thr kinases that were originally identified through genetic screens in yeast for rapamycin-resistant mutants (Heitman et al., 1991). mTOR belongs to the phosphoinositide 3-kinase-related kinase (PIKK) family of protein kinases, which also includes DNA-PK, ATM, ATR, and SMG1 (Cutler et al., 1999). In order to inhibit mTOR, rapamycin must be complexed to the peptydyl-prolyl cis/trans isomerase, FKBP12. This complex targets the FKBP12-rapamycin binding domain (FRB), just N-terminal to the kinase domain (reviewed in (Gingras et al., 2001b). mTOR is unusual among protein kinases in that the docking region for at least some substrates is located on an associated binding-partner, as opposed to the mTOR polypeptide itself (Avruch et al., 2009). Indeed, progress in understanding this kinase has revealed that mTOR actually forms two distinct kinase complexes, termed mTORC1 and mTORC2, that have unique substrates and regulation (Ma and Blenis, 2009; Wullschleger et al., 2006). mTORC1 is composed of mTOR, Raptor and GβL (LST8). It is the rapamycin-sensitive kinase responsible for inactivation of 4E-BPs by phosphorylation. The rapamycin-insensitive mTORC2 complex comprises mTOR, Rictor, GβL and SIN1. mTORC2 was found to be the elusive PDK2 that phosphorylates Akt at Ser473 in the hydrophobic motif to promote its full activation for downstream signalling (see section 1.8.1) (Guertin et al., 2006; Jacinto et al., 2006; Sarbassov et al., 2005b). mTORC2 kinase activity also targets PKC and RAC1 and is involved in regulation of the cytoskeleton (Jacinto et al., 2004; Sarbassov et al., 2004). Proline-rich protein 5 (PRR5, also called Protor) has also been demonstrated to associate with mTORC2 (Pearce et al., 2007; Woo et al., 2007). Although, the downstream effectors of mTORC2 are becoming well understood, it is unclear what regulates the activity of this complex.

### 1.8.1 mTORC1

Raptor is the defining member of the mTORC1 complex. Its association with mTOR is critical for the phosphorylation of 4E-BPs (Fonseca et al., 2007; Hara et al., 2002; Kim et al., 2002). Specific residues in the Raptor N-terminal RNC domain (Adami et al., 2007; Dunlop et al., 2009) allow it to mediate the recruitment of 4E-BPs by interacting with the C-terminal TOS motif (section 1.7.1) (Choi et al., 2003; Nojima et al., 2003; Schalm et al., 2003). A second major target of mTORC1 activity, similarly containing a TOS motif, are the S6 Kinases (S6K1 and S6K2) (Schalm and Blenis, 2002). These kinases phosphorylate ribosomal protein S6 upon mTORC1 activation and this correlates with increased rates of translation. S6K may also affect translation through phosphorylation of initiation factors (eIF4B in particular) and ribosome biogenesis (Fumagalli and Thomas, 2000). S6Ks regulate mTOR-mediated control of cell growth (Thomas, 2002). Surprisingly, the sequence contexts of mTORC1 phosphorylation sites in 4E-BPs and S6Ks are dissimilar. This suggests that mTORC1 targets are perhaps determined more by substrate recruitment (via a TOS motif) than by a specific phosphorylation site motif. More recently, the TOS motif-containing mTORC1 inhibitor, PRAS40, was identified. Overexpression of PRAS40 in cells or its inclusion in *in vitro* kinase assays blocked mTORC1-mediated phosphorylation of 4E-BP1 and S6K1, while its depletion by siRNA led to enhanced phosphorylation of these substrates (Fonseca et al., 2007; Oshiro et al., 2007; Sancak et al., 2007; Vander Haar et al., 2007; Wang et al., 2007). The inhibitory action of PRAS40 toward mTORC1 signaling is in-turn regulated by its growth factor-induced phosphorylation and association with 14-3-3 (Fonseca et al., 2007; Sancak et al., 2007; Vander Haar et al., 2007).

A model for the regulation of mTORC1 substrates has thus emerged whereby PRAS40 inhibits phosphorylation of 4E-BPs and S6Ks in the absence of nutrients or growth

factors by TOS-mediated competition for Raptor-binding. Upon stimulation, PRAS40 is phosphorylated and undergoes 14-3-3-dependent dissociation from Raptor. Other putative TOS-containing proteins have been described but are not well-characterised with respect to regulation by mTORC1 (Land and Tee, 2007; Lee et al., 2008; Parekh et al., 1999). Interestingly, mTORC1 has been found to associate with eIF3 in mRNA 5' cap-binding complexes, suggesting a more intimate involvement in the regulation of translation initiation than previously believed (Holz et al., 2005).

### 1.8.2 Regulation of mTORC1

As a critical regulator of translation and cell growth and proliferation, mTOR must integrate signalling inputs sensing growth factor/hormone receptor activation, nutrient availability, and energy status (Dunlop et al., 2009; Ma and Blenis, 2009; Wullschleger et al., 2006). Several of these inputs converge upstream of mTORC1 to regulate the GTP-loading of the small protein Rheb (Ras homologue enriched in brain) (Figure 1.5). Rheb-GTP is a potent activator of mTORC1 kinase activity *in vivo* and *in vitro*. Rheb-GTP may activate mTORC1 through its direct interaction with the complex (Avruch et al., 2009), perhaps increasing substrate recruitment (Sato et al., 2009). Alternatively, RhebB-GTP may activate the production of phosphatidic acid which in itself binds the mTOR FRB domain and is a positive regulator of mTORC1 (Sun et al., 2008). The highly conserved tumour suppressor composed of a heterodimer of tuberous sclerosis complex 1 and 2 proteins (TSC1 and TSC2) negatively regulates mTORC1. TSC1/TSC2 GAP activity promotes conversion of Rheb-GTP to inactive Rheb-GDP (Stocker et al., 2003).

It is the TSC1/TSC2 complex that is the site of convergence of signals from receptor-mediated activation of the PI3K-PDK1-Akt pathway and cellular energy status through

AMPK. Activation of various types of growth factor receptors signal to the lipid phosphoinositide 3-kinase (PI3K) which catalyzes formation of 3,4,5 phosphatidyl inositol triphosphate (PIP3) (Hay, 2005). PIP3 activates Phospholipid-dependent kinase 1 (PDK1), a process antagonized by the PTEN tumour suppressor, which in turn phosphorylates and positively regulates Akt/PKB (Maehama et al., 2001). Full Akt activation also requires direct input from mTORC2 (Sarbassov et al., 2005b). Phosphorylation of TSC2 on several sites by Akt inhibits its GAP activity toward Rheb and consequently relieves mTORC1 inhibition (Kwiatkowski and Manning, 2005). Several reports have also demonstrated MAPK- dependent inactivation of TSC1/TSC2 by phosphorylation (Ma and Blenis, 2009).

Adequate cellular energy, indicated by the ratio of AMP to ATP, is sensed by a negative regulator of mTORC1, AMPK (AMP-activated protein kinase). High AMP:ATP promotes AMPK-mediated phosphorylation of TSC1/TSC2, on sites independent of those targeted by Akt. This activates the GAP activity of TSC1/TSC2 to Rheb, opposing PI3K-PDK1-Akt output (Hahn-Windgassen et al., 2005). Other signalling pathways and metabolic alterations, including hypoxia, have been reported to affect TSC1/TSC2 activity toward mTORC1 (Ma and Blenis, 2009).

Nutrient availability imparts a major, TSC1/TSC2-independent regulation of mTORC1. Amino acids have been known to activate mTORC1 for some time (Hay and Sonenberg, 2004). Early observations that Rheb overexpression could compensate for amino acid starvation while TSC1/TSC2 inhibition could not were apparently at odds (Smith et al., 2005). Two recent studies have, at least in part, reconciled this discrepancy. The Rag GTPases were demonstrated to mediate the amino acid-dependent component of mTORC1 activation via localization of mTORC1 to Rheb-containing intracellular

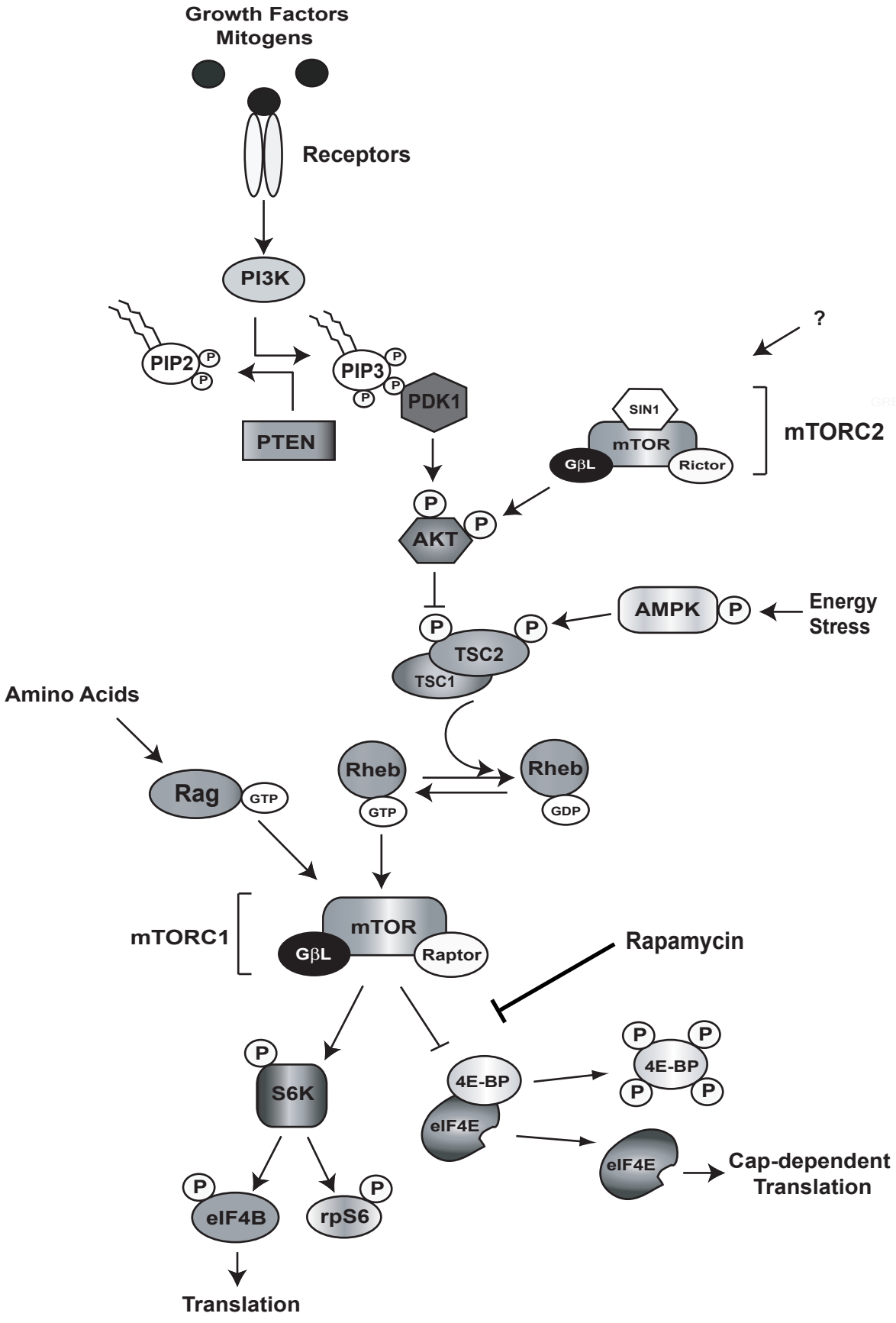



**Figure 1.5 Regulatory Inputs to mTORC1.**
mTORC1 is activated by Rheb-GTP which is antagonized by TSC1/TSC2. Inactivation of TSC1/TSC2 is mediated by PI3K-PDK1-Akt-dependent phosphorylation. Amino acid signaling to mTORC1 via Rag GTPases is critical for full activation and phosphorylation of downstream effectors. (Modified from R.J.O Dowling, 2009).

compartments. Interaction of RagGTP with Raptor is necessary for this effect (Kim et al., 2008; Sancak et al., 2008).

Posttranslational modification of mTORC1 is also becoming recognized as a mechanism of activity regulation. Until recently mTOR was known to be phosphorylated on three sites, one due to autophosphorylation, although with no defined functional significance (Cheng et al., 2004; Nave et al., 1999; Peterson et al., 2000; Sekulic et al., 2000). A fourth site, Ser1261, has now been demonstrated to induce mTORC1's phosphorylation of S6K1 and 4E-BP1 in a manner dependent on amino acids and Rheb activation (Acosta-Jaquez et al., 2009). Raptor is also phosphorylated on several residues. Phosphorylation of Raptor by Ras-MAPK-RSK (Carriere et al., 2008) or mTORC1 itself (Wang et al., 2009) enhances mTORC1 activity, while that by AMPK imparts negative mTORC1 regulation (Gwinn et al., 2008).

### 1.9 Translational Control in Learning and Memory

The processes of learning and memory require the synthesis of new proteins. The formation of memories is believed to rely on the property of neurons to modify the strength of their connections (synapses) in response to neuronal activity. Such activity-dependent changes are known as synaptic plasticity (Kandel, 2001). Cellular models of synaptic plasticity that represent maintained increases or decreases in synaptic strength are long-term potentiation (LTP) and long-term depression (LTD) (Kelleher et al., 2004b; Klann and Richter, 2007; Malenka and Bear, 2004). A structure within the temporal lobe called the hippocampus has been used extensively for studies of LTP and LTD. The hippocampus is particularly important for memories related to factual knowledge of space, time and objects. Memories and LTP both exhibit short-term (early or E-LTP) and long-term (late or L-LTP) phases. At the molecular level, short-term phases require modification of existing proteins, while it is the consolidation of the long-term phases that

is critically dependent on new protein synthesis (in addition to transcription) (Costa-Mattioli et al., 2009; Klann et al., 2004). LTP is induced by electrophysiological protocols that can specifically effect the early or late phases (LTP and LTD may also be elicited chemically), of which only the later phases are sensitive to protein synthesis inhibitors (Frey et al., 1988; Huang and Kandel, 1994; Nguyen et al., 1994). The coupling of these techniques and well-proven behavioural paradigms of learning and memory with transgenic mouse technology and molecular biology has led to remarkable progress in understanding the role of translation in synaptic plasticity. Importantly, general translational control mechanisms and the signaling pathways regulating them, including eIF2α phosphorylation (Costa-Mattioli et al., 2005; Costa-Mattioli et al., 2007), TSC maintenace (Ehninger et al., 2008), MAPK signaling (Kelleher et al., 2004a), and 4E-BP2 levels (Banko et al., 2006; Banko et al., 2007; Banko et al., 2005) have been elegantly demonstrated to regulate LTP, LTD and memory by genetic studies. Genetic deletion of 4E-BP2, the predominant 4E-BP in the mammalian brain, reduced the threshold for eliciting L-LTP and increased chemical-induced L-LTD, while normal L-LTP protocols led to L-LTP impairments (Banko et al., 2006; Banko et al., 2005). Furthermore, knock out mice exhibited impaired behaviour in several tasks, particularly those requiring contextual and spatial memory (Banko et al., 2007; Banko et al., 2005). It is thought that the enhancements in protein synthesis upon deletion of translation repressor 4E-BP2 engenders facilitated L-LTP with weak stimulation (E-LTP inducing stimulation), while increased protein synthesis evoked during strong L-LTP inducing stimulation or demanding behavioural tasks becomes detrimental. Several studies have demonstrated that mTORC1 activity is required in synaptic plasticity and memory consolidation, primarily based on the use of rapamycin which blocks L-LTP and some forms of memory consolidation (reviewed in (Costa-Mattioli et al., 2009). It is surprising however, that despite the importance of mTORC1 and 4E-BP2 in synaptic plasticity, the requirement

for mTORC1-mediated regulation of 4E-BP2 has not been specifically demonstrated. Local translation of specific mRNAs near active synapses, far removed from the cell body, also contributes to synaptic plasticity, thus implicating mRNA-specific translational control mechanisms in addition to general regulation (Sutton and Schuman, 2006).

### 1.9.1 Synaptic Transmission

Synaptic transmission is the mechanism by which neurons convey signals from one to another across a small cleft or synapse. The events of synaptic transmission are highly complex and, as such, a detailed description is beyond the focus of this work. However, a brief overview is warranted. Depolarization of the presynaptic neuron's axon, commonly during an action potential, causes fusion of neurotransmitter-containing vesicles with the presynaptic membrane. This allows neurotransmitter release into the synaptic cleft. Glutamate is a common excitatory neurotransmitter in the hippocampus. The response of the postsynaptic neuron to synaptic glutamate is mediated by ionotropic membrane receptors. Allosteric binding of glutamate induces opening of membrane channels that conduct the electric current into the postsynaptic cell. It is the temporal and spatial summation of these currents from thousands of postsynaptic sites that determines the potential of the neuron to fire (Kandel et al., 2000). The AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic) type of glutamate receptors mediate fast synaptic currents in the hippocampus. Indeed, their importance to LTP and LTD is highlighted by fact that these processes require insertion or removal of AMPA receptors at the postsynaptic membrane (Kessels and Malinow, 2009; Malinow and Malenka, 2002). A slow-current component of LTP, in some commonly studied types of hippocampal synapses, is mediated by a second glutamate receptor type. In addition to activity-dependent glutamate release, spontaneous fusion of neurotransmitter vesicles with the presynaptic membrane causes periodic small receptor-mediated currents called miniature excitatory postsynaptic

currents (mEPSC). Although mEPSCs are normally too small and infrequent to sufficiently depolarize the postsynaptic cell to elicit an action potential, they are often used to understand the biophysical properties and behaviour of a particular synapse (Otsu and Murphy, 2003).

## 1.10 Posttranslational Modifications

Many of the eukaryotic initiation factors and translation inhibitors contain sites of *in vivo* phosphorylation (Raught and Gingras, 2007). The functions of some of these are well understood, as for eIF2α or the 4E-BPs while others require further characterization. Moreover, it is likely that numerous other initiation factor phosphorylation events remain unidentified (Raught and Gingras, 2007). Excluding phosphorylation, there is a dearth of known initiation factor posttranslational modifications. Two occurrences effectively exhaust the known catalogue: eIF4G is posttranslationally cleaved during picornavirus infection, leading to shutoff of host protein synthesis (Etchison et al., 1982) and an eIF5A lysine is modified to hypusine, positively regulating the protein function in promoting cell growth and proliferation (Patel et al., 2009). Considering the preponderance of posttranslational modifications in eukaryotic cells it seems very likely that forms other than phosphorylation should target at least some of the proteins involved in translation initiation. However, the identity of such putative modifications and their substrates and functions are unknown.

### 1.10.1 Asparagine Deamidation

Deamidation is the conversion of intra-peptide asparagines into aspartates. This process is spontaneous (non-enzymatic) and occurs via a short-lived circular intermediate that is formed through the nucleophilic attack by the peptide bond amino group on the asparagine side-chain amide group (Figure 1.6) (Reissner and Aswad, 2003). The decay

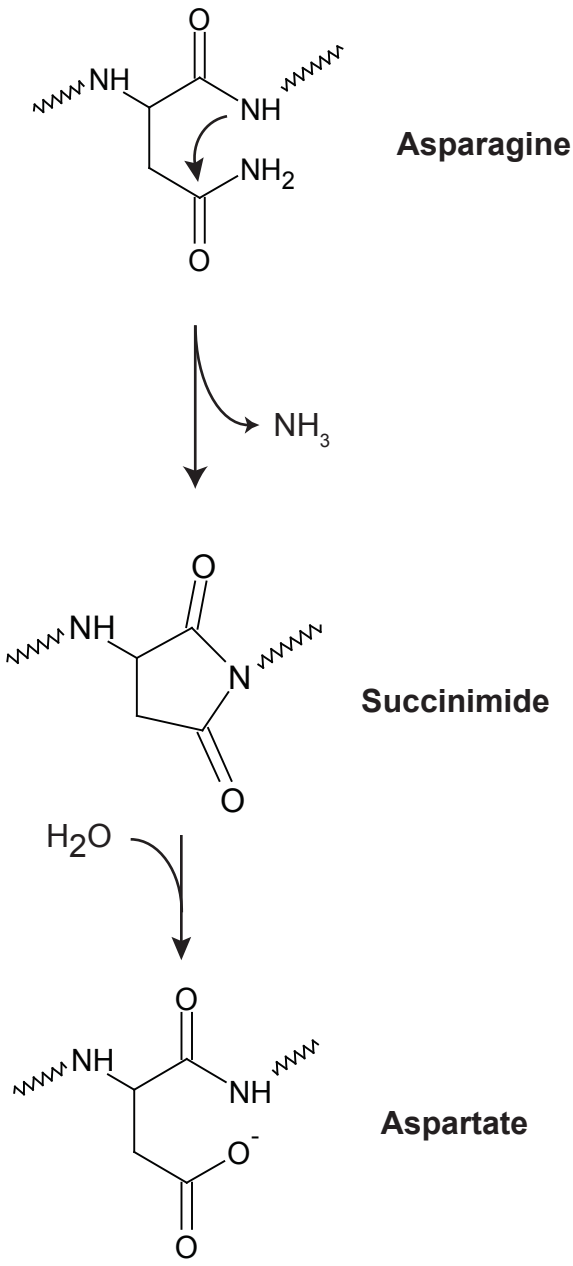



**Figure 1.6 The mechanism of spontaneous asparagine deamidation.** Asparagine conversion to aspartate occurs through a circular succinimide intermediate. Nucleophilic attack of the asparagine amide group by the C-terminal peptide bond amino group yields succinimide. This is quickly hydrolyzed to aspartate. Succinimide formation is rate-limiting in this process.

of the intermediate by hydrolysis actually yields two products, aspartate and its isomeric form isoaspartate, the latter of which will be discussed in chapter three. Knowledge of asparagine deamidation is more than a century old (Schulze and Bosshard, 1883), although it was believed to be a purification artifact until the 1960s. It was the work of Flatmark that elegantly provided the first demonstration of *in vivo* cytochrome c deamidation (Flatmark and Sletten, 1968; Flatmark and Vesterberg, 1966). A particularly convincing experiment utilized *in vivo* radioisotope labelling in rats to demonstrated that the deamidated forms contained the radiolabel that was originally incorporated into the unmodified protein (Flatmark and Sletten, 1968). Thus, deamidation results from a temporal conversion of an asparagine-containing polypeptide into an aspartate-containing peptide. The likelihood that an asparagine will undergo deamidation is largely determined by the flanking amino acid sequence. The residue immediately C-terminal in particular is a major determinant, with glycine being the most permissive neighbour (Robinson et al., 1973; Robinson and Robinson, 2001b). Location near flexible regions of the peptide also favours deamidation (Xie and Schowen, 1999). Indeed, the importance of context is underscored by the fact that the deamidation half-times of asparagines within different proteins range from days to years (Robinson and Robinson, 2001a, c, 2004). It is perhaps remarkable then that the amino acid analysis of 1465 proteins revealed that asparagines are distributed with high preference for or rejection of nearest neighbours (far more so than other amino acids), indicating that they may form conserved beneficial functional units (Robinson and Robinson, 1991). Such findings have prompted the molecular clock hypothesis which posits that deamidation events may be programmed, by maintenance of a favourable context, to occur at rates appropriate to influence biological processes. It adds that rapid deamidation events, if biologically deleterious, would have been otherwise eliminated by selective pressures (Robinson et al., 1970). Of the more than 200 proteins known to undergo *in vivo* deamidation, the function of some of those that have been well-

characterised appears to be mediated through altered protein:protein interactions. This is reasonable considering that deamidation imparts a negative charge to the protein, similar to phosphorylation. An excellent example is the deamidation of the antiapoptotic regulator Bcl-$X_L$. Antineoplastic treatment of susceptible cancer cells induces Bcl-$X_L$ deamidation and disrupts its ability to bind BH3 domain-only proteins, thus triggering apoptosis (Deverman et al., 2002; Zhao et al., 2008). Although deamidation has in the past suffered the stigma of being considered merely protein damage, it is becoming increasingly clear that this subtle modification can, in many cases, significantly impact cellular processes.

### 1.11 Rationale

Studies using 4E-BP2-/- mice that were generated in the Sonenberg laboratory demonstrated an important role for this translation inhibitor in some forms of memory and its cellular correlates, LTP and LTD (Banko et al., 2006; Banko et al., 2007; Banko et al., 2005). Other reports showed that 4E-BP1 was phosphorylated in a rapamycin-sensitive manner in cultured primary neurons shortly after plating (Kelleher et al., 2004a; Takei et al., 2001). As 4E-BP2 is the predominant 4E-BP in the adult mammalian brain, the focus on 4E-BP1 in these studies suggests that these *in vitro* developing neurons do not precisely recapitulate the relative *in vivo* expression levels of 4E-BPs of mature neurons. Further, signalling to mTORC1 is engaged upon LTP- or LTD-inducing stimulation (Costa-Mattioli et al., 2009). It is surprising though that little convincing evidence for LTP-induced phosphorylation of 4E-BPs, in particular 4E-BP2, in the mature hippocampus has been demonstrated. With this in mind we attempted to characterise the phosphorylation of 4E-BP2 in response to LTP-inducing stimulation. However, we were dismayed at our inability to detect significant levels of 4E-BP2 phosphorylation under any condition. This was especially troubling given that the

migration pattern of 4E-BP2 in SDS-PAGE was strikingly similar to that of multi-phosphorylated 4E-BP1. Further investigation strongly suggested that the 4E-BP2 forms in the brain are due to a novel modification appearing during early postnatal development. Thus, the work of this thesis was undertaken to identify and characterise this 4E-BP2 modification.

# Chapter 2

# Postnatal deamidation of 4E-BP2 in brain enhances its association with Raptor and alters kinetics of excitatory synaptic transmission.

## 2.1 Abstract

The eIF4E-binding proteins (4E-BPs) disrupt eIF4F complex formation, and thereby repress translation by precluding ribosome association with mRNAs. There are three 4E-BPs in mammals, of which 4E-BP2 is enriched in the brain and plays an important role in long-lasting synaptic plasticity and learning and memory formation. Here we describe asparagine deamidation as a novel posttranslational modification of 4E-BP2 in the brain. Deamidation is the spontaneous conversion of asparagines to aspartates. Two major sites of deamidation in 4E-BP2 were mapped to an asparagine-rich sequence unique to 4E-BP2. Deamidated 4E-BP2 exhibits increased binding to the mammalian Target of Rapamycin (mTOR)-binding protein, raptor, which leads to reduced association with eIF4E. 4E-BP2 deamidation occurs during postnatal development, concomitant with the attenuation of the activity of the PI3K-Akt-mTOR signalling pathway. Expression of deamidated 4E-BP2 in 4E-BP2-/- neurons yielded mEPSCs exhibiting increased charge transfer with slower rise and decay kinetics, relative to wild type 4E-BP2. We propose a function for 4E-BP2 deamidation as a compensatory mechanism for the reduced activity of the PI3K-Akt-mTOR module to facilitate translation and impact synaptic activity.

## 2.2 Introduction

Eukaryotic protein synthesis is primarily controlled at the initiation phase. A critical early event in this process is the association of the eukaryotic initiation factor 4E (eIF4E) with the mRNA 5' $m^7$GpppN-cap structure. eIF4E associates with eIF4G and eIF4A, to form a three-subunit eIF4F complex, which facilitates 40S ribosome recruitment to the mRNA (Gingras et al., 1999b; Pestova et al., 2007a). In mammals, an important regulatory function in this process is carried out by the eIF4E-binding proteins. The members of this family of three paralogs (4E-BP1, 2, and 3) compete with eIF4G for the same binding site on the dorsal surface of eIF4E (Mader et al., 1995; Marcotrigiano et al., 1999), and thereby inhibit translation initiation by preventing the interaction of eIF4E with eIF4G (Haghighat et al., 1995). The prototype protein, 4E-BP1, is the best characterized. The association of the 4E-BPs with eIF4E is abrogated by their hierarchical, multi-site phosphorylation (Gingras et al., 2001a; Mothe-Satney et al., 2000). The 4E-BPs exhibit disparate tissue expression (Lin and Lawrence 1996; Tsukiyama-Kohara et al. 2001). 4E-BP2 is predominant in the brain and its requirement for learning and memory has been studied extensively. Deletion of 4E-BP2 in mice engenders hippocampus-dependent memory impairments and alterations in long-lasting forms of synaptic plasticity (Banko et al., 2006; Banko et al., 2007; Banko et al., 2005).

The 4E-BPs are phosphorylated by the mammalian target of rapamycin complex 1 (mTORC1) (Gingras et al., 2001b; Proud, 2009; Yonezawa et al., 2004). In addition to the large serine/threonine kinase mTOR, this complex consists of GβL (LST8), and the mTORC1-defining member, raptor. Raptor, is a large scaffolding protein that recruits

mTORC1 substrates to the mTOR kinase domain through interaction with a substrate-specific TOR signaling motif (Hara et al., 2002; Kim et al., 2002; Schalm and Blenis, 2002; Schalm et al., 2003). In 4E-BPs, the TOS motif, which is located at the extreme C-terminus of the protein, is necessary for binding to raptor and consequently for phosphorylation on the rapamycin-sensitive sites (Ser65/Thr70) that are required for eIF4E release (Schalm and Blenis, 2002; Schalm et al., 2003). Recent studies, however, have indicated that the TOS motif alone is insufficient for efficacious 4E-BP1-raptor binding, thereby suggesting that other sequences in the 4E-BPs enhance this interaction (Choi et al., 2003; Eguchi et al., 2006; Lee et al., 2008).

mTOR controls cell growth and proliferation (Sarbassov et al., 2005a). mTOR integrates inputs from several signaling cascades communicating extracellular stimuli (PI3K-Akt pathway), intracellular energy status (LKB1-AMPK), cellular stresses (virus infection and hypoxia), and nutrient availability (Rag GTPases) into growth-promoting outputs, in particular protein synthesis (Sancak and Sabatini, 2009; Wullschleger et al., 2006). In neuronal systems, mTOR mediates several processes, including learning and memory (Blundell et al., 2008; Ehninger et al., 2008; Parsons et al., 2006), synaptic plasticity (Cammalleri et al., 2003; Tang et al., 2002), axon growth (Verma et al., 2005), dendritic protein synthesis (Gobert et al., 2008; Takei et al., 2004; Tsokas et al., 2005), and formation of the dendrite arbor (Brandt et al., 2007; Jaworski et al., 2005; Jossin and Goffinet, 2007; Kumar et al., 2005). Recent work demonstrated that mTORC1 activity declines during postnatal development in retinal ganglion neurons (Park et al., 2008).

Here we describe a novel, brain-specific posttranslational modification of 4E-BP2. Asparagine deamidation is the spontaneous (non-enzymatic) conversion of an asparagine into aspartate. In the mammalian brain 4E-BP2 undergoes deamidation on two asparagines in an asparagine-rich sequence, unique to 4E-BP2, located near the C-terminus of the protein. We demonstrate that this modification enhances the association of 4E-BP2 with Raptor. Further, we show that this modification occurs during early postnatal development. Expression of the deamidated form of 4E-BP2 in 4E-BP2-/- neurons altered the kinetics of miniature excitatory postsynaptic currents, thus indicating a functional significance in synaptic transmission. We suggest that 4E-BP2 deamidation may provide a compensatory mechanism to maintain cap-dependent translation despite attenuated PI3K-Akt-mTOR signaling in neurons.

## 2.3 Results

### 4E-BP2 in Mammalian Brain Resolves into Three Forms Independent of Phosphorylation

4E-BP2 protein from murine brain migrates as three forms in SDS-PAGE (Banko et al., 2005; Tsukiyama-Kohara et al., 2001), and thus is reminiscent of the well-characterized resolution of 4E-BP1 protein into several species in SDS-PAGE based on its state of multi-site phosphorylation (Gingras et al., 1999a; Graves et al., 1995; Lin et al., 1994). Considering the role of 4E-BP2 in synaptic plasticity and learning and memory processes (Banko et al., 2006; Banko et al., 2007; Banko et al., 2005), we wished to study its regulation by phosphorylation in the mammalian brain. We first determined that the hierarchical mode of 4E-BP1 phosphorylation, in which Thr37 and Thr46 precede Thr70

and Ser65 phosphorylation, is maintained in 4E-BP2. Mutation of either Thr37 or Thr46 to alanine precluded further phosphorylation events as evidenced by the reduced number of phosphorylated forms in two-dimensional isoelectric focusing (IEF)/SDS-PAGE, relative to the Thr70Ala or wild type proteins (Appendix 1, Figure S1). Surprisingly, however, we were unable to detect significant levels of phosphorylated 4E-BP2 from adult whole brain or hippocampal extracts by Western blotting with an antibody specific for the priming phosphorylation sites (Thr37/46) (Figures 2.1A and Appendix 1, Figure S2). Moreover, the slowest-migrating form is unique to the brain. We reasoned that if the slower migrating bands of 4E-BP2 in the brain represent uncharacterized hyperphosphorylated forms, we would expect reduced or abolished association with eIF4E, as is the case for hyperphosphorylated 4E-BP1. To address this, Far Western (FW) blotting was performed with a radiolabeled, recombinant eIF4E protein probe on brain lysates from adult wild type and 4E-BP2 null mice. eIF4E bound similarly to each of the three 4E-BP2 species (Figure 2.1B; compare signals from FW blot to lower panel WB blot), indicating that these forms do not comprise the canonical phosphorylated forms which do not bind to eIF4E. Further, λ phosphatase treatment of the wild type brain extract failed to alter the pattern of 4E-BP2 migration (Figure 2.1C; upper panel), despite efficacy towards phosphorylated threonines preceeding prolines as assayed by an antibody directed against phospho-Thr-Pro (Figure 2.1C; lower panel). Taken together, these results strongly suggest that 4E-BP2 in the adult mammalian brain is modified in some manner other than by phosphorylation.

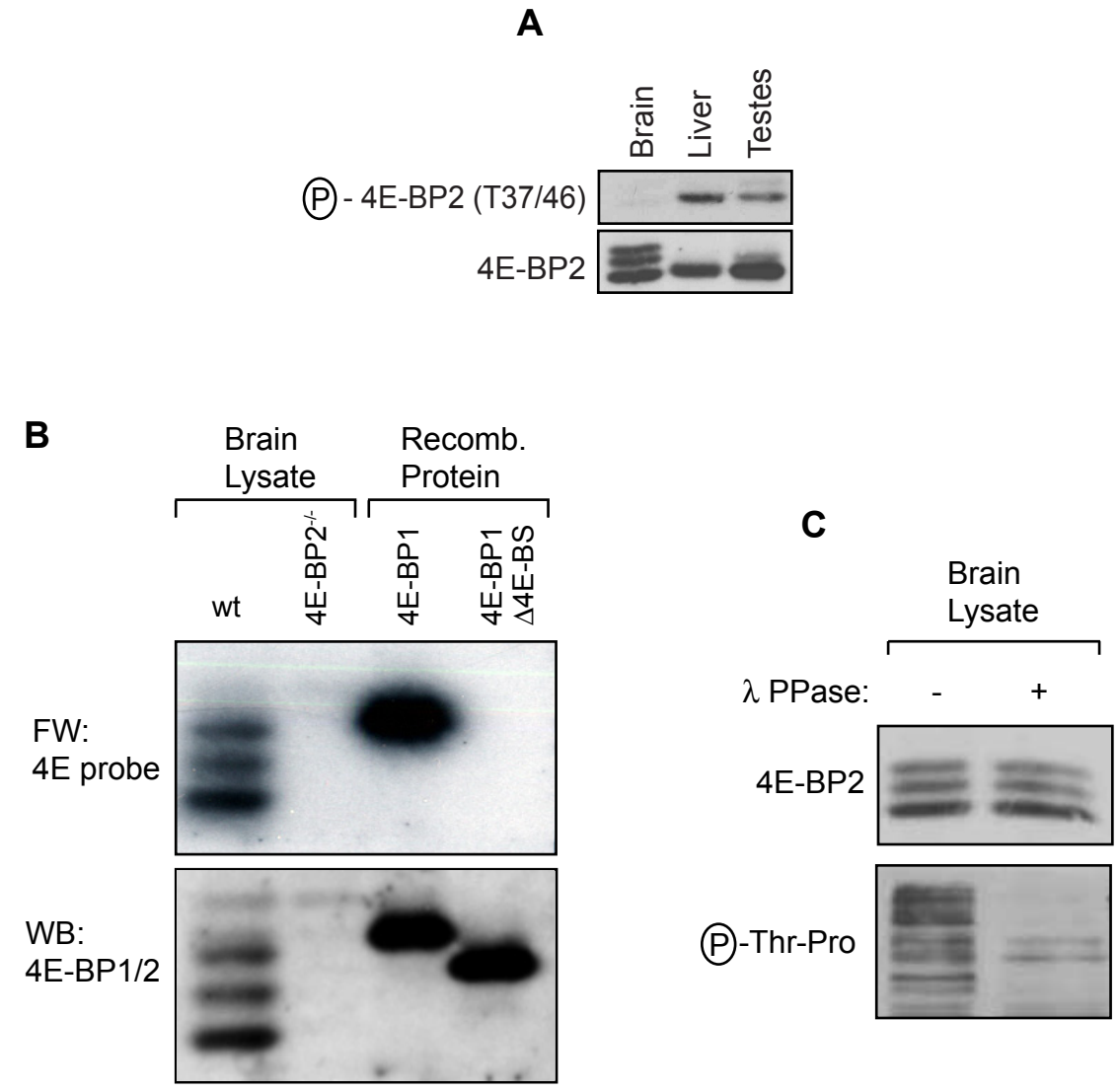



**Figure 2.1 The slow-migrating forms of 4E-BP2 in the adult mouse brain are not due to phosphorylation.**
(A) Western blotting failed to detect 4E-BP2 phosphorylation and a slow-migrating form of 4E-BP2 is unique to the brain. Lysates from tissues of 12 week-old mice were resolved by SDS-PAGE and phosphorylation of 4E-BP2 was detected with an antibody specific for T37/T46. The membrane was stripped and reprobed with 4E-BP2 antibody. (B) Far Western binding of eIF4E to the three brain-specific 4E-BP2 species. Radiolabeled recombinant FLAG-HMK-eIF4E protein was used as a probe to assay the interaction with 4E-BP2 protein in brain lysates (75μg) separated by 15% SDS-PAGE and immobilized on a nitrocellulose membrane. Recombinant wild type 4E-BP1 and 4E-BP1 lacking the 4E-binding site (Δ51-63) control for probe specificity. (C) 4E-BP2 from mouse brain is insensitive to phosphatase treatment. Brain lysates were incubated with λ phosphatase for 45 minutes at 30$^{o}$C followed by SDS-PAGE and western blotting for 4E-BP2. The membrane was stripped and reprobed with phospho-Thr-Pro motif antibody to confirm efficiency of the phosphatase treatment (lower panel).

**Asparagine Deamidation Causes the Slow Migrating Forms of 4E-BP2 in the Brain**

No alternatively spliced 4E-BP2 transcripts have been identified; consequently it is conceivable that a posttranslational modification accounts for the novel forms found in the brain. To identify this modification, endogenous 4E-BP2 protein was purified from adult murine brains and mass spectrometry was performed. This analysis detected asparagine deamidation in a region containing six closely-spaced asparagines near the C-terminus (Appendix 1, Figure S3). Deamidation is the spontaneous (non-enzymatic) conversion of asparagine residues to aspartates. The identity of the adjacent C-terminal amino acid (Robinson et al., 1970; Robinson et al., 1973; Robinson and Robinson, 2001b) and the proximity to flexible regions of the peptide (Xie and Schowen, 1999) largely influence this process. The rate of deamidation is also increased under alkaline conditions, thus facilitating analysis of susceptible residues (Deverman et al., 2002; Johnson et al., 1989; Robinson and Rudd, 1974). Purified recombinant 4E-BP2 was therefore treated over a range of alkalinity. Incubation for six hours led to the appearance of a single, slower migrating form in SDS-PAGE, while an additional species was noticed with protracted incubation (18 hours) at greater pH values (Figure 2.2A). This demonstrates that 4E-BP2 undergoes asparagine deamidation. A similar decrease in the electrophoretic mobility of Bcl-$X_L$ upon aspargine deamidation has been reported (Deverman et al. 2002). To confirm that deamidation is the modification found in the brain, mouse embryonic fibroblasts (MEFs) were starved of serum and amino acids to minimize 4E-BP2 phosphorylation and the cell lysates were subjected to neutral or alkaline conditions. A 4E-BP2 migration pattern similar to that described above was observed under alkaline conditions (Figure 2.2B). The lysates were then analysed alone or

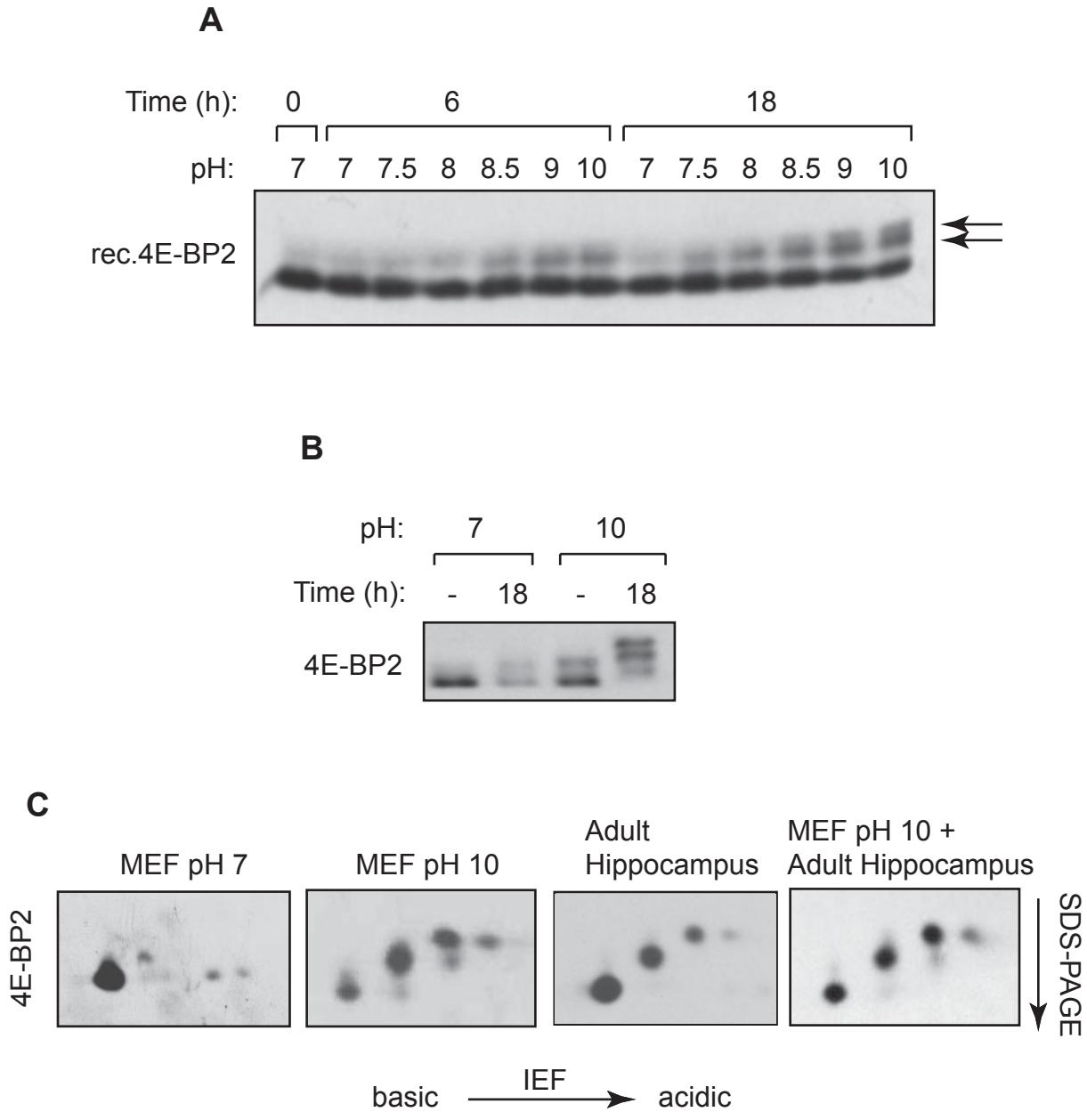



**Figure 2.2 4E-BP2 is susceptible to asparagine deamidation, which occurs spontaneously in the mammalian hippocampus.**
(A) Deamidation of recombinant 4E-BP2 is induced with elevated pH. Purified, recombinant 4E-BP2 was diluted in 0.15M Tris-HCl at the indicated pH and incubated for six or 18 hours. Proteins were resolved by 15% SDS-PAGE and analysed by Western blotting. Arrow heads indicate deamidated forms of 4E-BP2. (B) Deamidation of 4E-BP2 in cell lysates. MEFs were starved of serum for 24 hours, followed by incubation in PBS for an additional hour. The cells were then lysed at the indicated pH and the extracts were incubated for 18 hours prior to Western blotting. (C) 4E-BP2 deamidation caused by alkaline treatment or occurring spontaneously in hippocampus has identical migration pattern in two-dimensional isoelectric focusing/SDS-PAGE. Cells or adult hippocampi were homogenized and incubated as in (B). Seventy micrograms of each sample or a 1:1 mixture of pH10-treated MEF extract and adult brain (total 140μg) were analysed by IEF/SDS-PAGE. The electrophoretic dimensions applied to each sample are indicated.

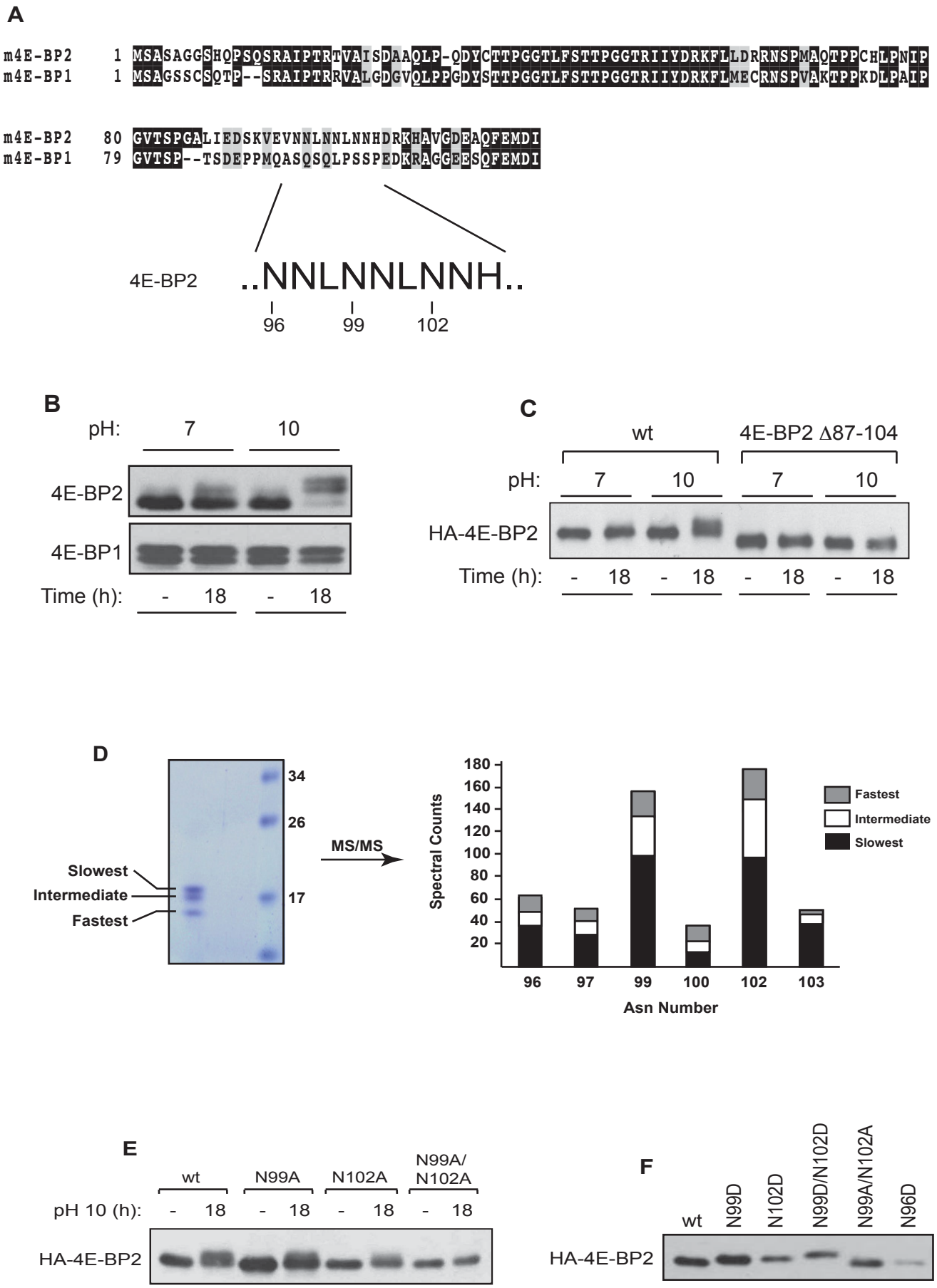
in a mixture with adult brain lysate by IEF/SDS-PAGE. Alkaline-induced deamidation of 4E-BP2 from MEFs produced a migration pattern similar to that observed in the adult brain (Figure 2.2C; compare the two middle panels). Strikingly, the migration was identical when the MEF and adult brain extracts were mixed, as evidenced by the perfect superimposition of the spots (Figure 2.2C; right panel). The normalization of signal intensity of the three principal forms indicated equal input from both lysates. These results confirm that 4E-BP2 in the brain is modified by asparagine deamidation.

**Asparagines 99 and 102 are the Sites of 4E-BP2 Deamidation**

We questioned whether asparagine deamidation is a general property of 4E-BPs or is unique to 4E-BP2. Alignment of the amino acid sequences of murine 4E-BP1 and 4E-BP2 revealed that 4E-BP2 contains six closely-spaced asparagines near the C-terminus (Figure 2.3A; the inset shows the numbered positions of these residues). Incubation of MEF lysates at alkaline pH caused a change in 4E-BP2, but not 4E-BP1, migration (Figure 2.3B); therefore deamidation is specific to 4E-BP2. Moreover, the 4E-BP2 banding pattern produced by this treatment was equivalent to that of brain lysate (Appendix 1, Figure S4). Next, 3HA-tagged wild type 4E-BP2 and a mutated version lacking the asparagine-rich region (Δ87-104) were expressed in HEK293 cells and alkaline treatment was performed. The deletion variant exhibited no reduction in electrophoretic mobility following incubation at pH 10, confirming that 4E-BP2 deamidation occurs on the six C-terminal asparagines (Figure 2.3C; the exogenously expressed proteins do not migrate in a pattern similar to endogenous 4E-BP2 owing to the presence of the 3HA-tag).

IEF/SDS-PAGE analysis of 4E-BP2 revealed two principal deamidation events in the adult brain (Figure 2.2C). To determine which asparagines undergo deamidation, recombinant 4E-BP2 was treated at high pH for 24 hours. The resolved species were then excised from the gel (Figure 2.3D; left panel) and analyzed by mass spectrometry (LC-MS/MS). Because deamidation is non-enzymatic and is an intrinsic property of an asparagine within a peptide, the susceptible residues determined in this analysis correspond to those occurring *in vivo*. As expected, the slowest migrating 4E-BP2 species contained the greatest amount of deamidation. While some deamidation was detected on all six Asn residues, a clear preference for aspartate formation was observed at positions 99 and 102 (Figures 2.3D and Appendix 1, Figure S5) as judged by spectral counting. Mutating both of these residues to alanines (N99A/N102A) confirmed this finding as it prevented the alkaline-induced deamidation shift (Figure 2.3E). The single alanine variants (N99A and N102A) reduced deamidation relative to the wild type protein but did not abolish it (Figure 2.3E). This suggests that there is no requirement for deamidation at one residue to precede that at the other. Asparagines 99 and 102 were then mutated to aspartates to replicate the *in vivo* deamidated 4E-BP2. As is the case for *in vivo* 4E-BP2 deamidation, the migration was reduced comparably by each of the single aspartate variants (N99D or N102D) and more so by N99D/N102D (Figure 2.3F), the form uniquely found in the brain. Notably, the migration of the N96D variant was unchanged, despite the fact that asparagine 96 has a similar sequence context to asparagines 99 and 102, further supporting our assignment of the deamidated residues. A similar finding was reported for human erythrocyte protein 4.1 in that only one of two proximal asparagines

**Figure 2.3 Deamidation of 4E-BP2 occurs on asparagines 99 and 102.**
(A) Amino acid sequence alignment of murine 4E-BP2 and 4E-BP1. Inset shows the asparagine-rich sequence of 4E-BP2 (B) 4E-BP2, but not 4E-BP1, is sensitive to deamidation induced by alkaline treatment in MEFs. MEFs were lysed and incubated as in (Figure 2B), followed by Western blotting against 4E-BP2 and 4E-BP1. (C) A 4E-BP2 variant lacking the asparagine-rich sequence is not deamidated by alkaline treatment. HEK293E cells were transfected with 3HA-tagged wild type 4E-BP2 or 4E-BP2 lacking residues 87-104. Cells were harvested 24 hours post-transfection, lysed at pH 7 or 10, incubated as indicated and western blotting was performed. (D) Mass spectrometry analysis indicates Asn99 and Asn102 are favoured for deamidation. Purified, recombinant wild type 4E-BP2 protein was *in vitro* deamidated in 0.15M TRIS, pH 10 for 24 hours at 37$^{o}$C. The modified protein was separated on 10-20% gradient SDS-PAGE gel, stained with GelCode Blue reagent (left panel), and excised bands were analysed by LC-MS/MS. (E) Mutation of Asn99 and Asn102, and not of Asn96, to aspartates recapitulates the slower electrophoretic mobility of deamidated 4E-BP2. The cDNA of human 4E-BP2 was mutated by substituting aspartates for asparagines as indicated and expressed in HEK293E cells as fusions to N-terminal 3HA epitope. Cells were harvested 24 hours post-transfection and Western blotting was performed. (F) Mutation of both Asn99 and Asn102 to alanines precludes alkaline-induced deamidation. HEK293E cells were transfected with the 3HA-tagged proteins as indicated (prepared as in (E)), cells were harvested 24 hours later, lysates were incubated as in (C) and Western blotting was performed.


A
m4E-BP2 1 MSASAGGSHQPSQSRAIPTRTVAISDAAQLP-QDYCTTPGGTLFSTTPGGTRIIYDRKFLLDRRNSPMAQTPPCHLPNIP
m4E-BP1 1 MSAGSSCSQTP--SRAIPTRRVALGDGVQLPPGDYSTTPGGTLFSTTPGGTRIIYDRKFLMECRNSPVAKTPPKDLPAIP
m4E-BP2 80 GVTSPGALIEDSKVEVNNLNNLNNHDRKHAVGDEAQFEMDI
m4E-BP1 79 GVTSP--TSDEPPMQASQSQLPSSPEDKRAGGEESQFEMDI
4E-BP2 ..NNLNNLNNH..
96 99 102
B
pH: 7 10
4E-BP2
4E-BP1
Time (h): - 18 - 18
C
wt
4E-BP2 Δ87-104
pH: 7 10 7 10
HA-4E-BP2
Time (h): - 18 - 18 - 18 - 18
D
34
26
17
Slowest
Intermediate
Fastest
MS/MS
Spectral Counts
180
160
140
120
100
80
60
40
20
96 97 99 100 102 103
Asn Number
Fastest
Intermediate
Slowest
E
wt
N99A
N102A
N99A/
N102A
pH 10 (h): - 18 - 18 - 18 - 18
HA-4E-BP2
F
wt
N99D
N102D
N99D/N102D
N99A/N102A
N96D
HA-4E-BP2

was subject to *in vivo* deamidation, despite both lying within a similar molecular context (Inaba et al., 1992).

**Deamidated 4E-BP2 Exhibits Enhanced Raptor-Binding and Reduced eIF4E-Association**

We considered the possibility that the proximity of the deamidation sites to the C-terminal TOR signaling motif might affect the interaction of 4E-BP2 with raptor. This is plausible in light of recent findings that the TOS motif in 4E-BP1 alone cannot account for efficient raptor-binding (Lee et al., 2008). Considerably more deamidated (N99D/N102D) 4E-BP2 co-immunoprecipitated with Myc-tagged raptor than did wild type or 4E-BP2 N99A/N102A (Figure 2.4A). Deletion of the TOS motif from 4E-BP2 (ΔTOS) abolished its interaction with raptor. We then wondered whether 4E-BP2 deamidation could affect its *in vivo* interaction with eIF4E. The amount of deamidated 4E-BP2 associated with eIF4E on $m^7$GDP resin was reduced in HEK293E cells (Figure 2.4B). As 4E-BP2 phosphorylation was not detected in mature brain, the association of 4E-BP2 and eIF4E was monitored in conditions favouring diminished 4E-BP2 phosphorylation owing to reduced mTORC1-activation. In the absence of serum (-FBS) alone or serum and nutrients (PBS) together, a marked reduction in eIF4E-bound deamidated 4E-BP2 was still observed (Figures 2.4C and 2.4D; expression of exogenous 4E-BP2 variants was reduced upon PBS treatment). This effect was dependent on interaction with raptor as deamidated 4E-BP2 lacking the TOS motif displayed a greater or equal association with eIF4E compared to wild type protein (Figure 2.4D). Precipitation of eIF4E from brain lysate confirmed these findings *in vivo*; comparison of

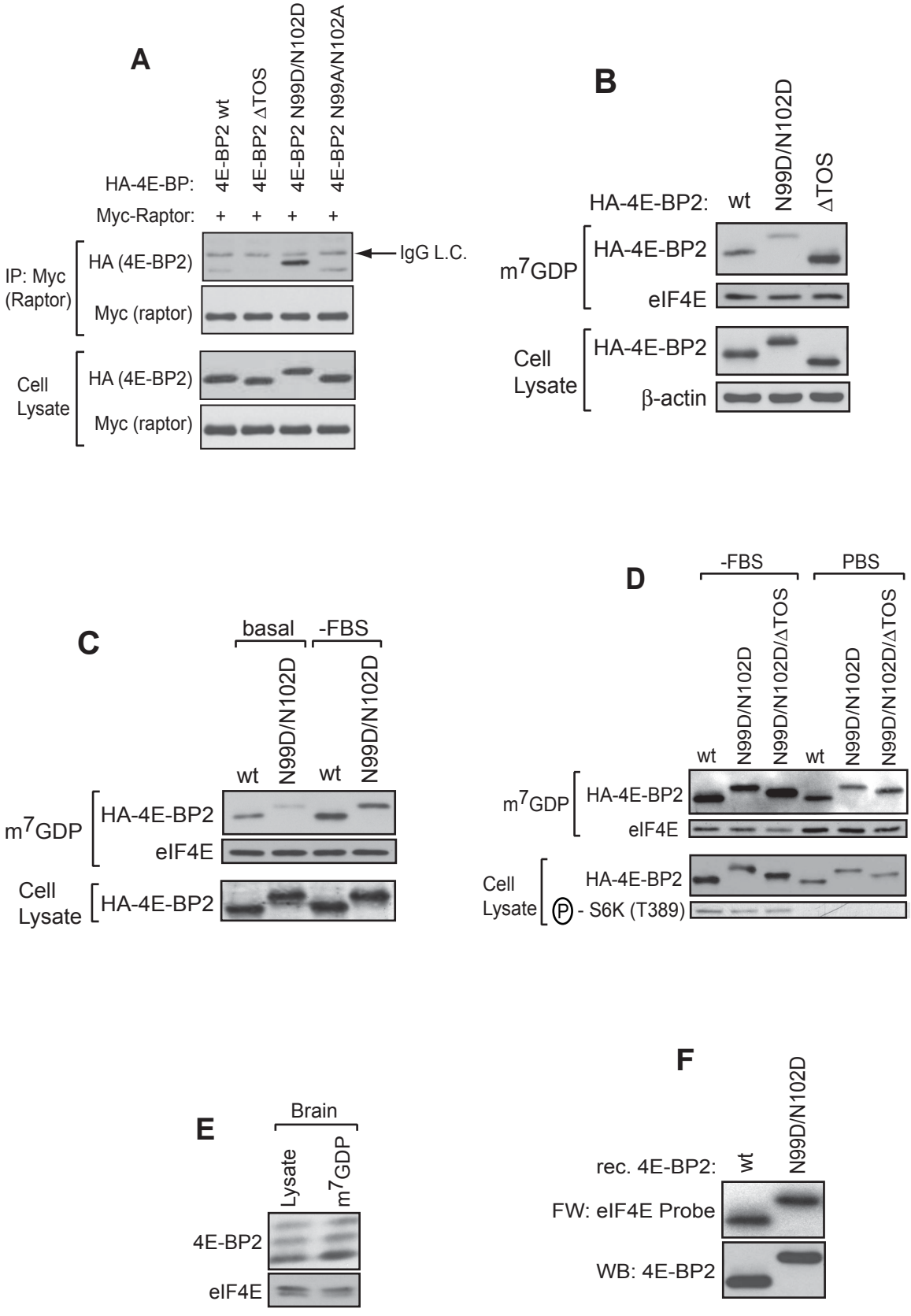
the ratio of deamidated 4E-BP2 to unmodified 4E-BP2 between $m^7$GDP pulldowns and lysates indicated that more unmodified 4E-BP2 (fastest migrating) was recovered with eIF4E than were the deamidated forms (Figure 2.4E). In agreement with these results, pull-down assays using recombinant 4E-BP1 variants as bait in cell lysates demonstrated increased eIF4E association with the variant containing a mutated TOS sequence relative to wild type 4E-BP1 (Wang et al., 2006). Far Western analysis with radiolabelled eIF4E of brain lysates (Figure 2.1C) or recombinant proteins (Figure 2.4F) yielded no difference in binding of eIF4E to wild type or deamidated 4E-BP2. As these assays involve the direct interaction between eIF4E and non-phosphorylated 4E-BP2 in the absence of Raptor, the results are consistent with the above finding that the attenuated association of deamidated 4E-BP2 with eIF4E is effected by raptor. Overall, this series of experiments suggest that the deamidation of 4E-BP2 increases its association with Raptor and, consequently, reduces binding to eIF4E relative to wild type 4E-BP2.

**Diminished 4E-BP2 Phosphorylation During Postnatal Brain Development Correlates With the Onset of Deamidation**

The absence of phosphorylated 4E-BP2 in the mature brain is at variance with earlier reports of robust phosphorylation of 4E-BP proteins in *ex vivo* dissociated neuron cultures (Kelleher et al., 2004a; Takei et al., 2001). These cultures are normally prepared from embryonic or early postnatal rodent brain regions and are often analysed less than 10 days after initial plating. The possibility of a developmental regulation of 4E-BP2 phosphorylation was therefore considered. Western blotting was performed on hippocampal lysates collected during early postnatal development. Phosphorylation at

**Figure 2.4 Deamidated 4E-BP2 exhibits increased raptor-interaction and reduced association to eIF4E.**

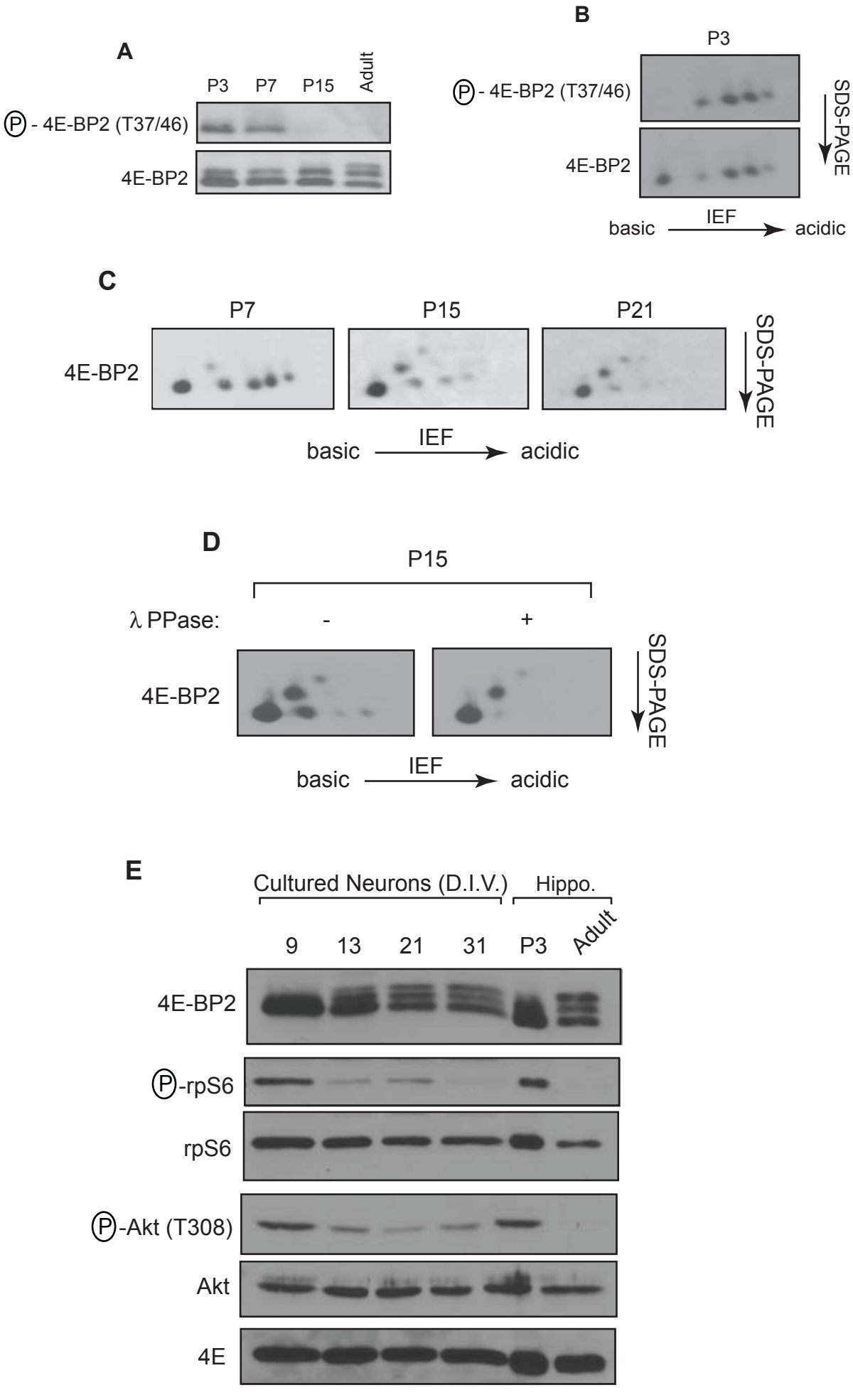
(A) Co-Immunoprecipitation of raptor and deamidated 4E-BP2 reveals enhanced association. HEK293 cells were transfected with Myc-tagged raptor and 3HA-tagged 4E-BP2 constructs as shown. Cells were harvested 24 hours post-transfection and 200μg of lysates were used for immunoprecipitated with anti-Myc antibody followed by Western blotting (7-15% SDS-PAGE). (B) Deamidated 4E-BP2 is more weakly associated to eIF4E in cells grown in complete medium. HEK293E cells were transfected with the indicated 4E-BP2-encoding plasmids and harvested 24 hours later. Lysates (200μg) were incubated with 30μl of $m^7$GDP agarose for 1.5 hours and Western blotting (15% SDS-PAGE) was performed. (C and D) Deamidated 4E-BP2 is more weakly associated to eIF4E in the absence of serum or nutrients and this is dependent on interaction with raptor. HEK293E cells were transfected and $m^7$GDP pulldowns and Western blotting were performed as in (B) after cells had been starved of serum for 24 hours or incubated in D-PBS for 1 hour. (E) $m^7$GDP pulldowns from brain lysates and Western blotting were performed as in (B) (F) Deamidation of 4E-BP2 does not affect eIF4E-binding in the absence of raptor. Far Western blotting was performed as in Figure 1C against recombinant wild type and 4E-BP2 N99/102D that were resolved by 15% SDS-PAGE. The same membrane was reprobed with anti-4E-BP2 (#11211) antibody (Western blot, lower panel).


A
4E-BP2 wt
4E-BP2 ΔTOS
4E-BP2 N99D/N102D
4E-BP2 N99A/N102A
HA-4E-BP:
Myc-Raptor: + + + +
IP: Myc (Raptor)
HA (4E-BP2)
Myc (raptor)
IgG L.C.
Cell Lysate
HA (4E-BP2)
Myc (raptor)
B
HA-4E-BP2: wt
N99D/N102D
ΔTOS
m7GDP
HA-4E-BP2
eIF4E
Cell Lysate
HA-4E-BP2
β-actin
C
basal
-FBS
wt
N99D/N102D
wt
N99D/N102D
m7GDP
HA-4E-BP2
eIF4E
Cell Lysate
HA-4E-BP2
D
-FBS
PBS
wt
N99D/N102D
N99D/N102D/ΔTOS
wt
N99D/N102D
N99D/N102D/ΔTOS
m7GDP
HA-4E-BP2
eIF4E
Cell Lysate
HA-4E-BP2
Ⓟ - S6K (T389)
E
Brain
Lysate
m7GDP
4E-BP2
eIF4E
F
rec. 4E-BP2:
wt
N99D/N102D
FW: eIF4E Probe
WB: 4E-BP2

Thr37/46 sharply receded during the first two weeks of postnatal brain development [postnatal days (P) 3, 7 and 15; Figure 2.5A]. Concomitantly, the slower migrating deamidated forms of 4E-BP2 appeared during this time frame and persisted in the adult. Exposure of the P3 lysate to high pH induced the appearance of the doubly deamidated (N99D/N102D) form, while in the adult the same treatment merely increased the intensity of the doubly deamidated band which is already present at this stage (Appendix 1, Figure S6). IEF/SDS-PAGE analysis of the P3 hippocampal lysate indicated that the phosphorylated 4E-BP2 species, which resolve in a horizontal pattern, are more abundant than unmodified 4E-BP2 (Figure 2.5B). Between one and three postnatal weeks, 4E-BP2 phosphorylation decreased to a fraction of the total pool, while at the same time the appearance of the deamidated forms was observed (slower migrating pattern, approximately $45^{o}$ above horizon) (Figure 2.5C). Phosphatase treatment of the P15 lysate abolished the horizontal modification series (Figure 2.5D), thereby confirming its assignment as multiple phosphorylation events, while the deamidated forms were unaffected.

We next asked whether the activities of components of the PI3K-Akt-mTOR signaling module are affected in a similar manner as 4E-BP2 during development. Brain lysates from P3 and adult mice were examined, as were lysates from dissociated hippocampal neuron cultures at several days following plating (days *in vitro*; DIV). The phosphorylation of both Akt (Thr308) and ribosomal protein S6 (Ser240/244), a downstream target of mTORC1, decreased dramatically in the adult brain as compared to P3 and this effect was mirrored in cultured neurons between days 9 and 31 (Figure 2.5E).

**Figure 2.5 Deamidation of 4E-BP2 occurs during early postnatal brain development and correlates with the loss of phosphorylation**.
(A) Western blot analysis (15% SDS-PAGE), using a phosphorylation-specific antibody to the priming sites of 4E-BP proteins (Thr37/46) and anti-4E-BP2 antibody (lower panel), of hippocampal extracts (30μg each) from several early postnatal days compared with adult. (B) IEF/SDS-PAGE followed by Western blotting of P3 postnatal hippocampal extracts. Extracts (70μg) were subjected to isoelectric focusing followed by 15% SDS-PAGE and Western blotting with antibodies as in (A). The electrophoretic dimensions are indicated. (C) Analysis as in (B) of hippocampal extracts from indicated postnatal days. (D) Phosphatase treatment of postnatal day 15 (P15) hippocampal extracts followed by IEF/SDS-PAGE and Western blotting. (E) SDS-PAGE analysis of lysates from dissociated hippocampal neuron cultures or brain from indicated days.


A
P3
P7
P15
Adult
Ⓟ - 4E-BP2 (T37/46)
4E-BP2
B
P3
Ⓟ - 4E-BP2 (T37/46)
4E-BP2
SDS-PAGE
basic
IEF
acidic
C
P7
P15
P21
4E-BP2
SDS-PAGE
basic
IEF
acidic
D
P15
λ PPase:
-
+
4E-BP2
SDS-PAGE
basic
IEF
acidic
E
Cultured Neurons (D.I.V.)
Hippo.
9
13
21
31
P3
Adult
4E-BP2
Ⓟ-rpS6
rpS6
Ⓟ-Akt (T308)
Akt
4E

Decremental S6 phosphorylation was also recently observed during neuronal retinal ganglion cell development (Park et al., 2008). Importantly, the deamidation of 4E-BP2 was observed in the cultured preparations, just as *in vivo* (Figure 2.5E; top panel). These observations, together with the raptor-dependent reduction of eIF4E-binding (Figures 2.4C and 2.4D), suggest the possibility of a compensatory function for 4E-BP2 deamidation by permitting protein synthesis in spite of reduced upstream signaling.

**Expression of Deamidated 4E-BP2 in 4E-BP2-/- Neurons Alters the Kinetics of Excitatory Synaptic Transmission**

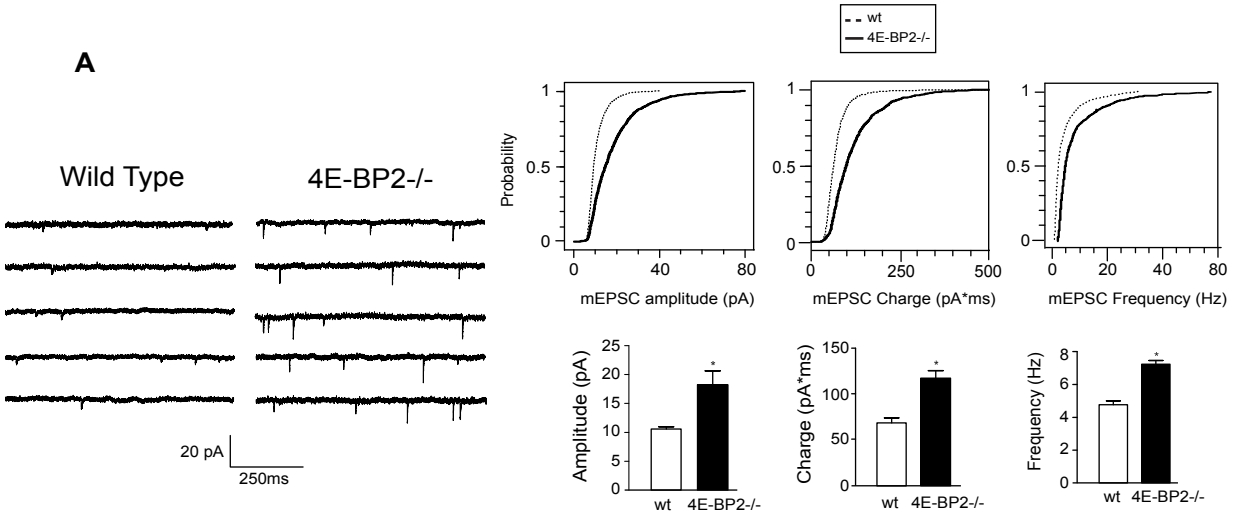
Since 4E-BP2 knock-out mice display altered hippocampal long-term synaptic plasticity (Banko et al. 2005, 2006), we wondered whether 4E-BP2 deamidation impacts excitatory postsynaptic transmission at synapses in mature hippocampal synaptic networks. To pursue this, miniature excitatory postsynaptic currents (mEPSCs) mediated by AMPA receptors were recorded at the soma of CA1 pyramidal neurons from organotypic hippocampal slice cultures. These measurements represent the postsynaptic current produced by the stimulus-independent release of single glutamate neurotransmitter vesicles. As a rule, mEPSC amplitude largely reflects the number of AMPA receptors in the postsynaptic membrane, frequency depends on both the probability of presynaptic transmitter release and the number of active synapses, while charge transfer is the cumulative ion flow through open receptor channels (Lisman et al., 2007). First, mEPSCs were compared between neurons from wild type and 4E-BP2-/- slices. 4E-BP2-/- neurons exhibited an approximately two-fold increase in the amplitude, frequency, and charge transfer of excitatory currents relative to wild type controls (Figure 2.6A). These results

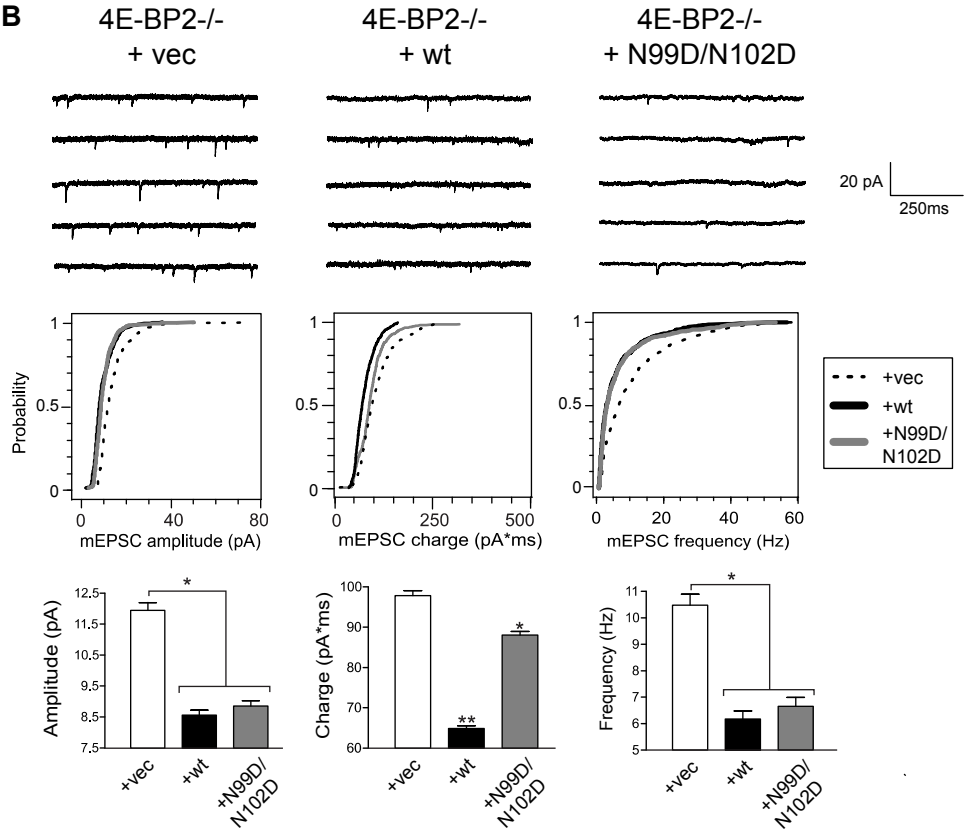
indicate that excitatory synaptic activity in pyramidal cells is increased in the absence of 4E-BP2. Next, the specific function of wild type 4E-BP2 or deamidated 4E-BP2 (N99D/N102D) proteins in mEPSC transmission was examined by their introduction into 4E-BP2-/- slice cultures. Identification of transfected cells was accomplished by co-expressing 4E-BP2 variants and eGFP from the same plasmid. Although, mEPSC amplitude and frequency were similarly repressed by expression of wild type or deamidated 4E-BP2 (Figure 2.6B), it is remarkable that the mEPSC charge transfer was only partially rescued by 4E-BP2 N99D/N102D. This incomplete repression suggests that deamidated 4E-BP2 has an altered effect on mEPSC kinetics. Indeed, our most significant observation is that mEPSCs in 4E-BP2-/- neurons expressing deamidated 4E-BP2 exhibit slower rise and decay times in comparison to those expressing wild type 4E-BP2 or GFP alone (Figure 2.6C). A role for 4E-BP2 deamidation is thus implied in the timing of excitatory postsynaptic signals independently of its effect on synaptic strength. Possible mechanistic explanations for this finding are addressed in the Discussion.

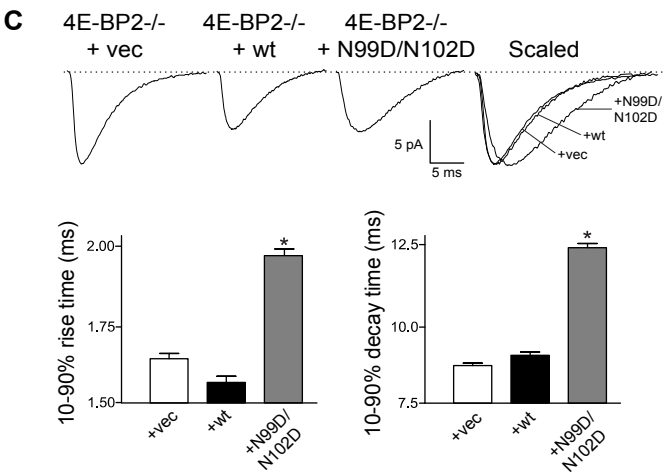
## 2.4 Discussion

We present here a developmentally patterned posttranslational modification of 4E-BP2 in the brain. Asparagine deamidation of 4E-BP2 during postnatal brain development converts asparagines 99 and 102 into aspartates. This elicits a gain of function activity by augmenting the association of 4E-BP2 with the mTORC1 scaffolding protein, raptor. Most strikingly, expression of deamidated 4E-BP2 in neurons from 4E-BP2-/- hippocampal organotypic slices cultures is associated with slower kinetics of miniature postsynaptic currents. Thus, our report describes both a novel, tissue-specific

**Figure 2.6 Expression of deamidated 4E-BP2 in 4E-BP2-/- neurons reduces elevated excitatory synaptic transmission but slows kinetics of mEPSCs.**
(A) Increased excitatory miniature synaptic activity in slices from 4E-BP2$^{-/-}$ mice. *Left*: Traces of mEPSCs fom 4E-BP2$^{-/-}$ and wild type mice. *Right*: Cumulative probability plots and summary bar graph for all cells showing increases in mEPSC amplitude, charge and frequency. Data are from 6 neurons per genotype with 200 events per neuron. (Data are means ± SEM. $P < 0.05$. Kolmogorov-Smirnov test). (B) Expression of deamidated or wild type 4E-BP2 in 4E-BP2-/- neurons. *Top*: mEPSC traces from 4E-BP2$^{-/-}$ organotypic slices transfected with empty vector (vec), wildtype 4E-BP2, and deamidated 4E-BP2 (N99D/N102D). The plasmid expresses eGFP from an IRES element. *Middle*: Cumulative probability plots of mEPSC amplitude, charge, and frequency. *Bottom*: Summary of effects on mEPSC amplitude, charge and frequency. Data are from 6 neurons per condition with 150 events per neuron. (* $P < 0.05$, ** $P < 0.01$. One-way ANOVA). (C) Partial repression of mEPSC charge by deamidated 4E-BP2 is associated with slower kinetics. *Left*: Averaged mEPSCs (150 events) from neurons transfected as in (B). Superimposed scaled responses demonstrate slower time course of average mEPSC in cells expressing deamidated 4E-BP2. *Right*: Summary bar graphs of rise and decay times. (N=6, $P < 0.01$. One-way ANOVA).


A
Wild Type
4E-BP2-/-
20 pA
250ms
wt
4E-BP2-/-
Probability
mEPSC amplitude (pA)
mEPSC Charge (pA*ms)
mEPSC Frequency (Hz)
Amplitude (pA)
Charge (pA*ms)
Frequency (Hz)


B
4E-BP2-/-
+ vec
4E-BP2-/-
+ wt
4E-BP2-/-
+ N99D/N102D
20 pA
250ms
Probability
mEPSC amplitude (pA)
mEPSC charge (pA*ms)
mEPSC frequency (Hz)
+vec
+wt
+N99D/
N102D
Amplitude (pA)
Charge (pA*ms)
Frequency (Hz)


C
4E-BP2-/-
+ vec
4E-BP2-/-
+ wt
4E-BP2-/-
+ N99D/N102D
Scaled
5 pA
5 ms
+N99D/
N102D
+wt
+vec
10-90% rise time (ms)
10-90% decay time (ms)

posttranslational modification of 4E-BP2 and functions for the modified protein in altering binding to mTORC1 and synaptic transmission. The genetic encoding of asparagines that are susceptible to deamidation appears to be selected for and may act as 'molecular clock' to regulate important biological events (Robinson et al., 1970; Robinson and Robinson, 1991; Robinson and Robinson, 2001b). Therefore, the fact that the C-terminal, deamidation sequence is perfectly conserved in 4E-BP2 in mammals but not in lower organisms, (Appendix 1, Figure S7) suggests a role in cognitive function.

The interaction of deamidated 4E-BP2 with raptor is enhanced relative to the wild type form. Altered protein-protein interactions have also been shown to be a consequence of deamidation of Bcl-$X_L$ and fibronectin (Curnis et al., 2006; Deverman et al., 2002). 4E-BP2 deamidation therefore appears to have an auxiliary function in the TOS-dependent binding of 4E-BP2 to raptor. This is consistent with recent findings of an insufficiency of the 4E-BP1 TOS motif for efficient Raptor-binding (Lee et al., 2008) and suggests that negative charges in the non-conserved C-terminal region of 4E-BPs, such as the constitutive phosphorylation of non-conserved Ser101 of 4E-BP1 (Wang et al., 2003), may enhance this interaction.

The diminution in eIF4E-binding of deamidated 4E-BP2 persists in conditions of reduced mTORC1 activity and is dependent on interaction with raptor. This raises the intriguing possibility that this postnatal 4E-BP2 modification compensates for reduced upstream signaling in the developing brain. This could permit sufficient translation required for numerous processes, including proper maintenance of synaptic activity. How does the

interaction of deamidated 4E-BP2 with raptor lead to reduced binding to eIF4E? It is possible that raptor spatially sequesters 4E-BP2 away from eIF4E. This is conceivable in light of previous reports demonstrating 4E-BP-dependent nuclear accumulation of eIF4E upon inhibition of mTORC1 (Rong et al., 2008) and raptor-dependent cytoplasmic to perinuclear localization of mTOR following release from amino-acid deprivation (Sancak et al., 2008). Thus, the partitioning of 4E-BP2 binding between eIF4E and raptor may favour interaction and co-localization with raptor at the expense of eIF4E upon deamidation. In neurons, such an effect could occur locally in dendrites as opposed to the cell body. Similarly, it is possible that enhanced phosphorylation of deamidated 4E-BP2 occurs at activated synapses in dendrites, consistent with the previous immunofluorescent detection of total 4E-BP2 in dendritic puncta (Tang et al., 2002) and DHPG-induced phosphorylated 4E-BP2 (T37/T46) in the Stratum radiatum (Banko et al., 2006). Yet, our inability to detect phosphorylated 4E-BP2 in the adult brain suggests that such local phosphorylation may be largely diluted and obscured in whole cell or tissue extracts.

Pyramidal 4E-BP2-/- neurons displayed enhancement in mEPSC amplitude, frequency, and charge transfer. Since expression of wild type or deamidated 4E-BP2 in post-synaptic 4E-BP2-/- neurons abolished the increase in amplitude and frequency, it is very likely that predominantly post-synaptic changes, such as surface AMPA receptor expression, mediate these enhancements (Malinow and Malenka, 2002). In contrast, charge transfer was only partially repressed by expression of deamidated 4E-BP2, as compared to wild type 4E-BP2, in 4E-BP2-/- neurons. Consistent with this finding, a salient feature of our electrophysiological analysis is that mEPSCs in 4E-BP2-/- neurons expressing

deamidated 4E-BP2 have slower kinetics (rise and decay times) relative to those expressing wild type 4E-BP2 or vector controls. This could favour enhanced integration of signals and, thus, plasticity as mEPSCs influence neuronal functions in firing, synaptic restructuring, and activation of postsynaptic signaling cascades (Carter and Regehr, 2002; McKinney et al., 1999; Murphy et al., 1994; Sharma and Vijayaraghavan, 2003).

How might deamidated 4E-BP2 influence mEPSC kinetics? One possibility is based on cable theory, which posits that the magnitude and kinetics of currents originating from distal synapses become attenuated to a greater extent, while approaching the cell body, than those more proximal to the soma (Bekkers and Stevens, 1996; Williams and Johnston, 1991). AMPA receptor-mediated mEPSC amplitudes recorded at the soma in hippocampal neurons are relatively independent of synapse location (Magee and Cook, 2000; Smith et al., 2003). Thus, expression of deamidated 4E-BP2 might favour currents emanating from distal dendritic regions. Wild type 4E-BP2, though, would not be expected to exhibit such spatial selectivity, since its expression did not alter mEPSC kinetics. A second possibility is that AMPA receptor channel gating (activation and deactivation/desensitization) is protracted. This could be due to alterations in the subunit composition of the tetrameric AMPA receptors (Jonas, 2000; Verdoorn et al., 1991) or the relative expression of AMPAR-associated auxiliary proteins (Milstein and Nicoll, 2008), both of which are known to influence postsynaptic current kinetics. In particular, transmembrane AMPA receptor regulatory proteins (TARPs) (Coombs and Cull-Candy, 2009; Milstein and Nicoll, 2008) and cornichons (Schwenk et al., 2009) interact with AMPA receptors and slow gating kinetics and increase charge transfer.

Recent work reported that mEPSCs block local dendritic translation in order to provide a rapid response to postsynaptic activity (Sutton et al., 2006; Sutton et al., 2007; Sutton et al., 2004). It was shown that increased protein synthesis and synaptic efficacy homeostatically compensate for tonic inhibition of action potentials that was coupled with mEPSC blockade. In acute measurements (30 minutes), however, we find larger and more frequent mEPSCs in the absence of a translation inhibitor, a context which engenders enhanced synaptic transmission (Figure 2.6A). The initial reports demonstrating a role for 4E-BP2 in synaptic plasticity found no change in basal synaptic function upon genetic deletion of 4E-BP2 in mice (Banko et al., 2006; Banko et al., 2005). Our finding of enhanced mEPSCs in 4E-BP2-/- organotypic slices is not at variance with this conclusion as our results are based on non-evoked spontaneous quantal events, whereas the observations of Banko et al. were induced by synaptic network stimulation. Thus, we have revealed both a novel modification of 4E-BP2 in the brain and a function for it in synaptic activity.

## 2.5 Materials and Methods

### Expression Vectors

Human 4E-BP2 cDNA was subcloned into pCDNA3-3HA (Imataka et al. 1997), using *EcoRI* and *XhoI* restriction enzymes, to express a fusion protein with three N-terminal HA tags. Deamidation-site point mutations in 4E-BP2 were generated by PCR mutagenesis and ligated into pcDNA3-3HA at the same sites. Constructs expressing 4E-BP2 point mutants N96D, N99D, N102D, N99/102D contain an additional *XbaI* restriction site and

N99A, N102A, N99/102A contain an additional *MscI* restriction site. 4E-BP2 Δ87-104 was generated by PCR mutagenesis and ligated as above. 4E-BP2 ΔTOS and 4E-BP2 N99D/N102D ΔTOS mutants lack the last 6 C-terminal amino acids (Gln-Phe-Glu-Met-Asp-Ile). 4E-BP2 wild type and mutant cDNAs were subcloned, using *EcoRI/XhoI*, into pGEX-6P-1 (Pharmacia) for recombinant GST-tagged protein production. Wild type and N99D/N102D were PCR-amplified and subcloned into pIRES-GFP plasmid, at *XhoI*/*EcoRI*, for neuronal expression.

**Antibodies and Reagents**

The following antibodies were obtained from Cell Signaling Technology: 4E-BP2 (#2845), 4E-BP1 (#9452), Phospho-4E-BP1 Thr37/46 (#9459), and Phospho-Threonine-Proline (#9391). Antiserum #11211, which cross-reacts with both 4E-BP1 and 4E-BP2, was previously raised in-house (Gingras and Sonenberg, 1997). HA antibody was purchased from Covance (MMS-101R). Myc (9E10) antibody was from Santa Cruz (#sc-40). 7-methylguanosine 5'-disphosphate ($m^7$GDP) - agarose resin was prepared as previously described (Edery et al., 1988). Adipic acid dihydrazide-Agarose, $m^7$GDP, and GDP were purchased from Sigma. λ protein phosphatase was purchased from New England Biolabs.

**Expression and Purification of Recombinant Proteins**

Recombinant 4E-BP proteins were expressed in *Escherichia coli* BL21 (DE3) as fusions to N-terminal glutathione S-transferase (Rong et al.) and purified on Glutathione Sepharose-4B resin (GE Healthcare). The 4E-BP moiety of the fusion proteins were

released by on-resin cleavage for 20h at 4°C with PreScission Protease (GE Healthcare) and stored at -80°C in 10% glycerol. Recombinant FLAG-HMK-eIF4E protein (Pause et al., 1994) was expressed in *Escherichia coli* BL21 (DE3). Cells were lysed by sonication (in 20mM HEPES-KOH pH 7.4, 100mM KCl, 0.5mM EDTA, 7mM β-mercaptoethanol, and 0.5mM PMSF), and eIF4E was purified on $m^7$GDP-agarose resin as previously described (Edery et al., 1988)

**Cell Culture, Transfections and Alkaline Treatments**

HEK293 or HEK293E cells were maintained in DMEM containing 10% or 5% FBS (Gibco), respectively. For transfections, 1 million cells/well were seeded in six-well culture plates. The following day cells were transfected for 3-5 hours with 1μg of DNA plasmid using the Lipofectamine/Plus reagents (Invitrogen). Cells were harvested 24 hours post-transfection. Alkaline treatments on cell extracts were performed as previously described (Deverman et al., 2002). Alkalinization of recombinant 4E-BP2 protein was carried out at 37°C in 0.15M Tris-HCl, pH 7.0 or 10.0, for the indicated time and stopped by addition of 5X Laemmli sample buffer.

**Phosphatase Treatments**

Whole brains or dissected hippocampi of mice at the indicated ages were homogenized in 1X λ phosphatase buffer containing 0.5 μg/mL leupetin and 1mM PMSF. Extracts were diluted to 2μg/μl in a total volume of 90μl. Aliquots (9μl) of the phosphatase were added and the samples were incubated at 30°C for 45 minutes. The reactions were stopped by

addition of 5X SDS-PAGE Laemmli sample buffer or isoelectric focusing rehydration buffer and samples were stored at -20$^{o}$C until use.

**Western Blotting and Two-Dimensional Isoelectric Focusing/SDS-PAGE**

Cell or tissue extracts for Western blotting were prepared in homogenization buffer (10mM $K_3PO_4$/1mM EDTA, 10mM $MgCl_2$, 50mM β-glycerophosphate, 5mM EGTA, 0.5% NP-40, 0.1% Brij 35, 0.1% sodium deoxycholate, 1mM sodium orthovanadate, 1mM PMSF, 1μg/mL leupeptin) and proteins were resolved by SDS-PAGE. Purified recombinant proteins were diluted with 5X sample buffer prior to SDS-PAGE. Following electrophoresis, proteins were transferred to 0.2μM nitrocellulose membranes and Western blotting was performed. Proteins were visualized by treatment with Enhanced Chemiluminesce reagent (Perkin Elmer) and exposure to X-Ray films (Denville Scientific Inc.). For 2D-isoelectric focusing/SDS-PAGE, concentrated protein samples were diluted in rehydration buffer (8M urea, 2% w/v CHAPS, 10mM DTT, 0.5% v/v IPG pH 3-10 buffer) and isoelectric focusing was performed on 13cm polyacrylamide gel strips with a pH 3-10 gradient (GE Healthcare) using the following protocol: 12 hours rehydration; 2 hours at 150V; 1 hour at 500V; 1 hour at 1000V; 4 hour at 8000V. Strips were then incubated in equilibration buffer (50mM Tris-HCl pH 8.8, 6M urea, 30% v/v glycerol, 2% w/v SDS, and 1% w/v DTT) for 15 minutes and horizontally applied to 16cm SDS-PAGE gels for electrophoresis, followed by Western blotting.

**Far Western Assay**

Flag-HMK-eIF4E probe was $^{32}$[P]-labeled, purified from unicorporated radioactivity, and Far Western blotting on nitrocellulose membranes was performed essentially as previously described (Gingras et al., 1996; Pause et al., 1994).

**Immunoprecipitation and m$^7$GDP Pull-down**

For immunoprecipitation, transfected cell extracts (200μg) prepared in homogenization buffer were mixed with 2μl of Myc antibody for 1.5 hours at 4$^o$C, followed by 1 hour incubation with 25μl of Protein G Sepharose (GE Healthcare). For m$^7$GDP pull-downs, 200μg of cell extract was mixed with 30μl of m$^7$GDP agarose for 1.5 hours at 4$^o$C. In either case, the resins were washed 4 times and proteins were eluted with 2X sample buffer followed by SDS-PAGE and western blotting.

**Mass Spectrometry Analysis of *In Vitro* Deamidated Recombinant 4E-BP2**

Duplicate Coomassie-stained samples of the slowest, intermediate and fastest 4E-BP2 bands were cut into 1mm x 1mm pieces and destained by several rinses with 50mM ammonium bicarbonate pH 7.9 (over 2h), followed by shrinking with acetonitrile. Gel pieces were rehydrated with trypsin/50mM ammonium bicarbonate (10ng trypsin/μl) and digested overnight at 37°C. Peptides were recovered by acetonitrile/formic acid extraction, lyophilized, and resuspended in 0.1% formic acid, 5% acetonitrile for loading onto the autosampler. Microcapillary Magic $C_{18}$ 100-Å, 5-μm silica (Michrom) columns (10cm, 75-μm inner diameter, 363-μm outer diameter; Polymicro Technology) were placed in-line with a Proxeon Easy Nano-LC system and a ThermoFinnigan LTQ-Orbitrap hybrid mass spectrometer. Each sample was subjected to a 2 hour LC gradient

(from 5% to 95% acetonitrile). The mass spectrometer was programmed for data-dependent MS/MS acquisition (one survey scan followed by four MS/MS of the most abundant ions). After sequencing the same species three times, the mass ± 1 Da was placed on an exclusion list for 3 minutes. Searches were conducted using X!Tandem, by comparing acquired data to the IPI human database, allowing for 1 missed tryptic cleavage site. The parent mass was monitored in the Orbitrap with a mass error of 100ppm, while the CID was acquired in the linear trap with a mass error of 0.4Da. Search parameters allowed for variable modifications (Met, Trp oxidation, and Gln, Asn deamidation). Spectra corresponding to the peptide *VEVNNLNNLNNHDR* were manually inspected for the proper assignment of the deamidated species.

**Organotypic hippocampal slice cultures, transfections, and electrophysiology**

Organotypic hippocampal slices were prepared as previously described (Lebeau et al., 2008). Slices were used for experiments after 13 to 15 days in culture. Biolistic transfection of neurons in organotypic slice cultures was performed as previously described (Bourdeau et al., 2007), using a Helios gene gun (Bio-Rad). Electrophysiological recordings were performed blind to identity of transfected plasmid in CA1 pyramidal neurons 48 hours later. For electrophysiology, slice cultures were transferred to a submerged recording chamber and continuously perfused (at 1 to 2 ml/min) with artificial cerebrospinal fluid (124 mM NaCl, 2.5 mM KCl, 1.25 mM $NaH_2PO_4$, 4 mM $MgSO_4$, 26 mM $NaHCO_3$, 10 mM dextrose, and 4 mM $CaCl_2$, saturated with 95% $O_2$ and 5% $CO_2$, pH 7.4). mEPSCs were recorded in whole-cell voltage-clamp mode using a Multiclamp 700A amplifier (Molecular Devices) in slices maintained at 30

to 32°C. Recording pipettes (4 to 6 M{Omega}) were filled with a solution containing 130 mM $CsMeSO_3$, 5 mM CsCl, 2 mM $MgCl_2$, 5 mM diNa-phosphocreatine, 10 mM HEPES pH 7.2 to 7.3, 2 mM ATP-Tris, and 0.4 mM GTP-Tris, 275 to 285 mOsm. DL-2-amino-5-phosphonovaleric acid (50 μM; Tocris Biosciences), GABAzine (5 μM; Tocris Biosciences), and tetrodotoxin (1 μM, Alomone Labs) were added to the extracellular solution to block NMDA, $GABA_A$ receptors, and action potentials, respectively. Data acquisition (filtered at 2 kHz, digitized at 20 kHz) and analysis were performed as previously described (Ran et al., 2009) using a personal computer equipped with pClamp9 software (Molecular Devices). Cells were maintained at a holding potential of -60 mV and series resistance routinely monitored. Threshold mEPSC amplitude was set at 3 pA, and typically 200 to 250 events were collected over a 15- to 25-minute period. For analysis, mEPSCs were detected with a running template (average of 20 events) with a well defined baseline. Data are represented as means ± standard errors of the means, unless otherwise mentioned, and statistical significance was set at $P < 0.05$. mEPSC parameters between groups were performed using Kolmogorov-Smirnov tests.

## 2.6 Acknowledgements

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## Connecting Text

Asparagine deamidation yields two products: aspartate and its isomer, isoaspartate. The latter is the major product and may be destabilizing to protein function. The enzyme Protein L-Isoaspartyl Methyltransfersase (PIMT), which is critical to nervous system function, catalyzes the conversion of isoaspartates to aspartates. Thus, we wished to determine if deamidated 4E-BP2 is a substrate for PIMT.

# Chapter 3

# Translational Repressor 4E-BP2 is a Substrate for Protein L-Isoaspartyl Methyltransferase in the Brain

## 3.1 Abstract

The eIF4E-binding proteins (4E-BPs) repress translation by disrupting eIF4F formation, thereby preventing ribosome recruitment to the mRNA. Of the three 4E-BPs, 4E-BP2 is the predominant isoform expressed in the mammalian brain and it plays an important role in synaptic plasticity and learning and memory. We have demonstrated that 4E-BP2 undergoes asparagine deamidation, uniquely in the brain, during early postnatal development. The deamidation process spontaneously converts asparagines into a mixture of aspartates or isoaspartates, the latter of which is considered to be destabilizing to proteins. The enzyme Protein L-Isoaspartyl Methyltransferase (PIMT) prevents isoaspartyl accumulation by catalyzing the conversion of isoaspartates to aspartates. PIMT exhibits high activity in the brain, relative to other tissues. We report here that 4E-BP2 is a novel substrate for PIMT. *In vitro* deamidated 4E-BP2 accrues isoapartyl residues and is a methyl-acceptor for recombinant PIMT. We also developed an antibody that recognizes 4E-BP2 specifically harbouring isoaspartyls at the deamidation sites, Asn 99 and Asn102. We used this antibody to demonstrate that endogenous 4E-BP2 in PIMT-/- brain lysates contains isoaspartyl residues. The steady-state levels of 4E-BP2 and its binding to eIF4E were not altered in the PIMT-deficient brain.

## 3.2 Introduction

Eukaryotic protein synthesis is primarily regulated at the step in which the ribosome is recruitment to the mRNA. The association of the cap-binding protein, eukaryotic initiation factor 4E (eIF4E), with the mRNA 5' $m^7$GpppN cap-structure initiates an ordered series of reactions culminating in 40S ribosome placement at the AUG initiation codon. eIF4E binds the mRNA cap as part of a larger complex, termed eIF4F, which includes the factors eIF4G and eIF4A. eIF4F recruits the ribosomal 43S complex (40S ribosome/ Met-$tRNA_i^{Met}$ -eIF2-GTP/eIF3) to the mRNA 5' untranslated region, (Gingras et al., 1999b; Pestova et al., 2007c; Sonenberg and Hinnebusch, 2009). In mammals, three eIF4E-binding proteins (4E-BP1, 2, and 3) antagonize translation initiation by preventing eIF4F formation through competition with eIF4G for a common binding-site on eIF4E (Haghighat et al., 1995; Mader et al., 1995; Marcotrigiano et al., 1999). Under cellular conditions favouring growth and proliferation, the 4E-BPs are hyperphosphorylated by the mammalian target of rapamycin complex 1 (mTORC1), which effects their release from eIF4E, and translation initiation proceeds following eIF4F formation (Gingras et al., 2001b; Proud, 2009; Yonezawa et al., 2004).

mTORC1 is composed of the kinase mTOR, GβL (LST8), and raptor, a large adaptor protein that recruits substrates to the mTOR kinase domain (Hara et al., 2002; Kim et al., 2002; Kim et al., 2003; Loewith et al., 2002). A TOR signaling (Fonseca et al.) motif common to mTORC1 substrates, including 4E-BPs, is necessary for the interaction with raptor (Lee et al., 2008; Nojima et al., 2003; Schalm and Blenis, 2002; Schalm et al., 2003). mTORC1 is regulated by several signalling pathways, including PI3K-Akt and

LKB1-AMPK, that converge on the tumour suppressor complex TSC1/2 to suppress its GTPase activity toward Rheb, a small GTP-binding activator of mTORC1 (Ma and Blenis, 2009; Wullschleger et al., 2006). Rag GTPases were recently shown to mediate the amino acid-dependent component of mTORC1-activation (Kim et al., 2008; Sancak et al., 2008).

Asparagine deamidation is a posttranslational protein modification comprising the non-enzymatic conversion of asparagines to aspartates. While this process is spontaneous, the propensity of different asparagines to undergo deamidation is dramatically dissimilar and is largely determined by the identity of the immediate C-terminal residue and the flexibility of the peptide backbone (Radkiewicz et al., 2001; Robinson and Robinson, 2001a; Xie and Schowen, 1999). Environmental factors, including pH, temperature, and ion concentration, can also significantly influence deamidation rates (Patel and Borchardt, 1990). *In vivo* deamidation has been reported for more than 200 proteins. Although this modification has often been viewed as undesirable degradation of aged proteins, important molecular and cellular functions of deamidated Bcl-$X_{L,}$ fibronectin, histone $H1^{o}$, cytochrome c, PKA, and rabbit muscle aldolase were described (Pepperkok et al., 2000; Weintraub and Deverman, 2007). The apparent evolutionary selection of sequences favouring asparagine deamidation (Robinson et al., 1970; Robinson and Robinson, 1991) and the coincidence of *in vivo* deamidation events with cellular processes suggest that deamidation may act as 'molecular clock' to regulate the onset of biologically significant events (Robinson et al., 1970; Robinson and Robinson, 2001b).

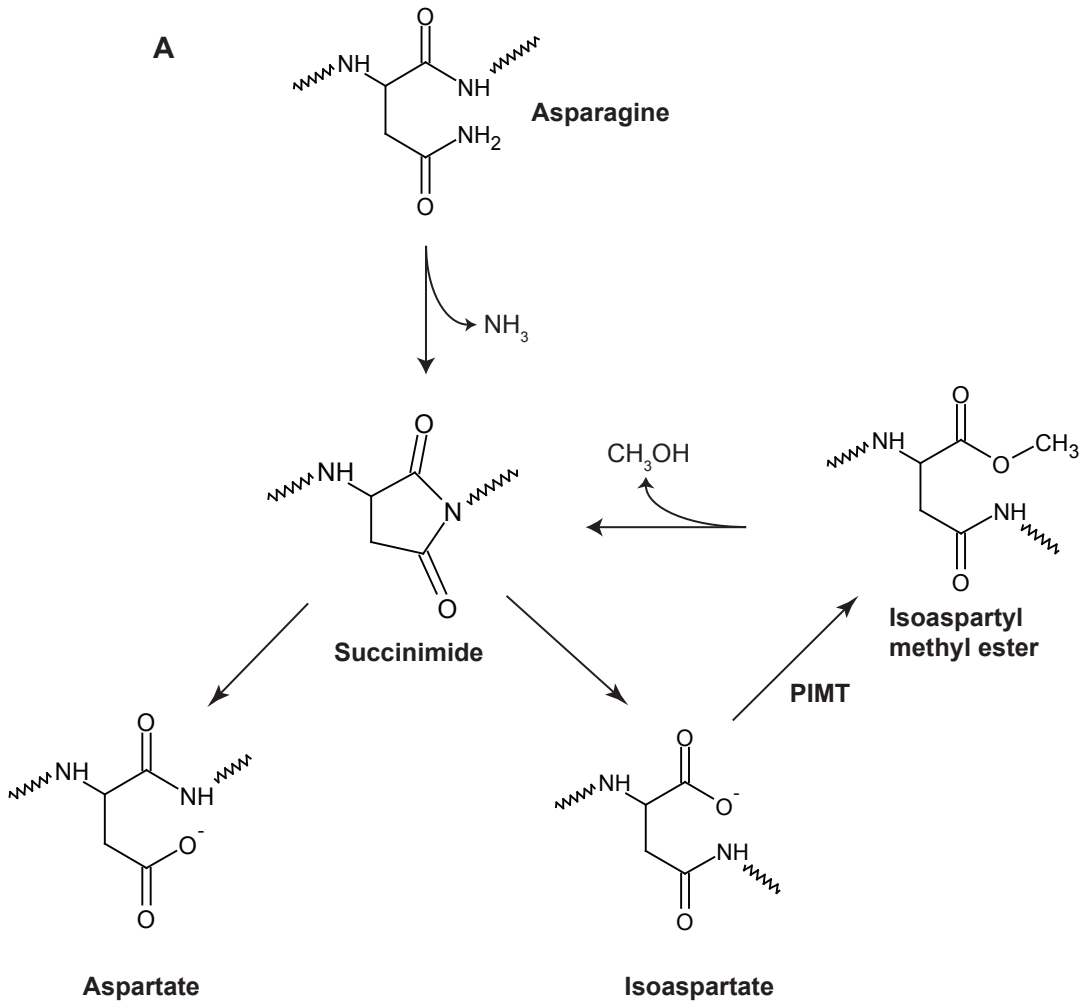
Deamidation occurs through nucleophilic attack by the C-terminal flanking peptide bond nitrogen toward an asparagine's side-chain amide group. This leads to the formation of a circular succinimide intermediate (Reissner and Aswad, 2003) (Figure 3.1A). The hydrolysis of this short-lived intermediate yields both aspartate (aspartyl) and isoaspartate (isoaspartyl) residues, the latter of which comprises 70-85% of the reaction product (Geiger and Clarke, 1987). The atypical peptide bond (β-linkage) of the isoaspartyl residue prevents normal protein folding and is therefore thought to represent a form of damage to proteins aged *in vitro* or that accumulate in some tissues (Clarke, 2003; Reissner and Aswad, 2003; Shimizu et al., 2005). To alleviate the potentially deleterious consequences of isoaspartyl formation, the enzyme Protein L-isoaspartyl methyltransferase (PIMT) converts these residues into the aspartyl form. PIMT catalyzes the transfer of a methyl group to the isoaspartyl carboxyl side-chain leading to reformation of the succinimide group (Aswad, 1984; Murray and Clarke, 1984) (Figure 3.1A). Iterative PIMT-catalyzed succinimide formation and decay leads to replacement of isoaspartyls with aspartyls (Brennan et al., 1994; Johnson et al., 1987; McFadden and Clarke, 1987). PIMT is ubiquitous although, it exhibits high expression and has the greatest number of substrates in brain (Kim et al., 1997; Vigneswara et al., 2006). The critical requirement for this enzyme in neuronal function is underscored by its genetic deletion in mice. Most strikingly, of a host of reported maladies, PIMT-/- mice display enlarged brains, suffer severe epileptic seizures, and die on average 6 weeks postnatal (Kim et al., 1997; Yamamoto et al., 1998). On the other hand, a transgenic fly line overexpressing PIMT exhibits increased lifespan (Chavous et al., 2001). Therefore, PIMT appears to play a role in longevity, possibly through its suppression of isoaspartyl content.

Owing to the importance of PIMT in the brain, recent independent studies were undertaken to globally identify its substrates in this tissue (Vigneswara et al., 2006; Zhu et al., 2006). The considerable discrepancy in the results between these studies suggests that still other PIMT substrates remain unidentified.

We recently discovered that translation repressor 4E-BP2, the predominant 4E-BP isoform expressed in the mammalian brain, undergoes brain-specific deamidation during postnatal development at a unique asparagine-rich sequence (Bidinosti et al., 2009). Considering this, we were interested to learn whether 4E-BP2 is a PIMT substrate.

### 3.3 Results

**GST-PIMT exhibits functional methyltransferase activity *in vitro*.** We previously reported that two 4E-BP2 asparaginyl residues (N99 and N102; Figure 3.1B) deamidate during postnatal development in the mammalian brain (Bidinosti et al., 2009). Considering the preponderance for isoaspartyl accumulation and the critical requirement for PIMT in the brain (Kim et al., 1997; Yamamoto et al., 1998), we hypothesized that 4E-BP2 would be a PIMT substrate. To address this, we first adopted an *in vitro* assay system based on the use of recombinant PIMT, similar to that used in previous studies (Reissner et al., 2006; Vigneswara et al., 2006; Zhu et al., 2006). To produce recombinant PIMT, the human PIMT isozyme II cDNA was subcloned into the pGEX-6P-1 vector for bacterial expression as a fusion protein to glutathione S-transferase at the PIMT N-terminus. Recombinant GST-PIMT was expressed in *E. coli* BL21 cells and purified to homogeneity (Figure 3.2A). The activity of GST-PIMT was then assessed by incubating



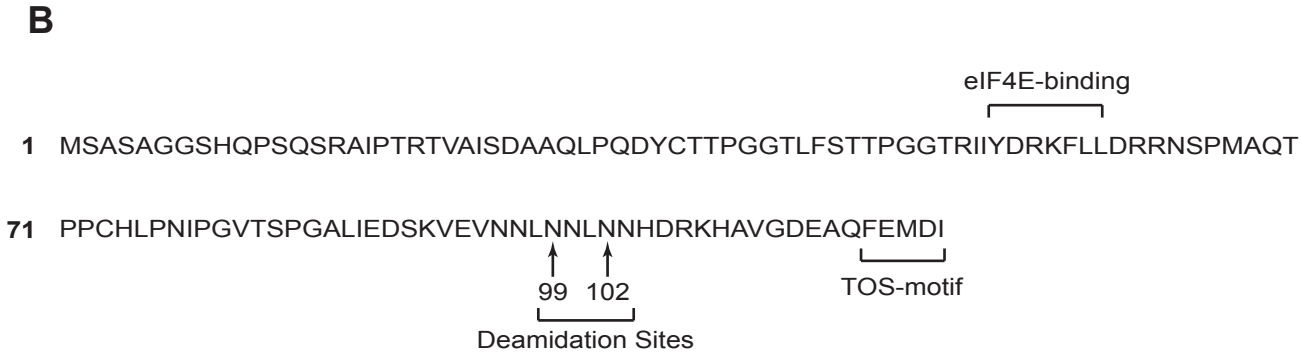




**Figure 3.1 PIMT repairs isoaspartyls formed during spontaneous asparagine deamidation in proteins.**
(A) The mechanism of aspartyl or isoaspartyl formation and repair of abnormal isoaspartyls by PIMT is depicted. Spontaneous asparagine deamidation yields a mix of aspartyls and isoaspartyls, of which the formation of the latter is favoured by approximately 3:1. The deamidation reaction proceeds through the metastable succinimide intermediate. PIMT catalyzes the transfer of a methyl group to isoaspartyls thereby forming the corresponding carboxyl-methyl esters. These rapidly revert to the original succinimide intermediate, consequently leading to de novo formation of aspartyls and isoaspartyls. Net accumulation of repaired aspartyls is accomplished iteratively. (B) Amino acid sequence of murine 4E-BP2. Deamidation sites, eIF4E-binding site, and TOS motif are indicated.

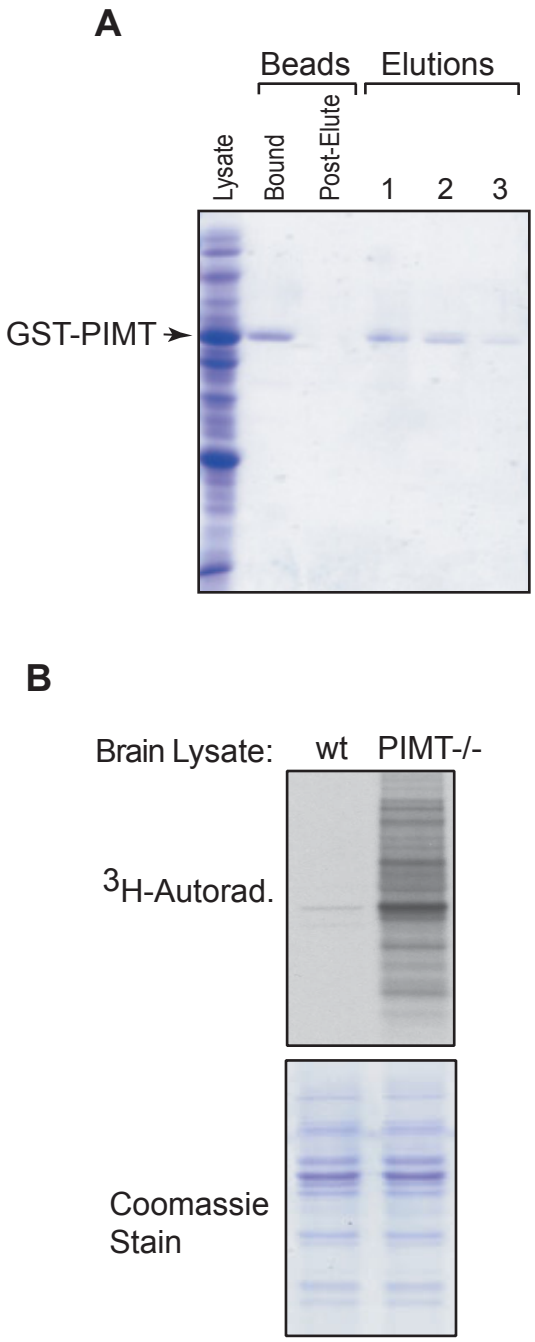



**Figure 3.2 Purified GST-tagged PIMT exhibits methyltransferase activity toward isoaspartyl residues in PIMT-/- brain lysates.**
(A) Expression and purification of GST-tagged PIMT. Human PIMT was expressed in *E. coli* as a fusion at the N-terminus to GST. Protein was purified on glutathione sepharose 4B resin and eluted in three fractions with 10mM glutathione. The crude lysate, glutathione resin before and following elution, and three elutions were resolved by 10% SDS-PAGE and proteins were visualized with Coomassie Brilliant Blue stain. The first two elutions were pooled and stored until use. (B) GST-PIMT catalyzes the transfer of methyl groups from [$^3$H]-labeled S-Adenosyl-L-methionine (AdoMet) to isoaspartyl residues accumulated in PIMT-/- brain lysates. 40μg of wild type or PIMT-/- lysates were incubated with 2.5μM GST-PIMT and 100 μM AdoMet (4μCi [$^3$H]) in 75 mM Na-MES pH 6.2, for 15 minutes at 30$^o$C. Lysates were separated on 4-12% BIS-TRIS PAGE and gels were processed for tritium detection.

it with wild type or PIMT-/- brain lysates and the tritiated methyl donor, AdoMet. As anticipated, GST-PIMT efficiently labelled the isoaspartyls that accumulate in PIMT-/- brains, relative to wild type, as was evidenced by the pronounced tritium signals across the entire molecular weight range of the PIMT-/- PAGE lane (Figure 3.2B). Similar results were previously obtained with different preparations of recombinant PIMT protein (Vigneswara et al., 2006; Zhu et al., 2006).

***In vitro* deamidated 4E-BP2 accrues isoaspartyls.** To determine whether 4E-BP2 deamidation produces isoaspartyls, in addition to aspartyl residues, the PIMT assay was adapted for methylation of *in vitro* deamidated 4E-BP2. Recombinant 4E-BP2 was subjected to alkaline-induced deamidation (pH 10) by incubation for 24 hours at 37$^{o}$C. This treatment produced similar quantities of unmodified, singly, and doubly deamidated 4E-BP2 (Figure 3.3; lower panel, middle lane). PIMT methylation was then performed on alkaline-treated 4E-BP2, untreated wild type 4E-BP2, and the deamidation mimic 4E-BP2 N99D/N102D (described in (Bidinosti et al., 2009). As expected, isoaspartyl groups were only detected in the wild type 4E-BP2 protein that was deamidated by alkaline treatment (wt+pH10) (Figure 3.3; upper panel). Thus, the formation of isoaspartyls during *in vitro* asparagine deamidation of 4E-BP2 causes it to become a substrate for PIMT. Importantly, GST-PIMT distinguishes between the isoaspartyls of alkaline-treated 4E-BP2 and the aspartyls at the same residues in 4E-BP2 N99D/N102D.

**A novel antibody recognizes 4E-BP2 specifically containing isoaspartyls at asparagines 99 and 102**. To further characterize the isoaspartyl formation in 4E-BP2, an

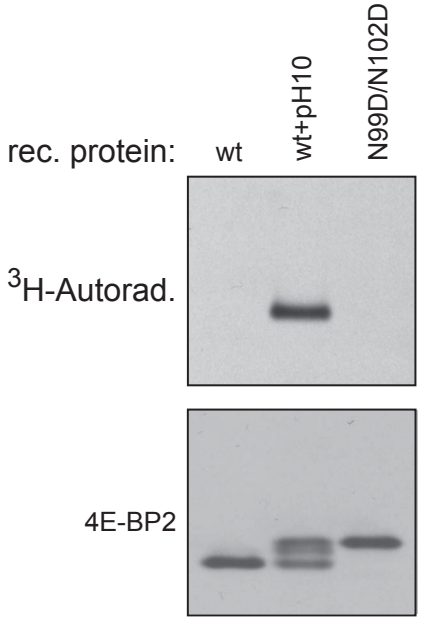



**Figure 3.3 *In vitro* deamidated 4E-BP2 accrues isoaspartyl residues and is a substrate for PIMT**.
Recombinant 4E-BP2 was alkaline deamidated in 0.15M TRIS pH 10 for 24h at 37°C. Wild type, alkaline-treated (wt+pH10), and the deamidated mimic (N99D/N102D) 4E-BP2 proteins were assayed for isoaspartyl content as in Figure 2B. One microgram of recombinant protein was incubated with 2.5 μM GST-PIMT and 50 μM AdoMet in 75 mM Na-MES pH 6.2 for 10 minutes at 30°C. Samples were divided and resolved by 4-12% BIS-TRIS PAGE ([$^3$H] detection; upper panel) or 15% SDS-PAGE (western blotting; lower panel).

antibody was produced to recognize 4E-BP2 harbouring isoaspartyls at both positions 99 and 102. The specificity of the immunopurified antibody was confirmed by Western blotting against recombinant 4E-BP2 proteins. The antibody, herein referred to as anti-Iso4E-BP2 (D99/D102), recognized only 4E-BP2 that had been deamidated by alkaline treatment (Figure 3.4A; upper panel), thereby confirming its specificity for isoaspartyl-containing 4E-BP2. The antibody similarly detected increasing amounts of isoaspartyls at positions 99 and 102 in recombinant wild type 4E-BP2 that had been 'aged' *in vitro* by incubation for several days at pH 7 (Figure 3.4B).

**4E-BP2 is an *in vivo* PIMT substrate in brain.** Our previous work demonstrated that 4E-BP2 undergoes deamidation, uniquely in the brain, during the first three murine postnatal weeks (Bidinosti et al., 2009). Thus, the isoaspartyl-specific antibody was used to determine whether 4E-BP2, the predominant 4E-BP expressed in the mammalian brain, is an *in vivo* substrate for PIMT in this organ. 4E-BP2 was immunoprecipitated from wild type or PIMT-/- brains lysates using anti-4E-BP2 antibody and proteins were resolved by 15% SDS-PAGE. Immunoprecipitation from 4E-BP2-/- brain lysates served as a negative control for antibody specificity. Western blotting with anti-Iso4E-BP2 (99/102) antibody indicated that only 4E-BP2 from PIMT-/- brains contained appreciable amounts of isoaspartyl residues (Figure 3.5). Faint signals in the wild type lane are likely due to cross-reactivity with unrelated proteins accumulated during the immunoprecipitation procedure, rather than with 4E-BP2 itself, as similar signals are also present in the 4E-BP2-/- lane.

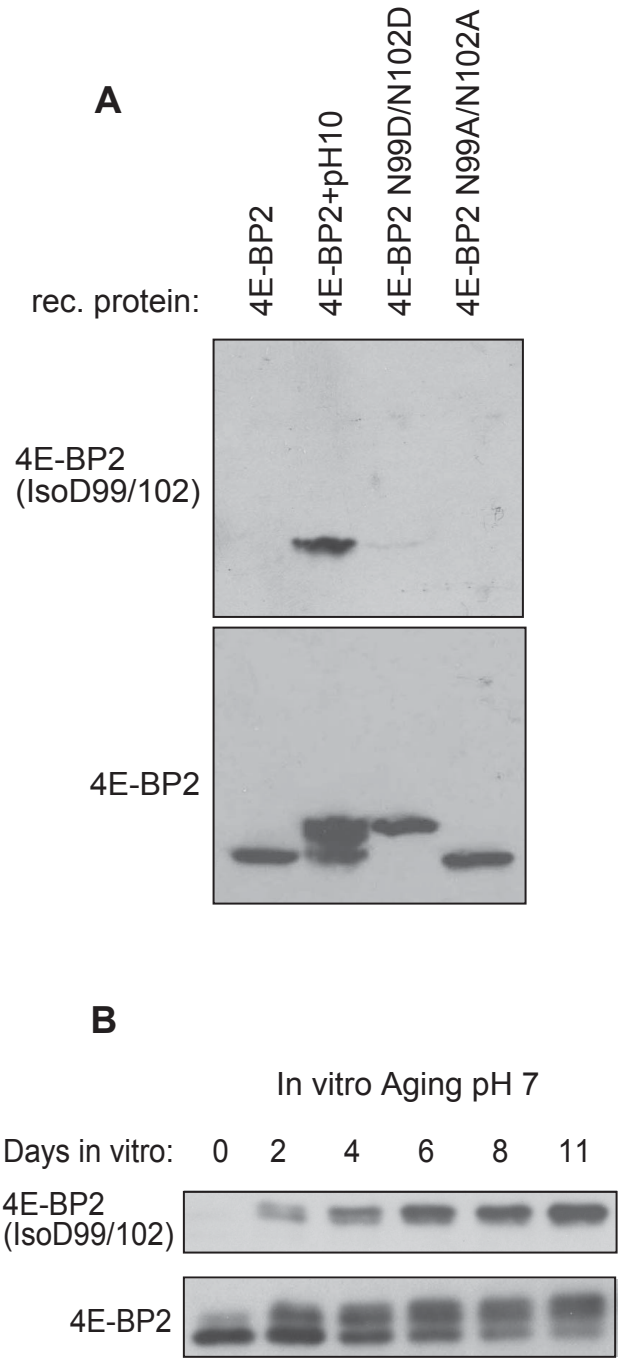



**Figure 3.4 An antibody raised in-house specifically recognizes 4E-BP2 harbouring isoaspartyl residues at positions 99 and 102.** (A) Western blot analysis with a purified antibody raised against a peptide containing the 4E-BP2 residues 99 and 102 in the isoaspartate conformation (Iso4E-BP2 99/102). Wild type recombinant 4E-BP2 protein was alkaline deamidated as in Figure 3 (wt+pH10). 0.5ng of each recombinant protein was resolved by 15% SDS-PAGE as indicated. The nitrocellulose membrane was stripped of primary antibody and reprobed with a 4E-BP2 specific antibody (lower panel). (B) Western blot detection of isoaspartyls accumulated in *in vitro* aged recombinant 4E-BP2. Recombinant protein was incubated at 37$^{o}$C in 0.15M TRIS pH 7 and sampled on the indicated days. Samples were resolved by 15% SDS-PAGE for Western blotting.

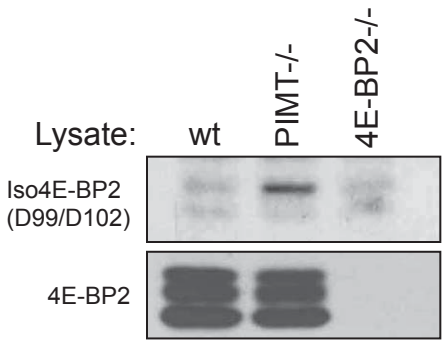



**Figure 3.5 4E-BP2 is an *in vivo* substrate for PIMT in the mammalian brain.** Western blot analysis using the Iso4E-BP2 99/102 antibody (Figure 4) against 4E-BP2 from wild type or PIMT-/- mouse brains. 4E-BP2 was immnoprecipitated from 200μg of whole brain extracts (7 weeks postnatal) and resolved by 15% SDS-PAGE. Immunoprecipitates from 4E-BP2-/- brain lysates controlled for antibody specificity.

**Isoaspartyl accumulation in 4E-BP2 does not alter stability or binding to eIF4E.**

Formation of isoaspartyl residues in some proteins has been associated with either increased propensity for proteasomal degradation (Bohme et al., 2008; Tarcsa et al., 2000) or aggregation (Shimizu et al., 2002). We therefore questioned whether the accumulation of unrepaired isoaspartates in 4E-BP2 in the PIMT-/- brain affects the stability of the protein. Western blotting on wild type and PIMT-/- brain lysates from animals aged approximately between six and 14 weeks postnatal indicated no apparent change in steady-state levels of 4E-BP2, thereby suggesting that 4E-BP2 is not subject to aberrant degradation or aggregation in the absence of PIMT-mediated repair (Figure 3.6A). We also wished to determine whether isoaspartyls affect the interaction of 4E-BP2 with its cognate binding-partner eIF4E, in the brain. To this end, eIF4E from age-matched wild type and PIMT-/- brain lysates was precipitated on an mRNA cap-analogue, $m^7$GDP, resin and the amount of associated 4E-BP2 was compared. Western blot analysis revealed no significant changes in the amount of 4E-BP2, including unmodified and deamidated forms, bound to eIF4E (Figure 3.6B).

## 3.4 Discussion

We report here that 4E-BP2 is a novel substrate of PIMT. Deamidation of endogenous 4E-BP2 in the PIMT-/- murine brain or of recombinant 4E-BP2 deamidated under alkaline pH or by *in vitro* aging caused the formation of isoaspartyl residues. Detection of isoaspartyls in 4E-BP2 in the PIMT-/- brain indicates that 4E-BP2 is an accessible intracellular substrate to PIMT and that PIMT normally acts to suppress isoaspartyl accumulation following deamidation in the wild type brain. 4E-BP2 deamidation occurs

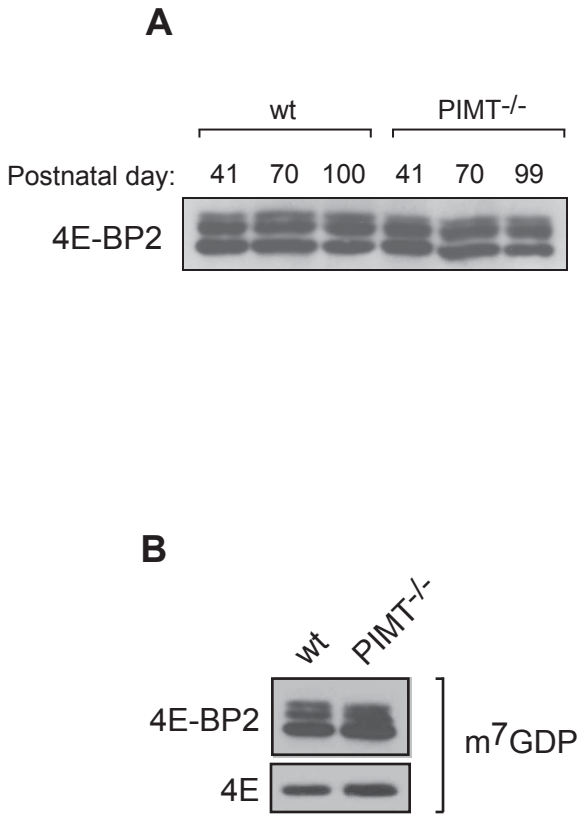



**Figure 3.6 Accumulation of isoaspartyl residues in 4E-BP2 in the brain does not alter protein stability or interaction with eIF4E.**
(A) The steady-state level of 4E-BP2 is unaffected in PIMT-/- brains as assessed by Western blotting. Brain lysates (30μg) from wild type or PIMT-/- mice of indicated ages were resolved by 15% SDS-PAGE. (B) The association of 4E-BP2 with eIF4E in wild type and PIMT-/- brains is unchanged. eIF4E/4E-BP2 complexes from 250 μg of brain lysates were recovered on mRNA cap-analog, $m^7$GDP, resin. Proteins were resolved by 15% SDS-PAGE, followed by Western blotting.

principally on two asparagines (N99 and N102) near the C-terminus (Bidinosti et al., 2009) and this leads to reduced migration of the deamidated species in SDS-PAGE. Thus, in the PIMT-/- brain the slow-migrating forms of 4E-BP2 will harbour 70-85% isoaspartyl content at each deamidation site, as this is the ratio favoured by succinimide hydrolysis. Our antibody directed to 4E-BP2 containing isoaspartyls at the deamidation sites (anti-Iso4E-BP2) recognizes the slowest migrating 4E-BP2 species formed by alkaline-induced deamidation (Figure 3.4). In using this antibody to demonstrate, by Western blotting, the accumulation of isoaspartyls in 4E-BP2 from PIMT-/- brain lysates (Figure 3.5), it was also shown that the 4E-BP2 signal from wild type brain was not above background levels (determined with the 4E-BP2-/- lysate). This indicates that PIMT-mediated repair of 4E-BP2 is sufficiently efficacious to maintain it in the aspartyl-containing form, at least as given by the limit of detection of this assay. More sensitive assays could potentially determine whether 4E-BP2 contains a basal level of isoaspartyls in wild type brain.

The appearance and accumulation of 4E-BP2 deamidation in murine brain in the early postnatal weeks roughly parallels that of PIMT expression (Shirasawa et al., 1995). This suggests that, in addition to 4E-BP2, numerous PIMT substrates accrue isoaspartyl residues during the same time frame and that increased PIMT expression is required to keep their levels in check. Indeed, the large number of isoaspartyls formed in the brain, relative to other tissues, rapidly increases within the first 100 postnatal days in PIMT-/- mice (Lowenson et al., 2001).

Studies aimed at cataloguing the complement of PIMT substrates in the brain did not identify 4E-BP2 as a substrate (Vigneswara et al., 2006; Zhu et al., 2006). A possible explanation for this is that 4E-BP2 from the PIMT-/- brain lysates yielded either a minor radiolabel signal from exogenous PIMT methylation or was not detected by protein staining in the two- dimensional IEF/SDS-PAGE analyses. Consequently, it would not have been identified. This is consistent with our observation, via large scale purification of 4E-BP2 from brain lysates, that 4E-BP2 is a relatively lowly expressed component of brain lysates (Bidinosti et al., 2009). However, 4E-BP2 is required for long-lasting forms of synaptic plasticity and learning and memory (Banko et al., 2006; Banko et al., 2007; Banko et al., 2005).

Isoaspartyl bonds tend to preclude protease activity (Bohme et al., 2008; Haley et al., 1966; Johnson and Aswad, 1990), yet evidence suggests that their accumulation in PIMT-deficient mice is maintained at a constant level in older animals by enhanced degradation and excretion of damaged proteins (Lowenson et al., 2001). We considered that isoaspartyl accumulation in 4E-BP2 in PIMT-/- mice might affect its stability or turnover and this would consequently be reflected by alterations in its steady-state levels. However, no changes were observed in 4E-BP2 levels upon comparing wild type and PIMT-/- brain lysates from mice aged between 40 and 100 days (Figure 3.6A). Thus, although no gross changes in 4E-BP2 expression arise from isoaspartyl formation in PIMT-/- brains in the first 100 postnatal days, it is possible that 4E-BP2 harbouring isoaspartyls would be degraded and excreted in older knock-out mice in which isoaspartyl content has reached a plateau. We also observed that deamidated 4E-BP2 from PIMT-/-

brain interacted with eIF4E to the same degree as that from wild type brain. The location of the deamidation-susceptible asparagines near the C-terminus of 4E-BP2 may be sufficiently distal to the eIF4E-binding site so as to not perturb the interaction with eIF4E (Figure 3.1B).

We describe here the novel generation of the GST-PIMT reagent. This recombinant protein retains the selective methyltransferase activity for isoaspartyl residues of previously reported preparations. This was indicated by the large number of proteins that were radiolabelled in PIMT-/- brain lysates relative to wild type (Figure 3.2B) (Kim et al., 1997; Yamamoto et al., 1998; Zhu et al., 2006) and the activity toward recombinant 4E-BP2 that was deamidated at pH 10 but not the deamidation mimic N99D/N102D (Figure 3.3). Moreover, in our experience, GST-PIMT has the advantage of facile and rapid preparation of large quantities of highly pure enzyme (Figure 3.2A), via batch incubation of Glutathione Sepharose-4B resin with bacterial lysate.

In summary, we have identified 4E-BP2 as a novel, endogenous substrate for PIMT-mediated repair of isoaspartyl residues that accrue by way of asparagine deamidation in postnatal brain development.

## 3.5 Materials and Methods

### Materials

S-[methyl-$^{3}$H] Adenosyl-L-Methionine (AdoMet; 81.9Ci/mmol) and unlabeled AdoMet were purchased from Perkin Elmer and Sigma, respectively. EN$^{3}$HANCE liquid autoradiography enhancer was from Perkin Elmer. 7-methylguanosine 5'-disphosphate ($m^{7}$GDP) - agarose resin was prepared as previously described (Edery et al., 1988). Adipic acid dihydrazide-Agarose and $m^{7}$GDP were purchased from Sigma. 4E-BP2 antibody (#2845) was from Cell Signaling Technology. eIF4E antibody (#610270) was from BD Biosciences.

**Vector construction and Recombinant Protein Expression and Purification**

Human PIMT cDNA was amplified by PCR with the addition of *EcoRI* and *XhoI* restrictions sites at the 5' and 3' ends, respectively. The PCR product was digested and ligated into the pGEX-6P-1 vector at the above sites in-frame with the upstream glutathione S-transferase sequence. Recombinant GST-PIMT fusion protein was expressed in *E. coli* BL21 (DE3) by induction with 0.1mM IPTG, followed by 3-4h growth at 30$^{o}$C. Protein was purified on Glutathione Sepharose-4B resin according to the manufacturer's recommendations (GE Healthcare) and eluted in fractions with 10mM glutathione in 50mM Tris-HCl pH 8.5. In the case of recombinant GST-4E-BP2 fusion proteins, the 4E-BP moieties were released, following binding to Glutathione Sepharose-4B, by on-resin cleavage for 20h at 4$^{o}$C with PreScission Protease (GE Healthcare). All purified proteins were stored at -80$^{o}$C in 10% glycerol.

***In vitro* Methylation of Isoaspartyls by Recombinant PIMT**

Methylation of brain lysates was performed similarly to that previously described (Zhu, 2006 #5). Briefly, 40μg of lysate was incubated with 2.5μM GST-PIMT, 100μM AdoMet (4μCi [$^3$H]; concentration and specific activity was adjusted with unlabeled AdoMet), and 75mM Na-MES (pH 6.2) in a total reaction volume of 27.5μl. For methylation of recombinant 4E-BP2, the wild type protein was *in vitro* deamidated in 0.15M Tris-HCl (pH 10.0) for 24h at 37$^o$C. The protein solution was then neutralized with 0.5M Na-MES and treated with 1mM DTT for 10 minutes. Control wild type and 4E-BP2 N99D/N102D recombinant proteins were similarly treated with 0.5M Na-MES and 1mM DTT immediately before use. Methylation reactions were then carried out in 25μl volumes containing 1μg of recombinant protein, 2.5μM GST-PIMT, 50μM AdoMet (4μCi), and 75mM Na-MES (pH 6.2). In both cases, reactions were for 15minutes at 30$^o$C and stopped by the addition of 5X Laemmli sample buffer. Samples were resolved on 4-12% Criterion XT Bis-Tris gels (BioRad). Gels were then treated with En$^3$HANCE$^{TM}$, dried and exposed to auto-radiographic film for tritium detection.

**m$^7$GDP Pulldown**

Brains were homogenized in 10mM Tris-HCl pH 7.4, 253mM sucrose, 1mM EDTA and 250 μg samples were incubated with 40μl of m$^7$GDP resin for 90 minutes at 4$^o$C. The resin was then washed 3 times with homogenization buffer and bound proteins were eluted with 2X Laemmli sample buffer, followed by separation by 15% SDS-PAGE and detection by Western blotting.

**SDS-PAGE and Western blotting**

Protein samples were resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were blocked in 5% powdered milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1h followed by incubation with primary antibodies for two hours at room temperature or overnight at 4$^{o}$C. Membranes were then washed with TBST three times and incubated with HRP-coupled secondary antibody for 1h at room temperature, washed again, and treated with enhanced chemiluminescence prior to detection on X-Ray film (Denville Scientific).

**Iso4E-BP2 (99/102) Antibody Production and Purification**

A peptide of amino acid sequence identical to that encompassing the deamidation susceptible region of 4E-BP2, with aspartates in β-linkages (isoaspartyls) replacing asparagines 99 and 102, was purchased from American Peptide. The peptide was conjugated to immunogenic Keyhole limpet hemocyanin (KLH) via an ectopic N-terminal cysteine using the Imject Maleimide Conjugation Kit (Pierce). Rabbits were immunized with the conjugated peptide, using Freund's Complete Adjuvant for first injection and Freund's Incomplete Adjuvant for subsequent injections, according to the McGill Animal Resources Centre protocol. For antibody purification, rabbit serum was incubated with the above peptide that had been previously conjugated to resin using the SulfoLink kit (Pierce). The antibody was eluted at low pH, dialyzed against 50% glycerol and stored at -20$^{o}$C.

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# Chapter 4

# Conclusion

## 4.1 General Discussion

This thesis describes the identification of asparagine deamidation as a novel, brain-specific posttranslational modification of 4E-BP2. The C-terminal asparagine-rich region, unique to 4E-BP2, was shown to be the sequence susceptible to deamidation. Importantly, mass spectrometry and mutational analyses confirmed that two of the six asparagine residues located there are the major sites of conversion to aspartate. With this new knowledge we were able to pursue the characterisation of the molecular and physiological function of deamidated 4E-BP2 that is presented in chapter 2. A corollary of asparagine deamidation is the formation of isoaspartates; harbouring this isomeric form of aspartate renders proteins natural targets for PIMT. In chapter 3, the identification of 4E-BP2 as a novel PIMT substrate is presented.

In chapter 2, we demonstrated that deamidation of 4E-BP2 enhances its affinity for Raptor. This interaction begets a reduction in its association to eIF4E that persists in the absence of 4E-BP2 phosphorylation. The finding that non-phosphorylated deamidated 4E-BP2 interacts more weakly *in vivo* with eIF4E than does wild type is surprising given the extensive literature defining the phosphorylation-induced release from eIF4E. However, we show with a TOS mutant that this new phenomenon is dependent on interaction with Raptor and suggest that Raptor might sequester deamidated 4E-BP2 in a subcellular location that contains little eIF4E. This could explain the reduction in eIF4E-binding and would be in line with the recent observation that Raptor mediates the amino acid-induced redistribution of mTORC1 to a subcellular compartment containing RHEB (Sancak et al., 2008). To substantiate this hypothesis, future studies will need to compare

the subcellular distribution of wild type and deamidated 4E-BP2. This will require the development of specific antibodies to deamidated 4E-BP2 for use in immunofluorescence and immunohistochemistry assays. Reliable subcellular fractionation methods would complement these studies. Interestingly, there appears to be no specific preference for deamidated 4E-BP2 in synaptosomes, neuronal fractions enriched in synapses (Bidinosti, M., Bramham, C., unpublished observations, not shown). Thus putative differences in subcellular expression are not likely to be at the level of, for instance, synapse versus cell body localization. As predicted from its increased interaction with mTORC1, deamidated 4E-BP2 is more highly phosphorylated *in vitro* by mTORC1 and, in serum-stimulated cells, exhibits facilitated release from eIF4E relative to wild type. Deamidated 4E-BP2 also more weakly inhibited translation of a reporter mRNA in comparison to wild type 4E-BP2. These data are included in Appendix 2 of this thesis.

4E-BP2 deamidation appears during early postnatal development. This, along with its gain-of-function in Raptor binding, parallels the developmental decline in mTORC1 activation, which has been observed elsewhere (Park et al., 2008). Taken together, these observations raise the intriguing possibility of a genetically-encoded mechanism to offset attenuated upstream signalling by lowering a barrier to translation initiation. Such a mechanism would be consistent with Robinson's molecular clock hypothesis of 'timed', biologically useful deamidation events (Chapter 1, section 1.10.1) (Robinson et al., 1970). Further supporting this reasoning, deamidation and other spontaneous protein modifications, including glycation, oxidation, racemisation and isomerisation, tend to accrue in long-lived proteins in aged organisms (Geiger and Clarke, 1987; Harding et al.,

1989; Hoenders and Bloemendal, 1983; Stadtman, 1992). However, the observation that 4E-BP2 deamidation occurs early postnatally, strongly hints at an adaptive function rather than age-related decay by passive modification. It is also noteworthy that the two sites of 4E-BP2 deamidation, Asn99 and Asn102, both contain asparagines as their C-terminal neighbours. A nearly identical single-site deamidation sequence was reported for a G protein (Exner et al., 1999). Glycine is the C-terminal residue most favourable to asparagine deamidation (Robinson et al., 1973; Robinson and Robinson, 2001b), while asparagine has an intermediate effect relative to the other amino acids. Considering this and the perfect conservation of the deamidation region in mammals, it is possible that this sequence is necessary to maintain the occurrence of 4E-BP2 deamidation precisely in a defined time frame.

Expression of deamidated 4E-BP2 in cultured hippocampal slices yields synaptic currents of reduced kinetics (Figure 2.6). This is significant in that current kinetics affect neuronal temporal summation, the integration of postsynaptic currents determining the overall membrane potential near the cell body, and therefore determines whether a neuron will significantly depolarize to fire (action potential). We examined currents specifically mediated by the AMPA type of postsynaptic receptor, which are responsive to the presynaptically released neurotransmitter glutamate. Although the reason for the reduction in current kinetics is unclear, it is tempting to speculate that diffenences in 4E-BP2-dependent translation rates impact the relative expression of AMPA receptor subtypes (Jonas, 2000) or their associated regulatory proteins (TARPs) (Milstein and Nicoll, 2008). Both of these factors are known to alter the kinetics of synaptic

transmission. Such a phenomenon would entail differential sensitivities of the mRNAs encoding these proteins to translation inhibition, perhaps through the degree of secondary structure in the 5'UTRs.

We demonstrated in chapter three that deamidated 4E-BP2 is a substrate for PIMT *in vitro* and *in vivo*. What is the significance of PIMT-mediated maintenance of deamidated 4E-BP2 in the aspartate form, as opposed to that containing isoaspartate? Given that 4E-BP2 deamidation increases its affinity for Raptor, it is reasonable to speculate that isoaspartates might negatively impact this gain of function. Consequently, PIMT-catalyzed conversion of isoaspartates to aspartates in the normal brain would maintain this enhanced interaction. Consistently, preliminary data (not shown) suggest that the *in vitro* interaction of isoaspartate-containing 4E-BP2 with Raptor is reduced to wild type levels. Although these results require confirmation, it appears that isoaspartates are disruptive to the enhanced Raptor-binding of deamidate 4E-BP2. However, in chapter 3 we show that there is no difference in the interaction of any form of 4E-BP2 with eIF4E between wild type and PIMT-/- brain lysates. Altered *in vivo* association of the deamidated forms with eIF4E might be anticipated based on the putative changes to the interaction with Raptor. Although further studies will be needed to rectify this potential discrepancy, one possible explanation is that secondary effects of PIMT removal *in vivo*, such as alterations in signaling pathway activation (Farrar et al., 2005), might have compensatory roles.

## 4.2 Future Directions

Several significant questions remain to be addressed. Perhaps the most conspicuous is why does 4E-BP2 deamidation occur nearly exclusively in the brain? To begin to address this it is worth recalling that deamidation is an inherent property of an asparagine's amino acid context that is influenced by environmental conditions. One possible explanation is that reduced protein turnover of 4E-BP2 allows deamidation to accumulate to a steady-state level not found in other tissues. Consistent with this idea, significant reductions in protein synthesis and turnover have been documented in the brain during the same postnatal time frame as the onset of 4E-BP2 deamidation (Johnson, 1976; Lajtha et al., 1992). To address whether 4E-BP2 turnover is reduced during the accumulation of deamidation, we are establishing a radiolabelling assay in cultured hippocampal neurons to measure protein turnover by pulse-chase assays coupled with 4E-BP2 immunoprecipitation. This culture system faithfully recapitulates the developmental increase in 4E-BP2 deamidation upon elapsed time in culture (Figure 2.5). Decreased rates of 4E-BP2 incorporation/degradation in cells cultured for longer periods would suggest that turnover is, at least in part, contributing to the accumulated deamidation. Another potential contribution to the appearance of 4E-BP2 deamidation is the regulation of intracellular pH. Basic conditions catalyze asparagine deamidation. The membrane ion transporter NHE1 (Na+/H+ exchanger) maintains elevated cellular pH levels by removing intracellular hydrogen ions in exchange for extracellular sodium ions. NHE1 overexpression increased cellular alkalinity that induced Bcl-$X_L$ deamidation and apoptosis of some cancer cell lines (Zhao et al., 2008; Zhao et al., 2007). Loss of NHE1 in mice reduced the intracellular pH of hippocampal CA1 neurons (Yao et al., 1999).

Thus, it is possible that NHE1-mediated pH regulation contributes to the onset of 4E-BP2 deamidation in neurons. To test this, NHE-/- and control brains, received from the lab of Gary Shull at the University of Cincinnati, will be analyzed by Western blotting. The amount of deamidated 4E-BP2 will be compared in NHE1-/- and control brains recovered from mice of different ages, the youngest of which is three weeks of age. At this early time point all deamidated forms are present.

Deamidated 4E-BP2 alters synaptic transmission (chapter 2). In view of this data, it is conceivable that learning and memory are impacted by 4E-BP2 deamidation. To pursue this hypothesis, we have established collaboration with Dr. Karim Nader of McGill University to perform behavioural analyses on 4E-BP2-/- mice expressing deamidated 4E-BP2 or controls. Exogenous expression of 4E-BP2 proteins in specific hippocampal neurons will be accomplished through intrahippocampal injection of viral expression vectors that are compatible with neuronal expression (Han et al., 2009; Nathanson et al., 2009). Importantly, the 4E-BP2-/- mice allow characterisation of the specific protein being expressed by eliminating the confounding effects of endogenous proteins. This *in vivo* expression system system will also be amenable to electrophysiological studies of LTP and LTD.

A third question is how deamidation enhances the interaction of 4E-BP2 with Raptor? Inspection of the 4E-BP2 amino acid sequence indicates that the C-terminal deamidation sites are adjacent to the TOS motif, the sequence required for Raptor-association. The sequence alignment of 4E-BP1 and 4E-BP2 C-termini (Appendix 3A) indicates that the

4E-BP2 deamidation site Asn102 aligns just next to the 4E-BP1 phosphorylation site, Ser101. Phosphorylation at this site, which is constitutive, has been demonstrated to be required for phosphorylation at Ser65 (Wang et al., 2003), the final site targeted in the 4E-BP1 hierarchy and thereby effecting its release from eIF4E. This suggests that negative charges near this region of the 4E-BPs might alter its ability to interact with Raptor. We examined this by co-immunoprecipitation of 4E-BP1, 4E-BP2, deamidated 4E-BP2 and controls, with Raptor. As expected, deamidated 4E-BP2 interacts much more strongly with Raptor than 4E-BP2. Strikingly, 4E-BP1 also showed a strong interaction with Raptor, similar in magnitude to that of deamidated 4E-BP2 **(**Appendix 3B). This suggests that 4E-BP2 normally interacts weakly with Raptor and that deamidation imparts a 4E-BP1 level of Raptor-binding. To address whether the C-terminal regions are involved in modulating Raptor-binding, these sequences were exchanged between 4E-BP1 and 4E-BP2. The C-terminus of 4E-BP1 mediated the enhanced interaction with Raptor as its fusion to the N-terminus of 4E-BP2 caused it to interact with Raptor to the same degree as wild type 4E-BP1 (Appendix 3C). We then tested whether a negative charge at the site of Ser101 of 4E-BP1 is important for association to Raptor. 4E-BP1 Ser101 was mutated to alanine or the phosphomimetic glutamate. The Ser101Ala mutant exhibited reduced Raptor-binding, while that of Ser101Glu (S101E) was greatly increased (Appendix 3D). Taken together, these preliminary observations are consistent with the idea that negative charges in the non-conserved C-terminal region of 4E-BPs are involved in strengthening the interaction with Raptor, and that, in the absence of deamidation, 4E-BP2 is deficient in this respect. Additional work is needed to confirm the validity of these results. In particular, a new series of mutants should be constructed in which the C-

terminus of deamidated 4E-BP2 is exchanged with 4E-BP1. Similarly, the C-terminus of 4E-BP1 containing Ser101 mutants should be exchanged with 4E-BP2. This will allow the molecular dissection of whether these sites specifically mediate the alterations in Raptor-binding or if other C-terminal features are involved. The responses of 4E-BP1 and 4E-BP2 to growth-factor stimulation will also be examined over time. This will determine if the decreased ability of 4E-BP2 to interact with Raptor leads to diminished or slowed phosphorylation and dissociation from eIF4E, relative to 4E-BP1.

### 4.3 Conclusion

Asparagine deamidation is a developmental modification of 4E-BP2 specific to the brain. The intact asparagine-rich deamidation sequence is conserverd only in mammalian 4E-BP2 in comparison to all known canonical 4E-BPs. This, together with the fact that 4E-BP2 is effectively the sole 4E-BP in the mammalian brain suggests a tantalizing link of 4E-BP2 deamidation to higher brain function. This is supported by our observation of altered synaptic transmission upon expression of deamidated 4E-BP2 in 4E-BP2-/- hippocampal neurons. However, much work is necessary to fully define the physiological role of 4E-BP2 deamidation in the nervous system. An out-standing question remains as to why 4E-BP2 deamidation occurs only in the brain despite the presence of the protein in several tissues. That 4E-BP2 deamidation appears to function by affecting its physical interactions with known binding partners is consistent with the affect of deamidation on other proteins. However, more studies and tools are required to fully work out the molecular details of how the deamidation sites affect the interaction of 4E-BP2 with Raptor, and consequently eIF4E. Moreover, how does PIMT-mediated repair of

isoaspartates impact these interactions? In conclusion, although the understanding of 4E-BP2 deamidation gained in preparing this thesis is still in its infancy, it is hoped that this knowledge will foster further studies leading to a fuller appreciation of this phenomenon and its physiological role.

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## Original Contributions to Knowledge

1) Identification of developmentally regulated deamidation of 4E-BP2 unique to the brain.

2) Identification of two major sites of asparagine deamidation near the C-terminus of 4E-BP2 and mutation of 4E-BP2 cDNA to replicate the deamidated protein.

3) Demonstration that deamidated 4E-BP2 exhibits enhanced interaction with Raptor. Expression of deamidated 4E-BP2 in neurons alters the kinetics of miniature synaptic currents.

4) Demonstration that deamidated 4E-BP2 is a substrate for PIMT. Development of a new antibody to recognize 4E-BP2 harbouring isoaspartyl residues at the deamidation sites.

5) Demonstration that wild type 4E-BP1 associates better with Raptor than 4E-BP2.

## Appendix 1 Supplemental Data to Chapter 2

### Purification of Endogenous 4E-BP2 and Mass Spectrometry Analysis

Fifty male C57BL/6 mouse brains (8-12 weeks postnatal; purchased from Harlan) were dounce homogenized in Buffer A (20mM Tris-HCl pH 7.4, 0.1M KCl, 1mM DTT, 1mM EDTA, 1mM EGTA, 50mM β-glycerophosphate, 1mM PMSF, 10mM NaF, 0.25 mM sodium orthovanadate, 1μg/mL leupeptin) and insoluble material was cleared by centrifugation at 27,000g for 20 minutes . The homogenate was diluted to 2.5mg/ml and powdered ammonium sulfate was slowly added to 35% w/v, with constant stirring. Insoluble proteins were precipitated by stirring on ice for 30 minutes and removed by centrifugation (as above). The solution was then brought to 60% w/v ammonium sulphate and insoluble proteins precipitated and pelleted as above. The pellet from the 35-60% ammonium sulphate cut, which contains 4E-BP2, was resuspended in Buffer B (20mM Tris-HCl pH 8.0, 50mM NaCl, 2mM EDTA, 1% NP-40, 50mM β-glycerophosphate, 1mM PMSF, 10mM NaF, 0.25 mM sodium orthovanadate, 1μg/mL leupeptin) and brought to 1.5mg/ml. The solution was boiled for seven minutes, centrifuged, and the cleared supernatant, containing 4E-BP2, was passed over a DEAE sepharose anion-exchange chromatography column. Two preclearing steps were performed prior to immunoprecipitation, each with 250μl of Protein G Sepharose for 1h at 4$^{o}$C. Immunoprecipitation with 250μl of 4E-BP2 antibody (Cell Signaling Technology), which was previously covalently conjugated to 100μl of Protein G Sepharose with DMP, was for 2h at 4$^{o}$C. The resin was washed four times and protein was eluted with 2% acetic acid (pH 2.2). Three successive elutions were pooled and lyophilized to dryness

overnight. Eluted protein was resuspended in 2X Laemmli sample buffer prior to separation by 15% SDS-PAGE. Proteins were visualized with GelCode Blue Stain Reagent (Pierce) and excised with a scalpel. Sequence analysis was performed at the Harvard Microchemistry and Proteomics Analysis Facility by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer.

**Supplemental Figure Legends**

**Figure S1. 4E-BP2 phosphorylation occurs in a hierarchical manner, similar to that of 4E-BP1.** HEK293 cells were transfected with plasmids encoding the indicated 3HA-tagged 4E-BP2 variants and phosphorylated forms were resolved by subjecting cell lysates to IEF/SDS-PAGE, followed by Western blotting with anti-HA antibody.

**Figure S2. Western blotting failed to detect 4E-BP2 phosphorylation in the adult brain relative to cultured cells.** Lysates from tissues of 12 week-old mice or HEK293E cells were resolved by SDS-PAGE and phosphorylation of 4E-BP2 was detected with an antibody specific for T37/T46. The membrane was then reprobed for 4E-BP2.

**Figure S3. Mass spectrometry analysis of purified, endogenous 4E-BP2 from brain indicates occurrence of deamidation in the asparagine-rich sequence.** Endogenous 4E-BP2 was purified from 50 mature mouse brains and analysed by tandem LC-MS/MS. The 4E-BP2 amino acid sequence and peptide coverage/analysis are shown for the slowest- migrating and unmodified 4E-BP2 species in the brain. The legend indicates putative modifications in the protein.

**Figure S4. *In vitro* deamidation of 4E-BP2 from cell lysates treated at high pH induces the same banding pattern as 4E-BP2 in the adult mouse brain.** MEFs were starved of serum and amino acids for 24h and then lysed at pH 10. Cell lysates were incubated at $37^0$C for 0 or 18h. Proteins were resolved by 15 % SDS-PAGE along with adult brain lysate and Western blotting was performed for 4E-BP2, followed by stripping of the membrane and reprobing for 4E-BP1.

**Figure S5. Representative MS spectra indicating the accumulation of 4E-BP2 deamidation in recombinant protein treated at pH 10 for 24h.** Prominent y-ions marked with asterisks denote the +1Da increases upon deamidation at N99 and N102 in the indicated species resolved in Figure 3D.

**Figure S6. Deamidation of 4E-BP2 is induced in postnatal day 3 hippocampal lysates and is present in mature hippocampus.** Hippocampi from P3 or adult mice were homogenized at pH 7 or 10 and incubated for the indicated times. Samples were analysed by 15% SDS-PAGE.

**Figure S7. Amino acid sequence alignments of 4E-BP2 (or single 4E-BP homologue) from several species.**

**Figure S1**

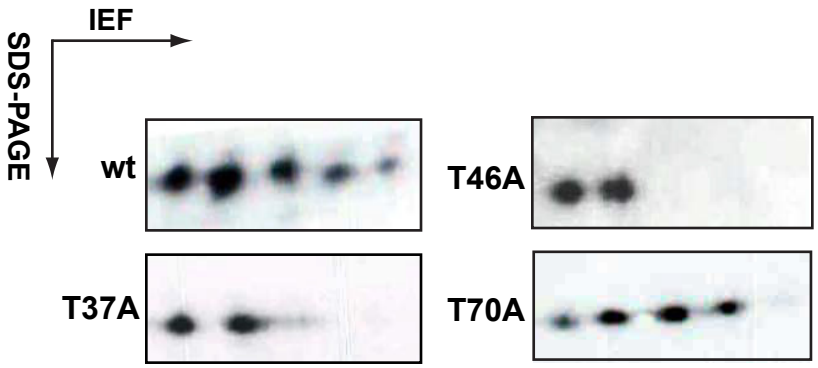
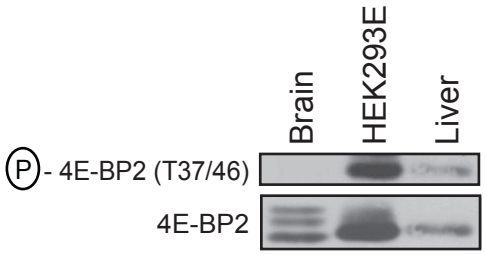


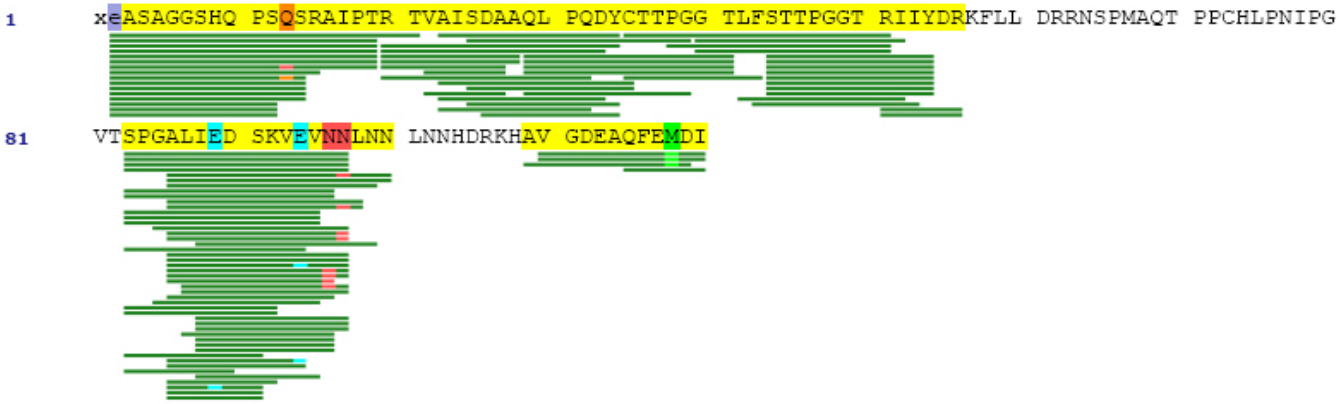
**Figure S2**



# Figure S3

## Modified Band (Slowest migrating)


## Unmodified Band (fastest migrating)

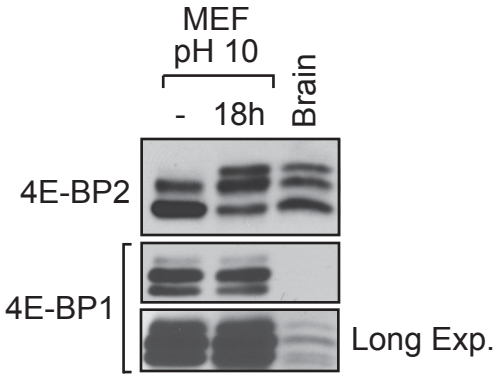



**Legend:**

Protein coverage — Peptide spectra

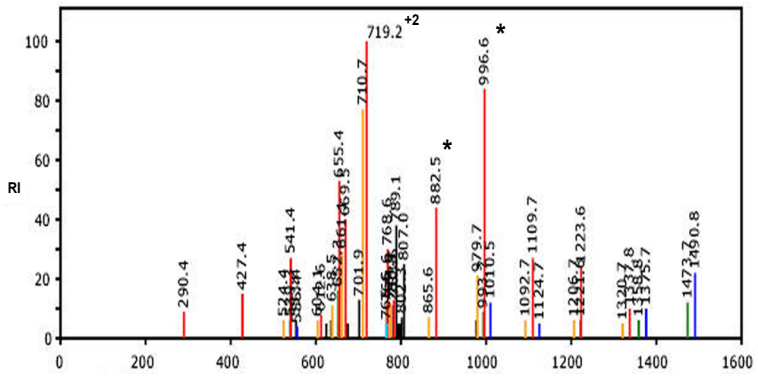
M*:+15.9949 Met sulfoxide (natural or lo pH artifact — CTRE#:+14.01570 Methylation (artifact or natural) — STQ^:+addition — NQ~:+0.98402 Deamidation — 2: e=Nterm-acetylated serine

**Figure S4**



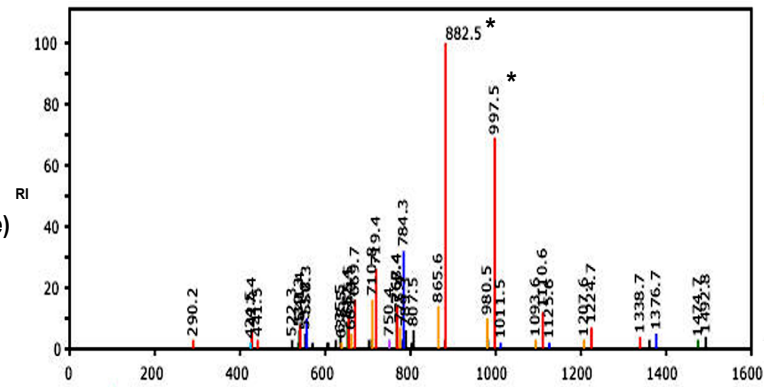
# Figure S5

**N99/N102 (fastest)**



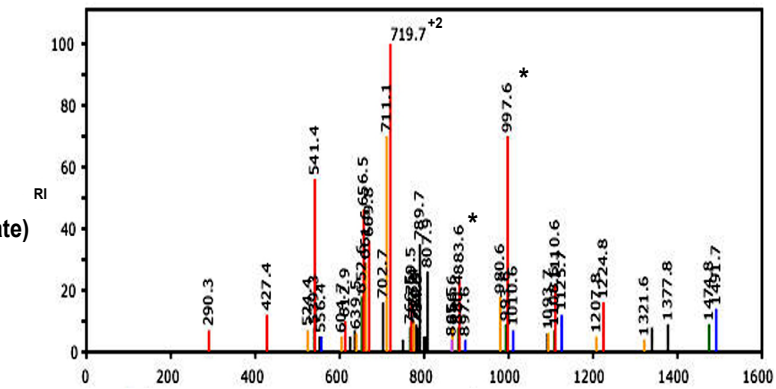

| bond | +1 y-ions | +1 b-ions |
|---|---|---|
| $V_1$ | $^{+2}$1566.742 | 100.076 |
| $E_2$ | $^{+2}$1436.699 | 229.118 |
| $V_3$ | $^{+1.2}$1337.631 | 328.187 |
| $N_4$ | $^{+1.2}$1223.588 | 442.230 |
| $N_5$ | 1109.545 | 556.273 |
| $L_6$ | 996.461 | 669.357 |
| $N_7$ | 882.418 | 783.400 |
| $N_8$ | 768.375 | 897.442 |
| $L_9$ | 655.291 | 1010.527 |
| $N_{10}$ | 541.248 | 1124.569 |
| $N_{11}$ | 427.205 | 1238.612 |
| $H_{12}$ | 290.146 | 1375.671 |
| $D_{13}$ | 175.119 | 1490.698 |

**N99D/N102 (intermediate)**



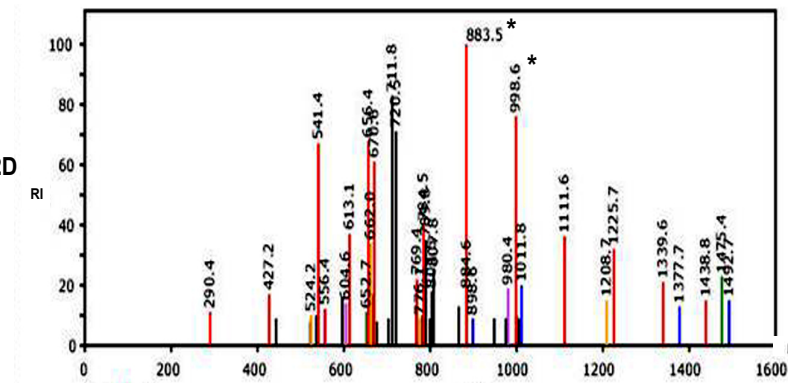

| | | |
|---|---|---|
| $V_1$ | 1566.726 | 100.076 |
| $E_2$ | $^{+2}$1437.684 | 229.118 |
| $V_3$ | $^{+1.2}$1338.615 | 328.187 |
| $N_4$ | 1224.572 | 442.230 |
| $N_5$ | 1110.529 | 556.273 |
| $L_6$ | 997.445 | 669.357 |
| $N_7$ | $^{+1.2}$882.418 | 784.384 |
| $N_8$ | 768.375 | 898.427 |
| $L_9$ | 655.291 | 1011.511 |
| $N_{10}$ | 541.248 | 1125.554 |
| $N_{11}$ | 427.205 | 1239.597 |
| $H_{12}$ | 290.146 | 1376.656 |
| $D_{13}$ | 175.119 | 1491.683 |

**N99/N102D (intermediate)**



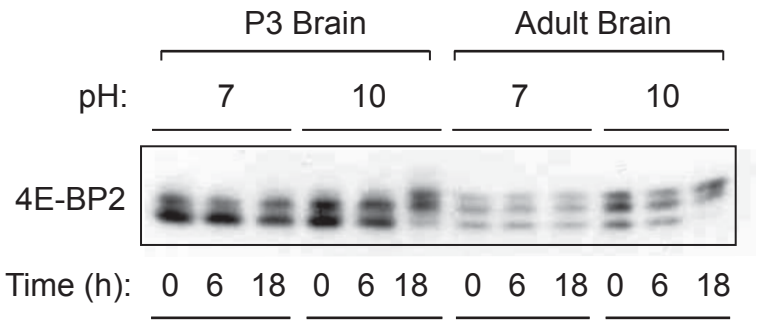

| | | |
|---|---|---|
| $V_1$ | 1566.726 | 100.076 |
| $E_2$ | $^{+2}$1437.684 | 229.118 |
| $V_3$ | $^{+2}$1338.615 | 328.187 |
| $N_4$ | $^{+1.2}$1224.572 | 442.230 |
| $N_5$ | 1110.529 | 556.273 |
| $L_6$ | 997.445 | 669.357 |
| $N_7$ | 883.402 | 783.400 |
| $N_8$ | 769.360 | 897.442 |
| $L_9$ | 656.275 | 1010.527 |
| $N_{10}$ | 541.248 | 1125.554 |
| $N_{11}$ | 427.205 | 1239.597 |
| $H_{12}$ | 290.146 | 1376.656 |
| $D_{13}$ | 175.119 | 1491.683 |

**N99D/N102D (slowest)**




| | | |
|---|---|---|
| $V_1$ | $^{+2}$1567.711 | 100.076 |
| $E_2$ | 1438.669 | 229.118 |
| $V_3$ | $^{+1.2}$1339.600 | 328.187 |
| $N_4$ | $^{+1.2}$1225.557 | 442.230 |
| $N_5$ | $^{+1.2}$1111.514 | 556.273 |
| $L_6$ | 998.430 | 669.357 |
| $N_7$ | 883.402 | 784.384 |
| $N_8$ | 769.360 | 898.427 |
| $L_9$ | 656.275 | 1011.511 |
| $N_{10}$ | 541.248 | 1126.539 |
| $N_{11}$ | 427.205 | 1240.582 |
| $H_{12}$ | 290.146 | 1377.641 |
| $D_{13}$ | 175.119 | 1492.668 |

**Figure S6**


P3 Brain
Adult Brain
pH:
7
10
7
10
4E-BP2
Time (h):
0 6 18 0 6 18 0 6 18 0 6 18

# Figure S7


human
chimpanzee
dog
cow
mouse
xenopus
zebrafish
chicken
drosophila
mosquito
human
chimpanzee
dog
cow
mouse
xenopus
zebrafish
chicken
drosophila
mosquito

**Appendix 2 Phosphorylation of deamidated 4E-BP2 is facilitated**

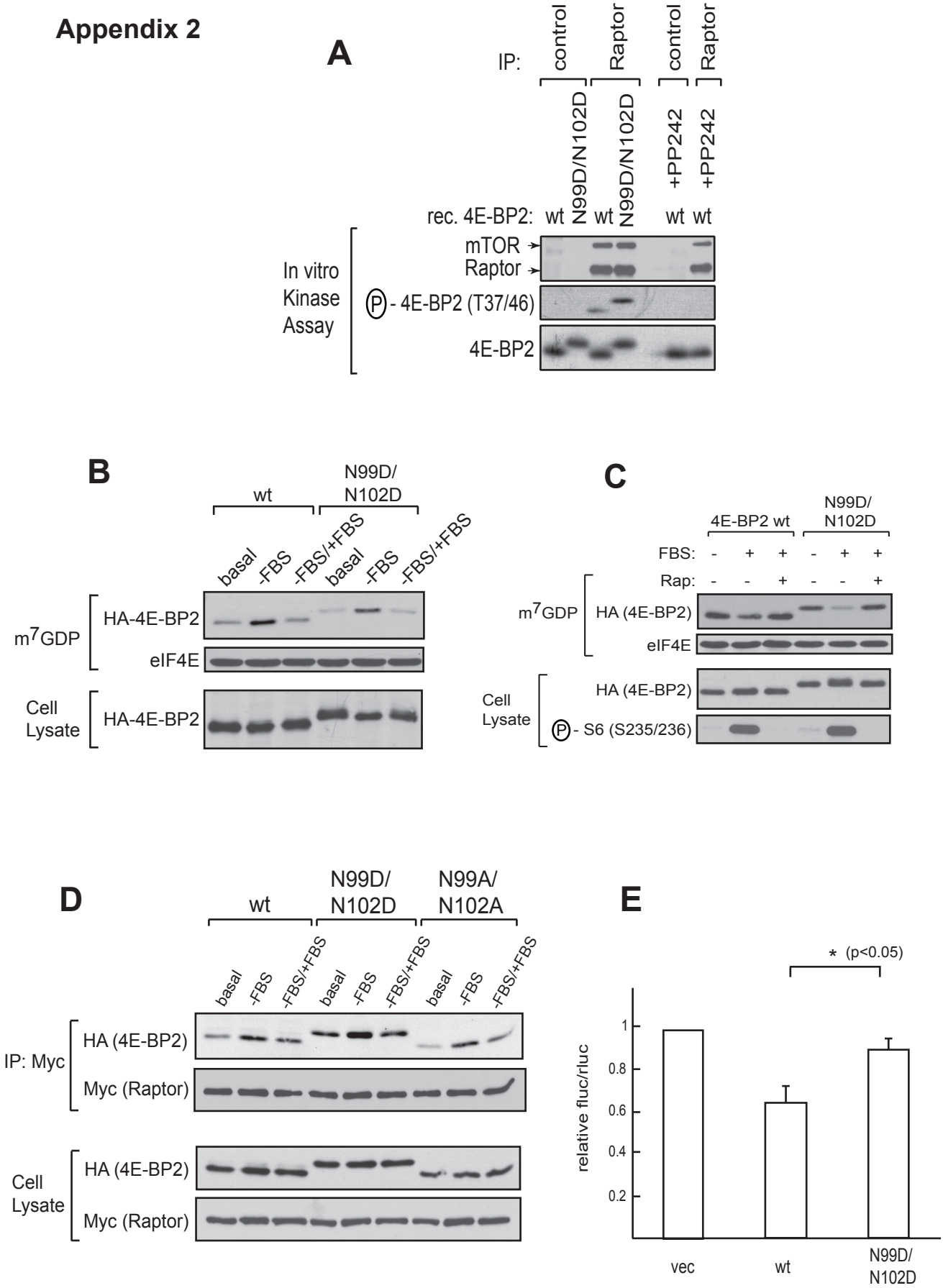
(A) Deamidated 4E-BP2 is more efficiently phosphorylated *in vitro* by mTORC1 relative to wild type 4E-BP2. mTORC1, enriched by Raptor immunoprecipitation, was used to phosphorylate recombinant 4E-BP2 proteins. Immunopreciptates were incubated with 150ng of recombinant protein and 250μM ATP for 20 minutes at 30$^{o}$C. Reactions were stopped by addition of 5X SDS-PAGE sample buffer. mTOR active-site inhibitor, PP242 (250μM) (Feldman et al., 2009)**,** was included were indicated to control for mTOR-independent kinase activities towards 4E-BP2.

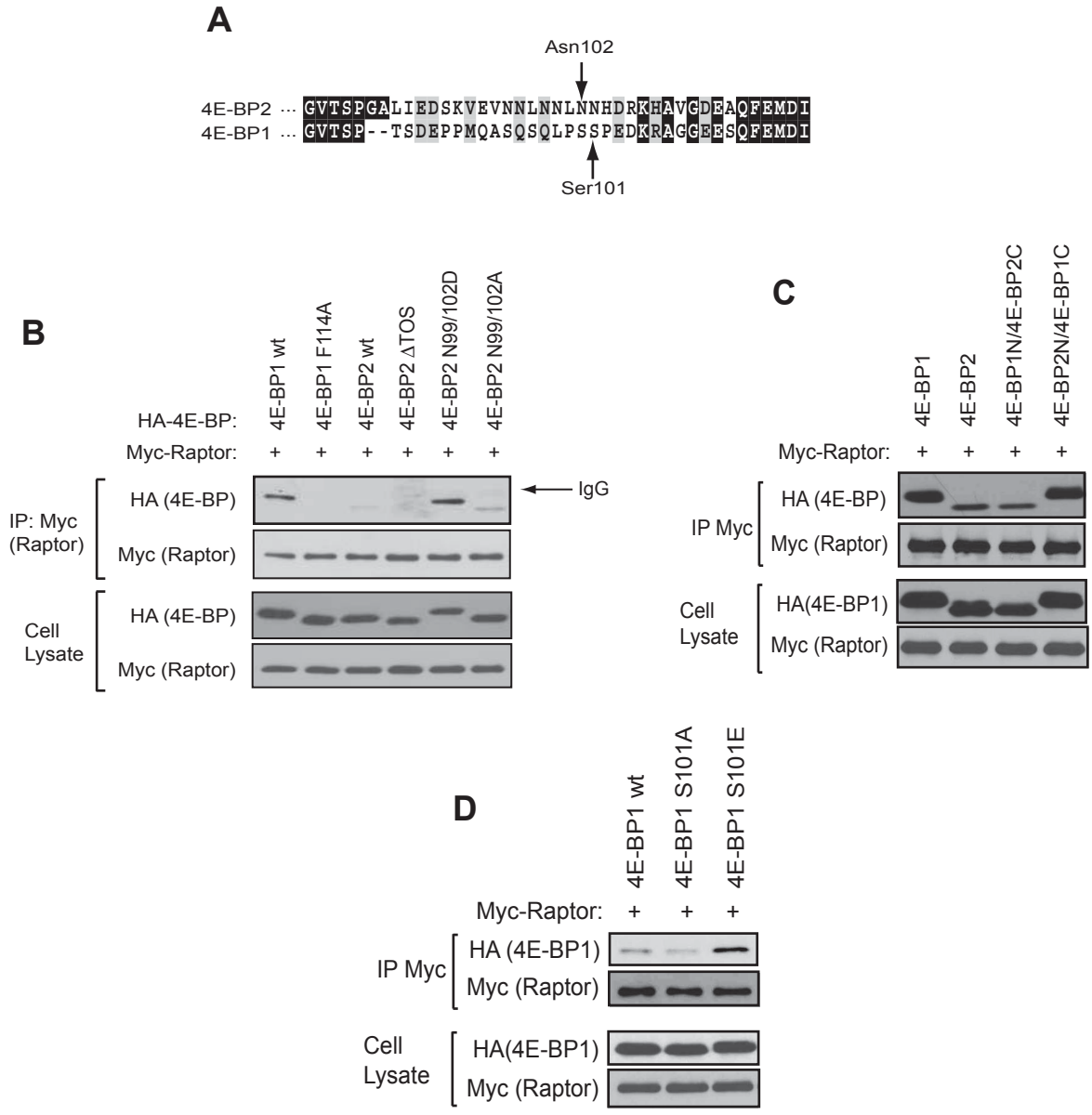
(B and C) Deamidated 4E-BP2 is more easily dissociated from eIF4E, in comparison to wild type, following serum stimulation in a rapamycin-sensitive manner. Cells were transfected with 4E-BP2-encoding plasmids, as above. After 24h, cells were starved of serum for an additional 24h, followed by stimulation with 10% serum (FBS) for 45 minutes. m$^{7}$GDP prescipitation of eIF4E complexes from cell lysates was then performed. Rapamycin (100nM) was added 15 minutes prior to serum-stimulation were indicated.

(D) Association of deamidated (N99D/N102D), wild type, or N99A/N102A 4E-BP2 with Raptor is regulated by serum-stimulation. Transfected cells were treated as in B and the interaction of 4E-BP2 variants with raptor was examined by immunoprecipitation of exogenously-expressed Raptor with anti-Myc antibody. Note that the enhanced interaction of deamidated 4E-BP2 with Raptor is maintained under all conditions examined.

(E) Deamidated 4E-BP2 displays attenuated translational repression towards a luciferase reporter mRNA harbouring a structured 5'UTR. HEK293E cells were transfected with 4E-BP2 variants as indicated and plasmids encoding firefly luciferase and renilla luciferase. Cells were harvested 24h post-transfection and relative luciferase activity was measured from the lysates. Firefly transcripts contain the 5'UTR of IRF-7 mRNA, which is sensitive to 4E-BP proteins (Colina et al., 2008). Renilla luciferase levels were used to normalize signals for transfection efficiency.

**Appendix 2**


A
IP:
control
Raptor
control
Raptor
N99D/N102D
N99D/N102D
+PP242
+PP242
rec. 4E-BP2: wt N99D/N102D wt N99D/N102D wt wt
In vitro Kinase Assay
mTOR
Raptor
Ⓟ - 4E-BP2 (T37/46)
4E-BP2
B
wt
N99D/ N102D
basal
-FBS
-FBS/+FBS
basal
-FBS
-FBS/+FBS
m7GDP
HA-4E-BP2
eIF4E
Cell Lysate
HA-4E-BP2
C
4E-BP2 wt
N99D/ N102D
FBS: - + + - + +
Rap: - - + - - +
m7GDP
HA (4E-BP2)
eIF4E
Cell Lysate
HA (4E-BP2)
Ⓟ - S6 (S235/236)
D
wt
N99D/ N102D
N99A/ N102A
basal
-FBS
-FBS/+FBS
basal
-FBS
-FBS/+FBS
basal
-FBS
-FBS/+FBS
IP: Myc
HA (4E-BP2)
Myc (Raptor)
Cell Lysate
HA (4E-BP2)
Myc (Raptor)
E
* (p<0.05)
relative fluc/rluc
1
0.8
0.6
0.4
0.2
vec
wt
N99D/ N102D




**Appendix 3 4E-BP2 weakly interacts with Raptor, relative to 4E-BP1. A link between 4E-BP2 deamidation and C-terminal 4E-BP1 phosphorylation in modulating Raptor-binding?**

(A) Amino acid sequence alignment of the C-termini of 4E-BP2 and 4E-BP1. 4E-BP2 deamidation residue Asn102 aligns next to constitutively phosphorylated Ser101 of 4E-BP1 suggesting a role for negative charges near this site in modulating the 4E-BP-Raptor interaction.
(B) Immunoprecipitation of Raptor reveals dramatically reduced interaction of 4E-BP2 in comparison to 4E-BP1. Deamidated 4E-BP2 exhibits enhanced Raptor-binding, similar in magnitude to that of 4E-BP1.
(C) Exchange of the C-terminal sequences between 4E-BP1 and 4E-BP2 inidcates that the non-conserved C-terminal region of 4E-BP1 mediates the enhanced interaction with Raptor.
(D) Disruption of 4E-BP1 Ser101 by mutation to alanine reduces interaction with Raptor. Conversely, the phosphomimetic form (S101E) enhances the Raptor-binding indicating that this site is involved in facilitating 4E-BP1's assocation with Raptor.