Investigating the potential interaction of RBM4 with Translational machinery in C2C12 cells undergoing myogenic differentiation.

Jed McDonald

D.Phil

The University of Sussex

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Declaration

I hereby declare that this thesis has not been and will not be, submitted in whole or part to another university for the award of another degree.

Jed McDonald 15th March 2012

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I would like to thank Simon for the opportunity to do this project and all of the help he has given me over the years and also for proof reading my work to correct my awful spelling and grammar.

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Abstract

Investigating the potential interaction of RBM4 with Translational machinery in C2C12 cells undergoing myogenic differentiation.

RBM4 has already been shown to be involved in myogenic differentiation and it has also been shown to interact with eIF4G and eIF4A in HeLa cells while under arsenite stress, what has not been investigated is whether RBM4 interacts with eIF4G or eIF4A or other parts of the translational machinery during myogenic differentiation. This work is divided into 3 parts that each focus on RBM4 during myogenic differentiation.

In the first section, I present data that looks at RBM4 expression levels total and both its isoforms RBM4a and RBM4b. Both total rbm4 and RBM4b increase in expression whereas RBM4a decrease as differentiation progresses. I also present data investigating potential regulation of RBM4 with data showing RBM4a mRNA expression decrease while RBM4b mRNA expression increases.

The second section focus on the potential role of p38MAPK kinase phosphorylation of serine 309 on RBM4 as a regulator of localisation of RBM4 and of RBM4 on general protein translation and its incorporating into the eIF4F complex and the data presented shows that RBM4 appears to not respond to p38 MAPK activity as observed in hela cells and that RBM4 is incorporated into eIF4F complex and that when overexpressed has a negative effect on RBM4 especially when it can not be phosphorylated on serine 309.

The final section focuses on RBM4 interaction with eIF4G and eIF4A both of which it binds during myogenic differentiation, but does not appear to bind eIf4G in vitro directly.

Abbreviations

4E-BP	eIF4E binding protein
ATP	adenosine triphosphate
C-terminal	carboxy terminal
CAD	C-terminal alanine rich domain
DAPI	4', 6-diamidino-2-phenylindole
cDNA	complementary DNA
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
dsDNA	Double stranded DNA
ECL	Enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
eEF	eukaryotic elongation factor
eIF	eukaryotic intiation factor
EMCV	Encephalomyocarditis virus
FAG	Fragment of eIF4G generated by apotosis
FCS	foetal calf serum
GAP	GTPase activating protein
GDP	guanine diphosphate
IRES	Internal ribosome entry site
kDa	kilodalton
MEM	Minimal essential medium
Met-tRNA$_i$	initator methionyl tRNA
MFC	Multi factor complex
MLC	myosin light chain

Mnk	MAP-kinase interacting kinase
mRNA	messenger RNA
MRF	myogenic regulatory factor
m^7GTP	7-methyl guanosine triphosphate
mTOR	mammalian target of rapamycin
MT	Mutant
MOPS	3-(N-morpholino) Pro panesulfonic acid
NLS	Nuclear localisation sequence
N-terminal	amino terminal
ORF	open reading frame
P-	phosphor
PABP	Poly (A) binding protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PK-	Protein kinase
RBM4	RNA binding motif protein 4
RBM4a	RNA binding motif protein 4a
RBM4b	RNA binding motif protein 4b
RNA	ribonucleic acid
RNP	ribonucleiceoprotein
Rpm	revolutions per minute
RRM	RNA recognition motif
SDS	sodium dodecyl sulphate
ssRNA	single stranded RNA
TBS	tris-buffered saline
TCA	tricholoacetic acid
TEMED	N,N,N',N'-tertamethyllethylenediamine

Tris	Tris(hydroxymethyl)-aminomethane
tRNA	Transfer RNA
UTR	untranslated region
WT	Wild-type

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-Chapter 1-

Introduction

1.1 The central dogma of gene expression in eukaryotes

Gene expression in eukaryotes is regulated at a multitude of points. To start with DNA is transcribed into pre-mRNA which is then spliced into mRNA and then exported into the cytoplasm. The mRNA can then be silenced, degraded by multiple different systems or translated into protein. The latter process can be also regulated at multiple steps (see later). Once the protein is made, its expression level is regulated by degradation or it can be re-localised (as a form of regulation) to decrease or increase its spatial expression. The main points of this process that I will focus on are splicing of the pre-mRNA and mRNA translation; specifically translation initiation.

1.2 Splicing of pre-mRNA into mRNA

Splicing take places after DNA has begun to be transcribed into pre-mRNA. A pre-mRNA contains both introns and exons (Wang & Burge, 2008), with an average of 8 exons per pre-mRNA (Faustino & Cooper, 2003). The process of splicing removes the introns to create mature mRNA. This process is controlled by the spliceosome which is made up of 5 RNAs (snRNAs) and a multitude of associated, regulatory proteins (Wang & Burge, 2008) which there are thought to be at least 145 distinct proteins (Zhou et al, 2002). These snRNAs are called U1, U2, U4, U5, and U6 and the spliceosome is thought to be one of the most complicated systems in cells (Zhou et al, 2002). The spliceosome interacts with the core splicing signals which are found in every intron, these being the 5'splice site (5'ss), the 3' splice site (3'ss) and the branch point sequence (BPS). The spliceosome does this by the U1 snRNA

binding to the 5'ss and the splicing factor 1 (SF1) binding the branch point in an ATP-dependent manner, to form the E' complex. The recruitment of the U2 auxilary factor (U2AF) heterodimer to the 3'ss changes the complex to the E complex. The E complex is then converted to the A complex by the replacement of SF1 with U2 snRNP at the branch point. U4/U6-U5 tri-snRNP associates with the complex converting it to B complex. This is then remodelled with the release of U1 and U4 snRNPs leading to the formation complex C which is catalytically active (Chen & Manley, 2009).

Splicing is not only a process which produces translatable mRNAs from immature mRNAs, it is a process that can give rise to many different isoforms of protein from a single gene, a process called alternative splicing. This ability allows proteomes to be larger than the genome that encodes them; 74% of multi-exon genes in humans are alternatively spliced. The most common splicing events are presented in figure 1.1. These events are often regulated by cis-regulatory elements which can be further dived into four categories; exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs) (Chen & Manley, 2009). These sites differ by their location and the proteins that bind them. PTB is known to bind the polyrimidine tract in the intron sequence and act as an inhibitor of the binding of U2AF, therby causing the exclusion of the 3' exon. Some of the elements are 100-200 bp away from the splicing site so a bind and block model will not work in such cases. A model for such sites suggest the formation of a loop of the pre-mRNA including the exon that will be excluded by the formation of bonds between two proteins situated either side of the exon (Chen & Manley, 2009). Splicing activators have been show

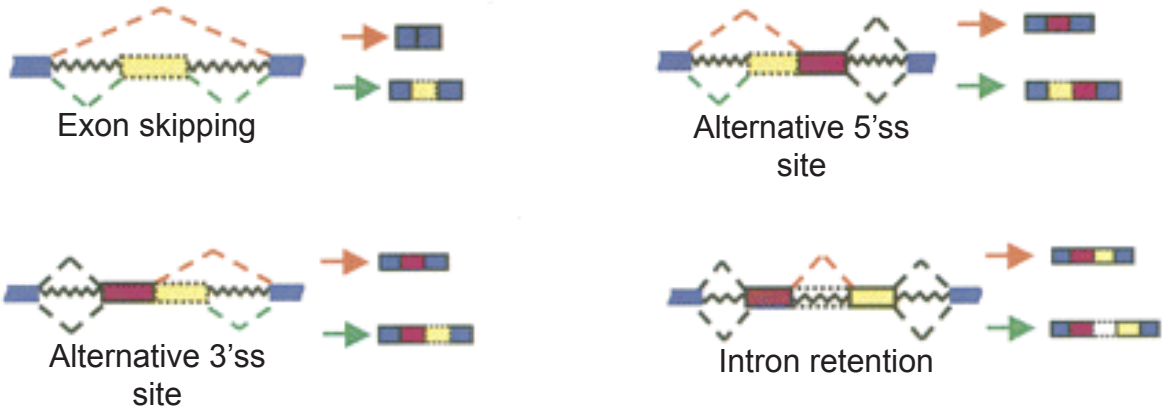


Figure 1.1 Major forms of potential alternative splicing. A selection of some of the major forms of alternative splicing events observed in mammilian cells. Coloured boxes = exons and Coloured dashed lines = potential splice events. Figure adapted from Chen & Manley (2009)

to work at least in part by binding ISE's and ESE's and causing the recruitment of the splicing machinery. An example of this is the T cell-restricted intracellular antigen 1 (TIA1) which binds a U rich region downstream of a weak 5' splice site and causes the recruitment of the U1 snRNP (Chen & Manley, 2009). Overall splicing is a complex system in which many proteins are required for the standard splicing and even more are required to regulate alternative splicing.

1.3.1 An overview of translation in eukaryotes

Translation is a process that converts information encoded in mRNA into protein which can then fulfil roles in the cell. Further modification of the protein by post translational modification can also be used to alter its function. Translation is split into three stages; initiation, elongation and termination. Translation initiation brings the initiator tRNA, the 40S and 60S ribosomal subunits together to form the 80S ribosome at the initiation codon of an mRNA (Pestova et al, 2001). This is followed by the elongation stage where the 80S ribosome moves along the mRNA adding amino acids to the nascent peptide (Preiss & W Hentze, 2003). In the final stage (termination) occurs when the elongating 80S ribosomal complex reaches a stop codon and the completed polypeptide is released and most likely the ribosome dissociates from the mRNA (Preiss & W Hentze, 2003). The initiation step will be discussed in detail here as it is related directly to my work.

1.3.2 Translation initiation

The initiation phase of protein synthesis is an intricate multi-step process whereby an 80S initiation complex is formed in a series of co-ordinated

protein:protein and protein:RNA interactions involving 5' m^7GTP cap structure and the 3' poly(A) tail as well as at least 12 eukaryotic translation initiation factors (eIFs) (reviewed by (Gingras et al, 1999); (Preiss & W Hentze, 2003). The process can be divided into four major sequential events: (i) recruitment of initiator methionyl tRNA (Met-$tRNA_i$) to the 40S ribosomal subunit; (ii) recruitment of the 40S ribosomal subunit to mRNA; (iii) 'scanning' of the 5' untranslated region (UTR) and initiation codon recognition; (iv) 60S ribosomal subunit joining at the start codon (Figure 1.2).

1.3.3 Recruitment of the 43S preintiation complex to the 5' end of the mRNA

The formation of the 43S preintiation complex requires eIF3 and eIF1A to keep the 40S subunit separate from the 60S subunit as the 80S ribosome is more favourable in physiological conditions (Preiss & W Hentze, 2003). eIF2, Met-$tRNA_i^{Met}$ and GTP form a ternary complex which then binds to eIF1, eIF1A, multi-subunit factor eIF3 and eIF5 forming the multifactor complex (MFC) (Asano & Sachs, 2007). This has been shown in yeast to form before binding to the 40S ribosomal subunit, indicating that Met-$tRNA_i$ binding to the small ribosomal subunit might be accomplished by a preassembled multi-initiation factor complex. The interactions within the MFC have been extensively analysed by a combination of *in vitro* binding assays, and purification of MFC sub-complexes formed *in vivo* by affinity-tagged eIF3 subunits lacking discrete binding domains for other MFC components (Verlhac et al, 1997); (Asano et al, 1998); (Asano et al, 2000; Asano et al, 1999); (Phan et al, 1998); (Hanachi et al, 1999). The results of these studies suggest that each of the three largest subunits of eIF3 (a,b and c) have binding sites for the

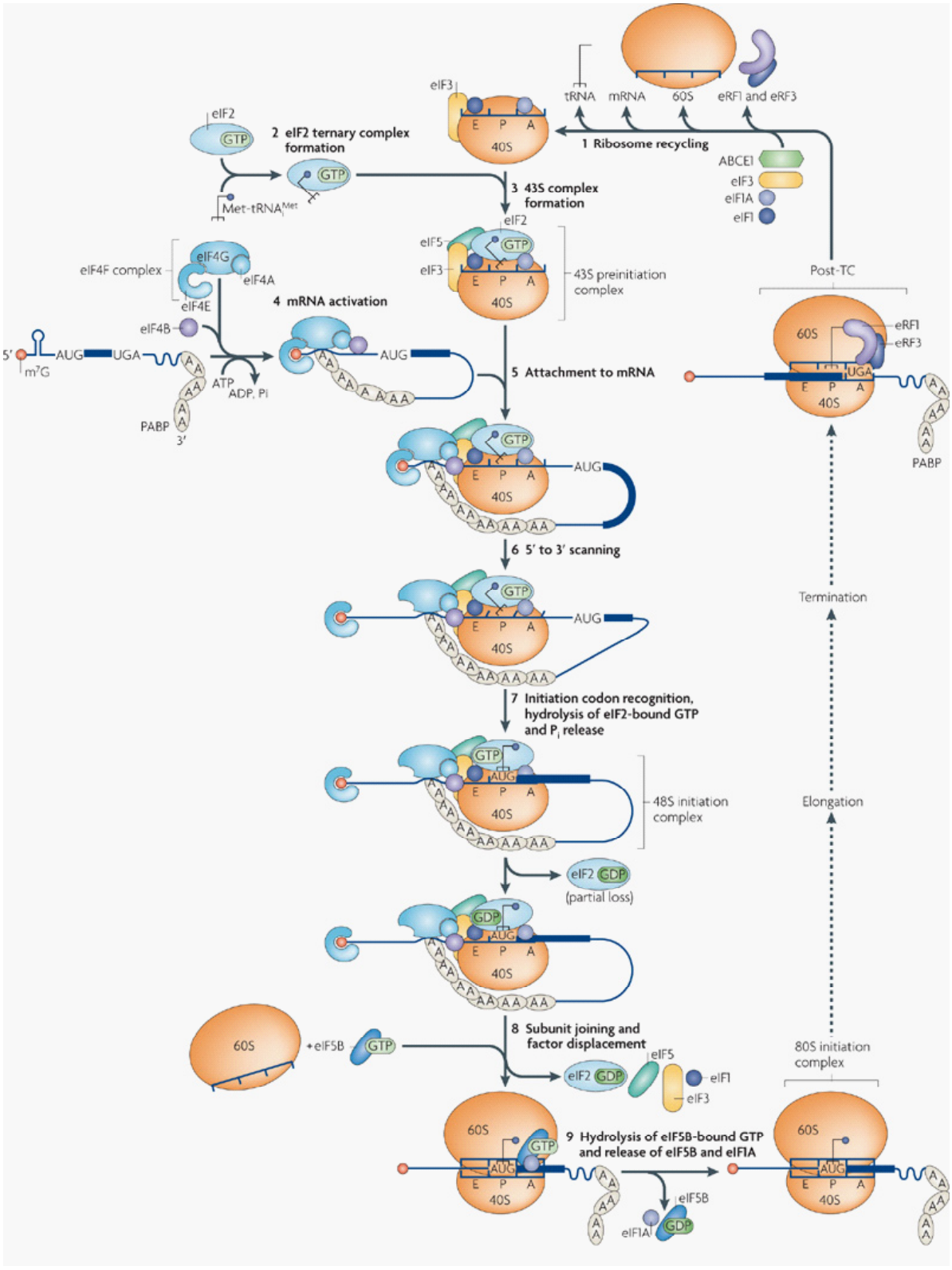


Figure 1.2 Overview of protein translation initiation. Methionyl-tRNAi binds to GTP bound eIF2, this then associates with the 40S subunit already associated with eIF1, eIF1A and eIF3 to form the 43S preinitiation complex. This complex is then recruited to mRNA via interaction between eIF3 and eIF4G (part of eIF4F). Scanning occurs along the mRNA in a 5'-3' direction until a start codon is reached at which point GTP hydrolysis occurs leading to the dissociation of eIF2 bound to the GDP, eIF5,eIF3 and eIF1, with recruitment of GTP bound eIF5B and the 60S subunit. The GTP bound to eIF5B is hydrolysed and leads to the release of eIF5B/GDP and eIF1A. The 80S subunit is now formed and can begin elongation. Taken from Jackson et al, (2011).

other two largest subunits. Further interaction studies established that the N-terminal domain of eIF3a and an internal segment of eIF3c can interact with the ribosomal protein, RPS04, a protein predicted to reside on the solvent side of the 40S ribosomal subunit, which is the opposite side to the 60S-interface side; (Spahn et al, 2001). In addition, the C-terminal domain of eIF3a can specifically interact with a short segment of 18S ribosomal rRNA positioned on the 60S interface side. Based on these findings, a 'wrap-around' model for MFC binding to the small ribosomal subunit was proposed (Valasek et al, 2003). According to this model, whilst eIF3 binds to the solvent side (Valasek et al, 2003); (Mayeur et al, 2003), it has access to the 60S-interface side of the 40S ribosomal subunit through the interaction of the C-terminal domain of eIF3a with the 18S rRNA, placing eIF1, eIF2, eIF5 at the 60S-interface (Siridechadilok et al, 2005).

eIF3 is required for the association of the 43S preintiation complex with eIF4F by binding to eIF4G, which is part of eIF4F (Asano & Sachs, 2007). eIF4F is a complex of eIF4E, eIF4G, and the helicase, eIF4A as shown in figure 1.3. This complex binds to the 5' cap of the mRNA and associates mRNA with the 43S preintiation complex (Asano & Sachs, 2007). Mammalian eIF4E is a 25-kDa protein that interacts specifically with the m^7GTP cap structure at the 5' end of cellular mRNAs and directs the eIF4F complex to the 5' end of the mRNA. Mammalian eIF4A is a 46-kDa protein exhibiting RNA-dependent ATPase (Grifo et al, 1984) and bi-directional RNA helicase activity (Ray et al, 1985); (Pause & Sonenberg, 1992); (Pause et al, 1994b); (Li et al, 1999). eIF4A is the prototype member of a large family of RNA helicases containing the specific DEAD box motif (Rogers et al, 2002). eIF4A helicase

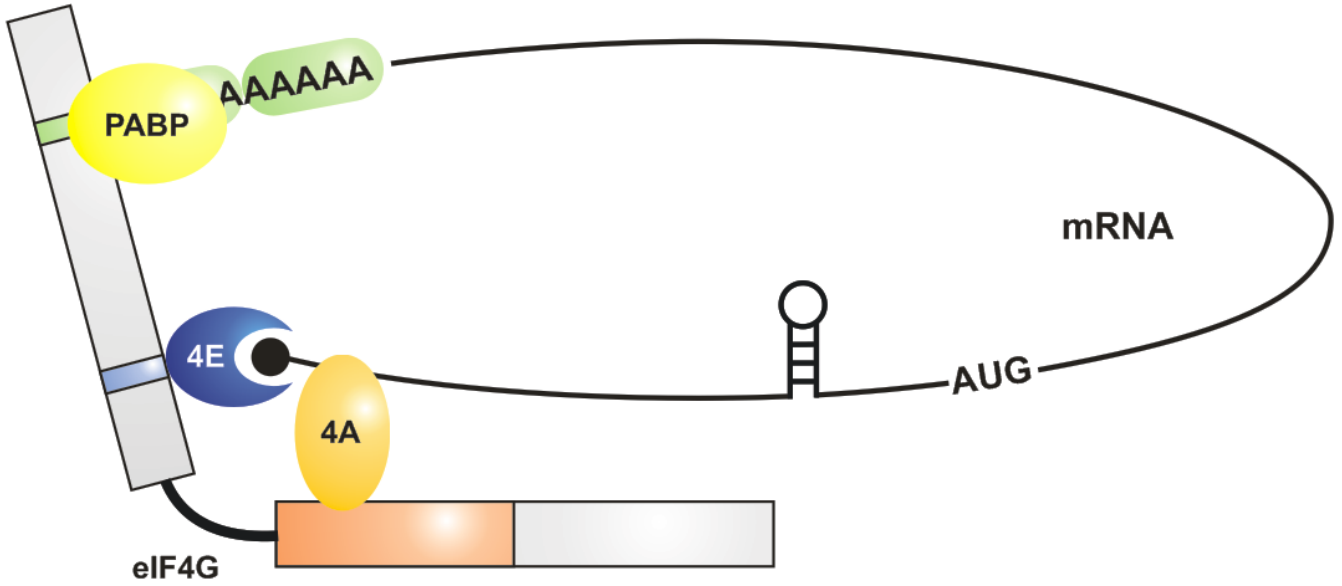


Figure 1.3 The eIF4F complex. The eIF4F complex is made up of the scaffolding protein eIF4G, the RNA helicase eIF4A and the mRNA cap binding protein eIF4E. Also shown here is the Poly A Binding Protein (PABP) and mRNA to illustrate the circularisation of mRNA.

activity is strongly enhanced by the co-factors eIF4B (Gingras et al, 1999) or eIF4H ((Richter-Cook et al, 1998); see later). The function of eIF4A is believed to be the unwinding of secondary structures in the 5' UTR of the mRNA to create an attachment site for the 40S ribosomal subunit (Ray et al, 1985). Mammalian eIF4G is a large protein that is thought to serve as an assembly platform for other components of the translational apparatus (reviewed in (Gingras et al, 1999); (Prevot et al, 2003)). Poly(A) binding protein (PABP),is a protein which binds to the mRNA 3' untranslated region (3'UTR) and eIF4G and causes the functional circularisation of the mRNA (Proud, 2007). This allows for translational regulation of some mRNAs whose poly(A) tail length varies in length depending on developmental conditions (Richter, 1999). Besides serving as a scaffold for the assembly of the translational machinery, eIF4G also influences the activity of other initiation factors. Binding of eIF4G to eIF4E markedly increases its binding affinity for the mRNA cap (Haghighat & Sonenberg, 1997) and the interaction of eIF4G with PABP has been suggested to increase the helicase activity of eIF4F (Bi & Goss, 2000b).

1.3.4 Ribosome scanning and start codon recognition

The 43S ribosomal complex is assembled near the 5' end of the mRNA and has to scan along the 5'UTR to reach the start codon which is usually AUG as part of a consensus sequence which is GCC(**A**/**G**)CCAUG**G**: the underlined region being the start codon and the bold letters being crucial bases; (Kozak, 1989). There are other start codons used to a lesser extent such as ACC (Cavener & Ray, 1991) and CUG, which codes for leucine rather than methionine (Schwab et al, 2004). eIF4A, eIF4B, eIF4F, eIF1 and eIF1A are required for scanning along the 5' UTR (Proud, 2007). eIF4A, eIF4B and

eIF4F are thought to be involved in remodelling the mRNA secondary structure in the 5' UTR to allow the 43S preintiation complex to scan past secondary structure that would have otherwise inhibit its progression (Oberer et al, 2005). eIF4A cycling in and out of eIF4F could be the source of movement during scanning (Preiss & W Hentze, 2003). eIF1 and eIF1A are thought to be required for affecting the structure of the 40S subunit to change the mRNA binding cleft or reposition the tRNA in the 43S complex (Pestova et al, 2001).

1.3.5 Recruitment of the 60S ribosomal subunit and recycling of the initiation factors

The final stage of initiation is the binding of the 60S subunit to form the 80S ribosomal complex. When the 43S initiation complex reaches the start codon, the codon and the anticodon on the tRNA bind, which may cause a conformational change in eIF2 which leads to hydrolysis of the GTP bound to it (Preiss & W Hentze, 2003). This hydrolysis step causes the dissociation of eIF2-GDP and possibly other factors. A second hydrolysis of GTP also occurs, promoted by the 60S subunit binding, causing the GTPase of the eIF5B to increase (Preiss & W Hentze, 2003). This hydrolysis promotes a decrease in the affinity of eIF5B for the ribosome and allows it to dissociate; it may also cause the release of eIF1A which it interacts with. Once the 80S ribosomal complex is formed elongation of the polypeptide chain can begin (Lee et al, 2002); (Pestova & Kolupaeva, 2002).

The GDP bound to eIF2 has to be replaced with GTP to allow the eIF2 to keep cycling through initiation complexes and performing its role in initiation

(Proud, 2007). This conversion is performed by eIF2B which is a guanine nucleotide exchange factor (GEF) which is a heteropentamer made up of α, β, γ, δ and ε subunits (Proud, 2007). eIF2Bε is the catalytic subunit and contains the binding site for eIF2 (Proud, 2007). This point is a crucial regulatory point and is controlled by eIF2α which can be phosphorylated on serine 51 which turns the subunit into a competitive inhibitor for eIF2 (Proud, 2007). This point of regulation is a global protein synthesis regulatory point as it does not control specific mRNA but rather regulates the total level of protein synthesis (Proud, 2007).

1.3.6 Cellular Internal Ribosome Entry Sites (IRESes)

Internal ribosome entry sites are regions in a mRNA that allows internal initiation of the translation and are found in the 5' UTR and in some cases inside the coding sequence of the mRNA (Komar & Hatzoglou, 2005). Internal initiation quite often does not require the 5' cap and involves either the direct recruitment of the 40S ribosomal subunit to the vicinity of the initiation codon or to the start codon directly (Komar & Hatzoglou, 2005). There is a belief amongst some workers in the community that these IRES activities may in fact be caused by cryptic promoters or different splicing patterns of mRNA (Kozak, 2005), but there has been much data that supports the existence of cellular IRESes (Komar & Hatzoglou, 2005). Consequently, protocols for determining IRES activity have been altered to take into account these factors (Bert et al, 2006).

IRESes are thought to be found in at least 3% of cellular mRNAs (Johannes et al, 1999), found to be mainly in mRNA that encode proteins regulating gene

expression in differentiation, development, cell cycle (G2/M phase) progression, apoptosis and stress (Komar & Hatzoglou, 2005; Spriggs et al, 2008). C-myc is an example of a mRNA reported to contain an IRES (Gerlitz et al, 2002). IRESes allow the cell to maintain the translation of the protein their mRNA's encode when general protein translation is decreased under such conditions following eIF2α phosphorylation (Komar & Hatzoglou, 2005); (Jopling et al, 2004). Some IRES are thought to have a reduced need for eIF4G,eIF4B, eIF2α and the p35 subunit of eIF3 (Komar & Hatzoglou, 2005). It has also been shown that eIF4A activity is absolutely essential for c-myc and BiP IRES activity (Thoma et al, 2004), but information about the requirement for eIF4A and initiation factors in other cellular mRNAs hosting an IRES is limited (Komar & Hatzoglou, 2005). In general, most mRNAs containing internal ribosome entry sites are not translated using their 5' m^7GTP cap as they often contain long and highly structured 5' UTRs, but there are cases, they can utilise both cap-dependent and –independent modes of translation (e.g. neurogranin mRNA) (Pinkstaff et al, 2001). Some IRESes are also not found close upstream of the start codon but are found inside the coding region. Such IRESes lead to translation of shortened proteins by IRES-driven translation, with full-length products derived from cap-dependent translation (Komar & Hatzoglou, 2005). FGF-2 is a good example of a protein regulated in this way, but the mRNA actually contains four IRES sites and produces different products as determined by the IRES used to initiate translation (Bonnal et al, 2003). C-myc is also thought to be regulated this way yielding two isoforms; one translated cap-dependently the other by a cellular IRES (Stoneley et al, 2000).

Cellular IRESes are thought to between 150 to 330 nucleotides in length, but some have been shown to be a small as 18 nucleotides. IRES have been shown to often contain pseudoknots, stem loop structures and 5'UTR's containing IRES's have also shown to be GC rich (Bert et al, 2006). However, no sequence homology has been found between different cellular IRESes (Komar & Hatzoglou, 2005). A proposed recruitment method is that the short IRES of 18 nucleotides found in the GTX mRNA is complementary to sequences in 18S rRNA ,potentially allowing binding of the 18S RNA and thus the rest of the 40S subunit to the IRES (Chappell et al, 2000). Also, tRNA-like structures found in some IRES are thought to potentially bind to the P or E sites on the 40S ribosome thus bind the 40S ribosome to the IRES (Fernandez et al, 2005). Proteins, called ITAFs (King et al, 2010), are thought to be involved in the regulation of IRES translation by interacting with the IRES and modulating its structure (Stoneley & Willis); (Bushell et al, 2006); (Lewis et al, 2007) and possible forming interactions with the ribosome directly (Kim et al, 2010; Majumder et al, 2009) and have been shown to up-regulate IRES activity.

PTB has been shown to be involved in regulating multiple cellular IRESs (Kim et al, 2010; Majumder et al, 2009). PTB has been shown to interact with hnRNP Q and upregulate the activity of an IRES in the mRNA coding for Rev-erb α, a protein crucial for the maintenance of cicardian rhythm in mice (Kim et al, 2010). PTB has also been found to interact with hnRNP L during amino acid starvation conditions and bind to the Cat-1 Arginine/Lysine Transporter mRNA and upregulate the IRES dependent translation of the mRNA (Majumder et al, 2009).

1.4 A detailed review of the components eIF4F structure function and regulation

1.4.1 eIF4E

eIF4E as mentioned before stimulates cap-dependent translation but different mRNAs have different dependencies on eIF4E. mRNAs with large amounts of structure in their 5' UTR have a greater dependency on eIF4E than those that have lesser structure (Robert et al, 2009). The three-dimensional structure of slightly truncated mouse and yeast eIF4E bound to the cap analogue m^7GDP was solved by X-ray crystallography and NMR, respectively (Marcotrigiano et al, 1997); (Matsuo et al, 1997). In addition, high-resolution crystal structures of human eIF4E bound to the cap analogues m^7GTP and m^7GpppA were determined (Tomoo et al, 2003). Collectively, these high-resolution structures revealed that the cap-binding protein shows the overall shape of a cupped hand or baseball glove. Cap-binding occurs in narrow hydrophobic slot on the concave surface of eIF4E by specific interactions with the m^7GTP moiety and with the 'downstream' phosphate groups. eIF4E associates with the mRNA *via* the binding with the cap and to eIF4G *via* a site on eIF4G which it also shares with 4E-BP1 (YXXXXLφ, where φ is either Leu, Met or Phe; (Asano & Sachs, 2007) (Robert & Pelletier, 2009).

eIF4E is a phosphoprotein and its major phosphorylation site is found at serine 209 in humans (Roux & Blenis, 2004). The phosphorylation of this site is implicated in protein synthesis regulation. The kinases responsible for this

are Mnk1 and Mnk2. A conserved region at the extreme C-terminus of eIF4G, the W2 domain, which shows homology to eIF5 and eIF2Bε (Aravind & Koonin, 2000), serves as a binding site for the eIF4E kinases, Mnk1 and Mnk2 (Pyronnet et al, 1999); (Morino et al, 2000). Mouse embryonic fibroblasts devoid of Mnk1 and Mnk2 show no eIF4E phosphorylation and this also appeared to have no effect on general protein synthesis (Ueda et al, 2004). However, another study has shown the inhibition of Mnk1 by the inhibitor, CGP57380, led to a reduction in Angiotensin-II-induced eIF4E phosphorylation and protein synthesis (Ishida et al, 2003). eIF4E phosphorylation at serine 209 has been shown to be required for the oncogenic properties of eIF4E (Furic et al, 2010); (Ueda et al, 2010). One reason for this could be that eIF4E phosphorylation leads to increased translation of mRNAs with long 5'-UTR by allowing the eIF4E to dissociate from the mRNA before it has finished scanning to allow more preintiation complexes to scan the mRNA (Raught & Gingras, 1999). These data suggest that even though eIF4E phosphorylation is not required in all cell types and situations, it is important in regulation of translation in response to certain situations.

4E-BP1 is also involved in the regulation of translation by its association with eIF4E, blocking the association of eIF4G with eIF4E (Figure 1.4) via the binding site YXXXXLφ (where φ is either Leu, Met or Phe) which they both share (Marcotrigiano et al, 1999).. This mutually exclusive association is regulated by hyperphosphorylation of 4E-BP1 by the mammalian Target Of Rapamycin Complex 1 (mTORC1) (Rapley et al), causing the disassociation of 4E-BP1 from eIF4E (Sonenberg & Hinnebusch, 2009). mTORC1 is involved

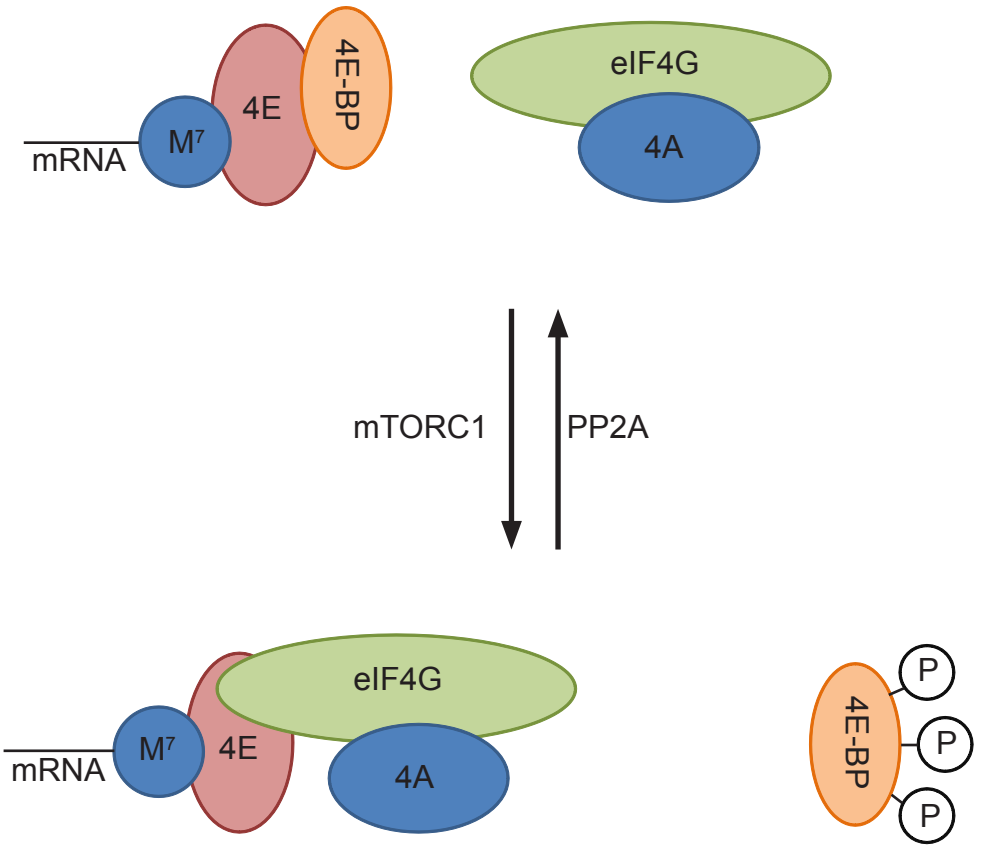


Figure 1.4 4E-BP binds eIF4E in a mutually exclusive manner inhibiting the formation of eIF4F. 4E-BP binds eIF4E inhibiting the interaction of eIF4E with eIF4G stopping the formation of eIF4F. When 4E-BP is hyperphosphorylated by mTORC1 it loses affinity for eIF4E and dissociates allowing eIF4G to bind to eIF4E

in integrating signals from extracellular stimuli, amino acid availability, oxygen and energy status of the cells, allowing the energy state of the cell to control translation rates (Sonenberg and Hinnebusch 2009).4E-BP1 is a member of a family of proteins which contains 4E-BP2 (Pause et al, 1994a) and 4E-BP3 (Poulin et al, 1998) 4E-BP2 and 4E-BP3 shares 56% (Gingras et al, 2001). and 57% (Poulin et al, 1998) sequence identity with 4E-BP1 respectivley and are most conserved in the eIF4E binding region. 4E-BP2 is phosphorylated on less residues than 4E-BP1 and appears to dissociate slower from eIF4E in response to phosphorylation than 4E-BP1(Gingras et al, 2001). Wheras 4E-BP 1 and 2 are expressed in most tissue types 4E-BP3 has a more restricted expression profile (Rong et al, 2008). All 3 isoforms bind eIF4E and inhibit its formation with eIF4G (Pause et al, 1994a; Poulin et al, 1998) which indicates that regulation at this point is important for cells to regulate their translation.

1.4.2 eIF4G

Two forms of mammalian eIF4G have been characterised, eIF4GI and eIF4GII. The predominant form, which probably accounts for 85% of eIF4G protein, is known as eIF4GI; the less abundant form is eIF4GII. In addition, eIF4GI itself consists of a family of five isoforms, which differ by the length and sequence of their amino-termini as shown in figure 1.5 (Imataka et al, 1998); (Byrd et al, 2002). The individual isoforms of eIF4G have been found to share similar, but distinct biochemical activities (Coldwell et al, 2004); (Coldwell & Morley, 2006); (Hinton et al, 2007b). The binding regions for several binding partners of eIF4G have been mapped by deletion and mutation analysis and are shown in figure 1.5. The amino-terminal fragment of eIF4G interacts directly with eIF4E (Mader et al, 1995); (Gradi et al, 1998; Imataka et al, 1998)

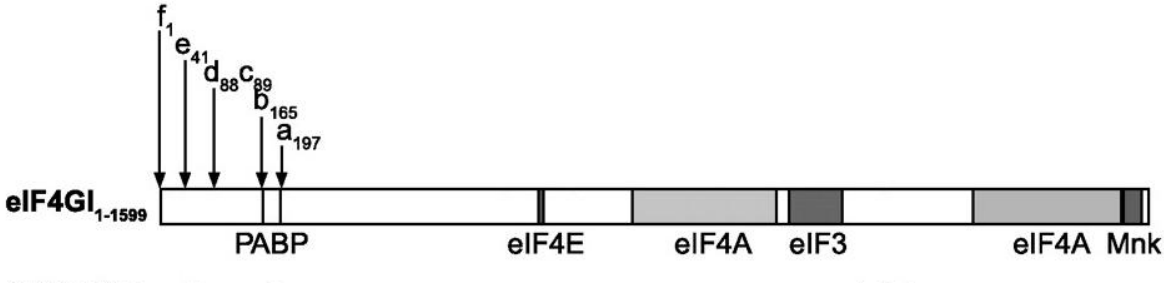


Figure 1.5 The isoform variants of eIF4GI and the binding sites of translation initiation factors. Isoforms of eIF4G are labeled a-f with their initiation sites marked. The binding sites of PABP, eIF4E, eIF4A, eIF3 and Mnk are labeled. Adapted from Morley and Coldwell (2006).

and PABP (Tarun & Sachs, 1996); (Tarun & Sachs, 1997); (Imataka et al, 1998); (Piron et al, 1998). PABP is a translation initiation protein and has been found to be important in circularisation of mRNA by binding the 3' poly(A) tails (Kahvejian et al, 2005) and up-regulating the translation of the mRNA (Doel & Carey, 1976); (Borman et al, 2000). The association of PABP with poly(A) tails has been found to increase the affinity of eIF4E for m^7GTP cap structures (Borman et al, 2000). Apart from eIF4GIa, all isoforms contain PABP binding sites; this isoform of eIF4GI was less effective at rescuing translation in cells depleted of eIF4GI using siRNA (Coldwell & Morley, 2006).

The conserved central fragment of eIF4G contains a binding site for eIF4A (Imataka et al, 1997); (Lamphear et al, 1995) and eIF3 (Lamphear et al, 1995); (Morino et al, 2000) and possesses RNA-binding activity (Goyer et al, 1993). This central segment constitutes the primary ribosome recruitment site (De Gregorio et al, 1999); (Ali & Jackson, 2001); (Morino et al, 2000) and its structure has been resolved by X-ray crystallography, demonstrating that it folds into 5 HEAT motifs (Marcotrigiano et al, 2001). The carboxyl-terminal fragment of eIF4G contains a second, independent binding site for eIF4A (Lamphear et al, 1995); (Morino et al, 2000); (Korneeva et al, 2001); (Li et al, 2001). eIF4G association with eIF3 is required to associate eIF4G with the 40S ribosomal subunit and its binding site is found in the middle domain of eIF4G between amino acids 975-1078. However, inhibition of eIF3 binding to eIF4G by mutation of the eIF3 binding site on eIF4G does not have a great effect on translation (Hinton et al, 2007a). eIF4G association with eIF3 has also been found to be synergistic with eIF4A binding (Korneeva et al, 2000) which could potentially indicate redundancy for the eIF3 binding site. The

eIF4A binding sites are found either side of the eIF3 binding site; one in the middle domain and one in the C-terminal domain (discussed later; (Parsyan et al, 2011).

1.4.3 eIF4A

eIF4A (also known as DDX2), is a DEAD box helicase (Oberer et al, 2005) whose activity is strongly enhanced by the co-factors eIF4B (Gingras et al, 1999) or eIF4H (Richter-Cook et al, 1998). The function of eIF4A is believed to be the unwinding of secondary structures in the 5' UTR of the mRNA to create an attachment site for the 40S ribosomal subunit (Ray et al, 1985) and to facilitate ribosomal scanning towards the AUG codon (Pestova & Kolupaeva, 2002); (Oberer et al, 2005); (Marintchev et al, 2009); (Parsyan et al). Dominant-negative forms of eIF4A inhibit the unwinding of 5' UTRs containing strong secondary structures more than ones containing weak secondary structures (Svitkin et al, 2001). eIF4A has 3 isoforms; eIF4AI, eIF4AII and eIF4AIII (Parsyan et al, 2011). The first two (eIF4AI and eIF4AII) share high sequence homology and are virtually identical in structure and function. The third isoform (eIf4AIII) is not thought to be involved in translation and cannot rescue cells that lack eIF4AI and eIF4AII (Rogers et al, 1999). However, eIF4AIII has a role in exon junction complex formation after splicing (Parsyan et al). eIF4AI and eIF4AII are 406 and 407 amino acids in length and both have RNA-dependent ATPase and ATP-dependent bidirectional helicase activity (Parsyan et al). eIF4A lacks RNA recognition domains and so has a weak affinity for mRNA which is sequence independent.

Structural work on eIF4A suggests that it exists in two states which are in equilibrium with each other; the open form and the closed form. The open form is the inactive form and the two RecA domains are positioned apart. Upon binding of ATP and mRNA, the gap between the two domains closes forming the catalytic site. Upon ATP hydrolysis, the domains separate releasing the mRNA for further cycles of enzymatic activity (Hilbert et al, 2011). Binding to eIF4G causes the equilibrium between closed to open to shift towards the active conformation. eIF4G does this by acting as a soft clamp for eIF4A, stabilising the closed conformation (Oberer et al, 2005). To do this two of the HEAT domains in the middle region of eIF4G bind to eIF4A *via* the C-terminal domain of eIF4A; the eIF4G also forms a weaker link with the N-terminal domain of eIF4A. (Parsyan et al). The association of the C-terminus of eIF4A with eIF4G is salt dependent; the N-terminal association is not salt dependent, indicating that the C-terminal domain binding is caused in part by charge (Oberer et al, 2005). This link between the two domains on eIF4A promoted by eIF4G helps stabilise the closed conformation (Parsyan et al, 2011). eIF4A is not only regulated by eIF4G; eIF4B and eIF4H also play important roles (Parsyan et al).

1.4.4 eIF4B and eIF4H

eIF4B and eIF4H has been shown to increase the helicase activity of eIF4A *in vitro* (Bi & Goss, 2000a; Rozen et al, 1990). eIF4B and eIF4H have been shown to form stable complexes with eIF4A which are mutually exclusive due to their overlapping binding sites (Rozovsky et al, 2008) and binding of eIF4H or eIF4B to eIF4A requires the association of ATP with eIF4A (Marintchev et al, 2009). eIF4B has the ability to bind single stranded RNA (Hinnebusch) but

the eIF4A, eIF4A/eIF4B and eIF4A/eIF4H complexes all have very similar RNAse protection footprints, indicating that they potentially do not bind mRNA (Rozovsky et al, 2008). Supporting this finding is evidence that one of the two eIF4B RNA Recognition Motifs (RRM) is thought to bind a region of the 18S RNA (Methot et al, 1996). This RRM has also been shown to be not required for binding of eIF4B to eIF4A in the presence of mRNA (Rozovsky et al, 2008). eIF4H on the other hand has been shown to bind immediately 5' to eIF4A on the mRNA which would mean it is behind the eIF4A with respect to the direct of translocation (Marintchev et al, 2009). The binding of eIF4H in this fashion could stop the mRNA re-annealing and promote processive and unidirectional translocation (Marintchev et al, 2009). eIF4B has also been shown to form associations with PABP increasing its affinity for the poly(A) region of mRNA (Le et al, 1997). PABP and eIF4A and the 18S RNA are not the only components of the initiation complex eIF4B forms associations with; in wheat, it also binds eIF4G and eIF3 (Parsyan et al, 2011).

1.4.5 Pdcd4

Pdcd4 is a 469 amino acid protein (Lankat-Buttgereit & Goke, 2009), and is known to shuttle between the nucleus and the cytoplasm (Lankat-Buttgereit & Goke, 2009). It consists of four domains (RBM1, RBM2, and two MA3 sequences) and binds mRNA. The RNA binding activity of Pdcd4 (Lankat-Buttgereit & Goke, 2009) has been localised to RBM1 and RBM2 in the N-terminus of the protein (Wedeken et al, 2010). These two regions are rich in lysines and arginines, both positive amino acid residues. (Wedeken et al, 2010). This RNA binding regions were found to be required for localisation of Pdcd4 to 48S pre initiation complexes as deletion of the complete N-terminus

of Pdcd4, or mutation of both RBM1 and RBM2 led to disassociation from mRNA (Wedeken et al, 2010). RBM2 was found to have a greater effect on binding of Pdcd4 to 48S preintiation complexes (Wedeken et al, 2010).

The two alpha helical MA-3 domains are highly conserved between species (Lankat-Buttgereit & Goke, 2009). These two MA-3 domains (MA3-c and MA3-m (Suzuki et al, 2008), have been shown to be important in allowing eIF4GI and II to interact with other proteins (Lankat-Buttgereit & Goke, 2009). As with eIF4G, Pdcd4 has been shown to bind eIF4A (Lankat-Buttgereit & Goke, 2009) and associate with the 48S preintiation complex. However Pdcd4 is not associated with polysomes (Wedeken et al, 2010). MA3-c has been shown to be able to compete with eIF4G for eIF4A binding and also to inhibit translation (Suzuki et al, 2008). Addition of recombinant Pdcd4 to an *in vitro* helicase assay has shown that it can inhibit helicase activity in a dose dependent manner (Lankat-Buttgereit & Goke, 2009), with both of the MA3 domains binding to the N-terminal domain of eIF4A (Suzuki et al, 2008). Mutational disruption of the MA3 domains led to a nearly complete disruption of the interaction between Pdcd4 and eIF4A (Lankat-Buttgereit & Goke, 2009) and also the association of Pdcd4 with the 48S pre initiation complex (Wedeken et al, 2010). MA3-m not only binds the C-terminal domain of eIF4A inhibiting translation by preventing the binding of eIF4A to eIF4G, it also competes with the binding of mRNA to eIF4F (Suzuki et al, 2008). Along with this, the Pdcd4 also seems to bind to eIF4G. One possible explanation for this is that Pdcd4 binds to eIF4G to localises itself to eIF4A in eIF4F or to increase its concentration in the region around eIF4A (Suzuki et al, 2008); (Lankat-Buttgereit & Goke, 2009).

Pdcd4 can be regulated by the phosphorylation of ser67 and ser457 by AKT/protein kinase B (Lankat-Buttgereit & Goke, 2009). The phosphorylation of these sites causes a nuclear translocation of Pdcd4 and a decreased function to act as an inhibitor to AP-1 mediated transcription (Lankat-Buttgereit & Goke, 2009). Pdcd4 is also up-regulated by v-myb and c-myb transcription factors and also during apoptosis induced by PMA, ionomycin and dexamethasone. Up-regulation of Pdcd4 is not observed in apoptosis caused by UV irradiation or topoisomerase inhibitors (Lankat-Buttgereit & Goke, 2009). Pdcd4 is also down-regulated in tumours and can be degraded by phosphorylation by p70 S6 kinase under the control of mTORC1 signalling. Phosphorylation marks Pdcd4 for degradation by recruiting a ubiquitin ligase, releasing eIF4A to promote translation initiation (Lankat-Buttgereit & Goke, 2009). Pdcd4 can also be down-regulated by miR-21 (Lankat-Buttgereit & Goke, 2009), a microRNA often over-expressed in tumour cells.

1.5 An overview of the protein RBM4

RNA binding motif 4 protein (RBM4) is an RNA binding protein (Lin & Tarn, 2005) which was originally discovered in *Drosophila* where it is referred to by the name LARK. Here it has been shown to have a role in controlling the circadian rhythm (Kojima et al, 2007) and regulation of eye development (Sofola et al, 2008). RBM4 can be found as two highly homologous isoforms, termed RBM4a and RBM4b, which are encoded by two separate genes. In humans, the genes are both found on chromosome 11q13 and in mice, on chromosome 19A (Markus & Morris, 2009). As shown in Figure 1.6, the whole of the gene encoding RBM4a is found within an intron of genomic sequence encoding RBM4b (Lai et al, 2003). Lai et al (Lai et al, 2003) postulated that

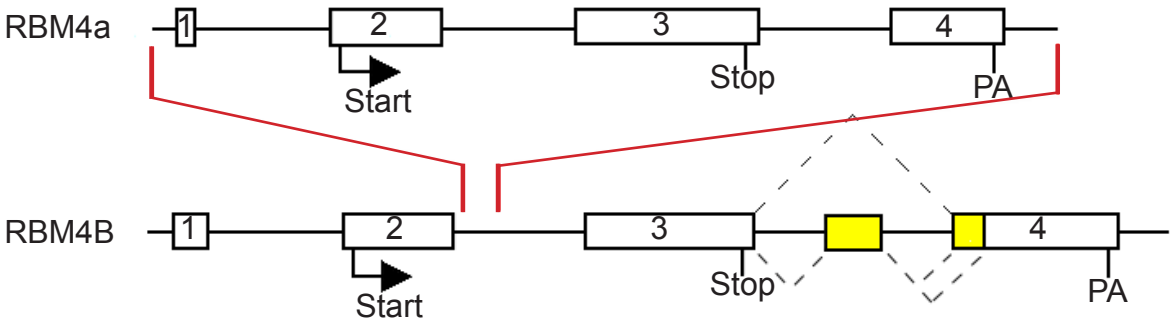


Figure 1. 6 RBM4a and RBM4b gene structure and sites of possible splicing. exons are indicated by boxes with numbers inside, The AUG start codon (Start) and stop codon (Stop) have been marked along with polyadenylation signal (PA) and as well as potential splicing exons (indicated by the yellow) boxes. RBM4a is contained within the two red line between exon 2 and 3 of RBM4b. exons are to scale, Introns are not to scale. adapted for Lai and Tarn (2003)

due to the high sequence homology and structural similarities, RBM4a and RBM4b had arisen from gene duplication. The only significant difference in sequence between RBM4a and RBM4b lies in their non-coding regions (Lai et al, 2003) which might allow alternative splicing to generate the three splice variants of RBM4b (Lai et al, 2003). RBM4 has been shown to have many different diverse functions and is present in many different subcellular compartments in the cell (Lin et al 2005). RBM4 has been shown to be involved in translation (Lin et al, 2007) alternative splicing (Kar et al, 2006), and miRNA silencing of mRNA (Lin & Tarn, 2009).These will be investigated later but all of these functions revolve around mRNA which RBM4 has the potential to bind to due to specific motifs in its structure (Markus & Morris, 2009).

1.5.1 Structure of RBM4

RBM4a and RBM4b are 361 and 357 amino acids long, respectively, and both contain two RRMs and a zinc finger in their N-terminus. This is summarised in Figure 1.7. The N-terminus shares the highest homology with LARK. The mouse and human C-terminal regions contain alanine rich repeats which are referred to as the C-terminal Alanine rich Domain (CAD). LARK on the other hand, contains several non-consecutive RS dipeptide repeats (Lai et al, 2003). However, the alanine rich repeats in the CAD and the non consecutive SR dipeptides appear to function in a similar way as binding sites for other proteins. RBM4 has been shown to shuttle between the cytoplasm and the nucleus this is also true of the CAD which indicates that it contains nuclear import and nuclear export signals or that the protein associates with a protein that does (Lai et al, 2003). Human and mouse RBM4a have a p38

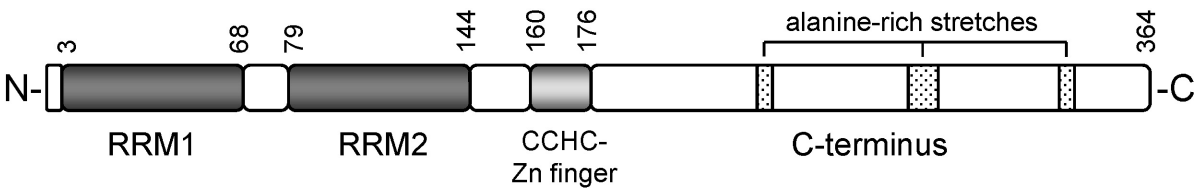


Figure 1.7 Domain Structure of RBM4 protein. RNA Recognition Motifs, zinc finger and alanine rich stretches are indicated. Image obtained form Markus (2008).

MAP kinase phosphorylation site at serine 309 and serine 306, respectively (Lin et al, 2007). These sites are also found in RBM4b protein (Markus & Morris, 2009).

1.5.2 Role of RBM4 in cellular functions

(i) RBM4 is involved in splicing regulation

RBM4 has been found to be involved in multiple splicing events. These include: the inclusion of Tau exon 10 (Kar et al, 2006); up-regulation of the alternative splicing of alpha tropomyosin leading to the up-regulation of the skeletal muscle specific isoform (Lin & Tarn, 2005); and the down-regulation of PTB and nPTB *via* exclusion of exon 11/10 followed by NMD degradation of the truncated isoforms (Lin & Tarn, 2011).

The inclusion of exon 10 into Tau, a protein involved in microtubule organisation in neurons, is regulated by RBM4. A delicate balance between tau exon 10 inclusion and tau exon 10 exclusion is maintained, which if disrupted leads to neurodegenerative disorders like dementia (Kar et al, 2006). Over-expression of RBM4 leads to up-regulation of exon 10 inclusion; the knock-down of RBM4 shows the reciprocal effect. RBM4 has been shown to bind to an intronic element of Tau but not to the mRNA. This site is believed to exist downstream of the 5' splice site and is believed to be made up of a CU rich region (Kar et al, 2006). RBM4 has been observed binding CU rich sequences in other systems (Lin et al, 2007).

Another protein that RBM4 has been shown to modulate by alternative splicing is α-tropomyosin. α-tropomyosin is a protein involved in regulation of

actin function in muscle cells and is required for muscle contraction. α-tropomyosin has two isoforms regulated by RBM4 called skeletal α-tropomyosin (SK) and smooth muscle α-tropomyosin (SM). Expression of RBM4 has a positive correlation with skeletal muscle isoforms and an inverse correlation with the smooth muscle isoforms. If RBM4 is over-expressed SK tropomyosin increases by as much as 500% and SM tropomyosin decreases by about 40%; the opposite is observed for knock-down of RBM4 (Lin et al, 2007). RBM4 has been shown to have CU-rich binding sites in intron 9a, termed CU1, CU2 and CU3. CU1 was also found to bind PTB competitively vs RBM4 and PTB has the opposite effect on splicing isoform selection to RBM4 (Lin & Tarn, 2005). CU2 was found to be the most important site for the effect of RBM4 effect on the splicing of tropomyosin pre-mRNA and PTB was found not to bind to this region (Lin & Tarn, 2005).

RBM4 has also been found to be involved in splicing regulation during differentiation. However, in this case, RBM4 supports PTB in alternatively splicing both neuronal PTB and PTB pre-mRNA. RBM4 causes the exclusion of exon 11 from PTB and exon 10 from nPTB (Lin & Tarn, 2011). RBM4 does this by binding to CU rich regions inside exon 10/11 and upstream and downstream in intronic regions. This binding pattern interferes with the spliceosome formation leading to the exclusion of the exon. In previous work RBM4 has caused inclusion of exons, the difference is believed to be caused by the binding of RBM4 to CU rich elements inside the exon alongside the upstream and downstream binding sites (Lin & Tarn, 2011). When generated, PTB-exon 11 and nPTB-exon 10 mRNAs are degraded by the NMD pathway and aberrant splice isoforms accumulate when NMD pathways are

inactivated. This would indicate that the splice event causes a nonsense mutation that leads to the mRNA degradation. (Lin & Tarn, 2011).

(ii) RBM4 is involved in miRNA-dependent mRNA silencing

RBM4 has also been found to be involved in miRNA-dependent silencing of gene expression, a system which is believed to regulate between 30-90% of all mRNA (Ding & Han, 2007). This is a system in which short 18-22nt non protein encoding RNA molecules (miRNAs) bind to the 3'UTR of target mRNAs. If the complementary sequence of the mRNA and the miRNA match exactly, the cell induces cleavage of the mRNA, as in plants. For sequences with close homology, this interaction between miRNA and mRNA leads to mRNA degradation (Pasquinelli, 2010). Recently characterised protein factors are required for this process (reviewed in (Kawamata & Tomari, 2010)) which are found in the complex called RNA-Induced Silencing Complex (RISC). This complex is assembled through paths that are dependent on Dicer, TAR (HIV) RNA Binding Protein (TRBP), and dsRNA-binding proteins of the AGO family and by other factors including helicases, nucleases and RNA binding proteins. (Fazi & Nervi, 2008); (Kawamata & Tomari, 2010).Once the miRNA is part of the RISC complex it associates with complementary binding site in the 3' UTR of target mRNAs, mRNAs are often subsequently concentrated in P-bodies. These structures are sites of mRNA storage but also contain a wide range of enzymes involved in RNA turnover, including de-capping enzymes, de-adenylases and exonucleases (Fazi & Nervi, 2008).

miRNAs are important in myogenic differentiation, with miR-1, miR-16 and miR-133 acting as muscle-specific miRNAs. miR-1 and miR-133 are

responsible for regulating apotosis in differentiating muscle cells with miR-1 down- regulating the pro-apoptotic factors, HSP60 and HSP70 post-transcriptionally. miR-133 down regulates Caspase-9 so that differentiating muscle cells do not die through receptor-independent apotosis. (Xu et al, 2007). miR-1 has also been shown to be responsible for regulation of myocyte enhancer factor 2C (MEF2C) an essential muscle transcription factor via direct post transcriptional down-regulation of histone deacetylase 4 (Gangaraju & Lin, 2009). miR-1 and miR-206 have been found to regulate the levels connexin 43, a protein involved in gap junction formation during myogenic differentiation (Gangaraju & Lin, 2009).

RBM4 was initially found to transiently localise to cytoplasmic granules, co-localising with Ago2, Dcp1 and GW182 (Ding & Han, 2007). These proteins are miRNP components and indicate that such localisation reflects the assembly of P-bodies. When RBM4 was over-expressed, it formed a RNAse resistant complex with Ago2 which was unaffected by the phosphorylation state of RBM4 (Lin & Tarn, 2009). This was observed in differentiating and proliferating cells but with a larger amount of Ago2 binding RBM4 during differentiation. This increase in complex formation was believed to be due to an increase in RBM4 shuttling into the cytoplasm where Ago2 is found (Lin & Tarn, 2009). This interaction allows RBM4 to recruit Ago2 to mRNAs containing CU rich elements resulting in translational inactivation of these mRNAs. If RBM4 is over-expressed, mRNAs with CU rich regions are preferentially down-regulated (Lin & Tarn, 2009). If Ago2 was knocked-down RBM4 was unable to down-regulate CU rich containing mRNAs. mRNAs that are associated with RBM4 have been shown to have lower levels of

association with eIF4E but not with eIF4A which can bind RBM4. RBM4 has also been shown to interact with miR-1 and miR-206 but not with muscle specific miR-16 (Lin & Tarn, 2009) or miR-133 (Xu et al, 2007).

(iii) RBM4 interacts with translation factors

RBM4 was found to be involved in the regulation of mPER1, a circadian rhythm protein found in mice. This protein has a cycle which is affected by the dark light cycle the animal is subjected to. This cycle pattern is very similar to RBM4 protein expression levels; over-expression of RBM4a or RBM4b leads to an up-regulation in mPER1 by 2.8 fold or 5 fold, respectively. A stem loop binding site for RBM4 has been discovered in the 3'UTR of mPER1. mRNA; binding of RBM4 inhibits translation in a bicistronic assay (Kojima et al, 2007). If this structure was disrupted, RBM4 would no longer bind to the mRNA and translational up regulation was lost (Kojima et al, 2007). However, point mutations that did not disrupt the structure had little effect on RBM4 binding or translational up regulation. The exact mechanism behind this inhibition was not determined (Kojima et al, 2007).

Another case of RBM4 affecting translation of a target mRNA has been reported for HeLa cells undergoing arsenite treatment. During arsenite treatment, the p38 MAP kinase signalling pathway is activated, resulting in the phosphorylation of RBM4 on ser309. This causes a re-localisation of RBM4 from the nucleus to the cytoplasm (Lin et al, 2007). Once in the cytoplasm, RBM4 binds to eI4A and eIF4G and recruits them to an expressed mRNA containing a Encephalomyocarditis Virus (EMCV) IRES sequence. This IRES requires eIF4A to initiate translation, with over-expression of eIF4A increasing

the activity of this IRES in reporter assays. If RBM4 was over-expressed in these transfected HeLa cells, it had a similar effect to eIF4A on the IRES activity. This is thought to be due to RBM4 recruiting more of the eIF4A to the IRES (Lin et al, 2007). It has also been postulated that when phosphorylated, RBM4 has a greater affinity for the EMCV IRES. There is some evidence of RBM4 binding cellular IRES structures, like those reported for c-myc and Bcl-2. Under these conditions, RBM4 can be found associated with these mRNAs. Increased RBM4 levels, or treatment of cells with arsenite increased c-myc and Bcl-2 protein levels (Lin et al, 2007). Like EMCV IRES, c-myc IRES activity has also been shown to be sensitive to levels of eIF4A (Thoma et al, 2004).

(iv) RBM4 regulation of circardian rhythm and eye development in *Drosophila*

RBM4 has been shown to be involved in the regulation of the cicardian rhythm of Drosophila (Huang et al, 2007; Huang et al, 2009; Sofola et al, 2008). LARK has been shown to have an expression profile that is regulated by the cicardian clock and when overexpressed caused an abnormal circardian rhythm (Schroeder et al, 2003). RBM4 has also been shown to interact with a mRNa encoding E74, which is a transcription factor required for regulation of the circadian clock. RBM4 up- regulates the expression of E74 without changing the expression of its mRNA, indicating a potential role in regulating translation (Huang et al, 2007). RBM4 has also been shown to regulate the development of Drosophila eye. It does this by forming a complex with dFMRP (Sofola et al, 2008) but how they mediate there effect is unknown.

1.6 Skeletal myogenic differentiation

Myogenic differentiation (Figure 1.8) is the process by which satellite cells terminally differentiate into multi-nucleated myotubes which then undergo further differentiation leading to fully functional muscle fibres (Grounds et al, 2002). The differentiation process is a highly organised switch requiring the cells to proliferate, exit the cell cycle and then begin expression of muscle specific proteins leading to fusion and subsequent production of contractile proteins (Hawke & Garry, 2001). Satellite cells are found below the basement membrane next to myofibres in a satellite cell-niche (Mauro, 1961). They express a key transcription factor, pax7 which is thought to be crucial in the development of satellite cells. These cells are normally in a quiescent state (Hawke & Garry, 2001), as indicated by a large nuclear to cytoplasmic ratio along with a smaller number of organelles and more heterochromatin (Charge & Rudnicki, 2004b). The satellite cells remain in this quiescent state until they are activated in response to numerous signals including myotrauma (Hawke & Garry, 2001), possibly due to a factor released by the damaged myofibres. The activation does not cause only local satellite cells to become active. More distant cells also become active and migrate towards the site of injury (Schultz & Jaryszak, 1985). These activated cells then go on to proliferate and are often referred to as adult myoblasts. Upon activation of satellite cells, Myf5 and MyoD are up regulated. These proteins which are Myogenic Regulatory Factors (MRF), drive the formation of a large pool of myoblasts which begin to differentiate by up-regulating myogenin and MRF4 protein levels. This is followed by increased levels of p21 which inhibits cell cycle progression, and

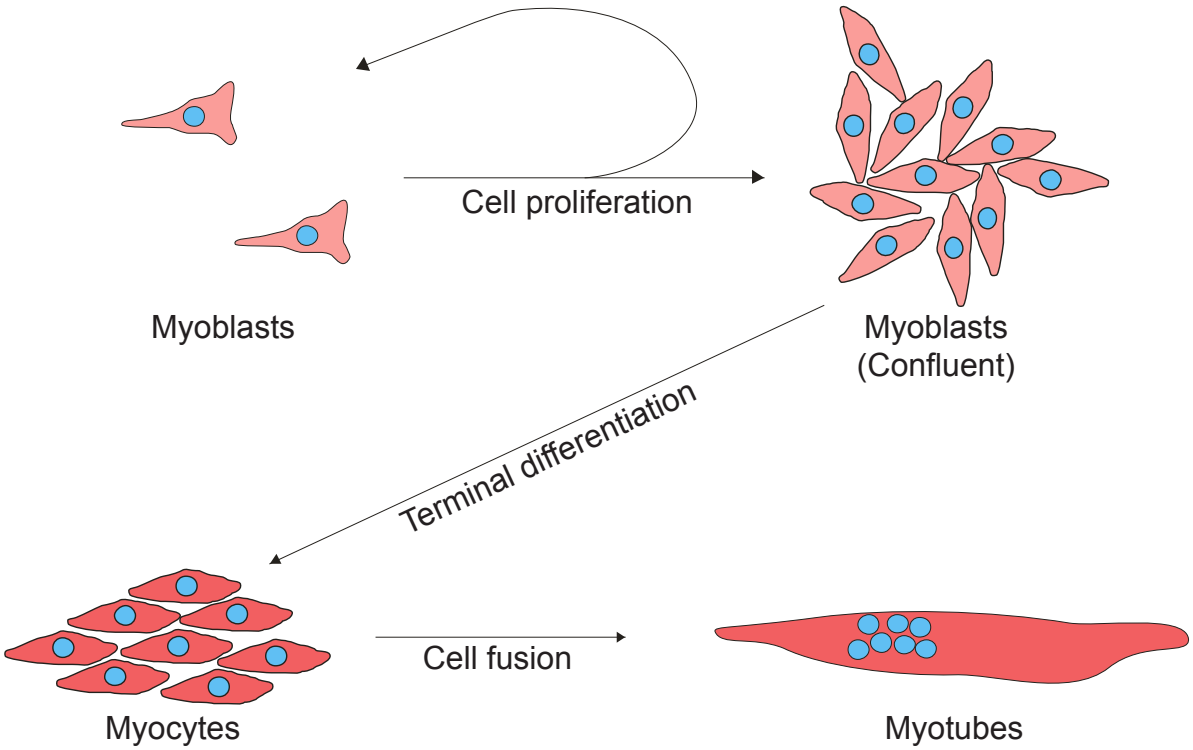


Figure 1.8 myogenic differentiation of C2C12 myoblast cells. C2C12 cells proliferate to confluenlency and once the cells are contacted inhibitiexd the cells begin to terminally diffferentiate and align into myocytes. These myocytes then begin to fuse to form multi nucleated myotubes.

is followed by fusion. M-cadherin and M-calplain are thought to play central roles in this latter process, promoting cell-cell interactions, cytoskeleton reorganisation and up-regulation of muscle specific proteins like myosin heavy chain (Charge & Rudnicki, 2004). Overall, myogenic differentiation is regulated by a number of specific signalling pathways like mTOR (Ge et al, 2009), p38 MAPK (Baeza-Raja & Munoz-Canoves, 2004), PKR (Alisi et al, 2008) calcineurin (Scicchitano et al, 2005) and CaMKIV (Xu et al, 2002). I will focus on the p38 MAPK pathway as this has been shown to induce phosphorylation of RBM4, the main focus of the work described in this thesis.

1.6.2 p38 MAPK in myogenic differentiation

p38 MAPK is a protein kinase primarily involved in signalling inflammatory and environmental stresses (Roux & Blenis, 2004). It has also been found that p38 MAPK signalling is required for myogenic differentiation (Wang et al, 2008). This effect is mediated by a sustained signalling via p38 MAPK (Alisi et al, 2008) which is different to inflammatory signalling via p38 MAPK which is transient (Alisi et al, 2008). p38 MAPK has a variety of different isoforms (α, β, λ and δ (Wang et al, 2008) and phosphorylation of their substrates require activation of p38 MAPK by phosphorylation of a Thr-Gly-Tyr motif which is found in the T-loop of the kinase (Roux & Blenis, 2004). p38 MAPK targets include phospholipase A2, the microtubule-associated protein Tau, the transcription factors ATF1 and -2, Sap-1, MEF2A, NF-κB, Elk-1, Ets-1, and p53. It has also been found that p38 MAPK can activate kinases such as MSK1, MSK-2, Mnk1, Mnk2, MK2 and MK3 in the stress pathways (Cuenda & Rousseau, 2007).

As mentioned above, p38 MAPK is required for myogenic differentiation(Alisi et al, 2008), and p38 MAPK activity is upregulated upon serum removal and is maintained throughout myogenic differentiation. The sustained activity of p38 MAPK during differentiation and the fact that there is not a parallel increase in JNK activity separate this responsce from stress related p38 activity which is more transient and has a parell activation of JNK (Wu et al, 2000). . The α, β, and λ isoforms (Wang et al, 2008) are important during myogenic differentiation and the activity of α and β isoforms increases in protein expression during differentiation (Alisi et al, 2008), a process essential for myogenic differentiation (Wang et al, 2008). One of the redundant roles of the isoforms is the up-regulation of E2F2 (transcription factor), which has been thought to regulate myogenin expression. The P38MAPKβ isoform has been found to be responsible for the up regulation of cyclin D3 which forms a complex with unphosphorylated Rb, CDK2, CDK4, p21 and Proliferating Cell Nuclear Antigen (PCNA). This complex has been associated with irreversible exit from the cell cycle which is a requirement for differentiation to occur (Wang et al, 2008). The p38MAPKα and β isoforms have been found to be phosphorylated and activated by N-cadherin signalling and led to an up-regulation of IGF-II protein expression, which is also crucial for myogenic differentiation (Lovett et al, 2006). The increase in phosphorylation of p38 MAPK has been found to be mediated by Abl (a non receptor tyrosine kinase (Bae et al, 2010)) and Cdo (a cell membrane protein that is involved in regulation of cell-cell interaction and is a cell surface receptors (Kang et al, 1998)), both of which if knocked-down reduce p38 MAPK phosphorylation and activity and lead to failed myogenic differentiation

(Bae et al, 2009). The two proteins are thought to ineract with each other, which allows activation of p38 MAPK pathway by the kinase activity of Abl (Bae et al, 2010).

Another role for multiple p38 MAPK isoforms is in the activation of NF-κB signaling by inducing the degradation of I-κB and activating p65 (forms a heterodimer with NF-κB). These effects lead to the up-regulation of IL-6 protein expression which is a promyogenic factor (Baeza-Raja & Munoz-Canoves, 2004). PKR is also involved in myogenic differentiation. It has been shown to cross talk with p38 MAPK and it regulates p38 MAPK kinase activity and is required for differentiation (Alisi et al, 2008). Previous examples mentioned have all indicated that p38 MAPK activity is required for differentiation but another group have found p38 MAPK activity to have a negative effect on late myogenic differentiation (post myotube formation), indicating a possible dual role for p38 MAPK during myogenic differentiation (Weston et al, 2003).

1.7 The Aim of this thesis

The aim of this thesis is to investigate the potential for RBM4 to regulate myogenic differentiation by directly influencing translation as RBM4 has been shown to up-regulate differentiation when over expressed and to interact with translation initiation proteins and to associate with IRES containing mRNAs. Previous work has shown that RBM4 shuttles from the nucleus to the cytoplasm in response to p38 MAPK signalling which is up-regulated in a sustained manor during myogenic differentiation. The work described here aims to investigate the interaction of isoforms of RBM4 with the translational apparatus using differentiating C2C12 myoblasts as a model system. This will be done by investigating the expression level of RBM4 total protein and its two isoforms RBM4a and RBM4b during myogenic differentiation, the association of RBM4 with the intiation factors eIF4G and eIF4A, the localisation of RBM4 during myogenic differentiation and the association between RBM4 and actively translating ribosomes.

-Chapter 2-

Materials and Methods

2.1 Chemicals and Biochemicals

2.1.1 Reagents and reagent suppliers

Fetal Calf Serum (FCS) was from Labtech International (UK) and all other materials for tissue culture were from Invitrogen. [^{35}S]-methionine was from MP Biomedicals (Brussels, Belgium). m^{7}GTP-Sepharose 4B resin and uncoupled resin were from GE Healthcare (UK). C2C12 and HeLa cells were from the European Collection of Cell Cultures (ECACC; UK) and American cell Type Culture Collection (ATCC; USA). Mammalian expression plasmids encoding wild-type *Homo sapiens* RBM4a and RBM4a containing a Ser309Ala mutation and bacterial expression vectors for *Homo sapiens* RBM4 Wild type and RBM4 RRM silenced mutant were a gift from Dr Woan-Yuh Tarn (Korea). All other kits and reagents were supplied by the companies indicated in the text and unless otherwise stated, all chemical were of analytical quality from Sigma Aldrich (UK).

2.1.2 Antibodies

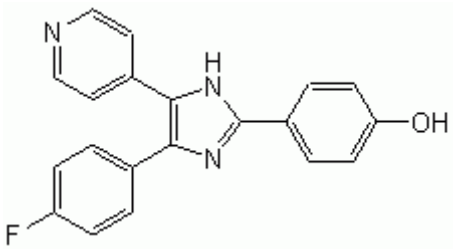
All primary antibodies used in this work are presented in Table 2.1. Anti RBM4a and anti RBM4b monoclonal rat antibodies where a gift from Dr Friedrich A. Grässer (Germany).

2.1.3 Cell permeable inhibitors

SB202190 (a p38 MAPK inhibitor shown below) was stored at 20mM in DMSO and used at 20µM (MERK Chemicals).

Table 2.1 Primary Antibodies						
Target	Predicted kDa	Antibody name	Immunogen	Dilution µl:µl	Donor animal	Supplier
RBM4 Total	40	RBM4 Total	RBM4 Homo Sapiens fusion protein	1:1000	Rabbit	Protein Tech
RBM4a	40	RBM4a	GST-RBM4a fusion protein	1:250	Rat	Dr Friedrich A. Grässer
RBM4b	40	RBM4b	Synthetic peptide: QSTTVTSHLNSTSVD corresponding to amino acids residues 255-269 of RBM4b	1:250	Rat	Dr Friedrich A. Grässer
eIF4A	46	Bloo4	Synthetic peptide: DLPANRENYIHRTGRGGRFGRK, corresponding to amino acid residues 348-369 of xenopus eIF4AI	1:1000	Rabbit	Dr Simon Morley
eIF4GI	220	Edith	Synthetic Peptide: RTPATKRTFSKEVEERSRERPSQP EGCR, corresponding to amino acid residues 1179-1206 of human eIF4GI	1:1000	Rabbit	Dr Simon Morley
PABP	70	Red2	Synthetic peptide: IPQTQNRAAYYPPSQIAQLRPS corresponding to amino acids 388-409 of human PABP	1:3000	Rabbit	Dr Simon Morley
eIF4E	25	Xavier	Synthetic peptide: TATKSGSTTKNRFVVC corresponding to amino acid residues 203-217 of human eIF4E	1:2000	Rabbit	Dr Simon Morley

Myogenin	34	Myogenin	Recombinant GST fusion protein corresponding to amino acids 30-224 of rat myogenin	1:1000	Mouse	BD Pharmingen
Actin	42	Anti-actin	Synthetic peptide corresponding to the carboxyl-terminal 11 residues, which is identical in most species	1:3000	Rabbit	Sigma-Aldrich
Caveolin-3	21	Caveolin-3	Synthetic peptide: MMTEEHTDLEARIIKDIH(C) corresponding to amino acid residues 1-18 of human caveolin-3	1:2000	Rabbit	Abcam
P-p38	38	Phospho-p38 MAP Kinase (Thr_{180}/Tyr_{182})	Synthetic phospho-peptide corresponding to residues surrounding Thr_{180} and Tyr_{182} of human p38 MAPK	1:500	Rabbit	Cell signaling technologies
Myosin heavy chain	204	MYC	Raised against synthetic peptide	1:1000	Mouse	National Hybridoma Bank wisconcen
c-myc	62	c-myc	Synthetic peptide AEEQKLISEE DLLRKRREQL KHKLE corresponding to C terminal amino acids 408-432 of Human c-Myc.	1:1000	Mouse	Abcam



2.2 Cell culture

2.2.1 C2C12 cell culture

C2C12 are mouse C3H muscle myoblast cells (Yaffe & Saxel, 1977). The cells used in this thesis have been passaged 12 times and where maintained for 12 further passages before fresh stocks were used. C2C12 cells were maintained at sub-confluent levels in C2C12 growth-medium at 37^{o}C with 5% carbon dioxide. Transfer of semi-confluent cells was achieved using trypsin-EDTA.

C2C12 growth medium	DMEM (Dulbecco's Minimal Essential Medium) supplemented with 20% (v/v) Foetal calf serum (FCS)

2.2.2 HeLa Cell culture

HeLa are human cervical epitheloid carcinoma cell (Gey et al, 1952). Hela cells were maintained at sub-confluent levels in HeLa growth-medium at 37^{o}C with 5% carbon dioxide. Transfer of semi-confluent cells was achieved using trypsin-EDTA.

HeLa growth medium	MEM (Minimal Essential Medium) with 10% (v/v) FCS, 2mM sodium pyruvate, 1x non essential amino acids and 2mM L-glutamine

2.2.3 Myogenic differentiation of C2C12 cells

Cells were seeded at 1 million cells on a 6cm plate in 3ml C2C12 growth medium (as described in Section 2.2.1) and incubated at 37^{o}C with 5% CO_2 for two days until completely confluent. Medium was aspirated and cells were briefly washed with serum-free culture medium (DMEM). 3ml of Differentiation Medium was added and was replaced every 24 hours of culture.

Differentiation Medium (DM)	DMEM with 10µg/ml transferrin, 10µg/ml insulin and 2% (v/v) horse serum.

2.2.4 Transfection of HeLa cells with cDNA encoding RBM4

Hela cells were plated out on 6cm plates at a density of 60,000 cells and were incubated at 37^{o}C with 5% CO_2 for 24 hours in 2ml of HeLa growth medium. Following this, between 3-6 µl Fugene (Roche, UK) was added to 100 µl serum-free MEM supplemented with 1-2µg of cDNA encoding RBM4 WT or RBM4 MT (S309A) (Lin et al, 2007), as outlined in the individual figure legends. This was mixed gently by tapping the tube and incubated for 20 mins at room temperature. The medium on the HeLa cells was reduced to 1ml and the DNA/Fugene complex was added in a drop-wise manner. The cells were

incubated for 24 hours at which point experiments could be conducted upon the transfected cells.

2.2.5 Transfection of C2C12 myoblasts with cDNA encoding RBM4

C2C12 cells were plated out at a density of 50,000 cells on a 6cm plate and incubated at 37^{o}C with 5% CO_2 in C2C12 growth medium. These were mixed gently by tapping the tube and incubated for 30 mins at room temperature. Following this, between 3-6 µl Fugene (Roche, UK) was added to 100 µl serum-free MEM supplemented with 1-2µg of cDNA encoding RBM4 WT or RBM4 MT (S309A), and incubation and transfection was carried as described as for HeLa cells in Section 2.2.4.

2.2.6 Inhibition of p38 MAPK in C2C12 undergoing differentiation

C2C12 cells were setup for differentiation as described in Section 2.2.3 and incubated in the absence or presence of 20 µM SB202109. The cell medium was changed for fresh differentiation medium every 24 hours also supplemented with 20 µM SB202109.

2.2.7 Arsenite treatment of cells

C2C12 cells were setup for differentiation as described in Section 2.2.3 and incubated in the absence or presence of 0.5 µM arsenite. The cells were then incubated for 30 mins before experiments were conducted upon them.

2.3 Cell lysis

2.3.1 Standard cell lysis

The cell medium was removed and replaced with 1ml of cold PBS (scaled up or down if bigger or smaller plates than 6cm plate were used) and the cells were scraped on ice and aspirated into cold 1.5ml microcentrifuge tubes. The cells were then isolated by centrifugation at 15,000 x g for 1 min at 4^{o}C. The supernatant was removed and the pellet resuspended in 100µl of lysis buffer and then supplemented with 0.5% (v/v) Igepal and 0.5% (v/v) Deoxycholic acid (volume scaled up or down if larger or smaller plates were used) followed by a vigorous vortexing. The ressupended cells were centrifuged at 15,000 x g for 10 mins at 4^{o}C and the recovered supernatant flash frozen in liquid nitrogen and stored at –80^{o}C.

Lysis buffer:	20mM MOPS (KOH) pH 7.2, 2mM benzamidine, 2mM $MgCl_2$, 2mM EGTA, 0.1mM GTP, 0.5 mM DTT, EDTA-free 1x protease inhibitor cocktail (Roche, UK).

2.3.2 Estimation of protein concentration (Bradford assay)

Coomassie dye (Bradford reagent; Bio-Rad UK) stock solution was diluted 1:5 with H_2O. 200µl of this was added per well to a 96 well plate. A standard curve was prepared by adding 0,0.5, 1, 2, 3 or 4µg of BSA per well (stock 10 mg/ml; New England Biolabs, UK). Extracts were diluted 1:2 to ensure the protein concentrations were in the range of the standard curve and 1µl was pipeted into each well, in triplicate. The colour reaction was quantified by

measuring absorbance (A_{620}) minus background absorbance (A_{405}) using the standard curve to calculate protein concentrations of the diluted extracts.

2.3.3 Sub-cellular fractionation of C2C12 myoblast extracts

Sub-cellular fractionation was performed using ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem, UK), as described in the supplied manual.

2.4 Western blotting

2.4.1 Sodium Dodecyl Sulphate-PolyAcrylaymide Gel Electrophoresis (SDS-PAGE)

Mini gels were used (Protean II, Bio-Rad, UK) and resolving gel was poured into the assembled apparatus and allowed to set for 30 mins under a layer of water-saturated butanol. The water-saturated butanol was removed and the stacking gel was poured into the mini gel followed by a gel comb and was allowed to set for 10 mins. Once the gel had set, the comb was removed and the gel was placed into the running rig. The central well was filled up with SDS running buffer and samples and markers were loaded (samples were diluted in sample buffer). The external chamber was then filled up with SDS running buffer to cover the base of the gels. The gel was then subjected to 100V and resolved until the loading dye was run off the end of the gel. To change the percentage of the gel to allow different size proteins to be resolved, acrlyamide concentrations were varied as described in individual figure legends.

Resolving gel:	10ml of separating gel mix (5ml per gel) 3.7ml pure H_20, 2.5ml 1.5M Tris/(HCl) pH 8.8, 50µl 20% (w/v) SDS, 3.7ml acrylamide /bisacrylamide mix (30%:0.8%), 50µl 10% (w/v) APS, 10µl TEMED.
Stacking gel:	3ml of stacking gel mix: 4.98% (w/v) acrylamide, 0.13% (w/v) bisacrylamide, 125mM Tris (HCl) pH6.8, 30µl 20% (w/v) SDS, 20µl 10% (w/v) APS, 10µl TEMED.
Sample Buffer:	1.4M β-Mercaptoethanol, 530mM Tris (HCl) pH 6.8, 0.85% (w/v) SDS, 42.5% (v/v) glycerol, 0.05% (w/v) Bromophenol blue and 0.05% (w/v) phenol red.
Running Buffer:	25mM Tris/ (HCl) pH 8.5, 0.16% (w/v) SDS, 192mM glycine.

2.4.2 Transfer of proteins to PDVF membrane

A semi-dry transfer was used for transferring proteins between the polyacrlyamide gels and PDVF membrane (GE Healthcare). Whatman 3MM chromatography paper was used during the process. A stack of Whatman paper was made up of four sheets soaked in Anode 1 buffer, two sheets soaked in Anode 2 buffer, one sheet of PDVF membrane hydrated in

methanol, the polyacrylamide gel and four sheets soaked in Cathode buffer. The stack was gently squeezed to remove air bubbles and excess fluid and placed in the transfer apparatus and 0.8mA/cm^2 was applied for 90 mins.

Anode 1 buffer:	0.3M Tris base, 20% (v/v) methanol.
Anode 2 buffer:	25mM Tris base, 20% (v/v) methanol.
Cathode buffer:	25mM Tris base, 40mM 6-NH_2 hexanoic acid, 20% (v/v) methanol **.**

2.4.3 Immunoblotting

After the protein transfer, the PDVF membrane was placed in blocking solution for 30 mins. Primary antibodies (Table 2.1) were diluted into blocking buffer and incubated with membrane overnight at 4^{o}C on a rocking platform.

TBS/Tween:	50mM Tris (HCl) pH7.4, 150mM NaCl, 0.5% (v/v) Tween-20.
Blocking solution:	3% (w/v) BSA, TBS/Tween.

2.5 Cell imaging

2.5.1 Immunofluorescence

Cells were washed once in 2ml warm PBS and then fixed with 4 % (v/v) paraformaldehyde for 20 mins. After washing three times with PBS, the cells were permeabilised for 5 mins in 0.1 % (v/v) Triton X100 and incubated for 1

hour in blocking buffer. The blocking buffer was aspirated, replaced with blocking buffer containing primary antibodies and incubated for 1 hour at room temperature. The cells were then washed three times in PBS and incubated with secondary antibodies and phalloidin (Sigma, UK; diluted in blocking buffer) for 1 hour at room temperature. The secondary antibodies were aspirated and the cells were washed three times with PBS. The fixed cells were then incubated with 1ml of 7.7 ng/ml DAPI (Sigma, UK) in H_2O for 5 mins. The cells were washed in distilled water three times and mounted for confocal microscopy using Mowiol mounting medium on coverslips, which were sealed with nail varnish and stored in the dark at 4^oC for at least a day before images were captured. Images of the cells were captured using a Zeiss axiovert LSM510 laser scanning confocal microscope.

Blocking buffer:	PBS with 3% (w/v) BSA.
Mowiol mounting medium:	0.2M Tris (HCl) pH 8.5, 33% w/v glycerol, 13% (w/v) Mowiol, 2.5% (w/v) 1,4-diazobicyol [2,2,2]-octane (DABCO).
4% paraformaldehyde solution:	PBS with 4% paraformaldehyde (v/v).

2.5.2 Analysis of confocal images

Colocalisation analysis was performed as described in Coste et al (2004). Correlation data was obtained using MBF ImageJ software (www.macbiophotonics.com). Briefly, pixel co-variation between individual channels was measured by linear regression using Pearsons Correlation Coefficient (Manders et al, 1993) and images were thresholded using Coste's

threshold; The Pearsons Correlation Coefficient was sequentially measured between two channel images at each individual pixel intensity from 255-0. The pixel intensity value where Pearsons Correlation Coefficient became zero (i.e. random correlation) was defined as the threshold, and any information below this intensity was disregarded from the colocalisation analysis.

The control for the Pearsons Correlation Coefficient was obtained using Coste's Randomisation Method; Briefly, the pixel distribution of one of the channel images was randomised according to half width point spread function. The Pearsons Correlation Coefficient was then obtained by comparing the randomised image to the non-randomised channel image. This procedure was repeated multiple times (n=200). Pearson's Correlation Coefficient for sample images was considered significant if it was above 0.5 and Coste's randomisation was close to 0.

2.5.3 Capturing light microscopy images

Images of cells in cell culture were captured using a Moticam 2000 2.0M pixel USB 2.0 attached to light microscope via a 10X objective and converted to gray scale in Adobe Photoshop.

2.6 Polysome gradients

Cell extracts were prepared using the standard lysis method as described in Section 2.3 and 200µl of extract was layered onto a 5ml linear gradient of 10-40% (w/v) sucrose (in sucrose buffer) and centrifuged for 60 mins at 192,000 x g in a SW55 Ti rotor at 4^{o}C. Optical density profiles were obtained by upward displacement of the sucrose gradient with 60% (w/v) pumping sucrose

through an ISCO UA6 gradient fractionator machine coupled to a UA5 spectrophotometer measuring the absorbance at 254nm. All steps of this protocol was performed under RNAse free conditions.

Sucrose Buffer:	20mM MOPS (KOH) pH 7.2, 75mM KCl, 2mM $MgCl_2$.
Pumping sucrose:	60% (w/v) sucrose in H_2O, phenol red (trace).

2.6.1 Phenol chloroform isolation of RNA from Polysome fractions

Phenol chloroform extraction was performed using Tri reagent as recommended by the manufacture scaled up for 4ml fractions. Tri reagent (12ml) was added to 4ml of pooled polysome fractions, incubated at room temperature for 5 mins and 3.2ml of 100% chloroform added followed by mixing. The samples were then incubated at room temperature for 15 mins, transferred to a 50ml Corex glass centrifuge tube and then subjected to 12,000 x g for 15 mins at 4^{o}C. The aqueous phase was transferred to a fresh Corex tube and 8.04ml isopropanol was added, the sample left to stand for 5 mins at room temperature and then subjected to 12,000 x g for 8 mins at 4^{o}C. The resulting pellet was washed in 16ml of 75% (v/v) ethanol and vortexed off the side of the Corex tube and then subjected to 7,500 x g for 5 mins at 4^{o}C. The supernatant was removed and the pellet was air dryed for 5-10 mins and re-suspended in 25µl of elution solution (pre-heated to 70^{o}C) from the RNA aqueous kit.

2.7 RNA extraction and processing

2.7.1 RNA isolation

RNA was isolated from cultured cells using RNAaqueous (Ambion, UK) as described in the manufacturer's instructions and briefly here. Cells were lysed in 100µl of lysis buffer and 50µl of ethanol was added followed by a vigorous vortexing. The lysate was then passed through a filter cartridge assembly, followed by 180µl of wash buffer 1 and two applications of 180µl each of wash buffer 2. This was carried out by centrifugation for 1 min in a microfuge and the flow through was discarded between each addition. An extra centrifugation step was performed to remove possible contaminants. The RNA was finally eluted using two elutions each of 10µl of water applied to the centre of the filter and centrifugation for 1 min.

2.7.2 Reverse transcription

Reverse transcription was performed using Improm-II reverse transcription systems (Promega, UK) as described in manufacturer's manual and briefly here. To make the reaction mixture, 4µl of Improm reaction buffer, 1.2µl of $MgCl_2$, 1µl of dNTP mix, 20U of recombinant RNAasein ribonuclease inhibitor and 1µl of Improm reverse transcriptase were combined and made up to 15µl with nuclease-free water. RNA (1µg) was diluted to 5µl with nuclease-free water and incubated at 70^{o}C for 5 mins followed by a 5 min incubation in ice water. The RNA was then added to the reaction mixture, mixed and incubated at 25^{o}C for 1 hour. The temperature was then increased to 42^{o}C for 1 hour to allow extension, then followed by a final increase to 70^{o}C for 15 mins to inactivate the reverse transcriptase.

2.8. QRT-PCR

cDNA was diluted 1: 20 for most mRNA targets and 1:1000 for 18S rRNA. Forward and reverse primers were diluted 1:66 from a 100µM stock. Master mix plus (22µl) was pipetted into each well of the strip tube and 3µl of diluted cDNA was also added. For the minus cDNA control, the cDNA was replaced with 3µl of sterile filtered distilled H_2O. The caps were placed on the tube strips and the tube strips briefly vortexed and placed into the Stratagene qRT-PCR machine. A programme was set up using standard qRT-PCR conditions, as described in the PCR manual for this machine, which had an extension and annealing temperature of 60^{o}C and a denaturing step of 90^{o}C with a 30 secs extension time.

Relative RBM4 mRNA abundance was derived from qPCR data using the relative quantification method as described in Willett et al, (Willett et al). 18S rRNA was chosen as a housekeeping RNA due to its long t_{50} and slow turnover. The formula 2^{-DDct} was used to calculate fold change in mRNA concentration where DCt (1), the calibrator is the Ct of the control sample normalised to the housekeeping 18S Ct, and DCt (2) is the unknown sample Ct normalised to the housekeeping 18S Ct.

DDCt is the difference between the sample DCt and the calibrator DCt .
As the PCR reaction is exponential, the natural antilog of the DDCt is then calculated to yield the fold change between the sample and the control.

Master mix plus:	2 x Master mix (Stratagene, UK) 500µl, primer mix 50µl and 330µl filter sterile H_2O
Forward RBM4a primer	GAGACTGCATTCCACAAGCA
Reverse RBM4a Primer	GGCTCCTCACTGAATCCAAA
Forward RBM4b primer	GACCATGTAGTGGCAAGCAA
Reverse RBM4b primer	CAACAAAACCCAATGGTCCT

2.9 Mini prep isolation of cDNA and recombinant protein production

2.9.1 Transformation of competent E.Coli

Competent *E.Coli* cells were inoculated with 1µl of plasmid solution and left on ice for 15 mins and then heat shocked for 45 secs at 42^{o}C. LB broth (Sigma, UK), pre-warmed to 37^{o}C was added and cells were left for 1 hour. Cells were then seeded out onto agar plates supplemented with selective antibiotic (either ampicillin 100µg/ml or kanamycin 50µg/ml, depending on plasmids resistance gene) and left overnight for colonies to form. Colonies were then picked and placed into LB containing selective medium and allowed to grow overnight.

2.9.2 Mini prep of plasmids

Mini preps were performed on *E.Coli* cultures using a mini prep kit (Qiagen, UK) as described in manufacturer's manual and briefly described here. The transformed *E.Coli* cells were centrifuged at 8,000 x g for 3 mins at room temperature, the supernatant removed and the recovered cells lysed in 250µl

of buffer P1. The lysed cells were pipetted into a microcentrifuge tube, 250µl of buffer P2 was added followed by 4-6 inversions to mix. Then 350µl of buffer N3 was added and immediately mixed by 4-6 inversions. The lysed cells were then centrifuged at 17,900 x g for 10 mins and the supernatant removed and pipetted into a QIAprep spin column. This was centrifuged for 30 to 60 secs in a microfuge and the flow-through discarded. Buffer PB (0.5ml) was added into the QIAprep column followed by centrifugation for 30 to 60 secs, with the flow-through discarded. This step was repeated using 0.75ml of buffer PE but with an extra centrifugation and discarding of flow-through to ensure no ethanol contamination of the sample. The DNA was eluted in 50µl of water, which was pipetted onto the centre of the QIAprep filter and left to stand for 1 min followed by centrifugation for 1 min in a microfuge.

2.9.3 Expression of RBM4 WT and MT (RRM silenced) His tagged in *E.Coli*

A small sample of frozen BL-21 *E.Coli* cells (stratagene) transformed with RBM4 WT and MT (RRM silenced) obtained from the Tarn group (Kar et al, 2006) was added to 5ml of LB and allowed to grow for 6 hours. Subsequently, this was added to 200 ml of LB and allowed to grow overnight for 14 hours at 37^{o}C at which point the cells were treated with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 1 hour. The cells were then harvested and protein isolated as described below.

2.9.4 Nickel agarose purification of RBM4 WT and MT (RRM silenced) His tagged protein

Cells were isolated using a Sorvall J6B centrifuge at 4,000rpm for 20 mins at 4^{o}C. The cells were re-suspended in 10ml of Buffer A1, 200Units of DNAse 1 added and the cells lysed by French press. The lysed cells were then centrifuged in a Sorvall SS34 rotor at 4^{o}C for 20 mins and 9,800rpm. The supernatant was removed and 1ml of 50% (v/v) NTA-Agarose resin/litre of cell culture was added to the supernatant which was then mixed end-over-end for 60 mins at 4^{o}C. The resin was recovered by centrifugation using a Sorvall RT6000 for 5 mins at 1,100rpm, then washed twice with 25ml Buffer A each. This process was repeated with Buffers B, C and D. The beads were then transferred to a 2ml microfuge tube and protein eluted with 0.75ml of Buffer E for 15 mins at 4^{o}C with end-over-end mixing. The resin was recovered by centrifugation in a microfuge and the supernatant removed. This process was repeated four times, the fractions pooled and then dialysed for at least 4 hours into Buffer F at 4^{o}C.

Buffer A1	40mM MOPS (KOH) pH7.2, 300mM NaCl, 2mM benzamidine, 20mM imidazole, 3.5 mM β-mercaptoethanol, 1x complete protein inhibitor cocktail (Roche), 1mM ATP.
Buffer A	40mM MOPS (KOH) pH7.2, 300mM NaCl, 2mM benzamidine, 20mM imidazole, 3.5 mM β-mercaptoethanol, 1x complete protein inhibitor cocktail (Roche).
Buffer B	40mM MOPS (KOH) pH7.2, 300mM NaCl, 2mM benzamidine, 20mM imidazole, 3.5 mM β-mercaptoethanol, 1x complete protein inhibitor cocktail (Roche) 1% (v/v) Igepal.
Buffer C	40mM MOPS (KOH) pH7.2, 1000mM NaCl, 2mM benzamidine, 20mM imidazole, 3.5 mM β-mercaptoethanol, 1x complete protein inhibitor cocktail (Roche) 1% (v/v) Igepal.
Buffer D	40mM MOPS (KOH) pH7.2, 300mM NaCl, 2mM benzamidine, 20mM imidazole, 3.5 mM β-mercaptoethanol, 1x complete protein inhibitor cocktail (Roche).

Buffer E	40mM MOPS (KOH) pH7.2, 300mM NaCl, 2mM benzamidine, 20mM imidazole, 3.5 mM β-mercaptoethanol, 100mM EDTA.
Buffer F	20mM MOPS (KOH) pH7.2, 25mM KCl, 10mM NaCl, 1.1mM $MgCl_2$ 7 mM β-mercaptoethanol, 2mM benzamidine.

2.9.5 Heparin-Sepharose purification of RBM4 WT and RRM silenced mutant his tagged protein

The dialysed RBM4 protein described above was added to 2 ml of 50% (v/v) Heparin-Sepharose beads (GE Healthcare, UK) and incubated for 1 hour at 4^{o}C with end-over-end mixing. The beads were then recovered by centrifugation and washed with 2ml dialysis buffer (Buffer F); this step was repeated three times. The protein was eluted by vortexing the recovered beads with Buffer F with either 100 mM, 250 mM, 500 mM or 1M NaCl in succession. Each elution was then dialysed into Buffer F for at least 4 hours at 4^{o}C, flash frozen in liquid nitrogen and stored at -20 ^{o}C.

2.9.6 FFQ anion exchange purification of RBM4 WT and RRM silenced mutant his tagged protein

The RBM4 protein was further purified using anion exchange chromatography using FFQ resin (GE Healthcare, UK). The dialysed RBM4 protein was added to 2 ml of 50% (v/v) FFQ-Sepharose beads and incubated for 1 hour at 4^{o}C with end-over-end mixing. The beads were then recovered

by centrifugation and washed with 2ml dialysis buffer (Buffer F); this was repeated three times. The protein was eluted by vortexing the recovered beads with Buffer F with either 100 mM, 250 mM, 500 mM or 1M NaCl in succession. Each elution was then dialysed into Buffer F for at least 4 hours at 4^{o}C, flash frozen in liquid nitrogen and stored at -20 ^{o}C.

2.9.7 Preparation of FLAG-tagged eIF4G^{a} from baculovirus-infected *SF9* cells

eIF4G^{a} is a form of eIF4G that was originally thought to reflect the whole open reading frame (Coldwell et al, 2006). However, this protein sequence lacks the PABP binding site subsequently found in the N-terminus of eIF4G^{e} and eIF4G^{f} (Coldwell et al, 2006). In conjunction with Prof. S. Morley (Sussex), *SF9* insect cells were infected with virus containing DNA encoding FLAG-tagged eIF4G^{a} at the optimum multiplicity of infection of 5 (Hinton et al, 2007). The cells were incubated for 72 hours at 27^{o}C and were harvested by centrifugation at 3,500 rpm at 4^{o}C in the Sorvall J6B centrifuge. The isolated cells were re-suspended in 20 ml of ice cold Buffer F (see Section 2.9.4) with 2X complete protease inhibitor cocktail (Roche, UK) and vortexed. The preparation was left on ice for 45 mins then centrifuged at 9,800rpm for 10 mins at 4^{o}C. The supernatant from this step was mixed with 50% (v/v) M2 affinity purified anti-FLAG Agarose resin (Sigma, UK) at 1ml/litre of culture, already pre-washed in Buffer F. This slurry was incubated for 2 hours at 4^{o}C, and the beads recovered by centrifugation at 1,500rpm for 5 mins in a Sorvall RT6000 at 4^{o}C, resuspended in 10ml Buffer F with protease inhibitors, washed by inversion ten to twelve times in the cold and then re-recovered by centrifugation. These washing steps were repeated until the supernatant was

essentially free of unbound protein. The beads were then re-suspended in Buffer G and transferred into 1.5ml microcentrifuge tubes and centrifuged for 1.5 mins at 13,000rpm to recover the beads. The eIF4G protein was then eluted with 1ml and then 5ml of Elution buffer. The final eluate was immediately adjusted to pH 7.2 with 2M Trizma base, flash frozen in liquid nitrogen and stored at -20 ^{o}C. All buffers used are as described in Section 2.9.4 above.

2.9.8 Preparation of $(His)_6$-tagged eIF4A protein from baculovirus-infected *SF9* cells

In conjunction with Prof. S Morley (Sussex), *SF9* cells (one litre) were infected with baculovirus encoding $(His)_6$-tagged eIF4A virus at the optimum multiplicity of infection of 2 and incubated in spinner flasks for 72 hours at 27^{o}C (Hinton et al, 2007). Cells were then harvested by centrifugation at 3,500rpm in a Sorvall J6B centrifuge at 4^{o}C, and the pellet re-suspended in 10ml per litre of Buffer D (Section 2.9.4), with 2x concentration of complete protease inhibitor cocktail. To lyse the cells, Igepal was added to 1% (v/v) final concentration, vortexed, and the lysate centrifuged at 9,800rpm in a Sorvall SS34 rotor at 4^{o}C for 30 mins. The resulting supernatant was added to 1ml of 50 % (v/v) Ni-NTA Agarose beads and mixed end-over-end at 4^{o}C for 1 hour . The Ni-NTA Agarose beads were washed with five times with 10ml each of Buffer D containing 1% (v/v) Igepal, followed by twice with 10ml of the same buffer containing 1M NaCl. This was followed by two washes of 10ml each with Buffer D containing 0.5M NaCl but with no Igepal. eIF4A protein was then eluted from the resin with Buffer E in two elutions each of 250µl. Fractions were pooled and dialysed against Buffer F for at least 4 hours at 4^{o}C, flash

frozen in liquid nitrogen and stored at -20 ^{o}C. All buffers used here are as described in Section 2.9.4.

2.10 Protein Interaction experiments

2.10.1 Immunoprecipitation of RBM4

Protein A/G (25µl of a 50% (v/v) slurry) magnetic beads (New England Biolabs, UK;) were added to 100 µl of 1mg/ml protein cell extract prepared by the standard lysis method as described above, and agitated for 1 hour at 4^{o}C. The extract was then subjected to a magnetic field and the supernatant aspirated into a new microfuge tube. Anti RBM4 antibody (5 µl) was added and the mix agitated for 1 hour at 4^{o}C. Protein A/G magnetic beads (25µl) were added to the pre-cleared extract which was then agitated for a further hour at 4^{o}C. The magnetic beads were washed three times with 1ml of IP wash buffer and the recovered protein eluted in 30µl SDS-PAGE sample buffer.

IP wash buffer:	150mM NaCl, 10mM Tris (HCl) pH7.4, 1mM EDTA, 1mM EGTA, 0.2mM sodium ortho vanadate, 0.2mM PMSF, 1% (v/v) Triton X-100, 0.5% (v/v) Igepal.

2.10.2 RNAse A and T1 degradation of RNA

Cell extracts had 10µl of RNAse cocktail (Ambion) which was deemed in excess of normal levels to ensure complete digestion as instructed in manual.

The cells extracts were then incubated on ice for 1 hour to allow digestion to occur experiments were then conducted as required.

2.10.3 m^7GTP-Sepharose-mediated purification of eIF4E

Firstly, 50% (v/v) m^7GTP-Sepharose 4B resin was centrifuged for 5 mins at 10,000 × *g* and the storage buffer discarded. M^7 Buffer (containing 1 mg/ml cytochrome *c*) was added to the resin to bring it back to the original volume. Cell extracts containing equal protein concentration were added to the washed resin, and incubated at 4 ºC with gentle shaking for 15 mins. The optimum conditions were achieved using 30 µl of 50% (v/v) resin per 30 µg protein (data not shown). The resin was subsequently isolated by centrifugation at 10,000 × *g* for 5 mins at 4 ºC. The resulting supernatant was aspirated and non-specifically bound proteins removed by washing the resin twice with 200 µl of M^7 Buffer . Finally, bound proteins were eluted by boiling the Sepharose beads in SDS-PAGE sample buffer for 5 mins and centrifugation at 10,000 × *g* for 5 mins. The supernatants were stored at -20 ºC.

M^7 Buffer:	20mM MOPS (KOH) pH 7.2, 25mM KCl, 2mM benzamidine, 7mM β mercaptoethanol , 1 mM $Mg(CH3COO)_2$, 0.1 mM GTP, 0.25% (v/v) Igepal, 10 mM NaF, 1 µM microcystin.

2.10.4 eIF4G and RBM4a in vitro binding assay

Recombinant RBM4a WT and eIF4G protein (3µg) isolated as described above, were mixed together in 1ml of Interaction buffer and incubated at 4^{o}C for 1 hour with gentle mixing. M2 Anti FLAG antibody (5µl) was added and the incubation continued for another hour. Protein G magnetic beads (50µl of 50% (v/v) slurry) were subsequently added and incubated for a further hour. The beads were then recovered using the magnetic recovery system and washed with 1 ml of Interaction buffer. This wash was repeated three times and the proteins bound were eluted using 50µl of SDS-PAGE sample buffer and vortexing.

Interaction buffer:	100mM NaCl, 50mM Tris (HCl) pH 6.9.

2.10.5 Supplementation of extracts with recombinant proteins

Extracts were obtained using the lysis protocol above in Section 2.3.1 and aliquots containing 250 µg total protein were diluted into 1 ml of Interaction buffer. To this, 3 µg of either eIF4A, RBM4 WT or RBM4 MT (RRM silenced) recombinant protein was added and the tubes were allowed to incubate for 1 hour at 4^{o}C with gentle mixing. Subsequently, 50µl of 50% (v/v) NTA-nickel Agarose beads were added and allowed to incubate for 1 hour as before. Once this was completed, the beads were recovered by centrifugation in a cooled microfuge and washed three times with 1ml each of Interaction buffer. The recovered proteins were eluted using 50µl of SDS-PAGE sample buffer as described Section 2.4.1.

2.11 [^{35}S]-methionine labelling of cellular proteins

Prior to harvesting, C2C12 myoblasts were pulse-labelled with [^{35}S]-methionine (10 µCi/ml) for 30 mins at 37^{o}C with 5% carbon dioxide. Cells were then harvested with the lysis conditions described in Section 2.3.1. Protein concentrations of extracts were determined as described above (Section 2.3.2) and 5 µl aliquots were also spotted onto Whatman filter paper. The papers were air-dried and transferred to 10% (v/v) TCA containing 5 mM unlabelled methionine for 15 mins, then boiled in 5% (v/v) TCA to degrade any radioactively-labelled tRNA. Once cooled, the filters were washed once in 100% IMS, once in acetone, dried and subjected to liquid scintillation counting. Incorporation of radioactive methionine into protein was expressed as cpm/µg protein.

2.12 Coomassie stain

Acrlyamide gel trimmed of stacking gel was placed into Coomassie stain for 30 mins on a rocker at room temperature. Stained gels were then placed into Coomassie destain on a rocker and incubated at room temperature until bands appeared and background stain decreased. The destain was replaced at regular intervals.

Coomasie stain:	1g coomasie briliant blue, 100ml glacial acetic acid, 400ml methanol, 500ml dH_2O.
Coomasie destain	100ml glacial acetic acid, 200ml methanol, 700 ml dH_2O

-Chapter 3-

Investigation of RBM4 protein expression and regulation during myogenic differentiation

3.1 Introduction

RBM4 has been shown to be involved in regulating myogenic differentiation via the miRNA system (Lin & Tarn, 2009), and in part by altering the splicing of PTB. As the total level of RBM4 protein increases slightly as differentiation progresses (Lin & Tarn, 2011), RBM4 may still have other as yet undetected or defined roles during the differentiation process. One such possible role could be that RBM4 possesses the ability to recruit initiation factors to specific mRNAs to up-regulate or down-regulate specific protein expression. In support of this is the finding that in HeLa cells in response to arsenite stress, RBM4 has previously been shown to recruit eIF4A and eIF4G to mRNAs that are thought to contain an Internal Ribosome Entry Site (Lin et al, 2007). Arsenite treatment up-regulates signalling via the p38MAPK pathway, a process which also happens during differentiation (Wang et al, 2008) and has been also been shown to inhibit translation of mRNAs that contain CU rich elements in their 3' untranslated region (UTR) (Lin et al, 2007). RBM4 has been shown to be phosphorylated as differentiation progresses when it is over-expressed in differentiating cells (Lin & Tarn, 2009). The same authors also showed that RBM4 protein phosphorylation increases as differentiation progresses (Lin & Tarn, 2011). The elevation of RBM4 phosphorylation during differentiation probably reflects elevated p38MAPK pathway, and RBM4 contains an identified p38MAPK phosphorylation site (Lin et al, 2007). These previous investigations could indicate that RBM4 may be recruiting eIF4G and eIF4A to specific mRNAs by phosphorylation of RBM4 during myogenic

differentiation akin to what has been seen in HeLa cells in response to arsenite.

What has not been investigated is the relative contribution of the isoforms of RBM4 (RBM4a and RBM4b) in myogenic differentiation. Most published work has focussed on RBM4a and total RBM4. The work described in this chapter will focus on characterising RBM4a and RBM4b protein expression profiles during differentiation. I will describe work investigating their translational efficiency, protein degradation and mRNA expression levels. A bioinformatics investigation will also be presented to highlight the differences and similarities between the two RBM4 isoforms.

3.2 Bioinformatics investigation of the RBM4a and RBM4b proteins

Sequence alignment of RBM4a and RBM4b protein shows that they are highly homologous (Figure 3.1) with an amino acid sequence identity of 87%; the highest homology is found in the N-terminal half of the proteins which have a sequence identity of 99%. The N-terminal half allows RBM4 to bind mRNA via its two RNA Recognition Motifs (RRMs) and its CHCC zinc finger (Markus & Morris, 2009). The C-terminal half has a sequence identity of 75% and contains a p38MAPK phosphorylation site which in *Homo sapiens* is found at Serine 309 (RBM4a); in *Mus musculus,* the phosphorylation site is found at Serine 306 (RBM4a) and Serine 302 (RBM4b). In addition, the C-terminus also contains regions of alanine rich repeats of unknown function. When mouse RBM4a protein is compared to human RBM4a (Figure 3.2) a high sequence homology is also observed (96% homology), especially in the N-terminal half of the protein. This suggests that any possible functional

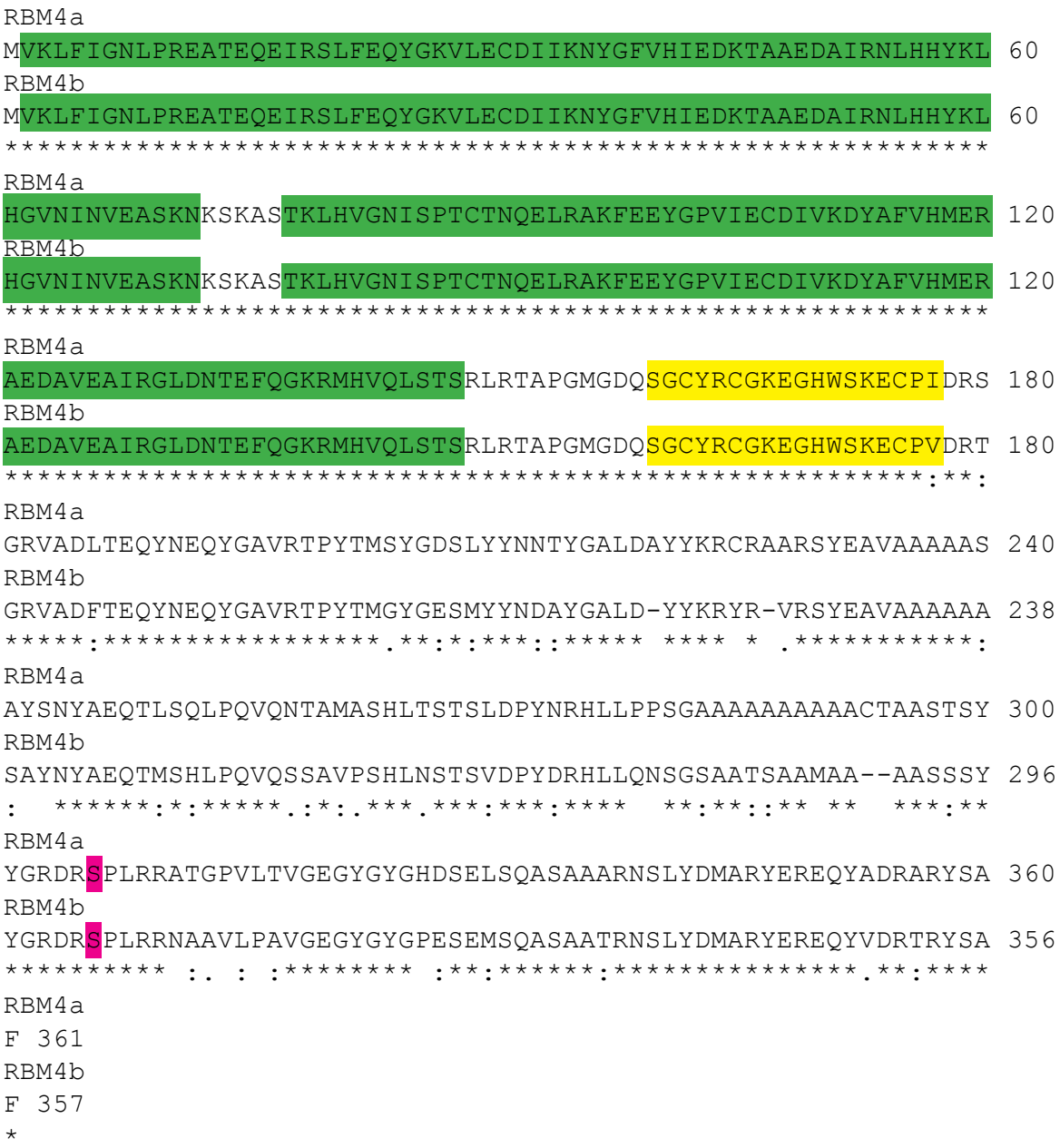


Figure 3.1. RBM4a shares high amino acid sequence homology with RBM4b. RBM4a and RBM4b *Mus musculus* protein complete sequences sourced from PUBMED were aligned to determine sequence homology by using ClustalW2 software. Stars signify a perfect match whereas double dots show similar amino acids and single dots show those with lower similarity. Green indicates RRM domains (RNA Recognition Motifs), yellow indicates Zinc finger structures and purple indicates the p38 MAPK phosphorylation site.

Amino Acid sequence identity = 87%

N-terminus sequence (1-180) identity = 99%

C-Terminus sequence identity = 75%

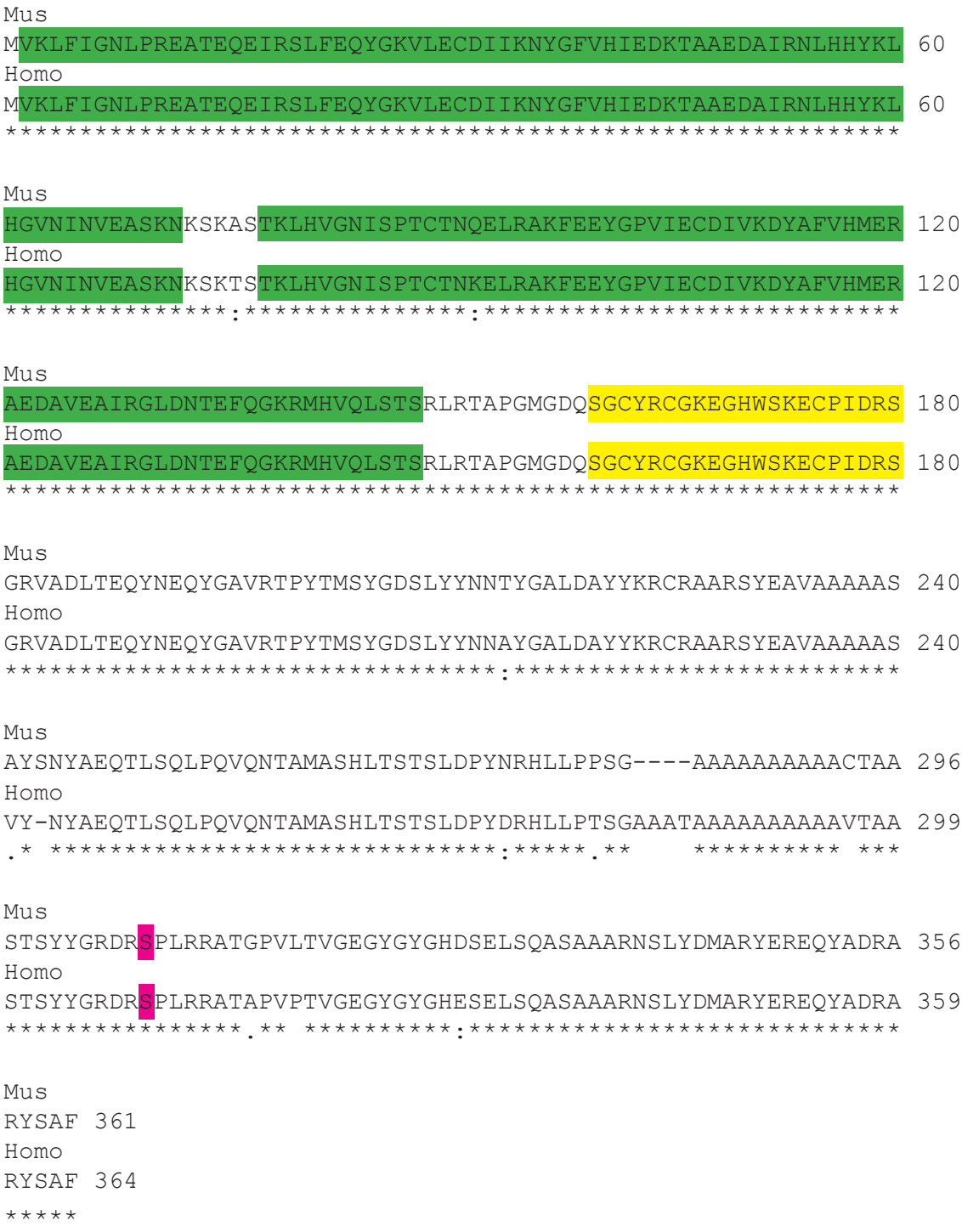


Figure 3.2. RBM4a from *Mus musculus* shares high amino acid sequence homology with RBM4a from *Homo sapiens*. RBM4a *Mus musculus* (Mus) and *Homo sapiens* (Homo) protein sequences sourced from PUBMED were aligned as before to determine the level of sequence homology using the ClustalW2 software. Stars signify a perfect match whereas double dots show similar amino acids and single dots show those with low similarity. Green indicates RRM domains (RNA Recognition Motifs), yellow indicates Zinc finger structures and purple indicates the p38 MAPK phosphorylation site.

Amino acid sequence identity = 96%

N-Terminus sequence (1-180) identity = 99%

C-Terminus sequence identity = 93%

difference in proteins function is more likely to be ascribed to the C-terminal half of the protein. A prosite scan was performed on mouse RBM4a to identify any putative motifs; none were found but some post translational modifications were predicted, including phosphorylation sites for casein kinase II (found throughout), protein kinase C (found only in C-terminus), and a single cAMP dependent protein kinase site in the C-terminus. There were also a predicted N-myristoylation site, an N-glycosylation site and an amidation site. The p38MAPK phosphorylation site observed by (Lin et al, 2007) was not predicted in the prosite analysis which supports the idea that this software does not predict all motifs and phosphorylation sites.

3.3 C2C12 myogenic differentiation markers

RBM4 is thought to be involved in myogenic differentiation as investigated by (Lin & Tarn, 2009). However, before I could look at the possible role for RBM4 in differentiation and translation initiation in C2C12 cells, the system had to be characterised and markers for myogenic differentiation had to be investigated to provide a means to identify the progress the cells are making through the differentiation process. A selection of proteins known to be muscle-specific and up-regulated during myogenic differentiation were selected for analysis, including myogenin (Cuenda & Cohen, 1999), αβ-crystalin (Kamradt et al, 2002), and caveolin-3 (Madaro et al). Myogenin was selected as it is a transcription factor responsible for up-regulating the expression of many different proteins required for differentiation and if not expressed, causes defects in the mid to late stages of differentiating myoblasts (Lin & Tarn, 2009). Caveolin-3 was selected because it is a muscle-specific scaffolding protein crucial for the regulation of caveolae by recruitment

of lipids, signalling molecules and other structural proteins; specific and defined mutations in caveolin-3 have been associated with four different human, muscle-specific diseases (Fanzani et al, 2007). αβ-crystalin was selected for analysis as it is involved in the regulation of the dynamic nature of microtubles which are thought to be required to maintain cell stability in myotubes (Sakurai et al, 2005).

Initially, C2C12 cells were grown to confluency, as described in Section 2.2.3. Cells were then placed into differentiation medium and induced to differentiate; samples were prepared for SDS-PAGE and Western blot analysis, as described in (Section 2.4). As shown in Figure 3.3A, myogenin expression was first detected at 24 hours and continued to rise to a peak at 48 hours after the induction of differentiation. αB-crystalin and caveolin-3 were expressed later in the differentiation process at 48 hours, with expression increasing to 72 hours. eIF4E was used as a loading control as previous work had shown that the protein levels do not change significantly during differentiation. Alongside protein markers there are visual indications of myogenic differentiation in cells. As seen in Figure 3.3B, with confluent C2C12 cells at 0 hours of the differentiation process, cells are mononucleated and are referred to as myoblasts; after 24 hours they have exited the cell cycle and are beginning to line up. At 48 hours of differentiation, cells have lined up and some have begun to fuse, with larger numbers of the myoblasts having fused at 72 hours to form multinucleated myotubes.

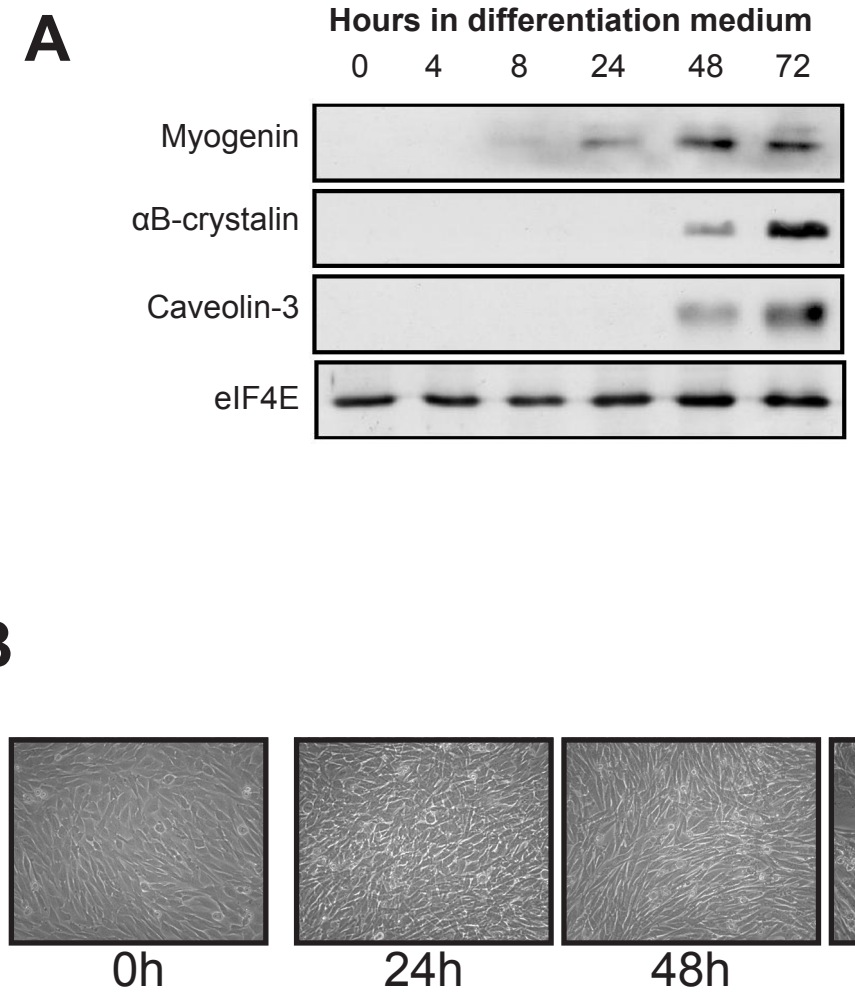


Figure 3.3. Expression level of myogenic markers during myogenic differentiation. Panel A. C2C12 cells were grown to confluency and their medium was changed to differentiation medium, as described in Materials and Methods Section 2.2.3. Cells were then harvested at 0, 4, 8, 24, 48 and 72 hours post addition of differentiation medium and extracts prepared as in Section 2.3.1. Total protein (10μg) was then analysed by SDS-PAGE and membranes probed with the antibodies shown above and visualised using an ECL system, as described in Materials and Methods Sections 2.4 . These data are from a single experiment but are representative of those obtained in 5 separate experiments. Panel B. Cells were induced to differentiate as above and images taken at 0, 24, 48 and 72 hours using a Moticam 2000 with a 10X objective.

3.4 RBM4 protein and isoform expression levels change during differentiation

Published work by Lin et al., (Lin & Tarn, 2009) has shown that total levels of RBM4 protein increased during differentiation of C2C12 myoblasts. To investigate any difference in expression between the two isoforms of RBM4 (RBM4a and RBM4b) during differentiation, initially their combined expression level was determined in C2C12 cells using antibodies that recognize both RBM4 isoforms. C2C12 cells were grown to confluency, as described in Section 2.2.3 and then induced to differentiate as described in section 2.2.3. As shown in Figure 3.4, Western blot analysis of extracts prepared from a number of independent experiments showed that RBM4 protein expression increase significantly at 24 hours and 48 hours, with the greater increase being at 48 hours when compared to the loading control PABP. These findings were in agreement with those presented previously (Lin & Tarn, 2009). The increase in total RBM4 protein level at early times was followed by a small, but significant decrease in levels at later times of differentiation. The progression of the differentiation process was confirmed in these experiments both by monitoring the expression of caveolin-3 (figure3.4) and visually, using microscopy (data not shown).

As there are two isoforms of RBM4, this increase in RBM4 protein expression could reflect a change in either isoform or in both isoforms of protein. To address this, I obtained isoform-specific antibodies raised against RBM4a or RBM4b (Pfuhl et al, 2008) and determined the isoform protein

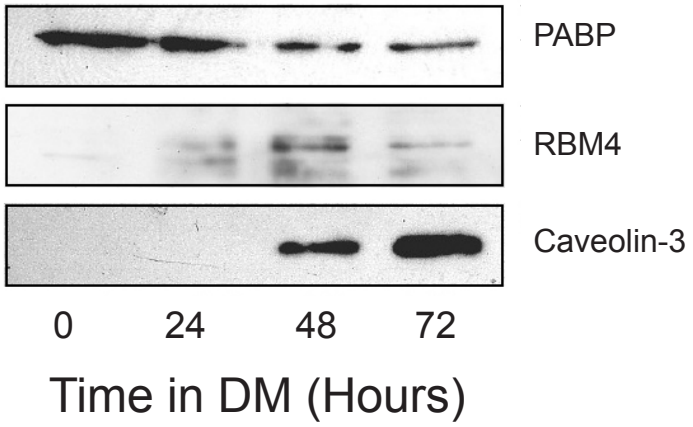


Figure 3.4 Total RBM4 protein expression increases during C2C12 myogenic differentiation. C2C12 cells were grown to confluency and their medium was changed to differentiation medium as described in Materials and Methods Section 2.2.3. Cells were then harvested at 0, 24, 48 or 72 hours post addition of differentiation medium. Aliquots of extract containing 10μg of protein were then analysed by SDS-PAGE and Western blotting, the membrane probed with antibodies shown and visualised using an ECL system, as described in Materials and Methods (Section 2.4). These data are representative of three separate, independent experiments.

expression during differentiation. As shown in Figure 3.5, RBM4a protein expression increased at 24 hours of differentiation. However, after this time its expression profile relative to total RBM4 protein levels diverge greatly, with RBM4a protein expression reducing at 48 followed by another reduction to around 40% of the starting levels at later times. Compared to this, the RBM4b expression profile matches what was seen with total RBM4 protein; at 24 hours a small increase is seen followed by a greater increase at 48 hours, with a small decrease at 72 hours. These data indicate that the RBM4b isoform is the protein with the greatest abundance during differentiation (Figure 3.4 vs Figure 3.5); however, this does not mean that RBM4a is not involved in modulating myogenic differentiation at some level.

3.5 RBM4a and RBM4b mRNA are also differentially expressed during differentiation

Protein expression in cells can be modulated by many different pathways; one of these is an up-regulation of transcription of the encoding mRNA. To investigate if RBM4a or RBM4b are regulated in this manner their mRNA expression levels were analysed during differentiation using q-RT PCR and isoform-specific primers (Figure 3.6). When compared to the 18S RNA expression, RBM4a mRNA levels were found to decrease at every time point relative to the undifferentiated cells, falling to 20% of the starting level. In contrast, RBM4b mRNAs levels (once again compared to 18S RNA) increases significantly at 24 hours to a peak at 650% relative to undifferentiated cells, falling to around 200% of control level at 72 hours after the induction of differentiation. 18S rRNA was chosen as a comparison as its turnover is low (Retz & Steele, 1980) and levels have been seen previously not to change

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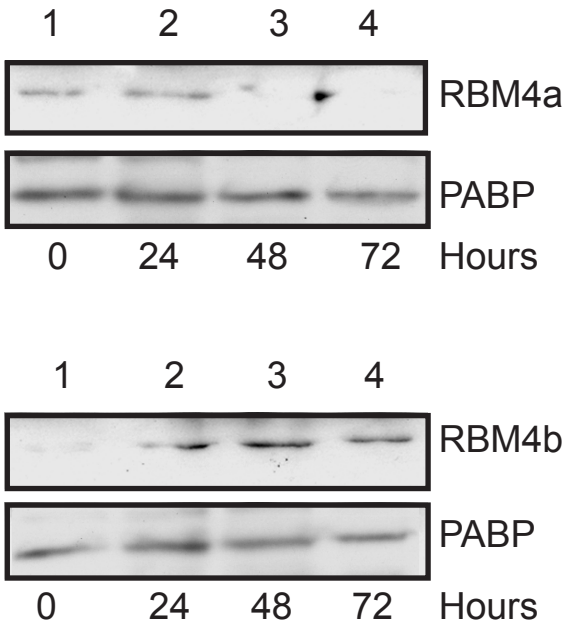
1
2
3
4
RBM4a
PABP
0
24
48
72
Hours
1
2
3
4
RBM4b
PABP
0
24
48
72
Hours

B

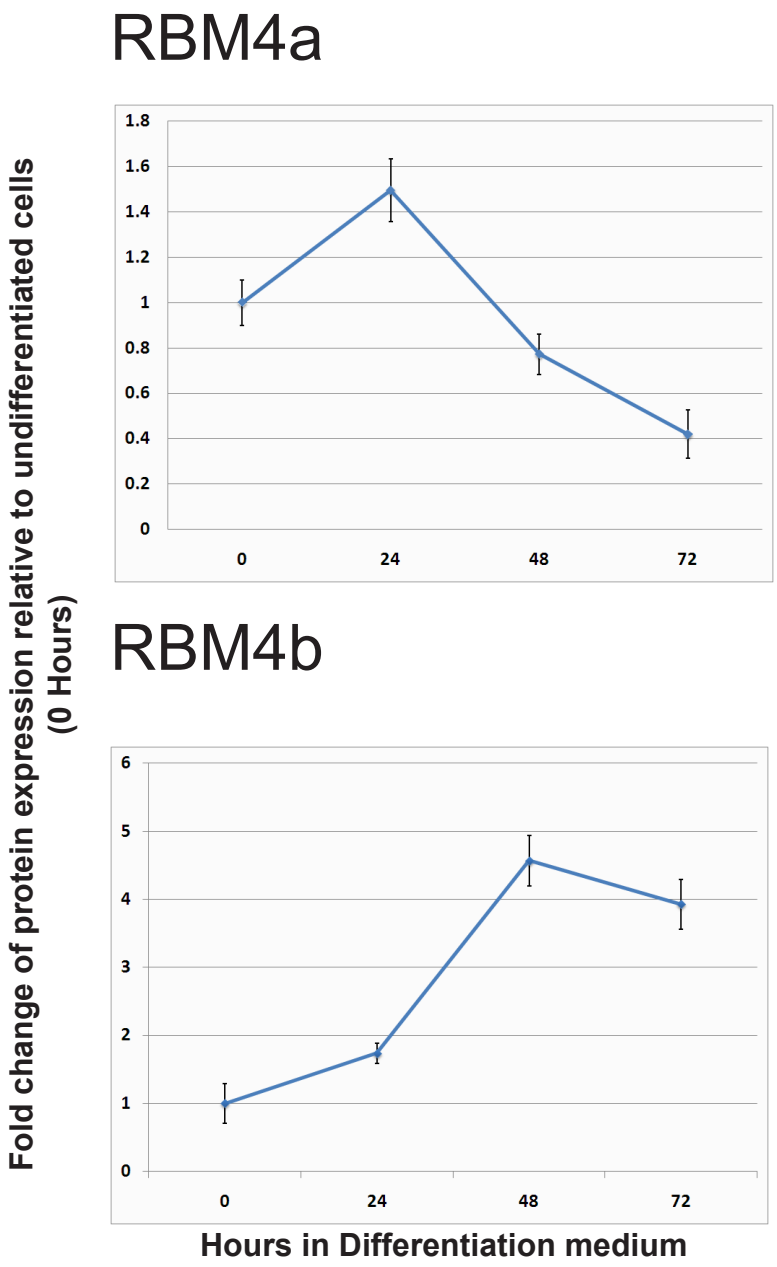

Figure 3.5 RBM4a and RBM4b are differentially expressed during myogenic differentiation. Panel A. C2C12 cells were grown to confluency and differentiated as described in the Materials and Methods Section 2.2.3. The cells were harvested at the indicated time points and aliquots of extract containing 10µg protein were analysed by SDS-PAGE and Western blotting using antibodies raised against the specific isoforms of RBM4. Panel B. Experiments were repeated twice and densitometry was performed using ImageJ to determine changes in protein expression levels normalised to PABP relative to undifferentiated cells (set at 1.0). Errors bars are standard error of the mean.

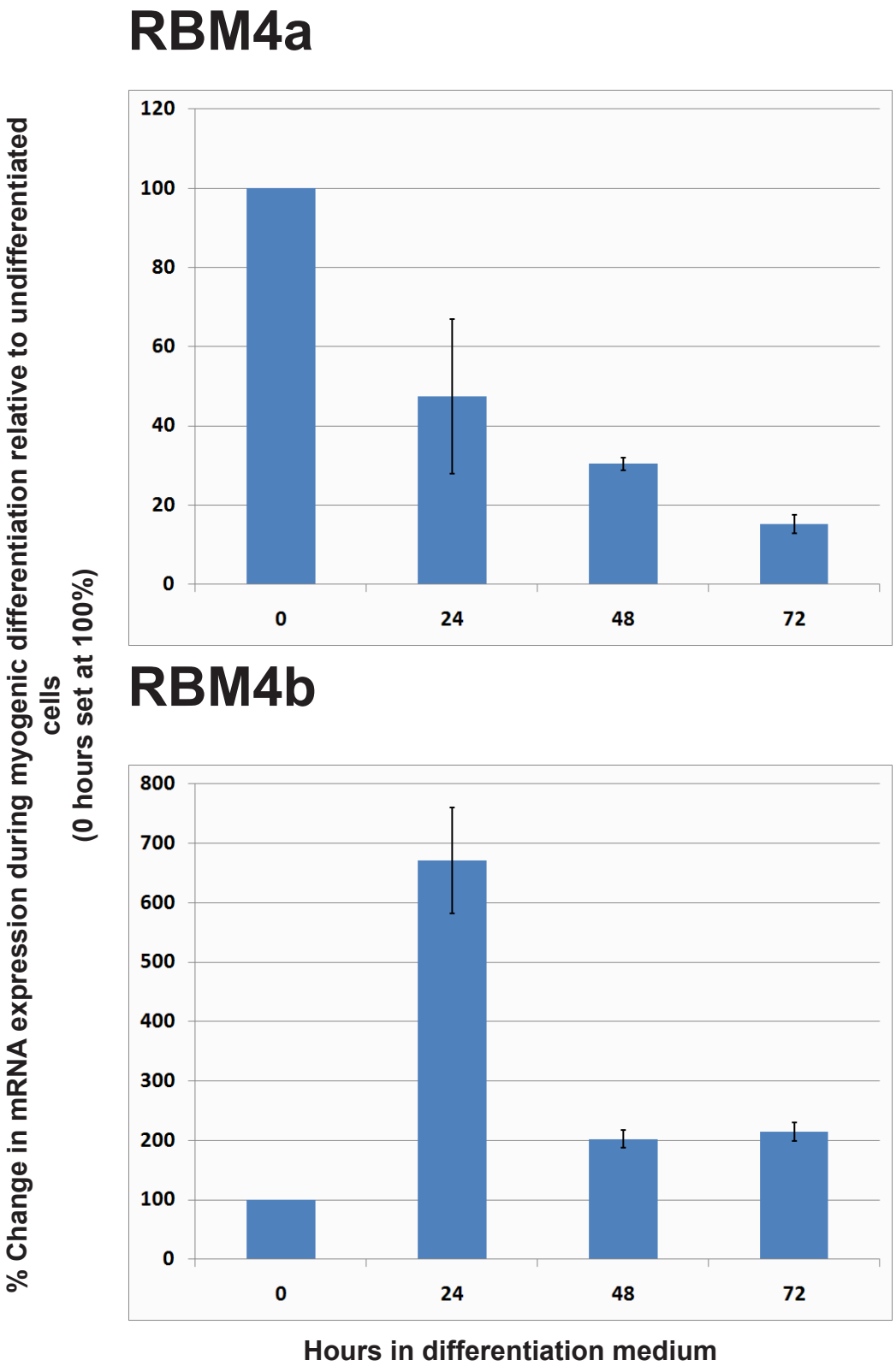

Figure 3.6. Differential expression of RBM4a and RBM4b mRNA during differentiation. C2C12 cells were prepared and differentiated as described in the Materials and Methods. mRNA was isolated and converted into a cDNA library as described in the Materials and Methods Sections 2.7.1 and 2.7.2 at the time points indicated. To quantify the mRNA, the library was then analysed by q-RT PCR as described in Materials and Method Section 2.8. Graphs are derived from 3 separate experiments with the error bars showing standard error of the mean.

dramatically in the differentiating cells within these time periods (data not shown).

Therefore, RBM4a mRNA expression levels (Figure 3.6) when compared to RBM4a protein expression (Figure 3.5), generally appear to follow a similar pattern during differentiation; a decrease in both the level of protein and mRNA. At 24 hours of differentiation there did appear to be an increase in RBM4a protein levels when RBM4a mRNA were shown to fall; the reasons for this are unclear. RBM4b mRNA expression levels (Figure 3.6) peaked 24 hours before RBM4b protein expression (Figure 3.5) RBM4b mRNA then decreases to 2 fold the starting expression, whereas RBM4b protein increase at 48 hours and reduces slightly at 72 hours. These data indicate that changes in RBM4a and RBM4b mRNA levels could be important for the regulation of their protein levels but other factors such as the half life of the protein or translation efficiency may be important in regulating total protein levels.

3.6 RBM4a and RBM4b mRNA loading onto polysomes changes during differentiation

The data presented in Figure 3.6 indicated that RBM4b mRNA may be actively recruited for translation early during the process of myogenic differentiation. In light of the fact that the antiserum was not of high enough titre to be used to immunoprecipitate RBM4 protein isoforms from cell extracts (data not shown), the loading of RBM4a or RBM4b mRNA onto polysomes was be used as a way of predicting protein expression rates. The polysome loading of RBM4a and RBM4b mRNA was measured as described in the Materials and Methods Section 2.8. Briefly, extracts prepared at different

times following the induction of differentiation were fractionated on sucrose gradients to resolve the mRNA in mRNPs/monosomes from the mRNA in polysomes. The polysome fractions from each time point were collected, pooled and q RT-PCR was performed using specific primers for RBM4, RBM4b and 18S rRNA. 18S rRNA level was also measured to observe any change in number of total number of ribosomes in the polysomes fraction.

The data presented in Figure 3.7 shows that the recovery of RBM4b mRNA in the actively translating polysomes fractions partially echoes the protein expression profile as seen in Figure 3.5. In both cases, relative to undifferentiated cells, the polysome loading of mRNA (Figure 3.7) and the protein expression show their greatest increases at 48 hours. However, this correlation does not hold for the 24 and 72 hour time points where protein expression is increased with no change in polysome loading of mRNA. The differences seen at 72 hours could be due to changes in protein degradation rates; even though the polysome loading of mRNA has decreased and potentially the RBM4b synthesis rate has decreased, the protein levels might not change due to increased stability of the RBM4b protein. In contrast, RBM4a mRNA loading onto polysomes does not match RBM4a protein expression in any way. At both 24 and 72 hours of differentiation, there was a large increase in RBM4a mRNA loading efficiency onto polysomes which did not correlate with protein levels at these times. One potential reason for this is that the degradation rate of RBM4a protein could have increased dramatically at later times of differentiation, meaning that any RBM4a that is produced at 24 and 72 hours is destroyed rapidly before it can accumulate. An alternative explanation for this could be that the RBM4a mRNA is loaded onto pseudo-

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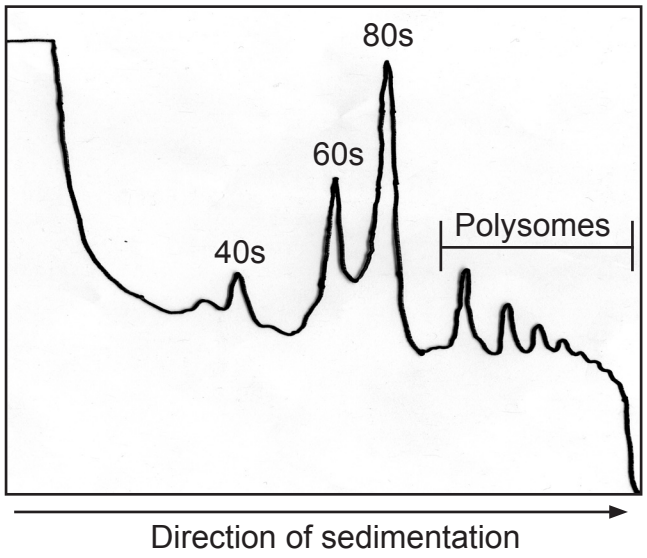

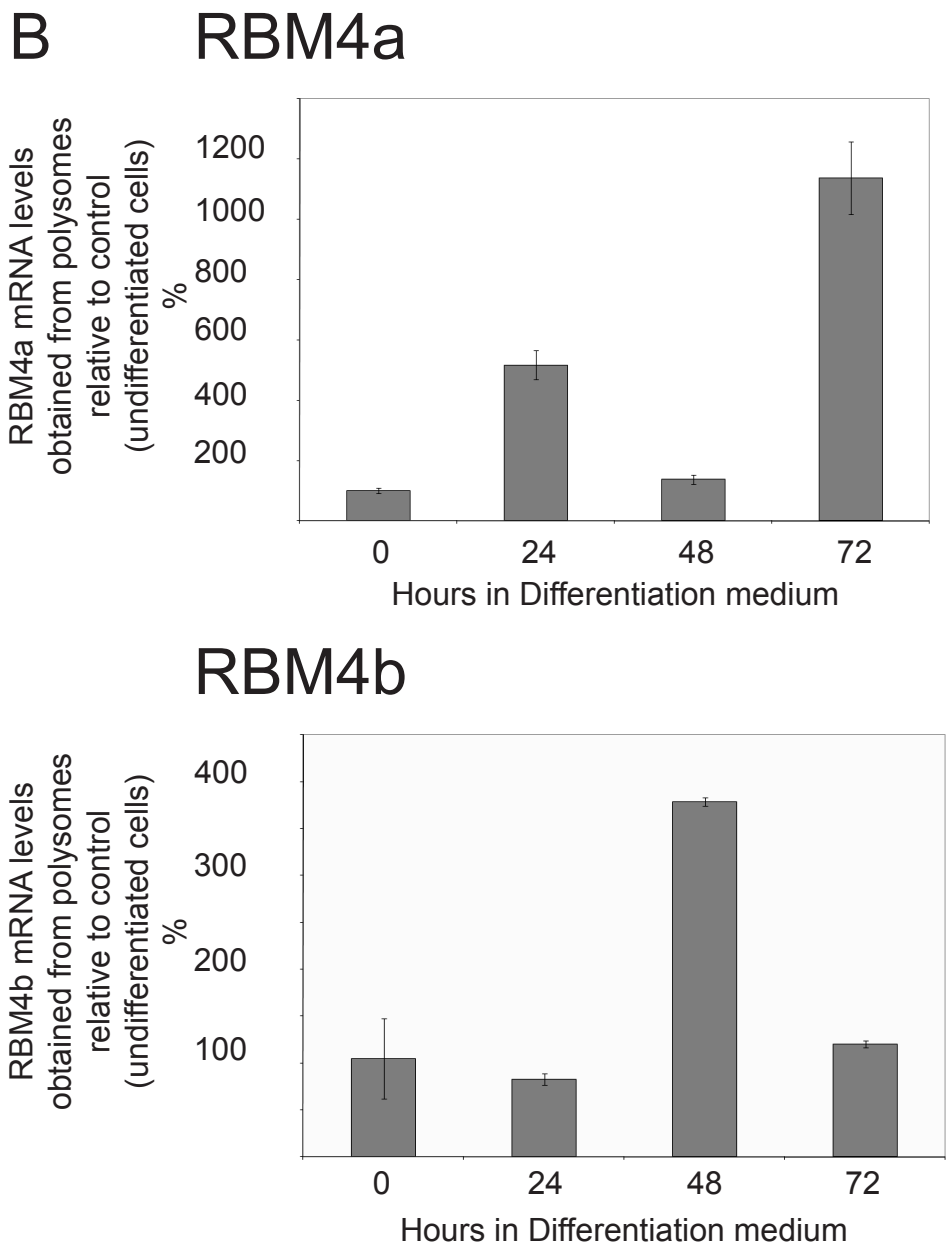

Figure 3.7. RBM4a and RBM4b mRNA loading onto polysomes during differentiation. C2C12 cells were induced to differentiate as described in Materials and Methods Section 2.2.3. The cells were harvested at indicated time points by the standard lysis method as described in Materials and Methods Section 2.3.1 under RNAse free conditions. The extracts were then fractionated by sucrose density centrifugation (Panel A) to obtain polysomes as described in Materials and Methods Section 2.6. The RNA was extracted from the polysomes and converted into a cDNA library as described in Materials and Methods Section 2.7.1 and 2.7.2. The cDNA was then analysed by q RT-PCR using primers designed to target RBM4A or RBM4b (Panel B) as described in Materials and Methods Section 2.8.1. N= 2 error bars are standard error of the mean.

polysomes at 24 and 72 hours. Pseudo-polysomes, as described by Thermann (Thermann & Hentze, 2007), are dense miRNPs these have the same sedimentation characteristics as polysomes. However mRNAs that are incorporated into these pseudo-polysomes are arrested in the elongation phase and not translated. miRNAs have been shown by Thermann (Thermann & Hentze, 2007) to lead to specific miRNAs to be incorporated into these pseudo-polysomes. Further work would need to be carried out to verify this, but it does suggest that RBM4a is partially regulated by miRNAs.

3.7 The 5' and 3' UTRs of RBM4a and RBM4b mRNAs show low sequence homology

The differences seen in the differential mRNA loading of RBM4a and RBM4b onto polysomes led to a sequence investigation of their 5' and 3' untranslated regions (UTR) as these can influence polysome loading and translation efficiency (reviewed in (Wilkie et al, 2003)). The 5' UTRs were compared (Figure 3.8A) and they were shown to share low sequence homology, with the 5'UTR for RBM4b mRNA being significantly shorter. However, interspersed amongst the area of low homology are some areas that have a higher level of homology. These higher homology areas could be conserved motifs but further analysis using a motif scanner suggested that this was unlikely to be the case (data not shown). This does not mean that such motifs do not exist as sequence analysis is not perfect. Furthermore, mRNA motifs: protein interaction can be sequence-dependent, structural-dependent or a mixture of both (Gupta & Gribskov, 2011). What the 5'UTRs from RBM4a and RBM4b do share in common is that they are both predicted to form quite stable structures, one of which is presented (Figure 3.8B). All of

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RBM4a   ATTTTAGCGTTTTGTCAGAACCGTCCGCGCTGCAAGGAGGAGGACCTGCAGGTATCCATG 60
RBM4b   ---------------CAGG-CAGCGCGCACTC---------GCGCGTGCGTGAGCTGGCG 35
                       ***  * *  *** **          *  * ***  *       *

RBM4a   CGGTGAGATACTCCACGTTCTTCCACTGTGTTCTTTTCTCTGTTAAAAAAACTTACCTGA 120
RBM4b   CG-CGAGAAA----GCGCCCGGTCGC---------------GCCGA------------GG 63
        **  **** *     **  *   * *                *   *            *

RBM4a   CTCCGGTGGGTGTCAGGGTTGGCATAGTGGGGTTGCGGTCTG-CGC---ACCCGTCCCTG 176
RBM4b   CTCGAGCGGCCGTCGCCATT----TTGTAGGGTT-CTCTCTGACGCGGGACCCGCCGCC- 117
        ***  * **  ***    **    * ** ***** *  **** ***   ***** * *

RBM4a   AGAGCCGACATCGGTCCTCGACTTAGTGCGGCTGTGTGGAG 217
RBM4b   ACCGCCGGCACCA---CCCGG---AG-GCTCTTGTCAGG-- 149
        *  **** ** *    * **    ** **   ***  **
                                  ▲
                                  AUG
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B

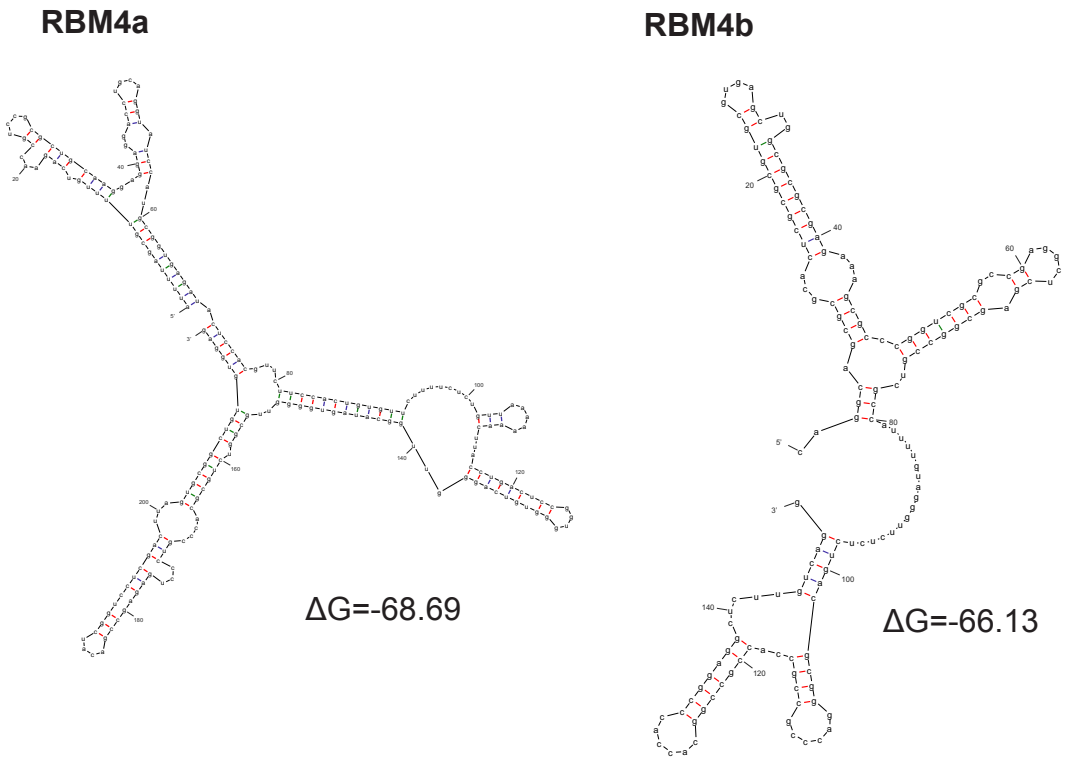


Figure 3.8 RBM4a and RBM4b 5' UTRs share little homology. Panel A. The nucleotide sequence of the 5'UTR from the two isoforms of *Mus Musculus* RBM4 were aligned using the Clustal Lalign programe. Stars indicate a match between the two sequences. The AUG start codon is indicated. Panel B. Sequences of the 5'UTRs of RBM4a and RBM4b where analysed by mFOLD and the most structurally stable structures where chosen to demonstrate potential secondary structures.

the predicted structures produced by the mFOLD software were highly structured. Obviously, the existence of any of these structures would have to be proven by RNA probing experiments (Ehresmann et al, 1987). The reason their structural complexity is important is that the 5' UTR structure can modulate translation efficiency by modulating the requirement of initiation factors involved in unwinding of complex 5' UTRs like eIF4A (Svitkin et al, 2001). The 3' UTRs of these mRNAs showed an even lower overall level of homology as shown in Figure 3.9, with the RBM4a 3' UTR showing only 24% the length of the RBM4b 3'UTR sequence (respective lengths of 149nt and 617nt). This huge difference in length allows for plenty of scope for the presence of isoform-specific regulatory motifs such as a miRNA binding site.

3.8 Discussion

The majority of work on the role for, and the expression of RBM4 during differentiation has been directed at the total RBM4 or the RBM4a isoform (Lin & Tarn, 2011b) My work described in Figure 3.4 has shown that the protein expression level of the RBM4 isoforms is differentially regulated, with RBM4b potentially accounting for the majority of the rise in total RBM4 protein expression observed during differentiation. This would lead to concerns with previous studies using over expression of RBM4a alone to study the effect of RBM4 on differentiation (Lin & Tarn, 2011b). Due to the highly conserved amino acid sequence of RBM4a and RBM4b, it is more likely that there could be little or small functional differences between the isoforms. However, a potential reason for having two isoforms of RBM4 is a greater ability to regulate RBM4 protein expression via their highly divergent 5' and 3' UTRs. This idea along with the large number of different systems RBM4 has been

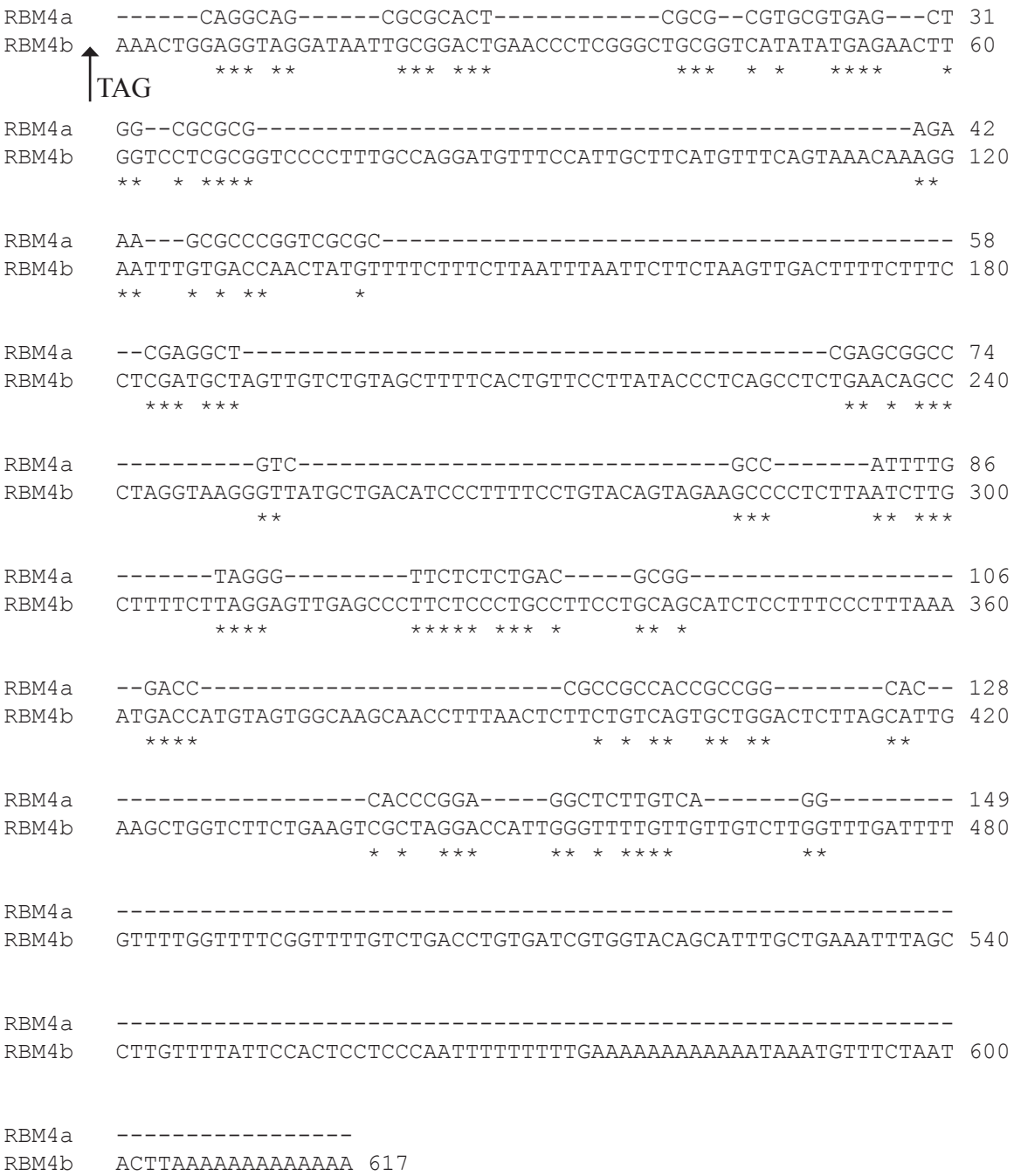


Figure 3.9 *Mus Musculus* RBM4a and RBM4b 3' UTRs share little homology. The nucleotide sequence of the 3'UTR from the two isoforms of RBM4 were aligned using the Clustal Lalign programe. Stars indicate a match between the two sequences. The TAG stop codon is indicated.

shown to be involved in, would indicate that regulation of RBM4 protein level is very important for the cell. Little is known about the rate of synthesis of RBM4 in C2C12 cells during differentiation. Unfortunately, due to the low titre of the anti-RBM4 isoform-specific antisera, I was unable to measure this directly by using [^{35}S] methionine labeling of the protein in cells followed by immunoprecipitation and autoradiography. Instead, I was limited to analysing the association of mRNA with polysomes as an indication of possible translational efficiency. The disagreement between the polysome loading of the mRNA encoding RBM4a and protein expression of RBM4a (Figures 3.7 and 3.5) can be potentially explained by some of the RBM4a mRNA going into actively translating polysomes at 24 and 72 hours in differentiation medium and some going into pseudo-polysomes which are not actively translated (Thermann & Hentze, 2007). This does not account for the fact that the level of RBM4a mRNA found in polysomes or pseudo-polysomes goes up even though the total mRNA expression of RBM4a decreases as differentiation progresses. Indeed, total RBM4a mRNA drops to 20% of the starting level whilst the polysome/pseudo-polysome loading increased by 8-fold. In contrast, the data presented in Figure 3.7 shows that the recovery of RBM4b mRNA in the actively translating polysomes at early times is reflected in protein levels (Figure 3.5). However, this correlation does not hold for the later time points where protein expression is increased with no change in polysome loading of mRNA. This may reflect changes in RBM4 protein degradation rates. Unfortunately, I was unable to investigate protein degradation rate changes between myoblasts and myotubes; the levels of RBM4a protein were too low to be detected at 48 or 72 hours and RBM4b protein expression was too low

in undifferentiated cells for such an experiment approach. To further investigate the regulation of the RBM4 isoforms new antibodies would have to be sourced to allow for immunoprecipitation of labeled protein to allow me to perform pulse chase assays in the presence of cycloheximide.

In summary, the data presented in this chapter has shown that the RBM4 isoforms, RBM4a and RBM4b, are differentially regulated and potentially have different functions during differentiation. it is predicted that these differences in function will most likely be reflected in isoform specific protein complexes. Protein-protein interactions of RBM4 and proteins involved in translation initiation is the focus of the data presented in Chapter 5.

-Chapter 4-

Investigation of RBM4 localisation during myogenic differentiation

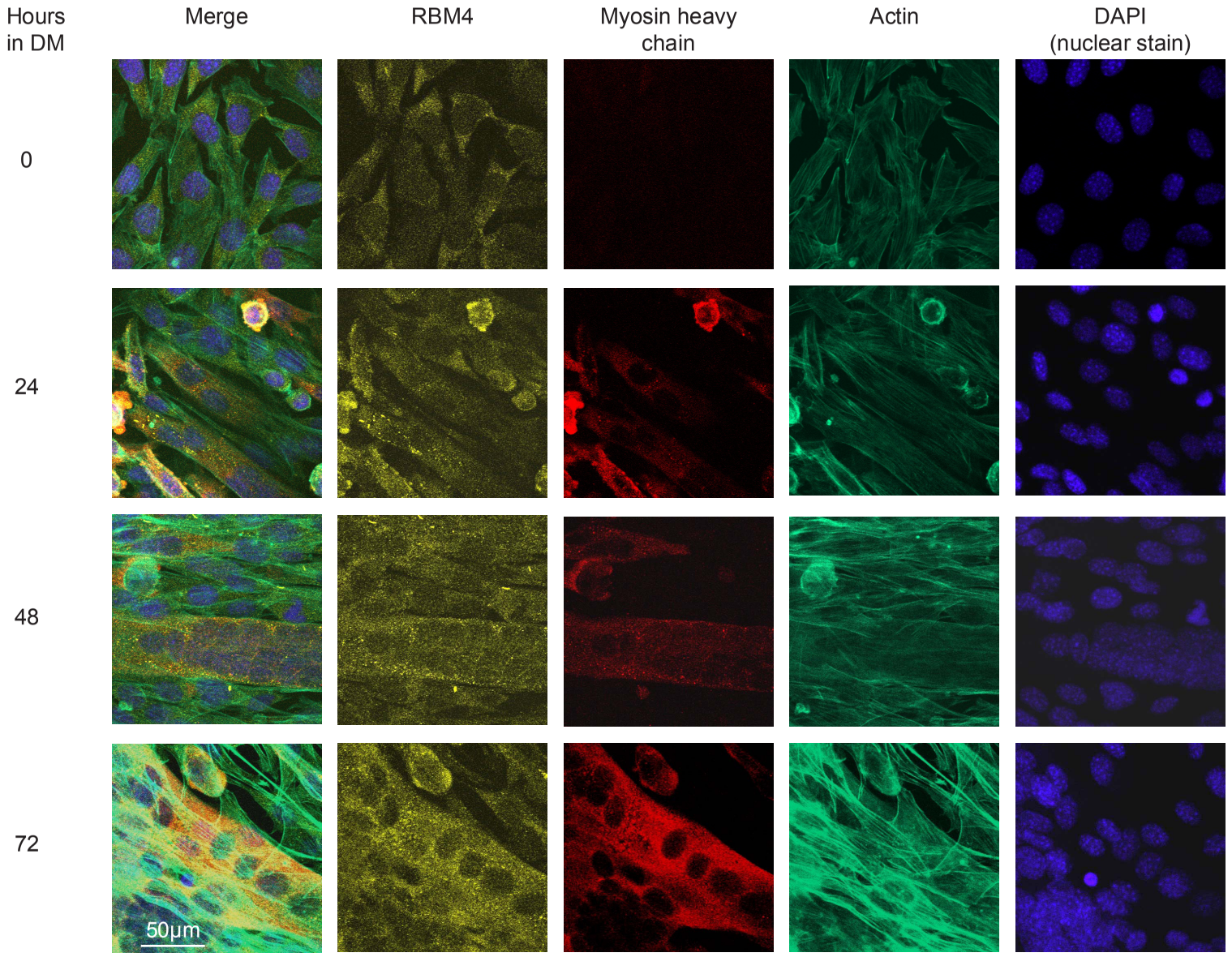
4.1 Introduction

RBM4 has been observed to have a predominantly nuclear localisation in HeLa cells (Lin et al, 2007). However, following treatment of the cells with arsenite, a proportion of RBM4 was re-localised to the cytoplasm where it has been shown to affect the level of expression of specific proteins. A role for p38MAPK signalling in the subcellular localisation of RBM4 has been suggested following the observation that down-regulation of p38MAPK activity results in a predominantly nuclear localisation of RBM4 (Lin et al, 2007). p38MAPK has also been observed to be active during myogenic differentiation of C2C12 cells and inhibition of this signalling pathway leads to a decreased rate of differentiation (Wang et al, 2008). This has led me to investigate the localisation of RBM4 in C2C12 cells undergoing differentiation.

4.2 RBM4 protein localisation during myogenic differentiation

To investigate the localisation of RBM4 in C2C12 myoblasts, cells were grown to confluency and induced to differentiate for up to 72 hours. Cells were fixed, permeabilised and the localisation of actin (for the cytoskeleton), myosin heavy chain (a marker for differentiation (Matheny & Nindl, 2011), nuclei (stained with DAPI) and RBM4 were visualised by confocal microscopy. As can be seen in Figure 4.1 (and quantified from three separate experiments in Figure 4.2), in confluent cells before the induction of differentiation, RBM4 staining was diffuse, but the protein showed a slight predisposition for the cytoplasmic compartment. However, as differentiation progressed, the cytoplasmic levels of RBM4 protein increased significantly (Figure 4.2),

Figure 4.1. Localisation of RBM4 protein during myogenic differentiation. Cells were grown to confluency and their medium was changed to differentiation medium (DM) as described in Materials and Methods Section 2.2.3. The cells were fixed using 4% paraformaldehyde at the indicated time points and permeabilised with Triton X-100 before being incubated with indicated antibodies (RBM4 and Myosin heavy chain) and stains (phalloidin and DAPI). The samples were then incubated with fluorescently labelled secondary antibodies and visualised using confocal microscopy as described in Section 2.5.1 of the Materials and Methods. Images shown are representative of those obtained in 3 separate experiments.


Hours in DM
Merge
RBM4
Myosin heavy chain
Actin
DAPI (nuclear stain)
0
24
48
72
50µm

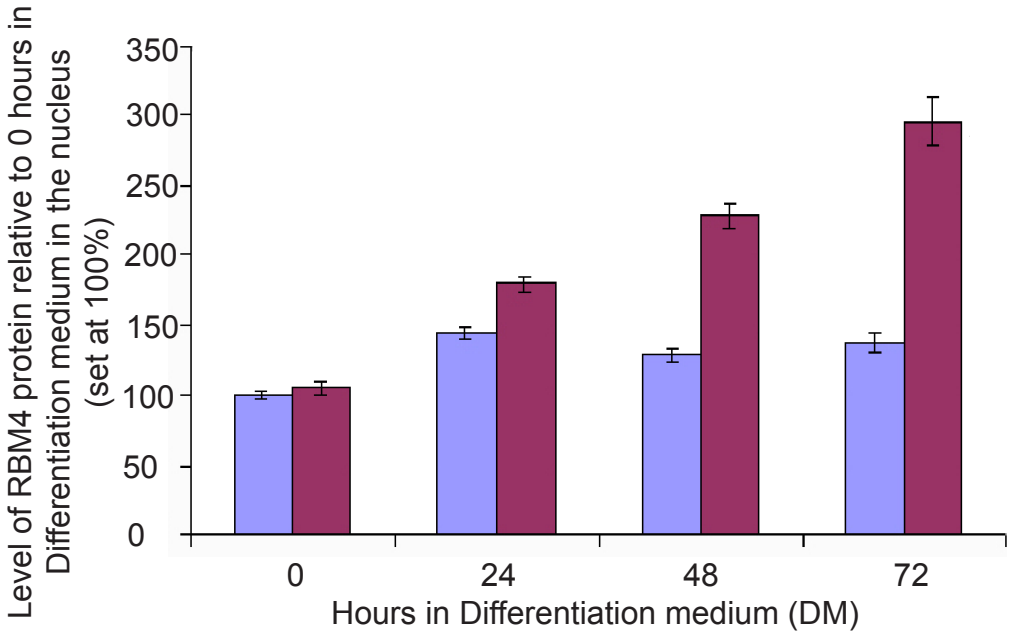


Figure 4.2. Quantification of cytoplasmic and nuclear localisation of RBM4 protein during myogenic differentiation. Images represented in Figure 4.1 were analysed for the density of total RBM4 found in the nucleus and cytoplasm; using ImageJ, 40 cells were analysed for each time point for both cytoplasmic and nuclear staining. The experiments were repeated 3 separate times and these data are the combined results of those experiments. Error bars are standard error of the mean. Blue = Cytoplasmic and Purple = Nuclear

showing an enrichment in the perinuclear region at 48-72 hours when multi-nucleated cells were clearly visible (Figure 4.1). The RBM4 staining was granular in appearance, an observation made in other studies (Lin & Tarn, 2009). This pool of RBM4 is believed to have a role in miRNA regulation of gene expression (Lin & Tarn, 2009). In contrast, the nuclear RBM4 levels initially increased significantly at 24 hours (Figure 4.2) but did not continue to increase in a manner seen for the cytoplasmic pool.

To further support these data, differentiating C2C12 cells were subjected to sub-cellular fractionation and the location of RBM4 visualised by Western blotting. As described in the Materials and Methods, this sub-cellular fractionation yields three fractions: a cytoplasmic fraction; a membrane bound protein fraction; and a nuclear fraction. As shown in Figure 4.3, fractionation was performed on cells undergoing a time course of differentiation with cell extracts prepared at 0, 24, 48 and 72 hours after the induction of differentiation. Antibodies against myc and caveolin-3 were used to demonstrate the purity of the nuclear and cytoplasmic fractions, respectively. As predicted, myc protein was only detected in the nuclear fraction (Figure 4.3A, lanes 3 and 6). In contrast, caveolin-3 can be observed in the cytoplasmic fractions (lanes 1 and 4) and to a greater extent the membrane bound fractions (lanes 2 and 5). These observations show that the sub-cellular fractionation was successful as caveolin-3 was not observed in the nuclear fraction and myc, which is a transcription factor, was found only in the nucleus. Western blotting of these fractions showed that RBM4 was found to be present in only the cytoplasmic fractions of cells harvested at 0, 24, and 48 hours of differentiation (Figure 4.3B, lanes 1, 4, and 7). The expression level

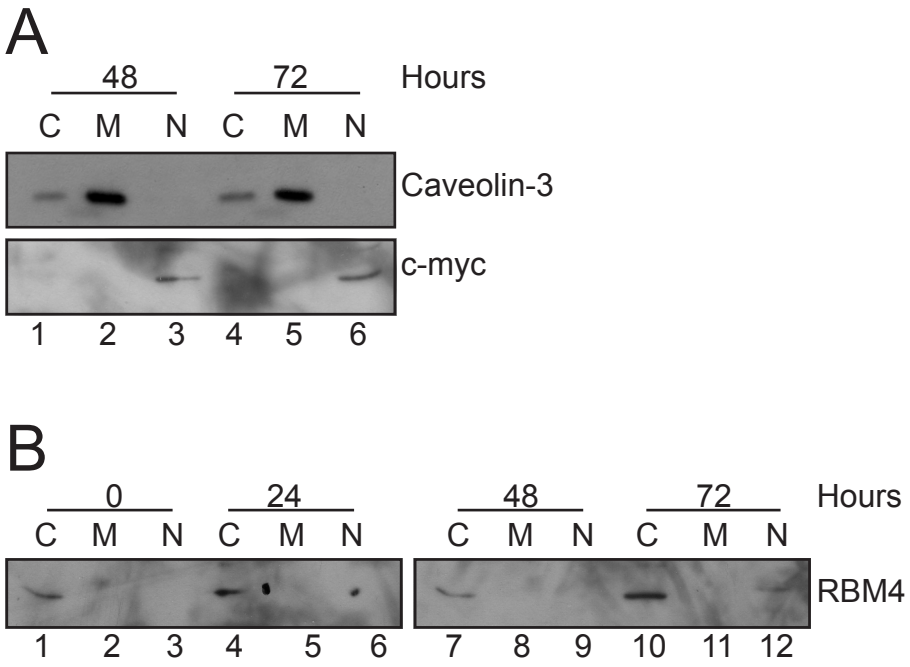


Figure 4.3. Localisation of RBM4 protein during myogenic differentiation as determined by sub-cellular fractionation. C2C12 cells were induced to differentiate as described in Materials and Methods Section 2.2.3. The cells were harvested at daily intervals as using the ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem) to separate cytoplasmic (C), membrane (M) bound and nuclear fractions (N), as described in Materials and Methods Section 2.3.3. Aliquots containing 10µg of each fraction were resolved by 12% SDS PAGE and subjected to Western blotting with the indicated antibodies, as described in Materials and Methods Section 2.4. Panel A shows Fractionation markers Caveolin-3 and c-myc. Panel B shows RBM4 sub cellular fractionation. N=2.

of the protein varied between each time point but as this experiment was set up to see the relative changes between compartments; this did not hamper interpretation of these data. Extracts prepared from cells induced to differentiate for 72 hours showed that the RBM4 signal in present in the cytoplasm as well in the nuclear fraction (Figure 4.3B, lanes 10 and 12). These data support the microscopy data in that a pool of RBM4 is nuclear although little evidence was obtained for the presence of the protein in the nuclear pool at other time points as shown by the microscopy. This difference could be caused by the detection method used between the two techniques; whilst immunoflorescence measured RBM4 level by volume, the sub-cellular fractionation and Western blotting measured RBM4 by mass of protein. The difference could also be caused by variations in the amount of protein loaded onto the gels at the different time points. This experiment would need to be repeated to ensure that the increase of the RBM4 protein in the nuclear fraction at 72 hours is reproducible. One interesting observation from Figure 4.3B is that the RBM4 protein found in the nuclear fraction at 72 hours (lane 12) appears to be migrating at a slightly higher molecular weight than that of RBM4 in the cytoplasmic fraction (lane 10). This could be because RBM4 has been phosphorylated or subjected to another post translational modification. RBM4 is known to be phosphorylated in response to p38MAPK signalling but this has only been demonstrated in HeLa cells when the protein was over-expressed and localised to the cytoplasm (Lin & Tarn, 2009).

4.3 p38MAPK is active during differentiation

Previous work has clearly shown that p38MAPK activity is required for myogenic differentiation. Treatment of C2C12 cells with the cell-permeable p38MAPK inhibitor, SB203580, blocked myogenic differentiation (Cowan unpublished observation) and knock-down of any of the p38MAPK isoforms also effectively blocked the differentiation process (Wang et al, 2008). To determined whether p38MAPK was active in my C2C12 cells under my culturing conditions, I examined the phosphorylation state of p38MAPK as an indicator of its activity; when p38MAPK is phosphorylated on Thr180/Tyr182 it is activated due to changes in its conformation (Roux & Blenis, 2004). As can be seen in Figure 4.4, p38MAPK phosphorylation is barely detected in undifferentiated cells (lane 1). However, at 24 and 48 hours after the induction of differentiation, p38MAPK is transiently activated (lanes 2 and 3 vs lane 1), declining by 72 hours to a level lower than in undifferentiated cells (lane 4 vs lane 1).

4.4 p38MAPK inhibition with SB202190 delays myogenic differentiation

To complement previous work with SB203580 (Cowan unpublished observation), I have investigated the effect of a more recently developed p38MAPK inhibitor, SB202190, on myogenic differentiation. Cells were grown to confluency and then induced to differentiate in the absence or presence of SB202190; the drug was replenished every 24 hours when the medium was changed. Figure 4.5 shows that in the absence of the drug, cells fuse to form myotubes which were visible at 48 hours and clearly evident at 72 hours after the induction of differentiation. However, inhibition of p38MAPK with

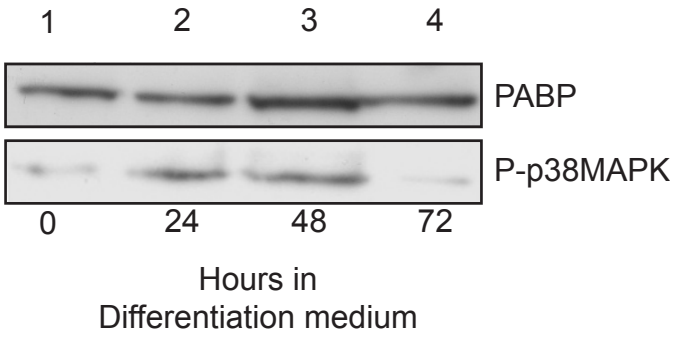


Figure 4.4. p38 MAPK is phosphorylated during myogenic differentiation. C2C12 cells were induced to differentiate as described in Materials and Methods Section 2.2.3. The cells were then harvested at daily intervals using the standard lysis conditions as described in Materials and Methods Section 2.3.1. Aliquots of extract containing 10µg of protein were resolved by a 12% SDS PAGE and subjected to Western blotting with the indicated antibodies.

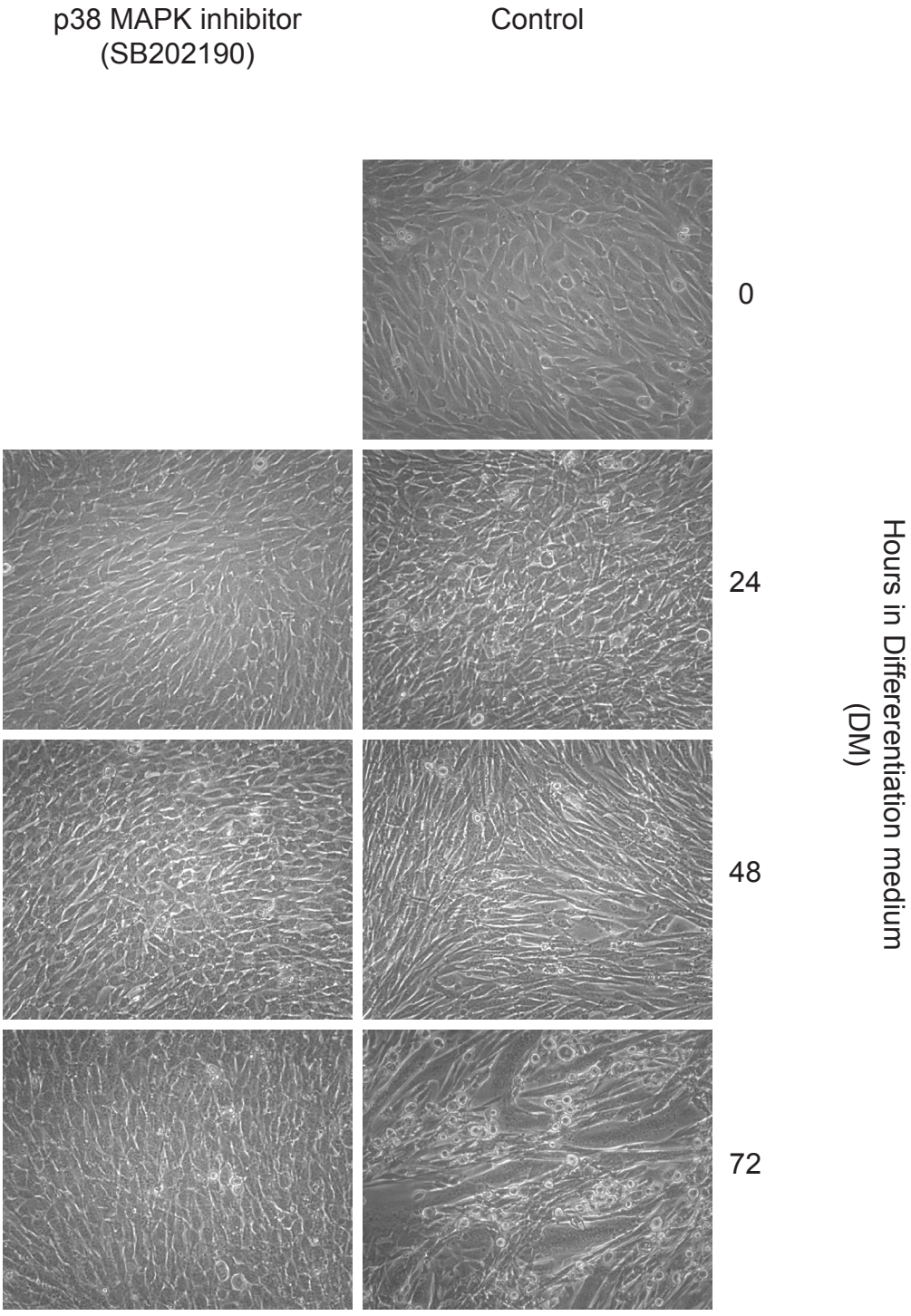


Figure 4.5. p38 MAPK inhibition by SB202190 inhibits the progression of myogenic differentiation. C2C12 cells were induced to differentiate in duplicate as described in the Materials and Methods Section 2.2.3. SB202190 (p38 MAPK inhibitor) was added to one half of the plates at 20μM final concentration as described in Materials and Methods Section 2.2.6. The cells were then imaged at daily intervals using a Moticam 2000 2.0M pixel USB 2.0 attached to a light microscope as described in Materials and Methods Section 2.5.3.

SB202190 did not allow cells to align or fuse into multi-nucleated myotubes, indicating that p38MAPK activity is required for efficient myogenic differentiation. Similar effects have been seen by other groups utilising the silencing of p38MAPK isoforms (Wang et al, 2008).

4.5 Does inhibition of p38MAPK affect RBM4 localisation?

As RBM4 localisation in HeLa cells has been shown to be regulated in part by p38MAPK activity (Lin & Tarn, 2009), I have investigated the localisation of RBM4 in C2C12 cells in the presence or absence of p38MAPK activity. C2C12 cells were grown to confluency and induced to differentiate for up to 72 hours in the absence or presence of SB202190. Cells were then fixed, permeabilised and the localisation of actin (for the cytoskeleton) and RBM4 visualised by confocal microscopy; nuclei were stained with DAPI. As can be seen in Figure 4.6, and in agreement with the data presented above, in undifferentiated cells RBM4 was distributed throughout the cell but more visible in the cytoplasm. The observed ratio between nuclear and cytoplasmic RBM4 levels appeared to be higher in these cells when compared with those shown in Figure 4.1. At 72 hours following the induction of differentiation with myotubes, RBM4 levels in the nucleus appeared to have decreased significantly and the levels in the cytoplasm increased dramatically. When cells were incubated in the presence of SB202190, actin staining showed that the cell shape had changed dramatically (the cells had lined up and become elongated compared to 0 hours) and RBM4 was predominantly cytoplasmic, with what appears to be slightly lower amount of RBM4 protein in the nucleus relative to undifferentiated cells. However, these data do not provide a conclusive result as to the role of p38MAPK in the localisation of RBM4 to the

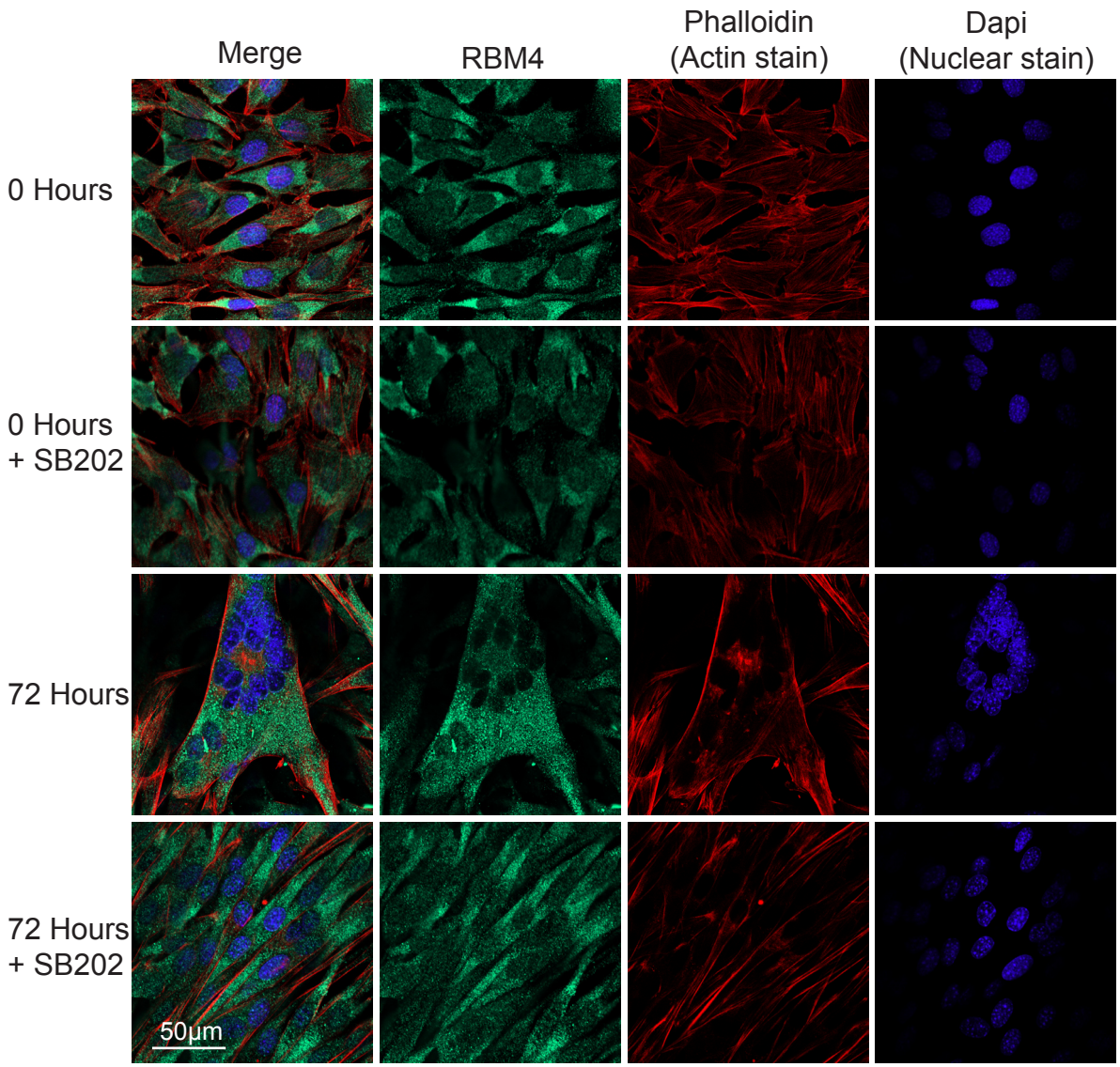


Figure 4.6. Does p38 MAPK inhibition effect the localisation of RBM4 during myogenic differentiation? C2C12 cells were induced to differentiate in duplicate as described in the Materials and Methods Section 2.2.3. SB202190 (shown above as SB202) was added to one half of the plates at 20µM as described in Figure 4.5. The cells were fixed, permeabilised and analysed using immuno fluorescence with antibodies and stains indicated and as described in Materials and Methods Section 2.5.1.

cytoplasm as SB202190 blocked both p38MAPK and differentiation. A different approach could be performed to see if p38MAPK affects RBM4 localisation during differentiation conducted by allowing cells to differentiate and an hour before harvesting, treating cells with p38MAPK inhibitor to see if this affects the acute localisation of RBM4 without interfering with the stage in differentiation reached. Unfortunately, time constraints did not allow me to carry out this experiment.

4.6 Optimisation of RBM4 over-expression in HeLa cells

As I wanted to investigate the role for phosphorylation in the localisation of RBM4 or its association with other cellular proteins, I obtained from the Tarn group (Lin & Tarn, 2009) a cDNA encoding wild-type (WT) human RBM4 and another with the phosphorylation site (Ser309) on human RBM4 mutated to alanine (MT). Initially, I optimised the transfection conditions for these constructs using HeLa cells. Figure 4.7 shows the effect on RBM4 protein expression following transfection of cells with two different amounts of WT or MT (S309A) cDNA encoding RBM4; 1µg was used in lanes 3,F 5, 6 and 7 and 2µg of the applicable construct used in lanes 4 and 7. Alongside the variations in construct amount, the Fugene transfection reagent levels were varied between 6µl (lanes 3 and 6) or 3µl (lanes 4, 5, 7 and 8). The transfected cells were then harvested after 24 hours and RBM4 levels visualised by Western blotting. Figure 4.7 shows that optimal WT RBM4 expression was obtained with 3µl Fugene and 1µg WT cDNA whilst MT RBM4 required 6µl Fugene and 1µg RBM4 MT cDNA. Another experiment was conducted to determine that the optimal time for RBM4 expression after transfection was 24 hours for both WT and MT RMB4 (data not shown).

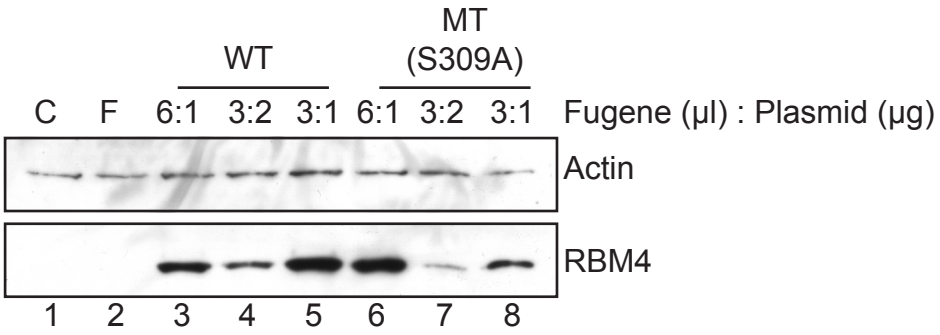


Figure 4.7. Optimisation of over-expression of RBM4 protein in HeLa cells. Hela cells were plated out at 60,000 cells per 6cm plate, allowed to proliferate for 1 day and then transfected with RBM4 WT or RBM4 MT (S309A) constructs as described in Materials and Method Section 2.2.4 using the quantities of Fugene (µl) and plasmid (µg) indicated. C contains no fugene and F contains 6µl of fugene. Cells were harvested after 24 hours using standard lysis conditions and aliquots containing 10µg of protein were resolved using SDS-PAGE and visualised by Western blotting using the antibodies shown as described in Materials and Methods section 2.4.

4.7 Over-expression of WT and S309A MT RBM4 in HeLa cells

To investigate if the phosphorylation of RBM4 affected its incorporation into initiation factor complexes, RBM4 WT and S309A MT were over-expressed in HeLa cells using the conditions determined above. Cell extracts were then prepared and the ability of the two forms of RBM4 to bind to eIF4E and associated proteins was determined using m^7GTP-Sepharose affinity chromatography. This resin binds eIF4E directly and any associated proteins can be recovered by limited washing of the resin and visualisation by Western blotting; a resin lacking the m^7GTP moiety was used as a control for non-specific binding to the scaffold resin. As shown in Figure 4.8, even with limited washing of the resin, eIF4E was recovered to a greater extent in tubes that contained m^7GTP-Sepharose than those with the 4B-Sepharose control resin (lanes 1, 3 and 5 vs lanes 2, 4 and 6). RBM4 was co-recovered with eIF4E (lanes 3 and 5), but not with the control resin even though lower levels of eIF4E were present (lanes 4 and 6); RBM4 protein was not recovered in the absence of transfection (lanes 1 and 2). These data show that the over-expressed RBM4 was not binding the Sepharose resin directly. Therefore, it appears that when RBM4 is expressed in HeLa cells, the protein can be incorporated into the eIF4F complex. It is unlikely to bind to eIF4E directly as previous work has shown eIF4E and RBM4s interaction to be sensitive to RNAse treatment (Lin et al, 2007). These data are in agreement with the findings of (Lin & Tarn, 2009). Furthermore, these data also indicates that mutation of Ser309 to alanine (and potentially phosphorylation at this site) does not influence the ability of RBM4 to associate with eIF4F. However, this does not mean that the phosphorylation site is not involved in regulating

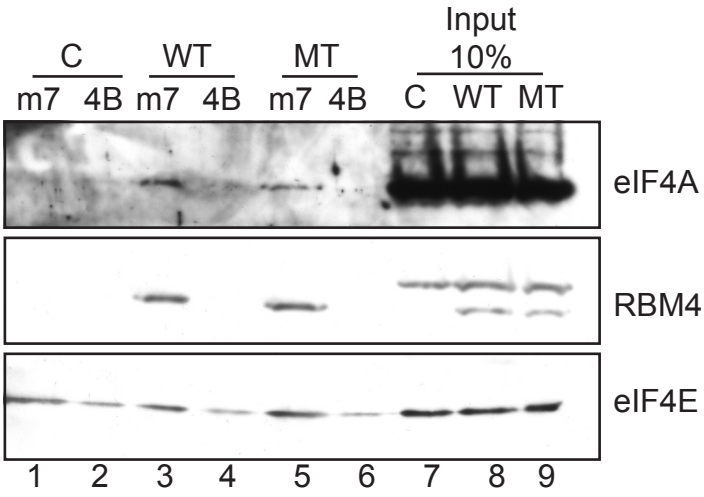


Figure 4.8. Does RBM4 associate with eIF4F when over-expressed in HeLa cells? HeLa cells were plated out at 60,000 cells per 6cm plate allowed to proliferate for 1 day and then transfected with RBM4 WT or RBM4 MT (S309A) cDNA (3µl of fugene and 1µg of RBM4 WT plasmid; 6µl fugene and 1µg of RBM4 MT plasmid). The transfected cells were then left for 1 day before the cells were harvested by the standard lysis conditions. The extracts were subjected to m^7GTP-Sepharose affinity chromatography as described in Materials and Methods Section 2.10.2. Each fraction was then resolved by 12% SDS PAGEand subjected to Western blotting with the indicated antibodies as described in the Materials and Methods Section 2.4. M7= m^7GTP-Sepharose beads; 4B = 4B-Sepharose beads (control); WT = RBM4 wild type transfected cells; MT= RBM4 mutant (S309A) transfected cells; C=untransfected cells

binding partners because the Ser309 may not be phosphorylated under these conditions. Further experiments would be needed to determine if the phosphorylation site is required. This would require a new mutation to be created which mimics phosphorylation at Ser309; mutating the serine into aspartic acid or glutamic acid which act as a phosphomimetics would be the approach to take.

4.8 The effect of RBM4 WT and MT (S309A) on translation rates in HeLa cells

As I have shown that RBM4 can interact with initiation factors, it is possible these interactions cause a global effect on translation rates in transfected cells. To investigate this possibility, HeLa cells were transfected in duplicate as described above with cDNAs encoding WT or MT (S309A) RBM4 alongside untransfected cells (C) or those exposed only to Fugene (F). After 23 hours, cells were pulse-labelled with [^{35}S] methinone for 1 hour and cell extracts were prepared. The level of RBM4 protein expression in these HeLa cells was visualised by Western blotting (Figure 4.9A). These data show that the level of WT RBM4 expression in these cells was relatively equal when the duplicates where compared (lanes 3 and 6). However, MT (S309A) expression was significantly lower in both MT (S309A) cases (lanes 4 and 5). As shown in Figure 4.9B, these cell extracts were also used to analyse the incorporation of [^{35}S] methionine into protein which is indicator of global translation (see Materials and Methods). The rate of protein synthesis was determined as cpm/µg protein and the rate in untransfected cells was set to 100%. These data show that Fugene alone causes a slight, but not significant inhibition of protein synthesis. When RBM4 WT was expressed, WT1 (Figure

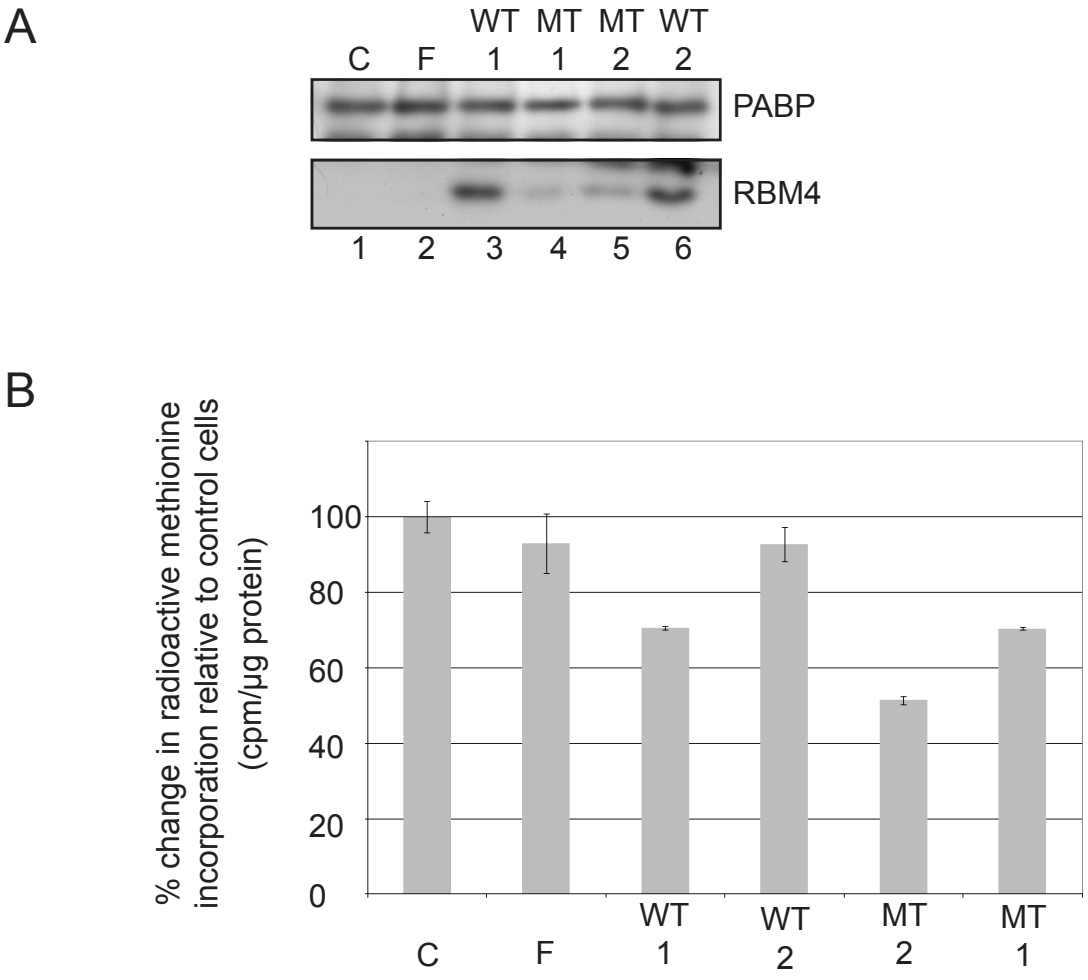


Figure 4.9. Does over expression of RBM4 have an effect on global translation in HeLa Cells? HeLa cells were transfected with cDNA encoding RBM4 WT and MT as described in Figure 4.8. After the 23 hour incubation [^{35}S] methionine was introduced to the medium a for 1 hour before harvesting using the standard lysis conditions. Panel A. Aliquots cointaining 10µg of each fraction was resolved by a 12% SDS page and subjected to Western blotting with the indicated antibodies as described in the Materials and Methods section 2.4. Panel B. The methionine incorporation into proteins was analysed as described in Materials and Methods Section 2.11 and the protein concentration was also measured (section 2.3.2) to allow methionine incorporation to be expressed as cpm/µg protein incorporation of radioactive methionine into protein at time 0 was set at 100%.

4.9A, lane 3) shows a significant drop in methionine incorporation into protein. However, WT2, which showed a similar level of RBM4 expression to WT1 (Figure 4.9A, lane 6), showed no difference in protein synthesis rates when compared to Fugene alone. MT2 (S309A) and MT1 (S309A) both show significant decrease in methionine incorporation into protein when compared to Fugene alone even though overall RBM4 protein expression is less than seen with WT RBM4. These data indicate that RBM4 may modulate general protein translation to a small degree and that the unphosphorylatable mutant has a greater effect on translation rates. This observation fits with the findings of (Lin & Tarn, 2009) who have proposed that unphosphorylated RBM4 binds to mRNA and inhibits its translation. One limitation of this experiment is that the RBM4 is transfected into a cell that does not contain a measurable level of RBM4 and so this response may not be physiological.

4.9 The localisation of over-expressed RBM4 WT and MT(S309A) in C2C12 myoblasts

The results presented above show that RBM4 has a predominantly cytoplasmic localisation during differentiation. My previous experiments to determine if the cytoplasmic localisation in C2C12 cells, required phosphorylation at Ser309 had limitations and was inconclusive. So another experiment was devised to address this question, where RBM4 WT and MT (S309A) protein was over-expressed in C2C12 cells. Attempts to generate plasmids encoding the murine forms of these proteins were unsuccessful so I had to use the human cDNAs described above. Initial experiments determined that a low transfection efficiency in C2C12 cells hampered an investigation using cell fractionation techniques (data not shown). However, I was able to

use cells transiently over-expressing RBM4 in conjunction with confocal microscopy from the pool of mixed cells. Figure 4.10 shows that both over-expressed WT RBM4 and MT (S309A) RBM4 had a nuclear localisation in sub-confluent myoblasts. To determine if RBM4 phosphorylation at Ser309 was required for the localisation of RBM4 to the nucleus in C2C12 myoblasts, the cells were treated with arsenite for 30 minutes at a level that had been titrated not to cause immediate apoptosis. Arsenite was used at this level as it activates the p38MAPK pathway which can then phosphorylate RBM4 on Ser309. These data show that following p38MAPK activation, WT RBM4 re-localised from the nucleus to granules in the cytoplasm. Whereas the MT (S309A) RBM4 also re-localised to the cytoplasm, it was not associated with distinct granules. These finding disagree with previous work from (Lin et al, 2007); the most likely sources of this difference the cell type used as the arsenite concentration was similar and the constructs for the WT and MT (S309A) RBM4 were obtained from Tarn (Lin et al, 2007). To allow for a greater understanding, SB202190 should have been used to determine if the arsenite was affecting the RBM4 localisation *via* p38MAPK signalling. Unfortunately, time constraints did not allow me to carry out this experiment.

4.10 Discussion

This experiments described in this chapter have shown that RBM4 is predominantly cytoplasmic in its cellular localisation and that the cytoplasmic level of RBM4 increases as differentiation progresses. A pool of RBM4 was found to be nuclear but this level only increased a small amount during myogenic differentiation. One interpretation of these data is that a level of

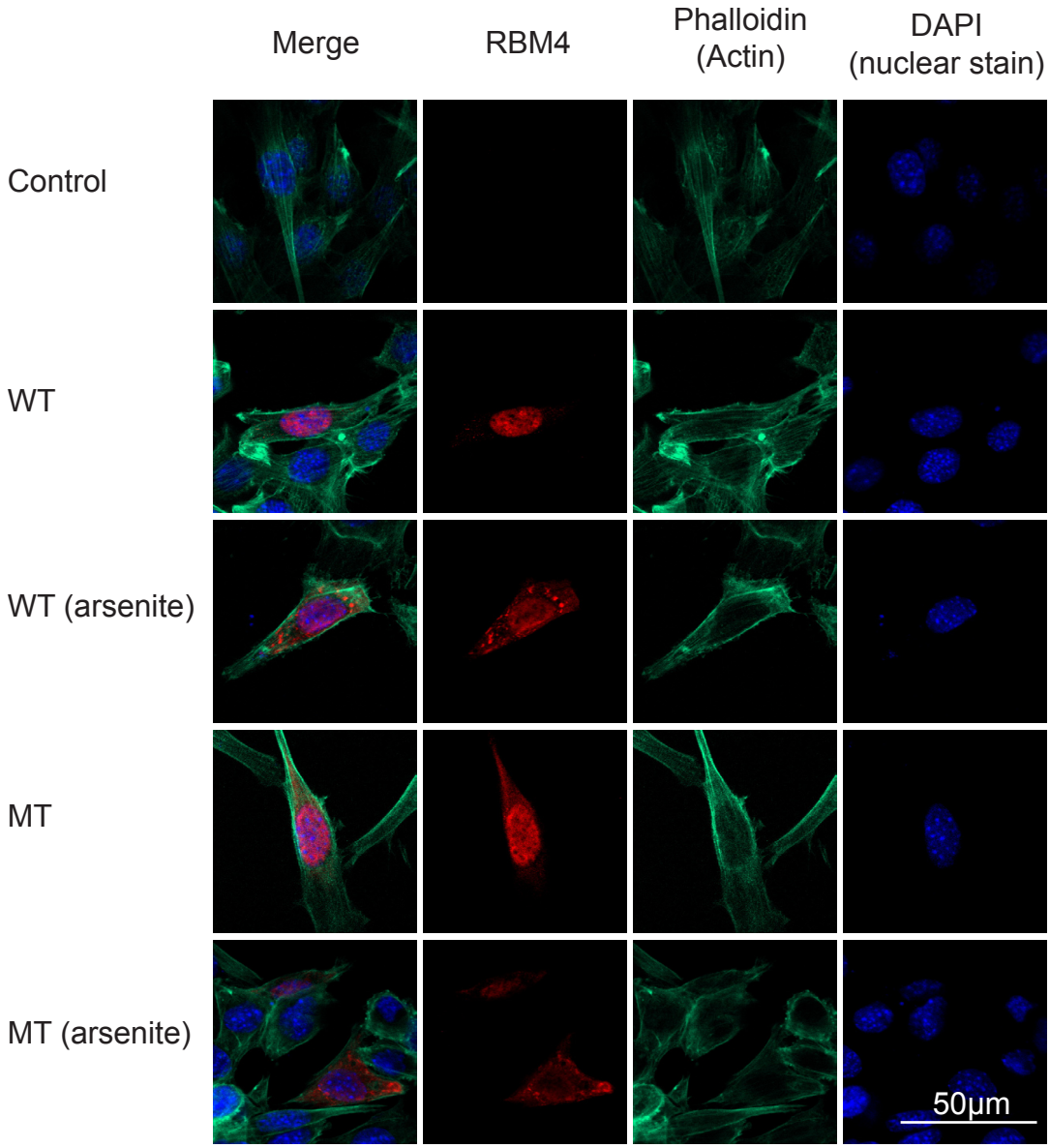


Figure 4.10. Does the RBM4 MT (S309A) protein localise differently to the wild type RBM4 in C2C12 cells? C2C12 cells were transfected in duplicate with plasmids encoding RBM4 WT and RBM4 MT (S309A) as described in Figure 4.8. The cells were incubated for 24 hours and then half of the plates were incubated with 0.5mM arsenite for 30 mins as described in Materials and Methods Section 2.2.7. These cells were then analysed by immunofluorescence using the indicated antibodies and stains as described in Figure 4.1.

RBM4 is required in the nucleus to promote pre-mRNA splicing and the demand for this protein does not change dramatically during differentiation. Interestingly, RBM4-mediated splicing of pre-mRNA regulates PTB pre-mRNA splicing in a way which reduces the expression of PTB protein. PTB has been shown to act antagonistically with RBM4 in controlling splicing, and the ratio of PTB to RBM4 has been found to be important in controlling such events (Lin & Tarn, 2011b). Therefore, even though RBM4 protein levels in the nucleus do not increase dramatically, it has been shown that the PTB protein levels actually decrease, altering the RBM4:PTB ratio and allowing the RBM4 to have greater effect on splicing reactions, as they share binding sites and have opposite effects on splicing to each other .

RBM4 has also been shown to have its localisation regulated by p38MAPK signalling (Wang et al, 2008) a process which has been shown to be required for differentiation. Investigating the role of p38MAPK signalling on RBM4 during differentiation has proven to be problematic. Inhibition of p38MAPK inhibited differentiation which changes so many variables such that determining if p38MAPK had a direct effect on RBM4 by this approach was impossible. Over-expression of RBM4 WT and the form where the Ser309 site was mutated to alanine (MT) was at too low transfection efficiency to allow for biochemical analysis, or for enough cells to be obtained at confluency to allow for differentiation experiments to be conducted on a large scale. Stable transfection of the C2C12 cells with vectors encoding RBM4 had the problem of rejection of the RBM4-encoding sequence whilst the cells maintained resistance to the selective agent without RBM4 expression (data not shown). Transient expression studies in conjuction with arsenite treatment to activate

p38MAPK cast doubts upon the role for p38MAPK signalling causing the re-localisation of RBM4 from the nucleus to the cytoplasm via phosphorylation of Ser309 in C2C12 cells. MT (S309A) RBM4 still re-localised from the nucleus; however it did not localise to granules as seen with the wild-type protein. The work in the next chapter describes an investigation into cytoplasmic proteins which interact with RBM4.

-Chapter 5-

Investigation of the putative interaction of RBM4 with translation initiation factors during myogenic differentiation

5.1 Introduction

When over-expressed in HeLa cells, RBM4 has previously been shown to interact with the translation initiation factors eIF4A and eIF4G via co-immuno precipitation (Lin et al, 2007) The way RBM4 is thought to interact with factors which promote translation initiation depends on the state of the cells. In unstressed cells, over-expressed RBM4 binds CU rich elements in target mRNAs and inhibits their translation by an unknown mechanism. This could potentially be regulated *via* the interaction of RBM4 with eIF4A (Lin et al, 2007). When HeLa cells are stressed with arsenite, over-expressed RBM4 is phosphorylated, interacts with eIF4A and eIF4G and recruits them to transfected EMCV IRES structures (Lin et al, 2007). However, at this time, it is not known whether RBM4 interacts with initiation factors during the process of myogenic differentiation. The work described in this chapter will address this using a variety of methods including: immunoprecipitation (IP) of endogenous protein; introduction and IP of recombinant proteins with endogenous proteins; and *in vitro* binding assays using expressed proteins.

5.2 eIF4A and eIF4G are co-isolated with RBM4 during differentiation

To investigate the potential binding of RBM4 to eIF4G and eIF4A in differentiating cells a co-IP was performed (Figure 5.1). The RBM4 recovered by the IP during differentiation matches the level of total RBM4 protein expression (as shown in Figure 3.4). However, the lanes showing RBM4 protein levels in 5% of the input into the IP show no apparent RBM4 protein at any time after the induction of myogenic differentiation. This is most likely

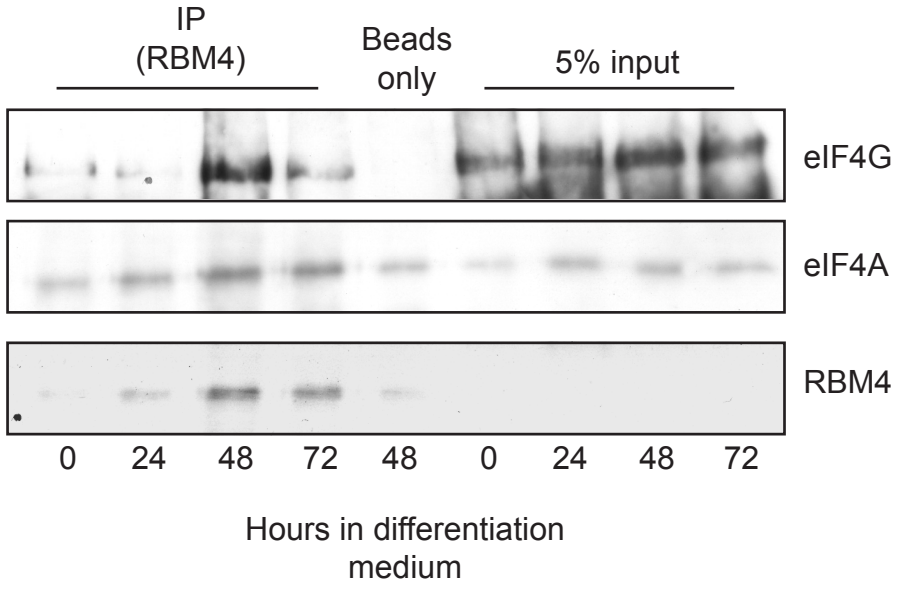


Figure 5.1 RBM4 forms complexes with eIF4A and eIF4G during myogenic differentiation. C2C12 cells were grown to confluency and induced to differentiate as described in Materials and Methods Section 2.2.3. The cells were then harvested at the time points shown above. Aliquots of extracts containing 100µg protein were used for immunoprecipitation (IP) of RBM4 using 5µg of a pan RBM4 antibody, as described in the Materials and Methods Section 2.10.1. The co-isolated proteins were visualised using Western blotting as described in Materials and Methods Section 2.4 with antibodies raised against eIF4A and eIF4G alongside the antibody used for the IP to show recovery of RBM4. Beads only shows IP using extract prepared at 48 hours but in the absence of RBM4 antibody. This figure is representative of results obtained in two separate experiments.

because the RBM4 protein expression level was too low for detection with the 5µg of total protein resolved on the gel. eIF4A is observable at a similar level in the 5% input at all time points. In contrast, the eIF4A co-recovered with the RBM4 increases in line with the level of recovery of RBM4, being maximal at 48 hours of myogenic differentiation. This could possibly indicate that RBM4 interacts with eIF4A in a dose-dependent manner. eIF4G shows a large increase at 48 hours, this then reduces to a significantly lower level at 72 hours whilst the total levels of eIF4G in the extracts does not change significantly. This rapid change in recovery of eIF4G which does not match the change in RBM4 protein level indicates that there could be a regulatory system affecting the interaction of RBM4 and eIF4G during differentiation.

To further investigate the interaction of RBM4 with eIF4A and eIF4G, the dependency on RNA for their interaction with RBM4 was tested. Figure 5.2 shows that treatment of a cell extract derived from cells induced to differentiate for 48 hours with an excess of RNAse A and T1 prior to IP did not affect the co-IP of eIF4A or eIF4G with RBM4. The eIF4G signal appears to be weaker in both RNAse-treated and control lanes when compared to that observed in Figure 5.1. Unfortunately, this was not taken further due to a shortage of the commercial antibody used for the study shown in Figure 5.1; subsequent batches of anti-RBM4 serum were not of high enough titre for immunoprecipitation. What these data indicate is that eIF4A interaction with RBM4 is potentially RNA-independent, but the experiment would need to be repeated to clarify if this is the case.

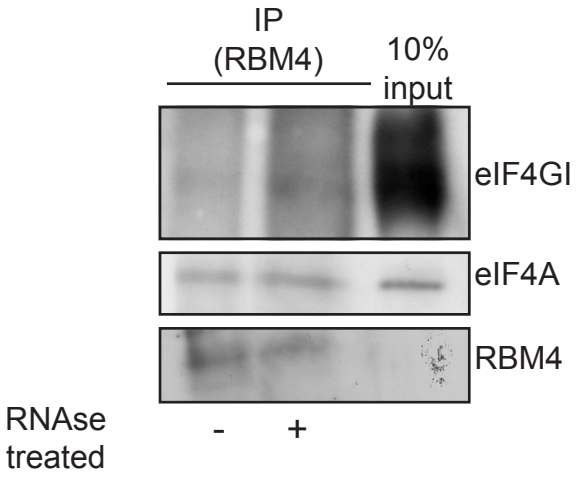


Figure 5.2 Co-isolation of RBM4 with eIF4A is resistant to RNAse treatment. C2C12 cells were grown to confluency, differentiated, then harvested at time points shown, as described in Figure 5.1. The extract was split in two, one half treated with excess RNAse A and T1 as described in Materials and Methods Section 2.10.3. IP of the extracts was performed using a pan RBM4 antibody as described in the Materials and Methods Section 2.10.2. The co-isolated proteins were visualised using SDS-PAGE and Western blotting (Section 2.4) with antibodies raised against eIF4A and eIF4G alongside the antibody used for the IP to show recovery of RBM4.

5.3 Expression and Purification of RBM4a for *in vitro* assays

To further investigate the interactions of eIF4G and eIF4A with RBM4, recombinant RBM4a was produced in bacteria to determine if eIF4A and eIF4G bind directly to RBM4a. A vector to allow for the recombinant expression of His-tagged RBM4a was obtained from Tarn (Lin et al, 2007). The vector was transformed into *E.Coli* and colonies were picked, grown up and induced. Cells were harvested from the cultures at 0, 1 and 2 hours and analysed by SDS-PAGE with a pan anti-RBM4 antibody. Figure 5.3 shows that the highest level of RBM4a expression was observed in Colony I (lanes 1,4 and 7) and the optimal induction time here was 1 hour (lane 4). There was some leaky expression of RBM4a seen without IPTG induction especially in Colonies I and III (lanes 1 and 3). The selected colony was then grown on a larger scale and expression of RBM4a induced with IPTG for 1 hour and cell extract prepared as described in Section 2.9.4. The cell extract was then subjected to Nickel Agarose affinity chromatography to isolated the His-tagged RBM4a following elution from the resin with imidazole. As shown in Figure 5.4, relative to the unbound material (FT, lane 1), elution from the resin resulted in the purification of recombinant RBM4a (lanes 2-6). Most of the non-specific bands have been removed except for a few faint bands observed in lanes 2 and 3. However, a proportion of the His-tagged RBM4a did not bind to the resin; either the tag was obscured in some way by the folding of the RBM4a protein or else interacting proteins prevented RBM4a binding to the resin. In an attempt to increase the yield of RBM4, I investigated whether RBM4 was being expressed and moved into insoluble inclusion bodies and therefore being discarded with the pellet during the lysis step. To recover the proteins in

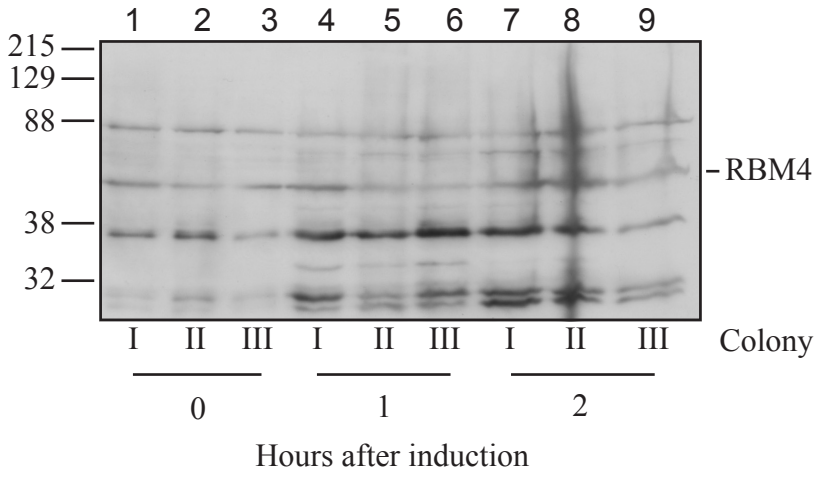


Figure 5.3 Colony selection for RBM4a expression. BL21 *E.Coli* were transformed with plasmids encoding RBM4a WT as described in Materials and Methods Section 2.9.1 and 3 colonies (CI-III) were picked. The cells were grown up as described in Section 2.9.3. The cells were then induced to express RBM4 protein by addition of IPTG for 0, 1 or 2 hours as described Section 2.9.3 and 1ml of cell culture removed, pelleted and lysed in 1ml of SDS-PAGE sample buffer. Aliquots containing 10µl of sample were analysed resolved on a 12% SDS-PAGE gel and analysed by Western blot with pan RBM4 antibody.

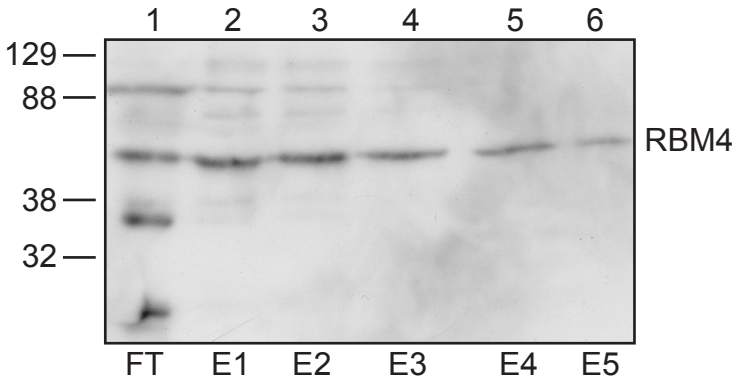


Figure 5.4. Nickel Agarose purification of RBM4a WT. A selected colony of BL21 *E.Coli* cells containing RBM4 WT expression plasmids (C-I as described in Figure 5.3) was amplified, and induced to express RBM4 protein by the addition of IPTG for 1 hour. Cells were lysed and RBM4 purified using Nickel Agarose, as described in Section 2.9.4. Samples of 10µl were resolved by SDS-PAGE and analysed by Western blot with a pan anti-RBM4 antibody.

the inclusion bodies guanidine hydrochloride was used. Figure 5.5A shows a flow diagram of 3 different methods employed for lysing the cells, resulting in Fraction GL (cells lysed in guanidine hydrochloride), Fraction GP (guanidine hydrochloride lysed pellet) and Fraction N (cells lysed by the original method). In each instance, RBM4a expressed in these samples was purified using Nickel Agarose and 5µg of recovered protein resolved by SDS-PAGE and Western blotting. As shown in Figure 5.5B, the greatest amount of RBM4a protein was recovered from the normal lysis conditions (lane 3) and very little RBM4a was recovered in the cell pellet recovered with guanidine lysis (lane 2). The RBM4a recovered from Fractions GL (lane 1) and GP (lane 2) had a higher amount of non-specific bands post Nickel Agarose purification (data not shown). Therefore, all of the elutions shown in Figure 5.4, lanes 2-6 were pooled for further use.

Analysis of this pooled material by SDS-PAGE and coomassie staining showed the presence of a number of non-specific bands not observed with Western blotting (Figure 5.6B, lane 1). To remove as many of these bands as possible before using the RBM4a for interaction studies, I tried to further purify RBM4a by using anion exchange chromatography with FFQ resin. This was used as different proteins have varying affinity for the resin as determined by their overall charge and the localisation of the charge on the protein. As can be seen in Figure 5.6A, when analysed by Western blotting, RBM4a binds the FFQ beads and can be eluted from the resin using NaCl. However, Figure 5.6B shows by coomassie staining that the overall protein purity of the sample remains low as another protein with a higher molecular weight makes up a large percentage of the protein in the sample.

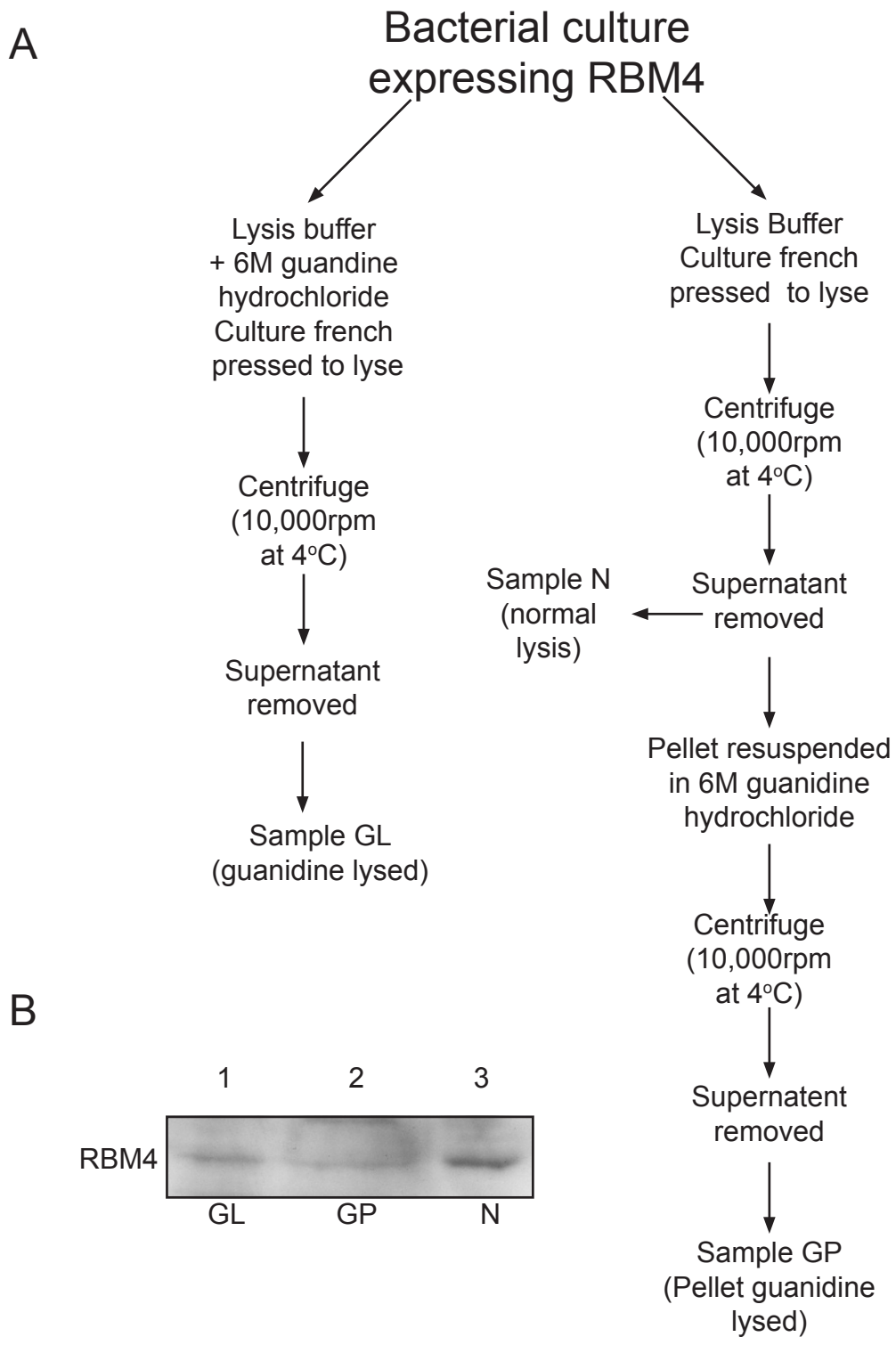


Figure 5.5 Optimisation of lysis conditions for recovery of RBM4 protein from *E.Coli*. BL21 *E.Coli* cells containing RBM4 WT expression plasmids were amplified as described in Figure 5.4. The cells were then lysed and purified as described in Section 2.9.4 with the alterations as shown in Panel A and 10µl of each sample was resolved by 12% SDS PAGE and visualized by Western blotting with pan anti-RBM4 antibody (Panel B).

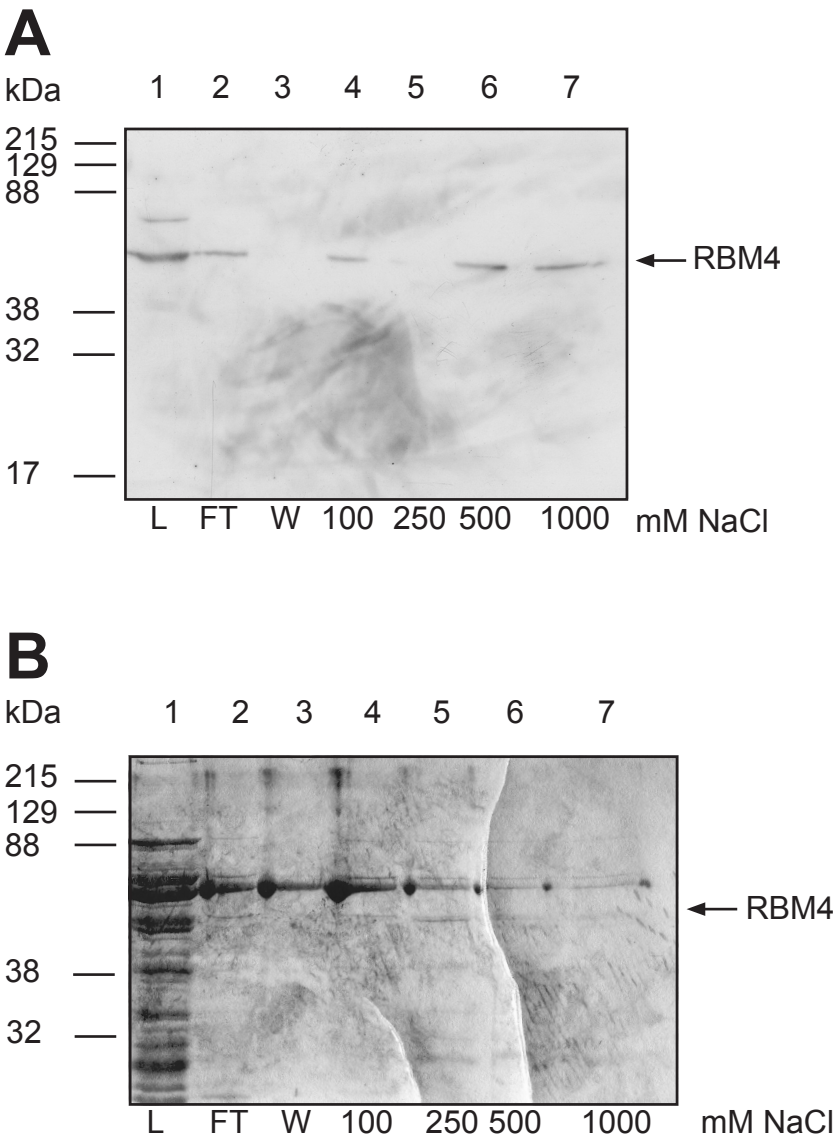


Figure 5.6 Purification of RBM4 by FFQ anion exchange chromatography. RBM4 previously purified by Nickel Agarose affinity chromatography was further purified by FFQ anion exchange chromatography, as described in Materials and Methods Section 2.9.6. Samples (10µl) were resolved by 12% SDS-PAGE and analyzed by Western blotting with pan anti-RBM4 antibody (Panel A) or coomassie staining (Panel B). L= Load; FT=unbound materials; W=Wash.

As the FFQ did not further purify RBM4a to a great extent (Figure 5.6B), another purification method was tried, which was Heparin-Sepharose. Heparin has similar characteristics to the backbone of mRNA and so should purify RBM4a by the RRMs having a high affinity for the heparin. The experiment was conducted as with FFQ but with heparin beads substituting for the FFQ beads. As shown in Figures 5.7A and B, RBM4a binds heparin but there was a significant amount of RBM4a observed in the flow through (lane 2) and to a lesser extent the wash (lane3). Western blotting (Panel A, lanes 5 and 6) and coomassie staining (Panel B, lanes 5 and 6) show that RBM4a is predominantly eluted from the resin at 250 and 500mM NaCl. The overall purity of RBM4a in these fractions was also higher than before as the major impurity that is seen in the FFQ isolation is still present but to a lower level. Therefore, fractions 5 and 6 were pooled and dialysed against buffer containing 100mM NaCl and used for experiments described in the rest of the chapter.

To complement this work, a vector encoding for a mutant form of His-tagged RBM4a was also obtained from Prof. Tarn (Lin et al, 2007) . This mutation (Kar et al, 2006) disrupts the RRMs by mutation of conserved aromatic amino acids in the binding region (Y37A, F39A, Y113A and F115A), thereby preventing mRNA binding. This vector was transformed into *E.Coli* and colonies were picked, grown up and induced, as described above. The His-tagged mutant RMB4a (RRM silenced) was then purified as described above using sequential Nickel agarose and Heparin chromatography steps, with the resultant Mt (RRM silenced) protein showing a similar level of purity to that of the wild-type protein (Figure 5.8).

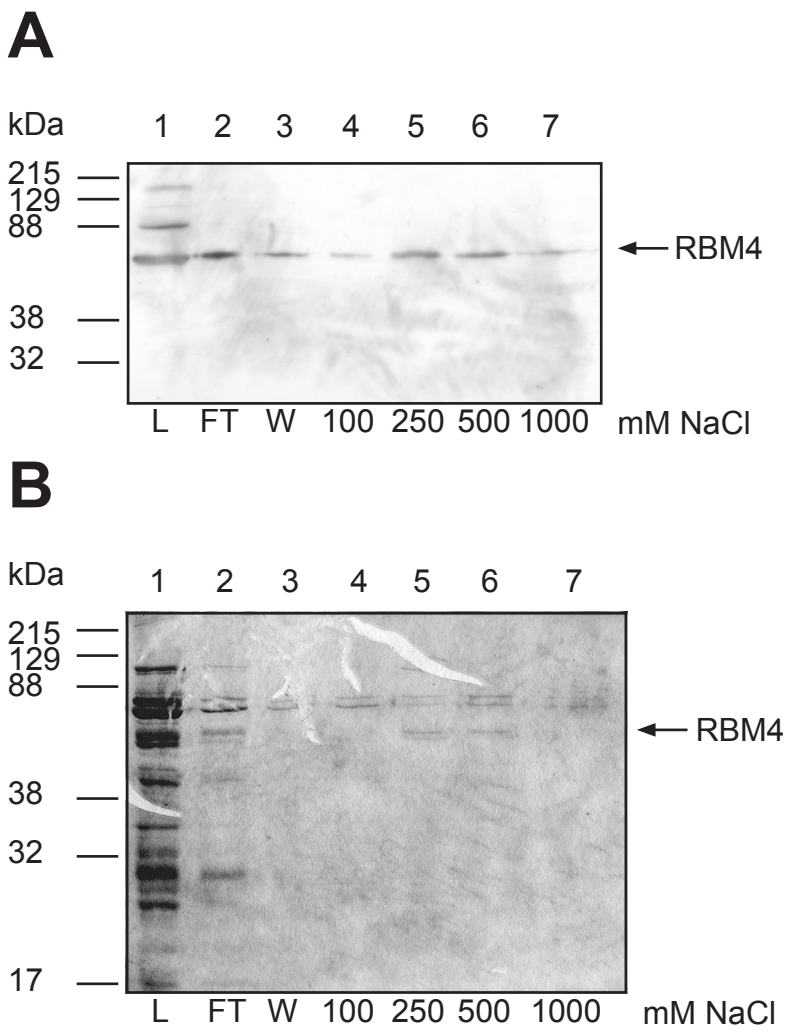


Figure 5.7 Purification of RBM4 by Heparin-Sepharose chromatography. RBM4 previously purified by Nickel Agarose affinity chromatography was further purified by Heparin-Sepharose chromatography, as described in Section 2.9.5. Samples (10µl) were resolved by 12% SDS-PAGE and analyzed by Western blotting with pan anti-RBM4 antibody (Panel A) or coomassie staining (Panel B).

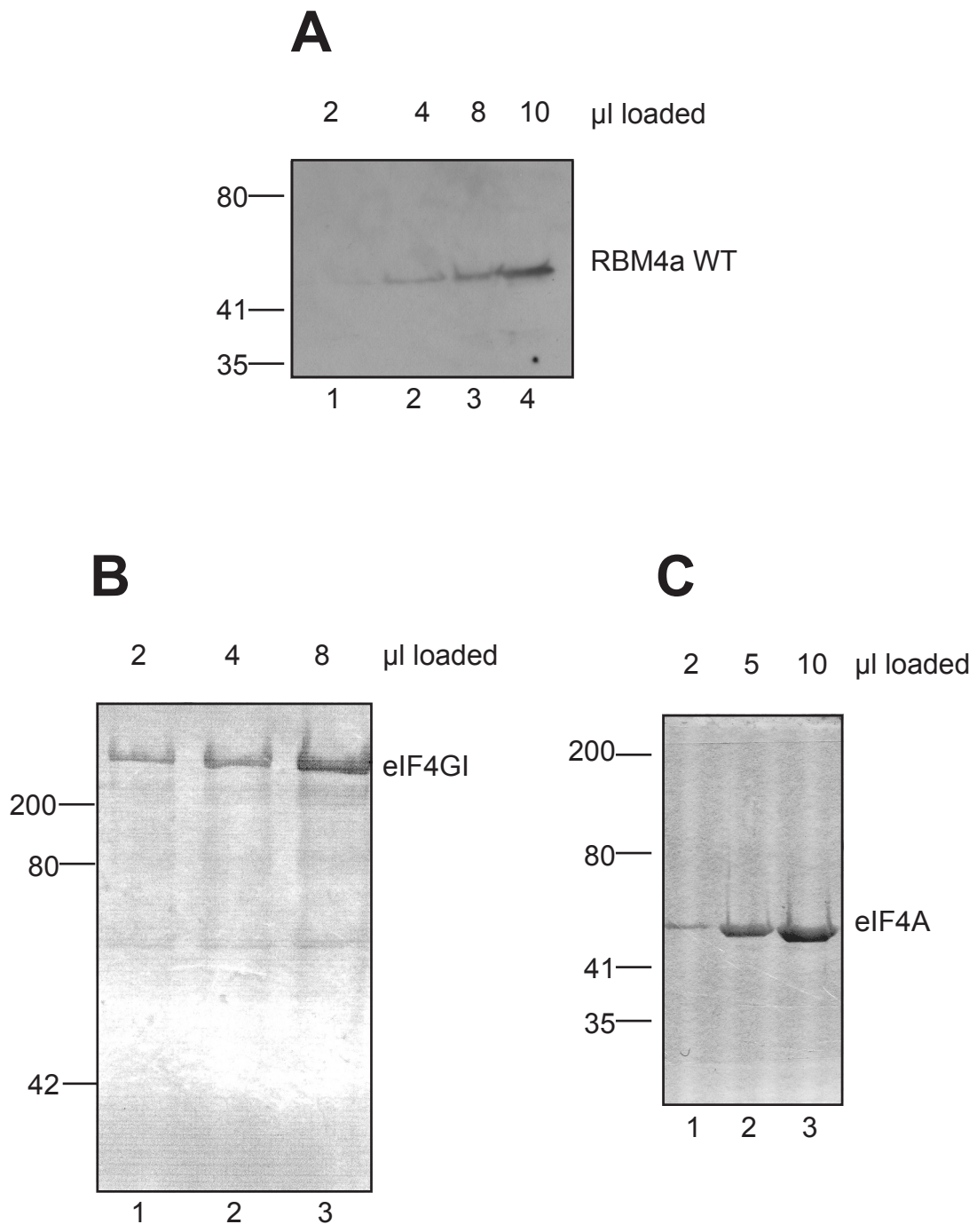


Figure 5.8 SDS-PAGE analysis of recombinant eIF4A, eIF4G and RBM4a WT. Aliquots of recombinant protein were resolved by SDS-PAGE as indicated and RBM4 was analysed by Western blot (Panel A), as described in Section 2.4 using a pan anti-RBM4 antibody. In addition, aliquots of eIF4GI (Panel B) and eIF4A (Panel C) were subjected to SDS-PAGE and analysed by coomassie staining as described in Section 2.12.

5.4 Recombinant RBM4a Wt and Mt (RRM silenced) can bind eIF4A and eIF4G

I have previously shown that RMB4 can interact with eIF4A and eIF4G (Figure 5.1) and it has also been observed to bind these proteins when over-expressed in cells (Lin et al, 2007). To analyse this interaction further, I have added recombinant, His-tagged wild-type (Wt), RRM silenced mutant protein (Mt) and eIF4A (purified as described in Section 2.9.8) into extracts prepared from confluent C2C12 cells (0 hour differentiating cells). Following recovery of the tagged RBM4a protein using Nickel Agarose beads, Western blotting showed that eIF4G could be isolated with RBM4a and eIF4A (Figure 5.9A, lanes 1-3). This interaction was not dependent on intact RRMs in the RBM4a as similar levels of eIF4G were recovered with the wild-type and RRM mutant proteins (compare lane 2 with lane 1). In addition, His-tagged wild-type (Wt) and RRM silenced mutant RBM4a protein were able to interact with eIF4A (Figure 5.9B, lanes 1 and 2). In the absence of added recombinant protein, but in the presence of cell extract (Panels A and B, lane 4), no significant amount of either eIF4G or eIF4A was recovered. In Figure 5.9B, the eIF4A blot has two sets of bands, with the upper band reflecting recombinant eIF4A. The eIF4A recovered in lane 3 was at levels sufficient to allow it to leak across into lane 2. RBM4 was not recovered from the extract using Nickel Agarose beads in the absence of added recombinant protein (Figure 5.9C, lane 2 vs lane1). These data indicate that the eIF4A and eIF4G can bind RBM4 in cell extracts. His-tagged eIF4A added to the cell extract was also able to co-isolate RBM4 (Figure 5.9C).

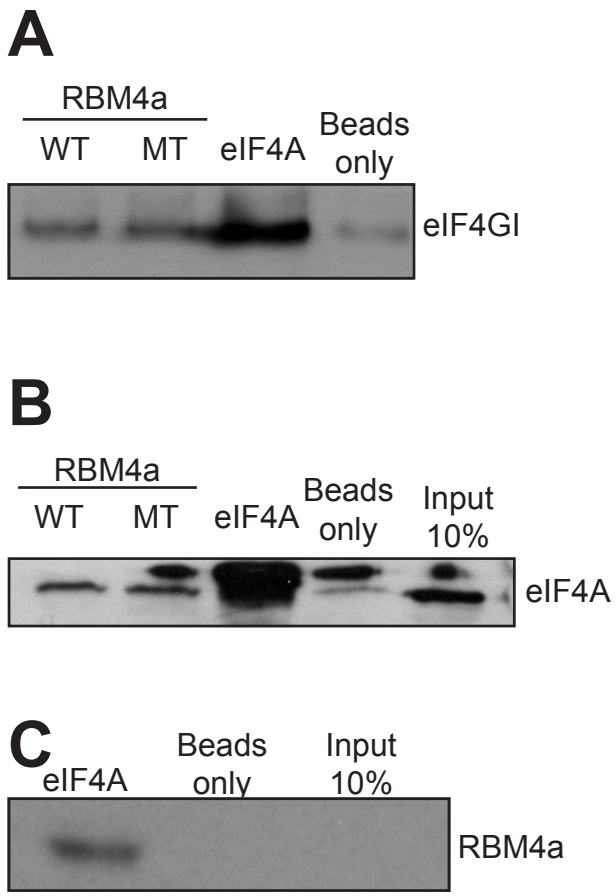


Figure 5.9 Recombinant RBM4 WT and RRM mutant (MT) protein form complexes with eIF4G and eIF4A when introduced into C2C12 cell extracts. Recombinant protein (3µg) was added to 250µg of cell extract from undifferentiated cells and incubated for 1 hr at 4°C, as described in Materials and Methods Section 2.10.5. The His-tagged recombinant protein were recovered by Nickel Agarose affinity chromatography by addition of 50µl of 50% Nickel agarose beads, the beads were washed with interaction buffer eluted using sample buffer. The recovered protein and co-isolated proteins were resolved by 12% SDS-PAGE and analysed by Western blotting with antibodies specific to eIF4GI (Panel A), eIF4A (Panel B) or RBM4a (Panel C). This figure represents data obtained from two separate experiments.

To investigate the binding of RBM4 and eIF4A further, cell extracts from differentiating cells were used in a similar experiment. Cell extracts were prepared from C2C12 cells induced to differentiate for different times (Figure 5.10A) and recombinant eIF4A was introduced into them (lanes 1-4), with a duplicate of each time point without eIF4A acting as controls (lanes 5-8). As before Nickel Agarose beads were used to isolate the His-tagged eIF4A from the extracts and the presence of either RBM4a or RBM4b was visualised by Western blotting. Figure 5.10A shows that in the absence of added His-tagged eIF4A, no recovery of RBM4a and RBM4b was observed (lanes 5-8). With added, tagged protein, both RBM4a and RBM4b were pulled down with eIF4A in cell extracts prepared from differentiating cells, with a peak of interaction observed in extracts from cells induced to differentiate for 24 hours (lane 2). As shown in figure 5.10B and in contrast, little RBM4a was recovered with eIF4A in undifferentiated cells (lane 1). In agreement with previous data (Figure 3.5) this probably reflects in part the low level of RBM4a protein expression at this time. In contrast to RBM4a, levels of RBM4b protein increased during the differentiation time course (Panel B vs Panel C). Both RBM4a and RBM4b where compared to a loading control which was PABP.

These data support the idea that eIF4A and eIF4G can form complexes with RBM4a and it even adds to this by showing that the RRM sites are not required for this interaction. This conclusion is supported by the data shown in Figure 5.2 as RNAse A and T1 treatment had little effect on complex formation. The RRMs could still be required for the formation of different complexes and to allow the RBM4 complexes to interact with mRNA or pre-mRNA. A complication with the data shown in Figure 5.10 is that protein

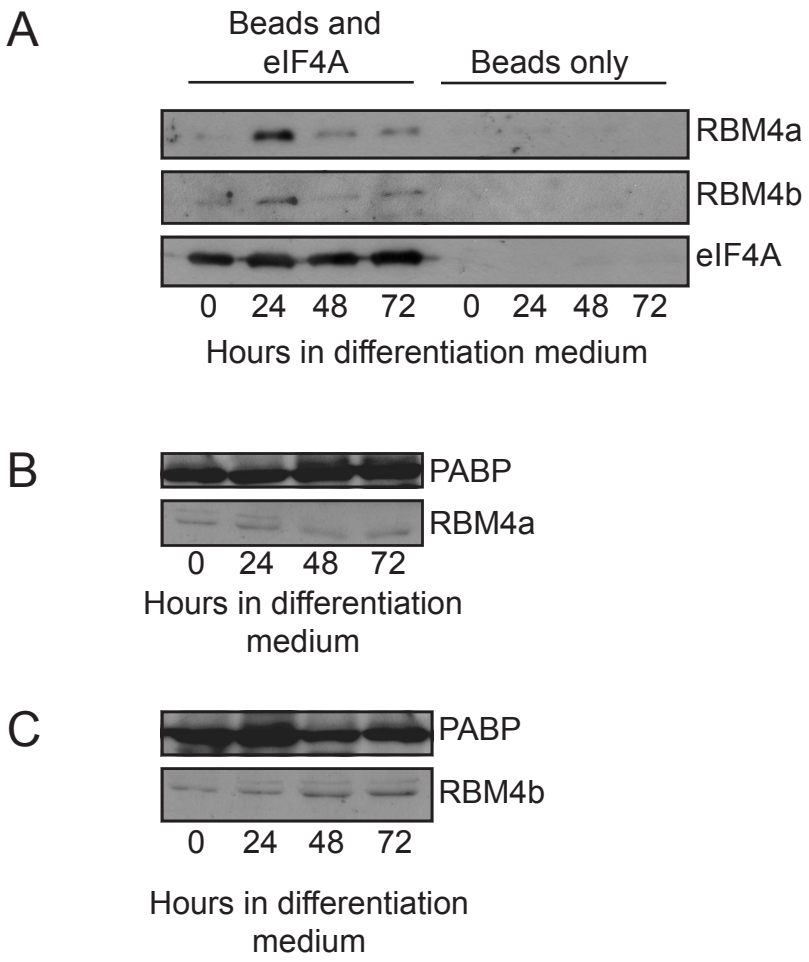


Figure 5.10 Is there a difference in the ability of recombinant eIF4A protein to form complexes with RBM4a and RBM4b when introduced into differentiating C2C12 cell extracts? Panel A Recombinant eIF4A protein was added to cell extracts and incubated as described in Figure 5.9 except that extracts were prepared from cells induced to differentiate for the indicated times. The recombinant protein was recovered by Nickel Agarose affinity chromatography as described in figure 5.9 and recovered protein and co-isolated proteins were resolved by 12% SDS-PAGE and analysed by Western blotting with indicated antibodies. Panels B and C. Aliquots of the total extract containing 10μg of protein were resolved by 12% SDS-PAGE and analysed by Western blot with antibodies indicated. This figure is representative of results obtained in three separate experiments.

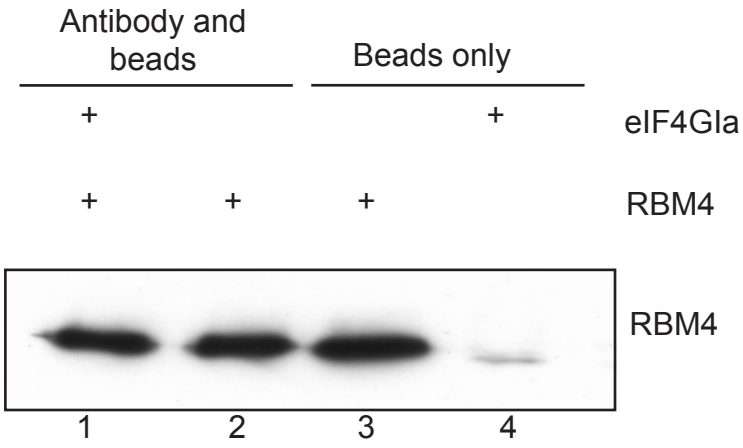


Figure 5.11 Does RBM4 bind directly to eIF4GI? Recombinant RBM4 (3µg) and 3µg of recombinant FLAG tagged-eIF4GIa were combined in 1ml of interaction buffer as described in Materials and Methods Section 2.10.4. Anti-FLAG antibodies were used to perform an IP as described in Section 2.10.4 and the recovered proteins resolved by 12% SDS-PAGE and analyzed by Western blotting with indicated antibodies.

beads or tubes non-specifically. Lane 4 shows eIF4G alone with beads and shows a small amount of RBM4a signal which is likely caused by protein from lane 3 leaking into lane 4.

As Figure 5.11 shows signal in all the control lanes as well as the actual experimental lane, this protocol needed to be optimised. To start with, the beads or tubes were found to be binding RBM4a (Figure 5.11 lane 3). To inhibit the binding of RBM4a to the tube walls siliconised tubes were used in the next experiment and also a greater amount of controls were used to try and determine the source of the background binding. FLAG-eIF4GIa was incubated with RBM4a in IP buffer (containing 100mM NaCl) and resulting complexes isolated and visualised by Western blotting for the presence of RBM4a. Figure 5.12 contains controls to account for binding of RBM4 non-specifically to the anti-FLAG antibody (lane 2), Agarose beads (lane 4) and tube walls (lane 6). Signal for RBM4 was seen in lanes 1, 2 and 4 and a very small amount in lane 6. Therefore, these data show that some of the non-specific binding of RBM4a seen in Figure 5.11 was caused by direct binding to the tubes. The assay still had problems with non-specific binding of RBM4a to the beads and potentially to the anti-FLAG antibodies themselves. This background binding could be caused by inadequate blocking of the beads with other proteins. In all the previous experiments the beads were blocked for 1 hour at 4^{o}C in 1mg/ml cytochrome C in IP buffer therefore, in the following experiment (Figure 3.13) this was increased to 5mg/ml Cytochrome C and the incubation time was increased to overnight to allow the cytochrome C to block the beads. In this experiment, the recovery of FLAG-eIF4GIa was also investigated to ensure that it was actually being pulled down by the anti-FLAG

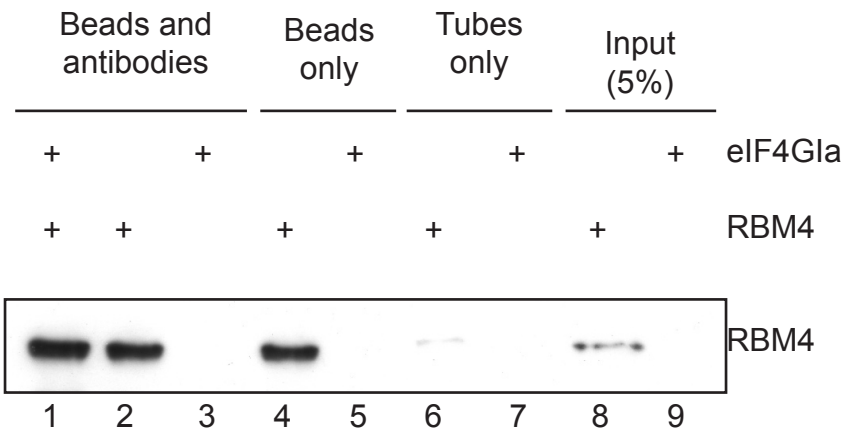


Figure 5.12 Does siliconised tubes decrease non-specific binding of RBM4 to tubes? The experiment described in Figure 5.11 was repeated except with the use of siliconised tubes.

complexes are already formed when the recombinant proteins were added to the cell extracts and this means that the proteins that are co-isolated with the recombinant protein have either come from complexes that have been altered by the addition of the recombinant protein or are part of a free pool of protein. It also appears to indicate that there is a larger pool of free RBM4a and RBM4b at 24 hours (Figure 5.10A) or that the complexes they are part of are more transient. The limitations of the experiments already described is that they only show that RBM4 is part of a complex with eIF4A and eIF4G which is RNAse resistant (potentially in the case of eIF4A and unknown for eIF4G) but it does not allow me to determine if this interaction was direct or indirect. To investigate whether RBM4 binds to either eIF4A or eIF4G directly the recombinant proteins would need to be assayed by an *in vitro* binding assay.

5.5 Optimizing in vitro binding assays for eIF4G and RBM4

To determine if RBM4 and eIF4G directly interact with each other, an *in vitro* binding assay was performed. To this end, FLAG-tagged eIF4GIa (Coldwell et al., 2006) was expressed in insect cells and purified as described in Section 2. 8.7 and figure 5.8B and C. FLAG-eIF4GIa was then incubated with RBM4a in IP buffer (containing 100mM NaCl) and resulting complexes isolated by the use of anti-FLAG antibodies bound to agarose which bind the tag on the N-terminus of the eIF4G. The beads were blocked for 1 hour at 4^{o}C with 1mg/ml cytochrome C in IP buffer prior to use and the presence of recovered RBM4a was visualised by Western blotting. Figure 5.11 shows that RBM4a could be co-isolated with FLAG-eIF4GIa under these conditions (lane 1). However, there is signal for RBM4 in the absence of added FLAG-eIF4GIa (lane 2) or anti-FLAG antibody (lane 3) indicating that RBM4a is binding to the

antibody beads. As shown in Figure 5.13 (lane 1), eIF4GIa was recovered efficiently in this assay compared to resin alone (lane 5). RBM4a was also recovered with eIF4GIa (lane 1). However, the RBM4 signal was actually highest in lane 4 which contained beads and no eIF4GIa. In addition, the recovery of RBM4a with the anti-FLAG antibodies beads in the absence of added FLAG-eIF4GIa (lane 2) was also greater than that seen in lane 1, suggesting the antibodies and eIF4G were actually blocking sites that RBM4 can bind to non-specifically in the assay. The increased levels and time of blocking indicate that cytochrome C had reduced the background in some of the lanes but crucially not in one of the controls.

To try and reduce non-specific binding to the resin, another approach taken was to increase the salt concentration in the binding buffer. However, this can also inhibit the interaction of the proteins with each other so this variable was optimised to try and reduce the background but not prevent protein complex formation. This experiment was carried out and it was determined that the salt concentration could be increased to 200mM NaCl from 100mM NaCl (data not shown). The increased salt concentration was used in the binding assays shown in Figure 5.14. These data show that increased salt reduced the binding of RBM4a to the resin alone (lane 5 vs lane 2). Lane 1 shows that RBM4a can be recovered with FLAG-eIF4GIa; however, there was still substantial binding of RBM4a to the anti-FLAG beads in the absence of added FLAG-eIF4GIa (lane 2) which could account for a large proportion of this binding. As the RBM4a was binding to the anti-FLAG antibodies I looked at potential causes of increased background here. Upon analysis of the RBM4a protein sequence, I found a region that had moderate homology with the

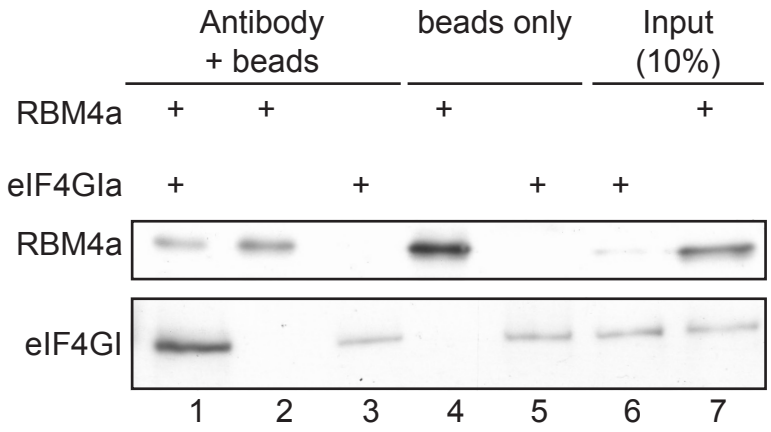


Figure 5.13 Does increased blocking of magnetic protein G beads reduce non-specific RBM4 recovery? The experiment described in Figure 5.12 was repeated except that the incubation of beads with cytochrome C was increased to 5mg/ml and to overnight.

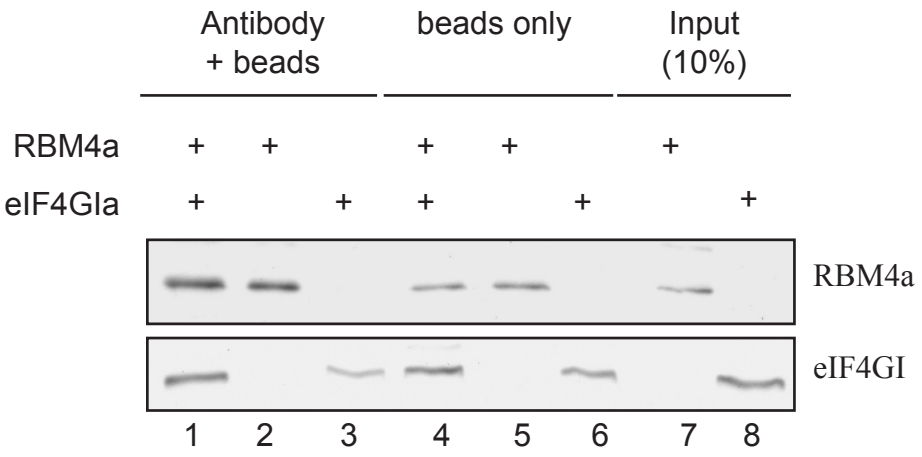


Figure 5.14 Does increased NaCl concentration reduce non-specific RBM4 recovery? The experiment described in Figure 5.12 was repeated except that NaCl in the interaction buffer was increased from 100mM to 200mM.

FLAG tag found on eIF4GIa (Figure 5.15). This could potentially cause RBM4a to bind to the anti-FLAG antibodies. This binding would not be as strong as eIF4GIa but could contribute some of the increased background observed in Figure 5.14, lane 2. To overcome this potential binding problem I obtained some triple-FLAG peptide (3xFLAG tag) to try and out-compete this binding by the antibodies. To determine what effect the FLAG peptide had on the binding of RBM4 to anti-FLAG antibodies, an experiment was set up with RBM4a alone with anti-FLAG antibodies and the beads in the presence of 0, 6.25, 12.5, 25, 50 and 100µg/ml triple-FLAG peptide. 100µg/ml was chosen as the top end as this is the stage at which the 3xFLAG peptides manual indicates elutes FLAG-tagged proteins from M2 FLAG affinity gels. Figure 5.16A shows that the peptide had little effect on the binding of RBM4a to the antibody. Alongside this, another experiment was carried out looking at the effect of the FLAG peptide on eIF4GIa recovery to ensure that the 3xFLAG peptide did not elute the eIF4GIa from the resin. This was set up in the same way as above but with added eIF4GIa in the presence of 0, 6.25, 12, 25, 50 and 100µg/ml triple-FLAG peptide. As shown in Figure 5.16B, FLAG peptide at 25-50µg/ml caused a slight reduction in the recovery of RBM4a with eIF4GIa (lanes 4 and 4 vs lane 1). Increasing the peptide concentration to 100µg/ml did not have any further effect on recovery of RBM4a but did reduce that of FLAG-eIF4GIa (lane 6 vs lane 5). The data shown in Figure 5.14 show a potential binding of eIF4G to RBM4 but attempts to reduce background further failed and more attempts to reduce this background would be needed to increase confidence in the validity of this interaction.

```
RBM4a      MVKLFIGNLPREATEQEIRSLFEQYGKVLECDIIKNYGFVHIEDKTAAEDAIRNLHHYKL 60
Flag       ------------------------------------------------------------

RBM4a      HGVNINVEASKNKSKASTKLHVGNISPTCTNQELRAKFEEYGPVIECDIVKDYAFVHMER 120
Flag       ------------------------------------------------------------

RBM4a      AEDAVEAIRGLDNTEFQGKRMHVQLSTSRLRTAPGMGDQSGCYRCGKEGHWSKECPIDRS 180
Flag       ------------------------------------------------------------

RBM4a      GRVADLTEQYNEQYGAVRTPYTMSYGDSLYYNNTYGALDAYYKRCRAARSYEAVAAAAAS 240
Flag       ------------------------------------------------------------

RBM4a      AYSNYAEQTLSQLPQVQNTAMASHLTSTSLDPYNRHLLPPSGAAAAAAAAAACTAASTSY 300
Flag       ------------------------------------------------------------

RBM4a      YGRDRSPLRRATGPVLTVGEGYGYGHDSELSQASAAARNSLYDMARYEREQYADRARYSA 360
Flag       ----------------------------------------------DYKDDD--DK----- 8
                                                           *: ::  *:

RBM4a      F 361
Flag       -
```

Figure 5.15 Alignment of FLAG peptide epitope and RBM4 sequences. The FLAG peptide sequence was aligned against RBM4a *Mus Musculus* protein sequence obtained from PUBMED using ClustalW2 software. Stars signify a perfect match whereas Double dots show similar amino acids and single dots show those with low similarity.

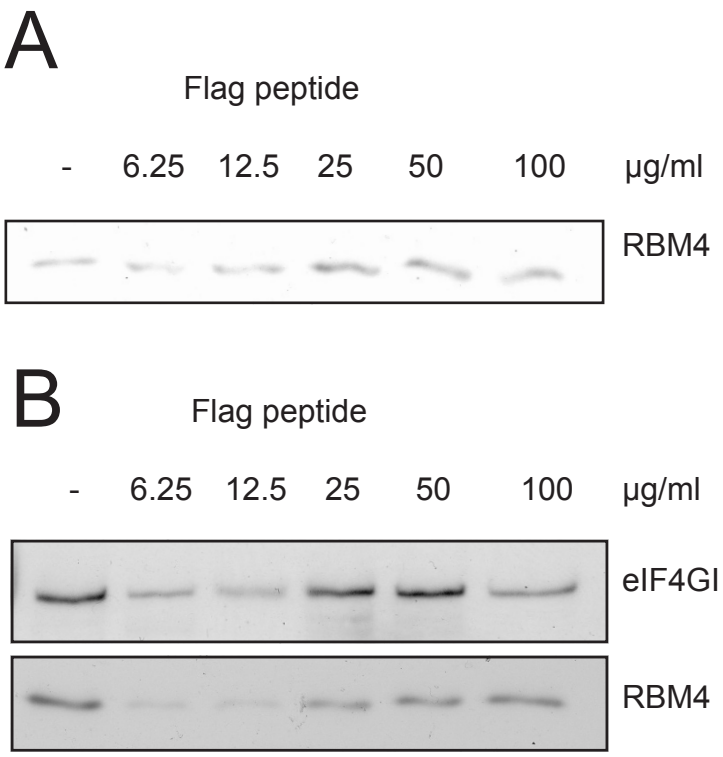


Figure 5.16 Does addition of FLAG peptide affect the non-specific recovery of RBM4a? 3µg of RBM4a alone (Panel A) or 3µg of each RBM4a and eIF4GIa (Panel B) were diluted into 1ml interaction buffer containing indicated levels of triple-FLAG peptide. Subsequently, M2 anti-FLAG antibody (5µl) and magnetic beads were added as described in Materials and Methods Section 2.10.4. The antibodies and co-isolated proteins were recovered and resolved by 12% SDS-PAGE and analysed by Western blotting using indicated antibodies.

5.6 eIF4GI, eIF4A and actively translating ribosomes are localised to the cell periphery during myogenic differentiation

As RBM4 has been shown to bind to eIF4A and eIF4GI during myogenic differentiation, I used confocal microscopy to determine their intracellular localisation to look for colocalisation. Figure 5.17 shows that eIF4GI has a diffuse cytoplasmic stain at 0 hours with very little eIF4GI observed in the nucleus. 48 hours after induction of differentiation the stain becomes more localised to the periphery of the cell, and as differentiation progresses to 72 hours, localisation of eIF4G becomes more continuous. eIF4A also shows a similar pattern during differentiation (figure 5.18) but across multiple images, the perphipheral stain was not as distinct. The eIF4A showed a greater degree of localisation to the nucleus except at 72 hours, with the stain more granular at 24, 48 and 72 hours of differentiation.

As eIF4GI were localised to the periphery of the cell during differentiation at later time points, the localisation of ribosomes and actively translating ribosomes was investigated using a method developed using puromycin to allow visualisation of ribosomes actively translating (Willett et al, 2011).

What was found using this technique was that ribosomes in differentiating cells generally had a diffuse granular stain in the cytoplasm at later time points (48 and 72 hours). However, the actively translating ribosomes, as indicated by the puromycin stain, were found enriched at the periphery of the cells. This was seen to the greatest extent in cells induced to differentiate for 48 hours (Figure 5.19) The finding that eIF4A, eIF4GI and actively translating

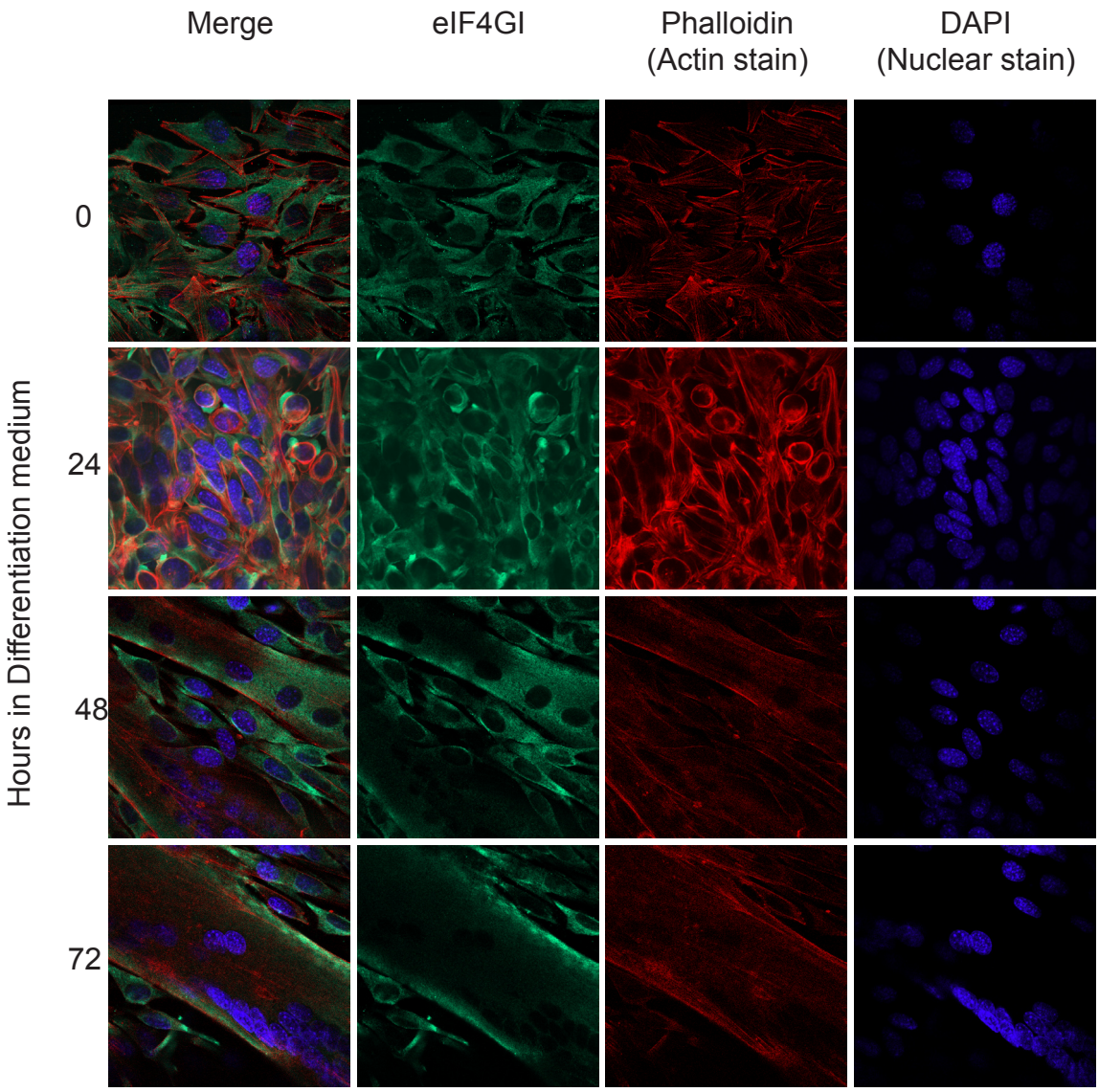


Figure 5.17 Localisation of eIF4GI during myogenic differentiation. Cells were induced to differentiate as described in Materials and Methods Section 2.2.3. The cells were then fixed and permeabilised before being incubated with indicated antibodies and stains at the indicated time points. Following incubation with fluorescently linked secondary antibodies, the samples were visualised by confocal miscroscopy as described in Materials and Methods Section 2.5.1.

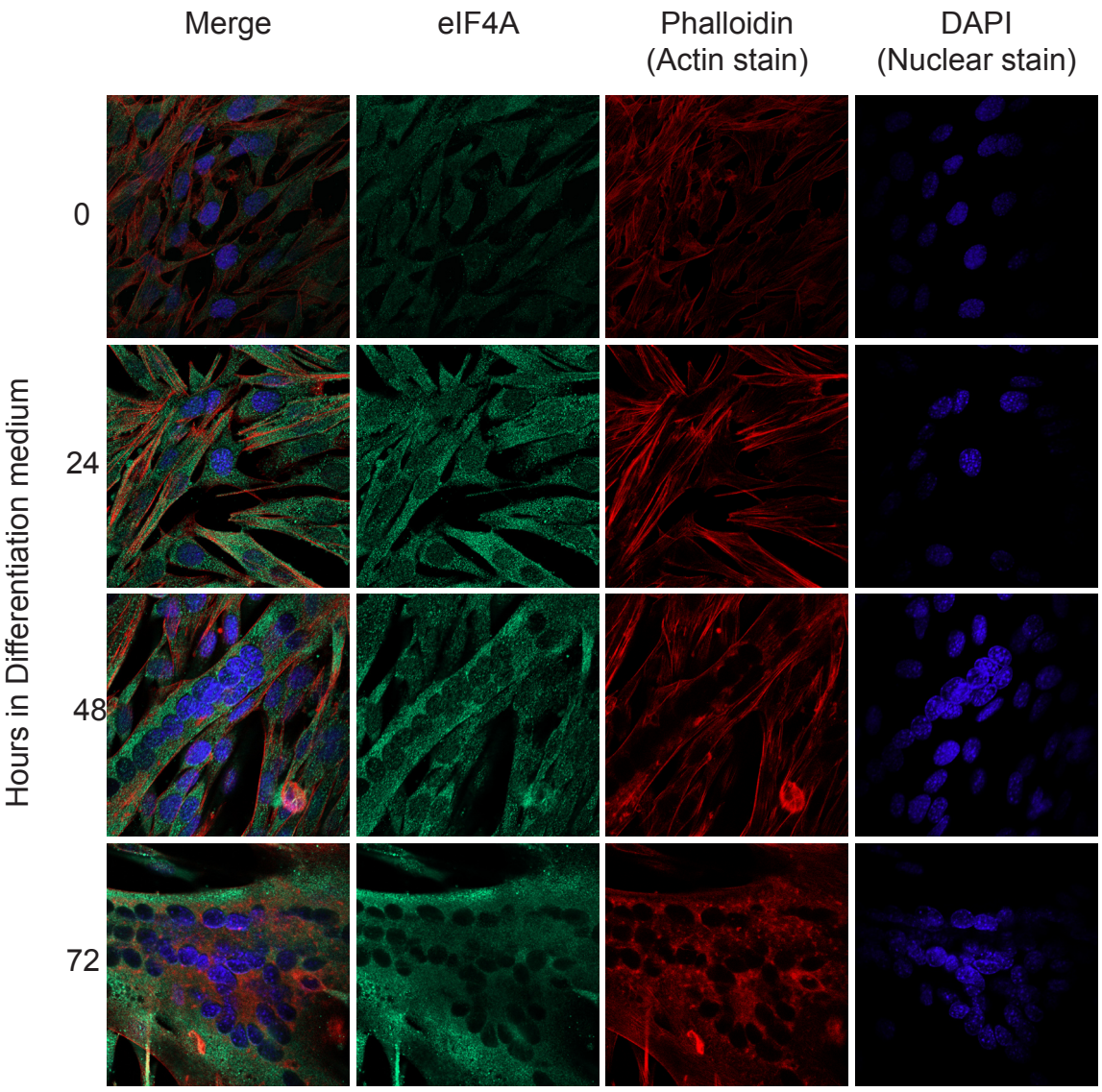


Figure 5.18 Localisation of eIF4A during myogenic differentiation. Cells were induced to differentiate, fixed and permeabilised before being incubated with the indicated antibodies and stains as described in Figure 5.17.

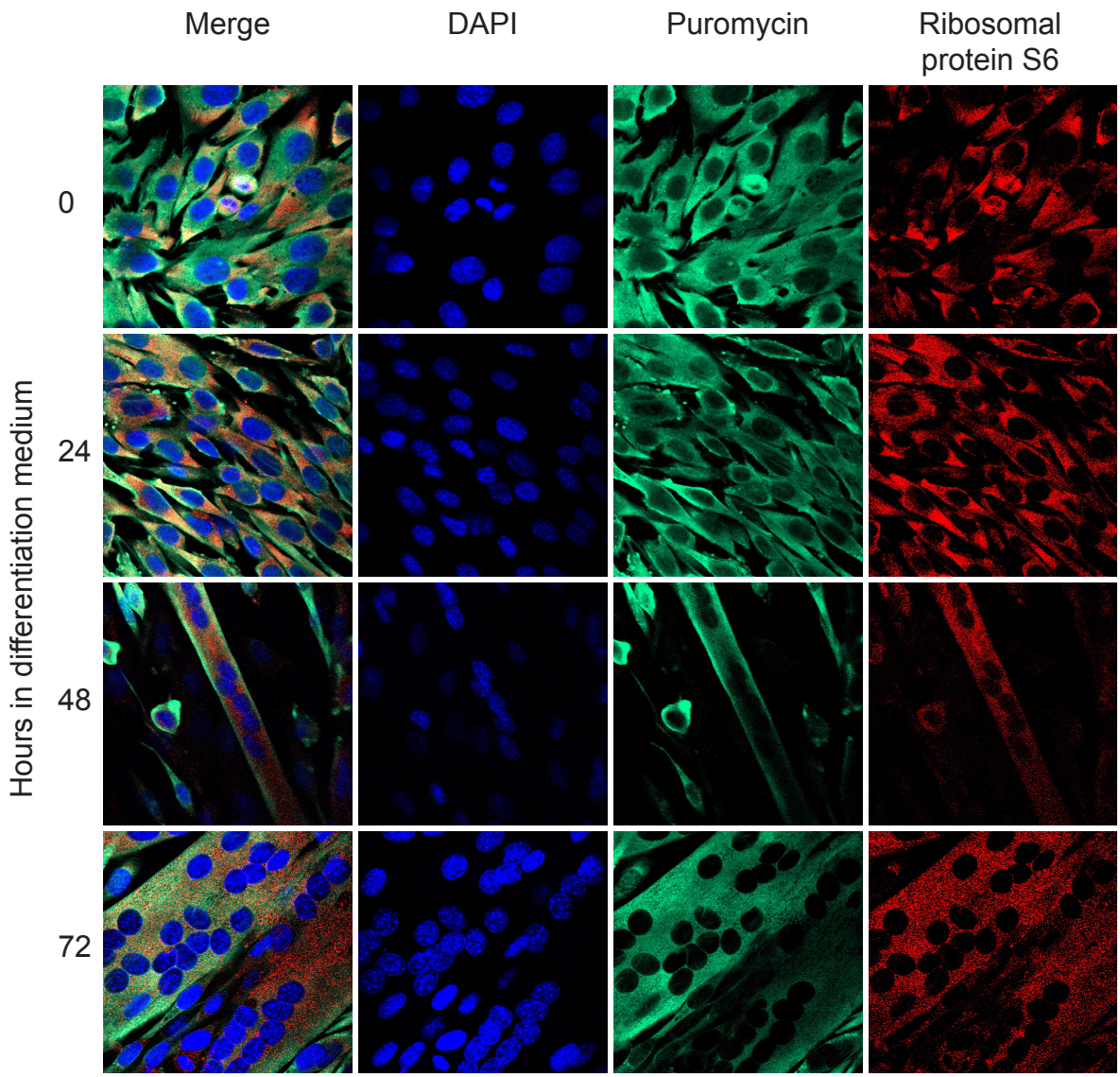
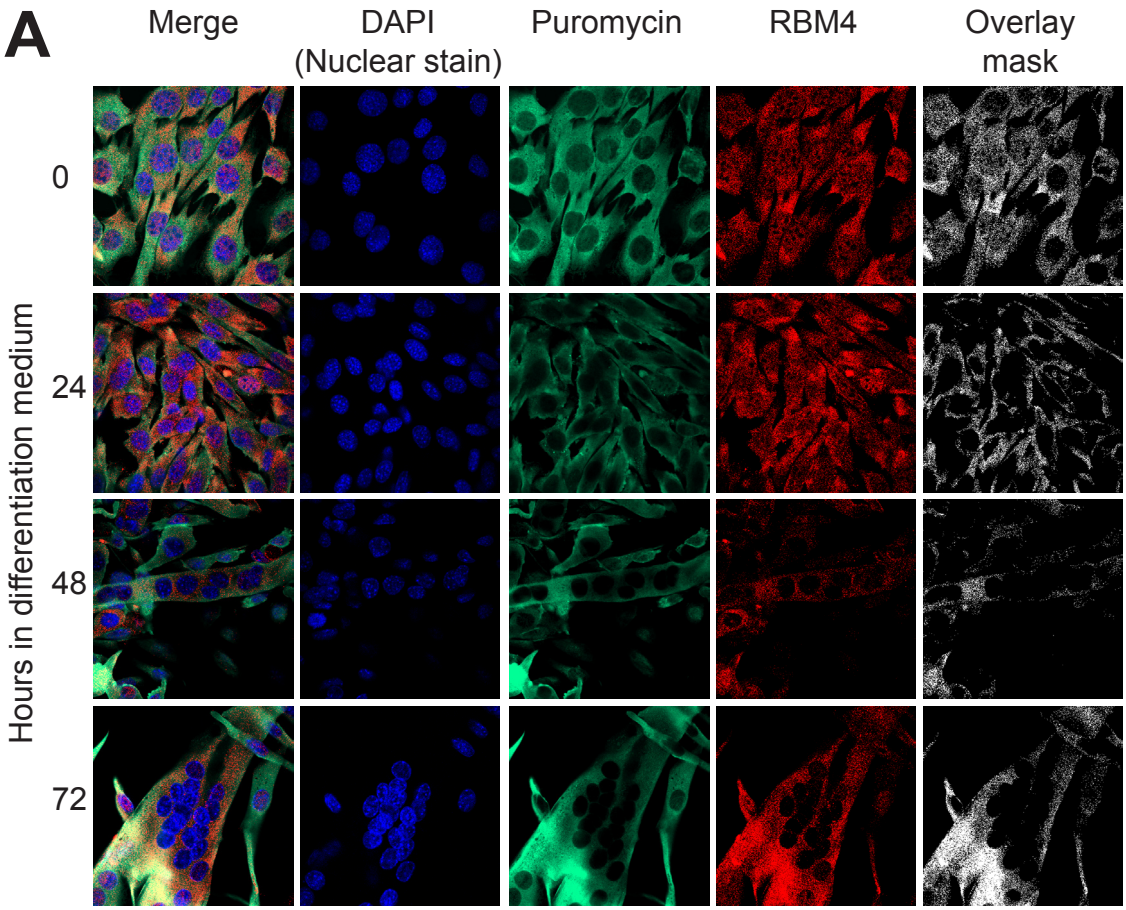


Figure 5.19 Actively translating ribosomes are localised to the cell periphery during myogenic differentiation. Cells were induced to differentiate as described in Materials and Methods Section 2.2.3. Before harvesting cells were incubated for 5 minutes with 91μM puromycin, (10mg/ml stock Sigma), 208μM emetine (10mg/ml Sigma) and 100μg/ml cycloheximide (Sigma). The cells were then harvested as described in Section 2.5.1 with 100μg/ml cycloheximide added to the pre-warmed PBS wash step. Antibodies and Stains were used as indicated.

ribosomes were localised to a similar region of the cell during differentiation indicates that local translation is required for some reason at these times. This could be to do with the fusion of cells that is occurring at these time points but further work would be required to investigate this.

5.7 RBM4 co-localises with actively translating ribosomes after 72 hours of myogenic differentiation.

As RBM4 has been shown to bind to translation intiation factors eIF4GI and eIF4A (Figure 5.1) The potential interaction of RBM4 with actively translating ribosomes was investigated. C2C12 cells were induced to differentiate and the co-localisation of RBM4 and puromycin positive ribosomes was detected by confocal microscopy as shown in Figure 5.20. At 0, 24, or 48 hours of differentiation RBM4 shows low levels of co-localisation with translating ribosomes. however at 72 hours of differentiation their co-localisation increased dramatically. The images were analysed for the co-dependency of the RBM4 and puromycin channels by linear regression with the Pearsons co-efficient, which yielded the data in Figure 5.20B. Pearson's values of 0.5 are deemed to be significant at 72 hours the Pearson's increases to a significant result of 0.571. The co-localisation of the RBM4 and actively translating ribosomes indicates that RBM4 may be potentially involved in modulating translation of certain mRNAs in a positive manner at 72 hours. In fact published data has shown RBM4 binding and up regulating c-myc and Bcl-2 translation which is believed to occur *via* IRES sites. C-myc is reported to be a negative regulator of myogenic differentiation (Miner & Wold, 1991); (La Rocca et al, 1994) and Bcl-2 is present at early stages of myogenesis but once mid stages has been reached and myotubes have begun to form Bcl-2 is



B Pearson's Coeffient of the co-dependency of RBM4 and Puromyocin during differentiation of C2C12 cells.

0h	r = 0.403 r (random) = 0.0±0.03	48h	r = 0.393 r(random) 0.0±0.049
24h	r = 0.381 r(random) = 0.0±0.03	72h	r = 0.671 r(random) = 0.0±0.003

Figure 5.20 Does RBM4 colocalise with active translation during myogenic differentiation? Cells were induced to differentiate as described in Materials and Methods Section 2.2.3. Before harvesting cells were incubated as in Figure 5.19. Panel A. Antibodies and Stains used as indicated and co-localisation was determined by digital subtraction of non-overlapping pixels. Panel B. These data show the Pearsons co-efficient (r) for the co-dependency of the RBM4 and puromycin channel during myogenic differentiation as described in the Materials and Methods Section 2.5.2.

no longer expressed (Dominov et al, 1998). Further work is required to determine which mRNAs are binding RBM4 at these sites in the myotubes.

5.8 Discussion

The work described in this chapter has focused on the binding interaction of RBM4 with initiation factors. Using two different methods, these data suggest that endogenous RBM4 forms complexes with eIF4A and eIF4GI in differentiating myogenic cells. These data are in agreement with studies from HeLa cells over-expressing RBM4 (Lin et al, 2007). The availability of eIF4GI to bind RBM4 also appears to be modulated as it has been shown to bind initiation factors to a greater extent at 48 hours of differentiation. In contrast, eIF4A appears to bind to RBM4 at all times (Figure 5.1). The interaction between RBM4 and eIF4A at 48 hours also appears to be RNAse resistant and therefore not dependent on mRNA (or the RNA component is well protected). As shown by optimised *in vitro* binding assays, the interaction of eIF4G with RBM4 appears not to be direct. When recombinant eIF4A was combined with extracts from differentiating cells, RBM4a and RBM4b were found to be recruited to it to a greater extent in extracts prepared from cells induced to differentiate for 24 hours. This potentially means that either there is a larger free pool of RBM4 at this time or that the complexes it forms are more easily disrupted by the recombinant protein. I believe the latter is the case as the maximal binding of RBM4a or RBM4b does not coincide with times of maximal RBM4a or RBM4b protein expression. To carry this work further, the IP RNAse A and T1 experiment shown in Figure 5.2 would need to be expanded and repeated but as mentioned before this would require finding a new source of antibody that works with IP. Another experiment that would

need to be done would be to look at the direct interaction of RBM4 with eIF4A via an *in vitro* binding assay. This was not possible as both protein possessed the same affinity tag; I was unsuccessful at cloning the murine RBM4 sequence into a different tagged vector and time pressures did not allow me to re-clone the eIF4A. Interestingly, RBM4 was found to be localised to actively translating ribosomes during myogenic differentiations at 72 hours without a significant localisation at earlier time points. This along with the interaction with eIF4A and eIF4GI gives a good indication that RBM4 is involved in regulation of translation during later times of myogenic differentiation especially at 72 hours.

-Chapter 6-

Discussion

The aim of the work described in this thesis was to determine if RBM4a or RBM4b played a role in the regulation of myoblast cell differentiation into muscle myotubes. Previous work in HeLa cells (Lin et al, 2007) has suggested that RBM4 interacts with the translational machinery but little is known about the role for this protein in myogenic differentiation. My data presented in this thesis show that total levels of RBM4 increase during differentiation (Figure 3.4). Whilst levels of RBM4b increase during differentiation, RBM4a protein expression decreased significantly (Figure 3.5). These data are in contrast to published findings where another group (Lin & Tarn, 2011) showed that RBM4 levels only increase by a small amount during differentiation. However, on closer examination of their data, the Western blots appear to be highly exposed which could indicate that the signal was outside of the linear range for this antibody. I have also examined the intracellular localisation of RMB4. My data shows that during differentiation, levels of RBM4 protein present in the cytoplasm increased, with levels found in the nucleus remaining relatively stable (Figures 4.1 and 4.2). Nuclear levels of PTB have been reported to decrease during differentiation (Lin & Tarn, 2011). Together these data may support the idea that a decrease in the ratio of RBM4 to PTB regulates splicing during myogenic differentiation. Further work investigating the relative expression levels of RBM4 and PTB in the nucleus may provide a better view of what is occurring as splicing occurs in the nucleus (Wang & Burge, 2008).

Using C2C12 cells induced to exit the cell cycle and differentiate, I have shown that RBM4 co-IPs with eIF4A and eIF4GI (Figure 5.1). The binding of RBM4 to eIF4GI and eIF4A most likely reflects recovery of RBM4b as levels of this isoform increase during myogenic differentiation (Figure 3.5). RBM4 interaction with initiation factors is interesting because as shown in Figure 5.1, the association of RBM4 with eIF4GI is not constant and peaks at around 48 hours following the induction of differentiation. In contrast, RBM4 binds to eIF4A at early times. This leads me to suggest that RBM4 is somehow interfering with the activity of eIF4A in an act of inhibition (Figure 6.1). RBM4 has been shown to cause down-regulation of cap-dependent translation by interacting with mRNAs containing CU rich elements. However, this effect on translation was only observed with cells transfected with reporter constructs and not with endogenous mRNAs (Lin et al, 2007). RBM4 could be functioning as an inhibitor by binding both CU rich elements and eIF4A as part of a complex attached to the mRNA, stopping the association of eIF4A in an active complex. After myoblasts have fused to form large myotubes, RBM4 forms a productive complex together with eIF4GI and eIF4A (Figure 5.1) which can drive translation of specific mRNAs (Figure 6.1). RBM4 has been shown to be involved in the selective activation of mRNA, having a direct role in the regulation of Period 1 (mPER1), a protein regulating circadian rythm in mice. Over-expression of RBM4a or RBM4b led to an up-regulation in mPER1 by 2.8 fold or 5 fold, respectively. Studies by Kojima *et al*., (2007) suggest that this is most likely through translational regulation, with RBM4 binding directly to a stem-loop *cis* element in the 3' UTR of the mPer1 mRNA. Alterations of RBM4 levels in cycling cells caused significant changes in circadian period,

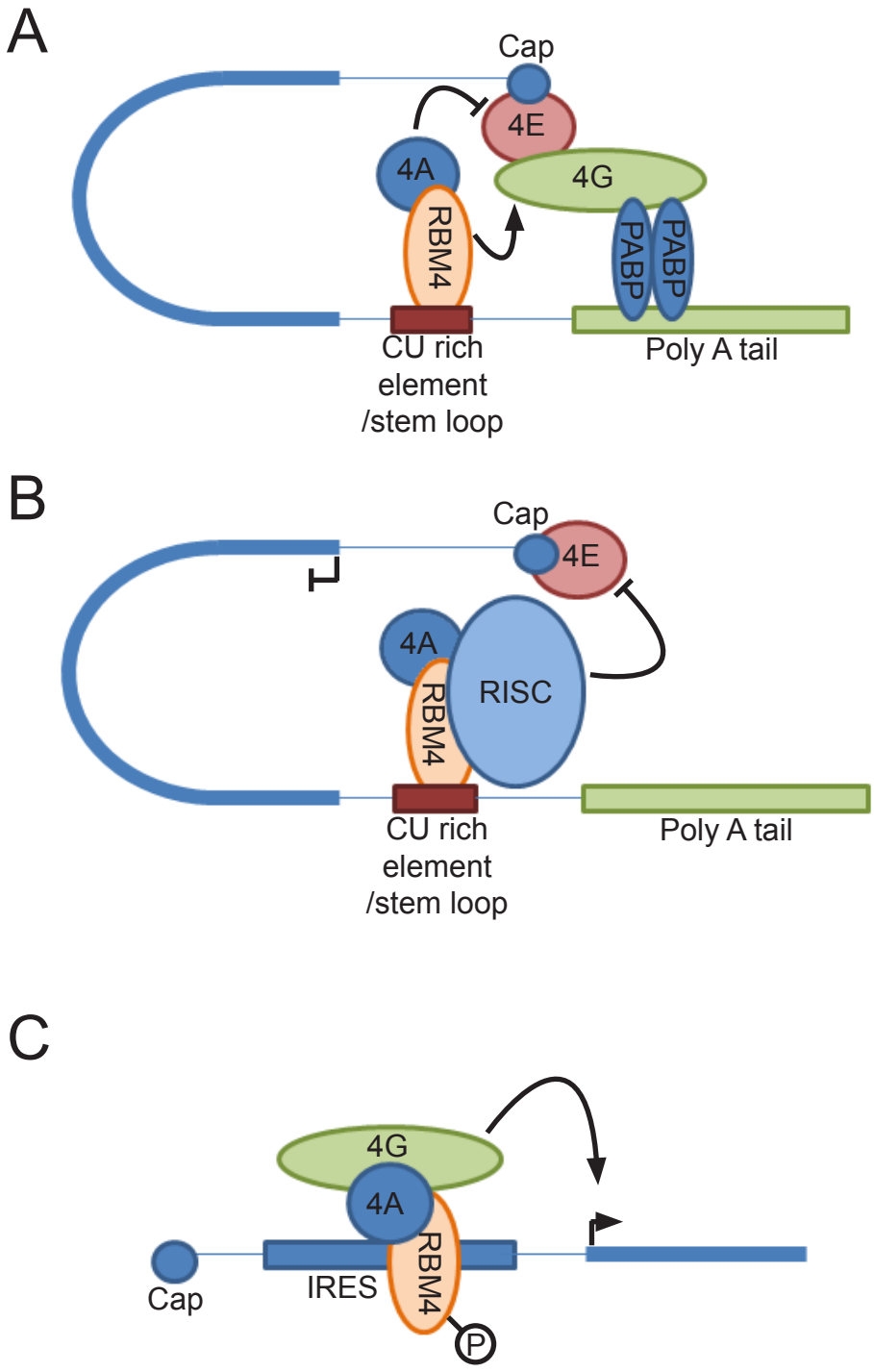


Figure 6.1 Model for RBM4 function in translational control in myogenic differentiation. (A) RBM4 binds to a CU rich element in the 3' UTR and associates with eIF4A and inhibits or activates cap-dependent translation. (B) RBM4 binds to a CU rich element as before but associates with eIF4A and the RISC complex leading to inhibition of cap-dependent translation. (C) RBM4 associates with eIF4A and eIF4G and recruits them to a cellular IRES.

with RBM4 knockdown by siRNA resulting in a shorter circadian period, and the over-expression resulting in a lengthened period (Kojima et al, 2007). However, the exact mechanism behind this inhibition was not determined

The study by Kojima *et al*., (2007) also suggested that RBM4 had a functional interaction with eIF4GI/PABP. In my work, I have shown that the interaction between the RBM4 and eIF4GI does not appear to be direct or too weak to detect (Figure 5.14). As this interaction has been shown to be RNAse resistant (Figure 5.2), it is most likely to be *via* another protein. RBM4 can also co-isolate with eIF4E but this was RNAse sensitive, indicating a bridging function of mRNA (Lin et al, 2007). Further work would focus upon determining if eIF4A can bind directly to RBM4, which domains on each protein were important for such an interaction and which other components of the initiation complex can be co-isolated with RBM4 during differentiation (Figure 6.1).

One possible link in the inhibition of translation mediated by RBM4 is Ago2. This forms part of the RISC complex, binds to eIF4E in a RNAse sensitive manner (Iwasaki et al, 2009) and directly to RBM4 (Hock et al, 2007). The interaction of RBM4 and Ago2 was reduced, but not prevented by RNase A was added (Hock et al, 2007), and RBM4 interaction with eIF4E has been shown to be RNAse sensitive (Lin et al, 2007). These data suggest that RBM4 could enhance the binding of Ago proteins to mRNAs by increasing the accessibility of miRNA target sites (Hock et al, 2007) and prevent eIF4E-dependent translation (Figure 6.1). This model is consistent with the report that if RBM4 is over-expressed, mRNAs with CU rich regions are preferentially down-regulated (Lin & Tarn, 2009). Another protein that functions as an

inhibitor of eIF4A is Pdcd4, which binds mRNA and ribosomal RNA. It is believed that such interactions increase the localisation of Pdcd4 to eIF4A (Wedeken et al, 2010). RBM4 could act similarly by binding CU rich elements in mRNA (Lin et al, 2007) and then binding and inhibiting eIF4A that associate with this mRNA (Figure 6.1).

At the later stages of differentiation, RBM4 also co-localises with actively translating ribosomes to a significant degree (Figure 5.20) adding more support for RBM4 being involved in the regulation of protein synthesis at the later stages of differentiation. Previous work from Lin *et al*., (2007) has shown that RBM4 acts in conjunction with eIF4A on IRES-mediated translation initiation. Following arsenite stress, RBM4 binding to cellular mRNAs encoding Bcl-2 and c-Myc was increased, suggesting that RBM4 may activate their expression either through IRES-mediated events or via an undetermined eIF4A-dependent mechanism (Figure 6.1). However, the role for eIF2α phosphorylation in this response remains to be addressed. Interestingly, neither Bcl-2 nor c-myc proteins are expressed at 48 hours following the induction of myogenic differentiation. c-myc is an inhibitor of myogenic differentiation and is not expressed at this time (Miner & Wold, 1991); (La Rocca et al, 1994). Bcl-2, an anti-apoptotic protein, is expressed at early time points (prior to myotube formation) but not at later time points (Dominov et al, 1998). These data suggest that while RBM4 has the potential to activate cellular IRESes, it would most likely not up-regulate these mRNA by this process. One possible mechanism in regulating RBM4 activity and function could be the phosphorylation at ser309 (Lin et al, 2007) following activation of p38MAPK signalling (Figure 4.4). During myogenic differentiation, there is an

increase of p38MAPK activity before and during the dramatic association of eIF4GI and RBM4 at 48 hours (Figure 4.4), at a time when RBM4 undergoes ser309 phosphorylation (Lin & Tarn). This phosphorylation may be a key switch in activity or function of RBM4 towards stimulating IRES-dependent translation by mediating the binding of RBM4 with eIF4GI (Figure 6.1). This correlation would need to be verified directly in my C2C12 system using phospho-specific ser309 RBM4 antibodies which could not be performed as no antibodies where available that worked with endogenous expression levels.

Another interesting finding is described in Chapter 5 regards the localisation of initiation factors in cells undergoing myogenic differentiation. Both eIF4GI and eIF4A show staining at the cell periphery at 48 and 72 hours after differentiation (Figures 5.18 and 5.19, respectively). This could potentially be to allow translation of proteins required at the cell membrane to facilitate cell fusion or communication. Further work would need to be done to fully understand the role of RBM4 in myogenic differentiation and to investigate the observed localisation of active translation to the cell periphery.

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