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**Expression and subcellular localisation of
poly(A)-binding proteins**

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Abstract

Poly(A)-binding proteins (PABPs) are important regulators of mRNA translation and stability. In mammals four cytoplasmic PABPs with a similar domain structure have been described - PABP1, tPABP, PABP4 and ePABP. The vast majority of research on PABP mechanism, function and sub-cellular localisation is however limited to PABP1 and little published work has explored the expression of PABP proteins.

Here, I examine the tissue distribution of PABP1 and PABP4 in mouse and show that both proteins differ markedly in their expression at both the tissue and cellular level, contradicting the widespread perception that PABP1 is ubiquitously expressed. PABP4 is shown to be widely expressed though with an expression pattern distinct from PABP1, and thus may have a biological function in many tissues. I have shown that PABP4 associates with polysomes and described interactions between PABP4 and proteins important for PABP1-mediated translational activation suggesting that PABP4 plays a similar role in translational regulation.

Translational inhibition is a common response to cell stress. Following certain cellular stresses e.g. arsenite, PABP1 localises to cytoplasmic stress granules, sites of mRNA storage. Here, I show that PABP4 is also a component of stress granules and have investigated the role of PABPs in stress granule formation. In contrast, I have shown that both PABP1 and PABP4 relocalise to the nucleus following UV irradiation in both human and mouse cell lines.

In order to understand the how the UV-induced change in PABP localisation may be regulated, I have investigated the regulation of PABP nuclear export. I have found that accumulation of PABP proteins in the nucleus after UV coincides with an accumulation of poly(A)⁺ RNA, indicative of a block in mRNA export, an effect which has not been previously characterized. My working model is that nuclear export of PABPs may be dependent on mRNA export, inhibition of which leads to nuclear accumulation of PABPs after UV, which may in turn augment translational inhibition.

Declaration

I declare that the work presented in this PhD is my own and has not been submitted for any other degree.

Hannah Burgess

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Abbreviations

4EHP - 4E-homologous protein

ARE – AU-rich element

ARC - autoregulatory complex

ARS - autoregulatory sequence

ATP – adenosine triphosphate

AGO - Argonaute protein

BARD1 - BRCA1-associated RING domain protein 1

bp – base pairs

BC1 – brain cytoplasmic 1

BRCA1 - Breast Cancer associated-1

CaMKII α - calcium/calmodulin dependent protein kinase II α

CBC - cap binding complex

CBP – cap binding protein

C/EBP β - CCAAT/Enhancer β

CHOP -C/EBP homologous protein

CPEB - cytoplasmic polyadenylation element binding protein

CPSF –cleavage and polyadenylation specificity factor

CRM1 - chromosome region maintenance 1

CstF - cleavage stimulatory factor

CTD - COOH-terminal domain of RNAPII

DAB - 3,3'-diaminobenzidinetetrahydrochloride

DAPI - 4',6-diamidino-2-phenylindole

DMEM - Dulbecco's minimal essential medium

DNA – deoxyribonucleic acid

DRB – 5,6-dichlororibofuranosylbenzimidazole

DSE - downstream sequence element

DSP – Dithiobis (succinimidyl propionate)

EDTA – ethylene diamine tetraacetic acid

eEF – eukaryotic elongation factor

eIF – eukaryotic initiation factor

eRF – eukaryotic release factor

EJC – exon junction complex

ELAV - embryonic lethal abnormal vision

EMSA - electrophoretic mobility shift assay

ER - endoplasmic reticulum

FAK - focal adhesion kinase

FISH – fluorescent *in situ* hybridisation

FRAP - fluorescence recovery after photobleaching

g – Grams

g – centrifugal force

G3BP - GTP-ase activating protein SH3 domain-binding protein

GADD34 - growth arrest and DNA damage gene 34

GAP - GTPase activating protein

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GCN2 - general control non-depressible 2

GEF - guanine nucleotide exchange factor

GFP – green fluorescent protein

GGR - global genomic repair

HA-tag – haemagglutinin

HGNC - Hugo Gene Nomenclature Committee

hnRNP - heterogeneous ribonucleoprotein

HRI - haem-regulated inhibitor

HRP – horseradish peroxidase

HSV1 - herpes simplex virus type 1

IF - immunofluorescence

IHC –immunohistochemistry
IP - immunoprecipitation
IPG – immobilised PH gradient
IPTG - isopropyl β -D-1-thiogalactopyranoside
IMP1 - insulin-like growth factor II mRNA binding protein-1
ITAF - IRES *trans*-acting factor

Kb – kilobase
kDa - kilodalton
KSHV - Kaposi sarcoma-associated herpes virus
KSRP - KH splicing regulatory protein

L - litre
LMB – leptomycin B
LB – luria broth

m⁷G - 7-methylguanosine
M – molar
MEF – mouse embryonic fibroblast
ml – millilitre
mM – millimolar
miRISC - microRNA-induced silencing complex
miRNA - Micro RNA
mRNA – messenger RNA
mTOR - mammalian target of rapamycin

NAB2 - nuclear polyadenylated RNA-binding protein 2
NES – nuclear export signal
NGD – no go decay
NMD - nonsense-mediated decay
NSD - non-stop decay
NSP3 - non-structural protein 3

OPMD - oculopharyngeal muscular dystrophy

ORF - open reading frame

PABP – poly(A)-binding protein

PABPN1 - Nuclear poly(A)-binding protein

PAGE - polyacrylamide gel electrophoresis

PAM – PABP-interacting motif

PAP – poly(A) polymerase

PBS – Phosphate buffered saline

PCR – polymerase chain reaction

PARN - poly(A)-specific ribonuclease

PBS – phosphate buffered saline

Pdcd4 - programmed cell death protein 4

PERK - PKR-like ER localized kinase

PFA - paraformaldehyde

pI – isoelectric point

PI3K - phosphoinositide 3-kinase

PKC - protein kinase C

PKR - protein kinase activated by double-stranded RNA

PP1 - protein phosphatase 1

PTC - premature termination codon

PVDF - polyvinylidene fluoride

RACK1 - Receptor for activated kinase 1

RNA – ribonucleic acid

RNAi – RNA interference

RNAPII – RNA polymerase II

RNase - ribonuclease

RNase MRP - RNase mitochondrial RNA processing

RNP – ribonucleoprotein

RoXaN - rotavirus X protein associated with NSP3

RRL - rabbit reticulocyte lysate

RRM - RNA-recognition motif

SAP - shrimp alkaline phosphatase

SDS - sodium dodecyl sulphate

SEM - standard error of the mean

shRNA – short hairpin RNA

SG – stress granule

siRNA – short interfering RNA

snoRNA - small nucleolar RNA

TAP - Tip-association protein

TBS - Tris-buffered saline

TCA - trichloroacetic acid

TCR - transcription coupled repair

TD-NEM - transcription dependent nuclear export motif

TEMED - tetramethylethylenediamine

TGF- β - transforming growth factor beta

TNF - tumour necrosis factor

TREX - transcription/export

tRNA - transfer RNA

TTP – tristetraproline

UNR - upstream of N-ras

UTR - untranslated region

UV - ultraviolet

VEGF - vascular endothelial growth factor

VHL - von Hippel-Lindau

VSV - vesicular stomatitis virus

WB - western blot

w/v – weight/volume

YB-1 - Y-box binding protein 1

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Chapter 1: Introduction

1.1 Eukaryotic gene expression and regulation

The complexities of eukaryotic life demand the precise regulation of gene expression. The central dogma of molecular biology explains gene expression as a multi-step process of DNA transcription to mRNA and mRNA translation to protein. It is now known that each step from gene transcription toward protein synthesis is not discrete, but intimately connected to the others with shared protein components and functions (Figure 1.1) (reviewed in (Bentley, 2005; Moore and Proudfoot, 2009; Orphanides and Reinberg, 2002).

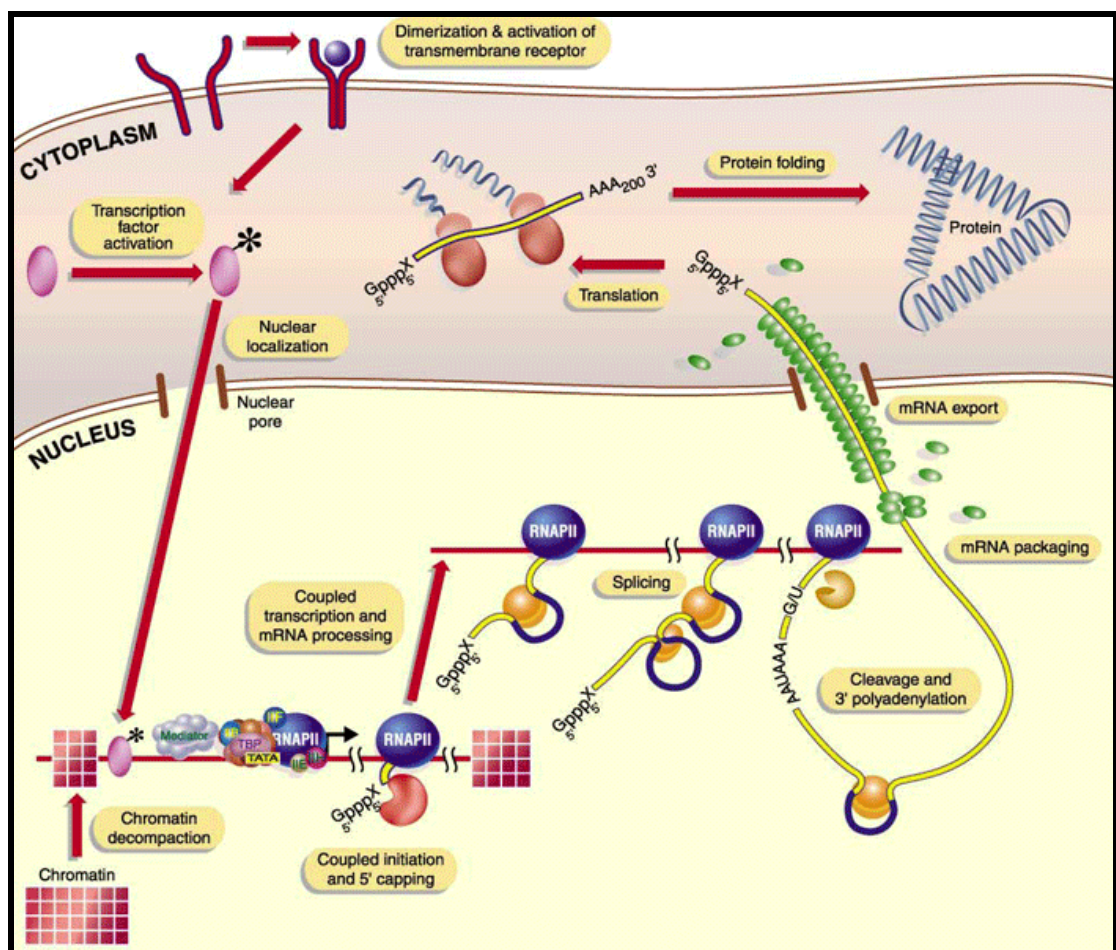


Figure 1.1 Eukaryotic gene expression. Transcription, splicing, capping, 3' end processing, mRNA nuclear export, turnover, localisation and translation are linked events with common protein components. Taken from (Orphanides and Reinberg, 2002).

1.1.1 Transcription

Many genes are regulated at the transcriptional level by factors that bind regulatory DNA sequences. The activity of transcription factors is tightly controlled by a myriad of pathways processing signals from the external and internal environment of the cell. Multiple mechanisms, such as histone modifications and the binding of activator or repressor proteins, affect recruitment of RNA polymerase (RNAP) enzymes to the gene (Reviewed in (Lee and Young, 2000)).

Protein-coding genes are transcribed to pre-mRNA by RNAP II. Following initiation of RNAP II transcription, a 7-methylguanosine (m^7G) cap is added to the 5' of RNA transcripts when they are 20-30 nucleotides long. In the first instance, the cap protects the transcript against degradation by exonucleases; but also provides a platform for protein interactions important for mRNA export and translation. In the nucleus the cap is bound by CBP20 and CBP80 known as the cap binding complex (CBC) (Proudfoot et al., 2002). As the polymerase moves along the gene, the elongation phase of transcription is also subject to regulation (Landick, 2006; Shilatifard, 2004).

1.1.2 Splicing

Concomitant with transcription, introns are removed from the nascent pre-mRNA by splicing. Splicing is accomplished by the spliceosome, a large complex of many proteins and five small RNAs (U snRNAs) which specifically binds splice sites at the 5' and 3' of introns and catalyses their removal (Reviewed in (Wachtel and Manley, 2009)). Alternative splicing of pre-mRNAs increases protein diversity by the splicing together exons in different combinations – a process that is influenced by the binding of positively and negatively acting factors (Reviewed in (Nilsen and Graveley, 2010)).

1.1.3 3' end processing

Proper 3' end formation involves cleavage of the new transcript and the addition of a polyadenylate tail (Figure 1.2). Two multi-subunit complexes, cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulatory factor (CstF) recognize conserved cis-elements in the transcribed RNA sequence and catalyse the cleavage reaction. Poly(A) polymerase (PAP) is also required for efficient cleavage and 3' addition of adenosine residues by PAP is stimulated by CPSF and the nuclear poly(A)-binding protein (PABPN1) (Mandel et al., 2008; Proudfoot et al., 2002).

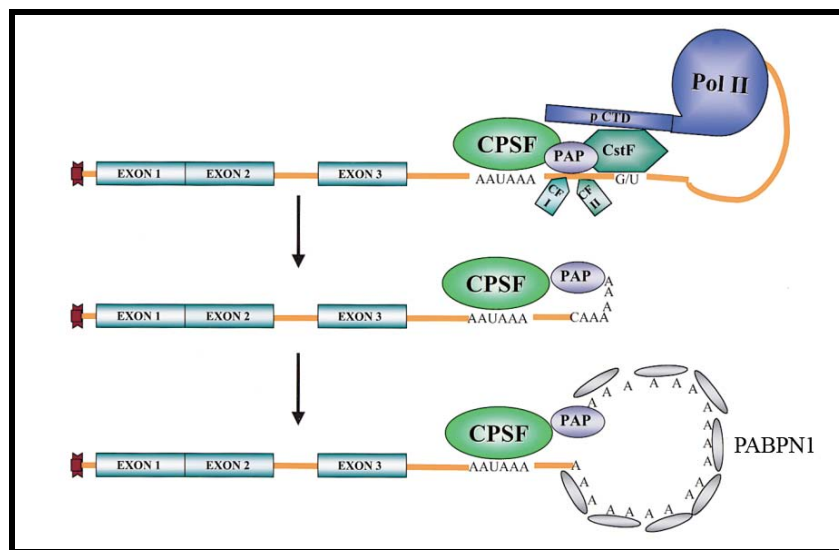


Figure 1.2 3' end formation involves pre-mRNA cleavage and polyadenylation
The conserved polyadenylation signal AAUAAA is recognised by CPSF and determines the site of cleavage 10-30 nucleotides downstream. The downstream sequence element (G/U) is bound by CstF which promotes RNA binding and cleavage by CPSF. Cleavage factors I and II and PAP also promote cleavage. PAP directs polyadenylation which is enhanced by CPSF and PABPN1 bound to the poly(A)-tail. Taken from (Proudfoot et al., 2002).

PABPN1 binds the newly formed poly(A) tail and stabilises PAP, preventing its dissociation and inducing processive addition of adenosines. When the poly(A) tail reaches a length of about ~250 nucleotides PABPN1 disrupts the stimulation of PAP by CSPF and hence can be viewed as a molecular ruler to measure poly(A) length (Kuhn et al., 2009). At least half of human genes possess more than one

polyadenylation site (Tian et al., 2005; Yan and Marr, 2005) and alternative poly(A) site usage represents a further mechanism to regulate gene expression and increase protein diversity (Danckwardt et al., 2008).

1.1.4 mRNA export

Processed mRNAs acquire a complement of bound factors that target them to and escort them through nuclear pores into the cytoplasm. These include splicing regulators such as some SR proteins which remain associated with exons and the exon junction complex (EJC) which is deposited on spliced mRNAs 20-24 nucleotides upstream of the exon-exon junction (Le Hir et al., 2000). The EJC is a dynamic complex consisting of the core proteins, eukaryotic translation initiation factor (eIF) 4AIII and the Y14:Magoh heterodimer both of which have RNA binding activity, and several more peripherally associated proteins such as Aly/REF (Reviewed in (Tange et al., 2004)). The TREX (transcription/export) complex associates with mRNA during splicing and consists of the THO complex, which is also involved in transcription elongation, Tex1, UAP56 and Aly/REF (Reed and Cheng, 2005; Strasser et al., 2002). TREX is thought to associate with the 5' most exon of the mRNA and impart directionality to export, so that mRNAs are transported through the nuclear pore 5' to 3' (Cheng et al., 2006). Aly/REF and SR proteins serve as export adapters and allow the mRNA to interact with the export receptor TAP (Tip-associated protein; also known as nuclear export factor1/NXF1) (Figure 1.3) (Walsh et al., 2010). Adapter protein binding to RNA and TAP has recently been shown to be mutually exclusive suggesting that adapter proteins are likely to transfer mRNAs to TAP, rather than bridging an interaction with TAP (Hautbergue et al., 2008). TAP forms a heterodimer with p15 (also known as Nxt1) and interacts with nucleoporins to facilitate translocation of the mRNA through the nuclear pore (Wiegand et al., 2002).

After entering the cytoplasm, TAP dissociates from the mRNA. In *S. cerevisiae* the DEAD-box helicase DBP5 has been shown to mediate this dissociation and contribute to further mRNP remodelling which is thought to ensure mRNA translocation is unidirectional (Lund and Guthrie, 2005) (Stewart, 2007).

DBP5 is stimulated by Gle1 and both proteins are thought to be localised to the cytoplasmic face of the nuclear pore complex via interactions with nucleoporins (Weirich et al., 2004; Weirich et al., 2006). Both DBP5 and Gle1 are conserved in humans and interact with nucleoporins suggesting this mechanism is also conserved in mammals (Kendirgi et al., 2005; Rodriguez et al., 2004).

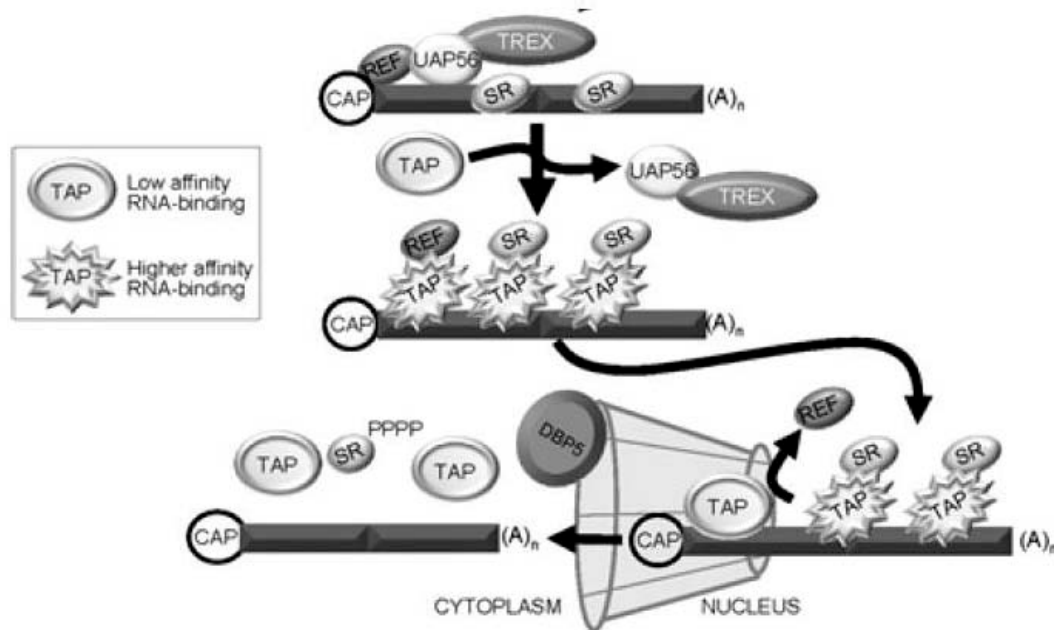


Figure 1.3 Simplified view of the role of adapters in mRNA export. The THO/TREX complex, EJC (not shown) and SR proteins are deposited on mRNAs as a consequence of splicing. Binding of these proteins by the export protein TAP increases the affinity of TAP for RNA. Interaction of TAP with nucleoporins mediates mRNA export, during which REF likely dissociates. After export dissociation of TAP is mediated by DBP5, in yeast at least. Adapted from (Walsh et al., 2010).

In the cytoplasm the cap binding complex is replaced by eukaryotic translation initiation factor (eIF) 4E and PABPN1 is replaced by cytoplasmic poly(A)-binding proteins (PABPs). This exchange is generally attributed to high cytoplasmic concentrations of eIF4E and PABPs (Carmody and Wentz, 2009). However, translation of the newly exported mRNA has recently been suggested to accelerate the exchange of PABPN1 for PABP1 and the interaction of CBP80 with importin- α suggested to be involved in the dissociation of the CBC (Sato and Maquat, 2009).

1.1.5 mRNA stability

1.1.5.1 Mechanism of mRNA turnover

In the cytoplasm, the stability of messenger RNAs is regulated by several mechanisms in order to control their availability for translation into protein. PABPs bound to the poly(A)-tail and eIF4E bound to the cap are generally regarded as having a protective role against degradation (Bernstein et al., 1989; Schwartz and Parker, 2000). Shortening of the poly(A) tail (deadenylation) is thought to initiate degradation of bulk mRNA (Reviewed in (Garneau et al., 2007). Two complexes, Caf1-CCR4 and PAN2-PAN3, are thought to mediate most eukaryotic cytoplasmic deadenylation. In humans and some other eukaryotes but not yeast, an additional deadenylase has been identified, PARN (poly(A)-specific ribonuclease), though its contribution to bulk deadenylation *in vivo* is not clear (Yamashita et al., 2005). Deadenylated mRNAs are committed to destruction when they are targeted by either the exosome, a 3'-5' exoribonuclease complex, or by decapping enzymes which leave the mRNA susceptible to 5'-3' degradation by the exoribonuclease XRN1 (Garneau et al., 2007) (Figure 1.4).

Alternative decay pathways have also been reported to operate on specific mRNAs such as deadenylation-independent decapping (e.g. (Badis et al., 2004) and endonucleolytic cleavage (e.g. (Gill et al., 2004). Deadenylation-independent decapping is also thought to be one pathway used for the degradation of nonsense-mediated decay substrates in yeast (Cao and Parker, 2003) and endonucleolytic cleavage is implicated in multiple mRNA surveillance pathways (see Chapter 1.1.5.3). Endonucleolytic cleavage by argonaute-2 also facilitates degradation of mRNAs targeted by short interfering RNAs (siRNAs)(Liu et al., 2004).

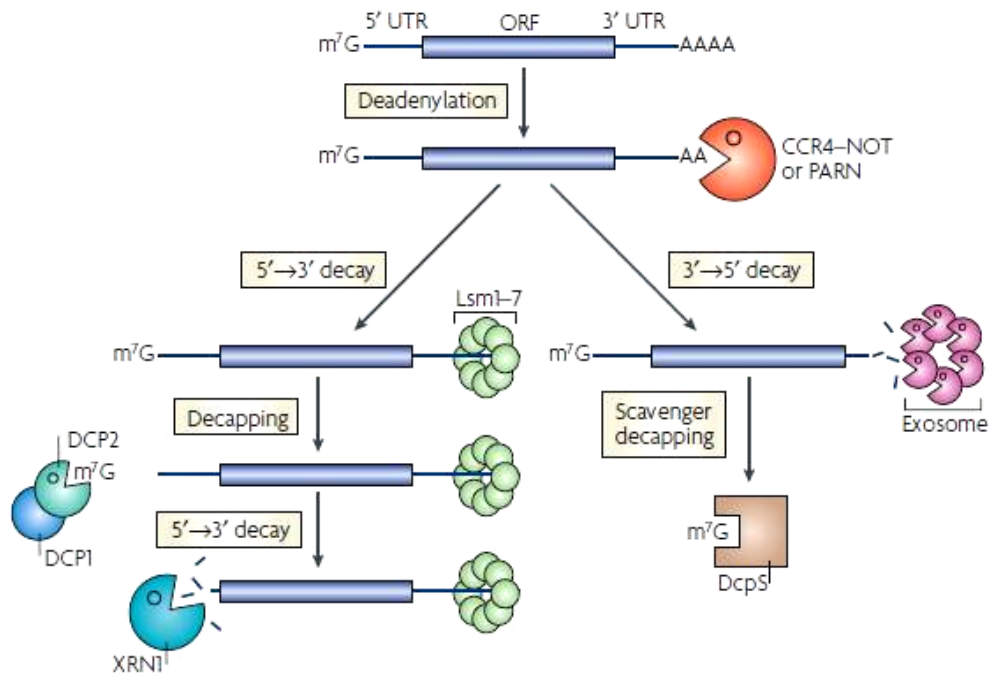


Figure 1.4 Deadenylation-dependent mRNA degradation The mRNA is first deadenylated, perhaps by CCR4-NOT or PARN. Following deadenylation, mRNA can be subject to decapping followed by 5'-3' decay, or 3'-5' decay. Decapping is achieved by the DCP1-DCP2 complex and is promoted by the Lsm1-7 complex which associates with the 3' end of the mRNA. The 5'-3' exoribonuclease XRN1 is then able to degrade the message. Alternatively, the deadenylated mRNA may be degraded by the exosome (3'-5') with hydrolysis of the remaining cap structure achieved by the scavenger-decapping enzyme Dcp5. Taken from (Garneau et al., 2007).

The location of mRNA degradation within the cytoplasm is subject to much debate. Many components of the canonical deadenylation-dependent decay pathway are enriched in cytoplasmic foci named processing bodies or P bodies, including deadenylases, decapping enzymes and XRN1 (Cougot et al., 2004; Sheth and Parker, 2003). P bodies are observed to enlarge and increase in number when cells are exposed to stress or when mRNA decay is impaired which is thought to reflect an increase in decay substrates (Cougot et al., 2004; Sheth and Parker, 2003). As decay intermediates have been found in P-bodies (Sheth and Parker, 2003), they are believed to be sites of active decay, however the steps that occur within P bodies, and the portion of cellular mRNA decay that occurs within them is not clear. Furthermore, mRNAs are able to return to polysomes from P bodies (Bregues et al., 2005), suggesting a broader role that might include mRNA sorting or storage. A

recent study however, indicated that decay may also occur on mRNAs that are being actively translated (Hu et al., 2009), further complicating the picture of where decay occurs and its links to translation.

1.1.5.2 Regulation of mRNA stability/turnover

The stability of mRNAs can be regulated by RNA binding proteins which bind to sequence elements, often but not exclusively located in the 3' UTR (Garneau et al., 2007). One of the best studied of these are AU-rich elements (AREs). The stability of ARE-containing mRNAs is regulated by ARE-binding proteins such as embryonic lethal abnormal vision (ELAV) proteins (e.g. HuR and HuD) which have a stabilising function and tristetraprolin (TTP) and KH splicing regulatory protein (KSRP) which recruit deadenylases and decay enzymes to the message (Garneau et al., 2007).

RNA-binding proteins may also act to prevent access by endonucleases. For example, the regulation of transferrin receptor (TfR) mRNA stability is mediated by five iron-responsive elements (IREs) in its 3' UTR. When intracellular iron concentration is high, TfR is degraded by endonucleolytic cleavage at a site between the IREs (Binder et al., 1994). When intracellular iron concentrations are low, the affinity of iron-regulatory protein (IRP) for RNA increases. IRP binding to IREs then occludes the cleavage site in the TfR 3' UTR and stabilises the mRNA (Schlegl et al., 1997).

mRNA degradation is also directed by microRNAs (miRNAs). miRNAs are ~22 nucleotide endogenous RNAs that down regulate expression of partially complementary target mRNAs (Reviewed in (Filipowicz et al., 2008). miRNAs are known to inhibit mRNA translation (see Chapter 1.3.3.4) and mediate deadenylation by Caf1-CCR4 (Behm-Ansmant et al., 2006; Eulalio et al., 2009). PABP1 has recently been found to be important for this deadenylation (Chapter 1.4.3.4)(Fabian et al., 2009; Walters et al., 2010; Zekri et al., 2009). Target mRNAs are subsequently degraded by the normal deadenylation-dependent degradation pathways (Behm-Ansmant et al., 2006). The interplay between translational inhibition and

destabilisation of miRNA targets however, is not yet clear; one recent report presented data suggesting deadenylation precedes translational inhibition (Beilharz et al., 2009) while another suggested the opposite (Fabian et al., 2009), though these apparently conflicting results may reflect the presence of multiple mechanisms for translational repression by miRNAs.

1.1.5.3 mRNA surveillance

mRNA surveillance mechanisms operate in the cytoplasm to mediate degradation of non-functional and potentially harmful mRNAs. Non-sense mediated decay (NMD) ensures that mutant transcripts containing premature termination codons (PTCs) are degraded in order to avoid the potentially deleterious consequences of producing truncated proteins. PTCs are thought to be detected by the mRNP composition downstream of the terminating ribosome (Rebbapragada and Lykke-Andersen, 2009; Shyu et al., 2008). An EJC downstream of a stop codon implies there is a coding exon downstream and marks the stop codon as a PTC promoting NMD (Palacios et al., 2004; Singh et al., 2007). In contrast, proximity of PABP1 downstream of a stop codon marks it as a valid termination point and antagonises NMD (Behm-Ansmant et al., 2007; Ivanov et al., 2008; Singh et al., 2008). Following PTC recognition the mRNA is rapidly degraded though the degradation pathway used however, appears to differ between species with deadenylation, decapping, and endonucleolytic cleavage by SMG6 implicated in human cells (Eberle et al., 2009; Huntzinger et al., 2008; Lejeune et al., 2003).

Non-stop decay (NSD) mediates the degradation of mRNAs without a stop codon. These may be generated by mutation or premature polyadenylation and result in translation by ribosomes through the coding region and into the poly(A)-tail, where they are thought to displace PABP1 and stall at the end of the message. Release of the stalled ribosome is mediated by Ski7 which also recruits the SKI complex (Ski2, Ski3, Ski8) and the exosome to facilitate 3'-5' mRNA degradation (van Hoof et al., 2002). In the absence of Ski7 however, removal of PABP1 is also thought to lead to deadenylation, decapping and 5'-3' degradation of NSD substrates (Inada and Aiba, 2005). Though this molecular mechanism was dissected in yeast,

non-stop mRNAs are also degraded in mammalian cells (Frischmeyer et al., 2002) suggesting conservation of the pathway.

A further surveillance pathway has recently been described in yeast. No-go decay (NGD) involves the endonucleolytic cleavage of mRNAs on which elongating ribosomes are stalled (Doma and Parker, 2006). NGD also functions in *Drosophila* cells (Passos et al., 2009), however the conservation and importance of this pathway in higher eukaryotes remains untested.

1.1.6 mRNA localisation

Regulation of mRNA localisation and its subsequent translation is an important mechanism to generate different distributions of proteins within cells. Localisation of mRNAs is important for patterning during embryonic development in many species and many mammalian mRNAs are now known to be specifically enriched at the pseudopodia of migrating fibroblasts, neuronal dendrites and mitotic spindles (reviewed in (Holt and Bullock, 2009)). Localisation of mRNAs can be achieved by cytoskeletal active transport of mRNA and anchorage at its destination site, and/or selective local protection from degradation. These mechanisms are generally mediated by trans-acting factors recruited to sequence elements often positioned in the 3' UTR of mRNAs (Holt and Bullock, 2009). Localised mRNAs are generally translationally silenced during transport by translational repressors which are inactivated by other proteins or signalling events at the mRNA's destination (reviewed in (Besse and Ephrussi, 2008)). For example, the calcium/calmodulin dependent protein kinase II α isoform (α CaMKII) regulates vesicle release at synapses and is one of the best characterised dendritically localised mRNAs (Gavis et al., 2007). Within its 3' UTR α CaMKII features two cytoplasmic polyadenylation elements (CPEs), which during transport are bound by CPEB which mediates translation repression via interaction with maskin (see Chapter 1.3.3.3)(Huang et al., 2003; Wu et al., 1998). Receptor activation at the synapse however, activates Aurora kinase resulting in CPEB phosphorylation, maskin dissociation and CaMKII α polyadenylation and translational activation (Huang et al., 2002)(see Chapter 1.3.3.5).

1.2 Eukaryotic translation

Translation can be viewed as three steps. Initiation as the ribosome is assembled on the mRNA and identifies and commits to a translation start codon. Elongation, as it moves processively along the message catalyzing the joining of amino acids supplied by transfer RNAs (tRNAs); and termination when the complete polypeptide is released upon recognition of a stop codon.

1.2.1 Initiation

Eukaryotic translation initiation is a complex multi-step process requiring numerous eukaryotic initiation factors (eIFs). Translation initiation on most RNAs is cap-dependent and can be considered as six key events which are described in detail below and illustrated in Figure 1.5.

1. Ternary complex formation

The first step in initiation is the formation of the ternary complex consisting of eIF2-GTP and the initiator Met-tRNA (Met-tRNA_i^{met}) (Figure 1.5 - stage 1). The function of this complex is to deliver Met-tRNA_i^{met} to the ribosome and to participate in start codon recognition. eIF2 is a heterotrimeric complex consisting of a large γ -subunit and smaller α and β subunits. The γ -subunit binds GTP and Met-tRNA_i^{met} and is homologous to elongation factor 1A (eEF1A) which delivers acylated tRNAs to the elongating ribosome (Pestova et al., 2007). The α -subunit is proposed to stabilise the interaction between eIF2 γ and Met-tRNA_i^{met} (Yatime et al., 2004) and is important for regulation of eIF2 activity (see Chapter 1.3.2.1). The β -subunit binds eIF2B and eIF5 (Asano et al., 1999). Sequence differences between the initiator Met-tRNA and the Met-tRNA which participates in translation elongation (Met-tRNA_m^{met}) prevent association of Met-tRNA_i^{met} with eEF1 and are important for eIF2-GTP binding (Drabkin et al., 1998; Farruggio et al., 1996).

eIF2B is a guanine nucleotide exchange factor (GEF) and stimulates exchange of GDP for GTP on eIF2 γ . eIF2-GTP has higher affinity for Met-tRNA_i^{met} compared to eIF2-GDP which is released at the end of initiation (Kapp and Lorsch, 2004a). eIF2-GDP is therefore inactive and must be converted to eIF2-GTP to participate in further rounds of initiation.

2. Binding of the ternary complex to the small (40S) ribosomal subunit to form the 43S complex

eIF1, eIF1A and eIF3 have been shown to promote binding of the eIF2-GTP-Met-tRNA_i^{met} ternary complex to the 40S ribosome (Chaudhuri et al., 1999; Majumdar et al., 2003). In budding yeast, eIF5 has also been shown to promote 43S complex formation and is able to interact with eIF1, eIF2 β and eIF3 simultaneously (Singh et al., 2004). Furthermore, it has been proposed that in yeast a pre-formed multifactor complex of eIF1, eIF2, eIF3 and eIF5 binds the 40S ribosome (Asano et al., 2000; Hinnebusch et al., 2007). In mammals, the interaction of eIF5 with eIF2 β and eIF3 is conserved (Bandyopadhyay and Maitra, 1999; Das et al., 1997) but an interaction with eIF1 has not been reported and its role in 43S complex formation has not been assessed. Moreover, in mammals, eIF1, eIF1A and eIF3 are suggested to be recruited to the 40S ribosomal subunit during ribosome recycling (Pisarev et al., 2007) (see Chapter 1.2.4) and so the order of translation factor binding to the 40S ribosome may differ between eukaryotic species.

3. Binding of the 43S complex to mRNA

43S pre-initiation complexes are able to bind an unstructured 5' UTR without additional factors or ATP (Pestova and Kolupaeva, 2002). However, most mRNAs do contain a degree of secondary structure and are thought to require additional factors, eIF4F and eIF4B to unwind the 5' UTR and prepare a landing site for the 43S complex (Pestova et al., 2007). The eIF4F complex consists of the cytoplasmic cap-binding protein eIF4E, the scaffold protein eIF4G and the DEAD-box RNA helicase eIF4A. eIF4E alone binds the cap weakly, however, the eIF4G-eIF4E

interaction strongly enhances the affinity of eIF4E for the cap (Ptushkina et al., 1998; von Der Haar et al., 2000). eIF4G also interacts with eIF3 (Lamphear et al., 1995) and PABP1 (see Chapter 1.4.3) and the interaction with PABP1 further enhances the affinity of eIF4E for the cap (Borman et al., 2000; von Der Haar et al., 2000; Yanagiya et al., 2009). The helicase activity of eIF4A is increased by its incorporation into eIF4F and is further stimulated by cofactors eIF4B or eIF4H (Rogers et al., 2001). eIF4H is homologous to a part of eIF4B and stimulates RNA unwinding by eIF4A to a similar degree (Rogers et al., 2001), however, it is not known whether these cofactors are recruited in mRNA specific or regulated manner.

The order of mRNA binding by eIF4F complex components is not solved. Given the low affinity of eIF4E for the cap, it has been suggested that eIF4E-eIF4G at least bind the cap together (Gingras et al., 1999). Conversely, eIF4G is able to bind 43S pre-initiation complexes suggesting it may be recruited to an eIF4E-bound mRNA with the 40S ribosome (Joshi et al., 1994).

In addition to unwinding the 5' of the mRNA, eIF4F is thought to promote 43S complex joining by the direct interaction between eIF4G and eIF3 (Imataka and Sonenberg, 1997; Lamphear et al., 1995) since the central portion of eIF4G which interacts with eIF3 and eIF4A is sufficient to direct ribosomal recruitment to an internal site in an mRNA to which it is tethered (De Gregorio et al., 1999). eIF3 also interacts with eIF4B (Methot et al., 1996) and another eIF4A interacting protein poly(A)-binding protein interacting protein 1 (PAIP1) (Martineau et al., 2008) and these interactions may also contribute to 43S complex recruitment.

4. Ribosome scanning

Once bound, 43S complexes scan the 5'UTR in a 5'-3' direction to find the AUG initiation codon (Pestova and Kolupaeva, 2002). This model is supported by the observation that secondary structure in the 5' UTR or repressor protein complexes can block scanning and impair detection of the AUG (Kozak, 1991; Paraskeva et al., 1999). Scanning is thought to be accompanied by continued unwinding of the mRNA since movement of the 40S ribosome on UTRs with even weak secondary structure requires ATP and eIF4F/eIF4B helicase activity (Pestova

and Kolupaeva, 2002). eIF1 and eIF1A are also required for scanning (Pestova and Kolupaeva, 2002), likely due to the conformational change their binding has on the 40S ribosome, which opens the mRNA binding channel (Passmore et al., 2007).

The fate of cap-associated factors during scanning is not clear. As eIF4F significantly enhances scanning on structured 5' UTRs (Pestova and Kolupaeva, 2002), eIF4G has been proposed to move with the scanning ribosome (Jackson, 2000; Jackson et al., 2010; Pestova et al., 2007), as depicted in Figure 1.5 (stage 4). Recent evidence indicates that eIF4E remains associated with the cap during scanning since it could not be identified in a cross-linking study more than two nucleotides from the cap (Lindqvist et al., 2008). Cross-linking of eIF4G however, was not detected more than twelve nucleotides from the cap suggesting it does not accompany the scanning ribosome (Lindqvist et al., 2008). By contrast, eIF4A, eIF4B and eIF4H were detected 52 nucleotides from the cap, though less efficiently with increasing distance, and the authors of this study suggested that multiple molecules of eIF4A with eIF4B/H may bind the 5' UTR and prepare the mRNA for scanning (Lindqvist et al., 2008).

5. Start codon identification

Scanning occurs until the ribosome reaches the first start codon in sufficiently good context. The optimal context was initially characterised by sequence comparisons and selective mutagenesis as GCC(A/G)CCAUGG with the purine at -3 and a G at +4 (relative to the start of the AUG codon) being the most important (Kozak, 1991). Later, further nucleotides downstream (at +5 and +6) were also found to effect the efficiency of initiation codon recognition (Boeck and Kolakofsky, 1994; Grunert and Jackson, 1994).

Although recognition of the AUG is directly conducted by complementarity with the Met-tRNA_i^{met} anticodon, eIF1 and eIF1A are also crucial for efficient and accurate start site selection (Fekete et al., 2007; Pestova et al., 1998; Pestova and Kolupaeva, 2002). Start codon recognition is followed by a number of other structural rearrangements within the scanning complex which lead to commitment to initiation at that codon. An interaction between eIF1A and eIF5 is induced, which

tightens the association of eIF1A with the 40S ribosome (Maag et al., 2006) and eIF1 is displaced, which leads to a conformational change of the complex, locking it on the mRNA (Maag et al., 2005; Passmore et al., 2007). Irreversible commitment to an AUG occurs after hydrolysis of eIF2-GTP and release of the inorganic phosphate (Algire et al., 2005). This reaction is mediated by eIF5, which is a GTPase activating protein (GAP) and is only able to activate the GTPase activity of eIF2 γ bound to 40S ribosomes (Das and Maitra, 2001). Premature hydrolysis of eIF2-GTP is prevented by eIF1 (Algire et al., 2005; Unbehaun et al., 2004) however, eIF1 displacement following codon-anticodon pairing releases this repression allowing eIF2-GTP hydrolysis. GTP hydrolysis reduces the affinity of eIF2 for the Met-tRNA_i^{met} and it is thought that this leads to partial dissociation of eIF2-GDP from the 48S complex (Kapp and Lorsch, 2004a; Pisarev et al., 2006).

6. Recruitment of the large (60S) ribosomal subunit to form an elongation competent 80S complex.

Joining of the 60S subunit and displacement of eIF1A, eIF2-GDP, eIF3 and eIF5 is mediated by eIF5B (Pestova et al., 2000). eIF5B is a ribosome-dependent GTPase (Pestova et al., 2000) which is thought to be recruited to the 48S complex by eIF1A (Fringer et al., 2007) and promotes the recruitment of the 60S ribosome subunit (Acker et al., 2006; Fringer et al., 2007). 60S joining is required for the displacement of residual eIF2-GDP, eIF3 and eIF5 (Unbehaun et al., 2004) and release of eIF1A (and eIF5B) is dependent on eIF5B-GTP hydrolysis (Acker et al., 2009; Pestova et al., 2000) after which the 80S complex is ready for translation elongation.

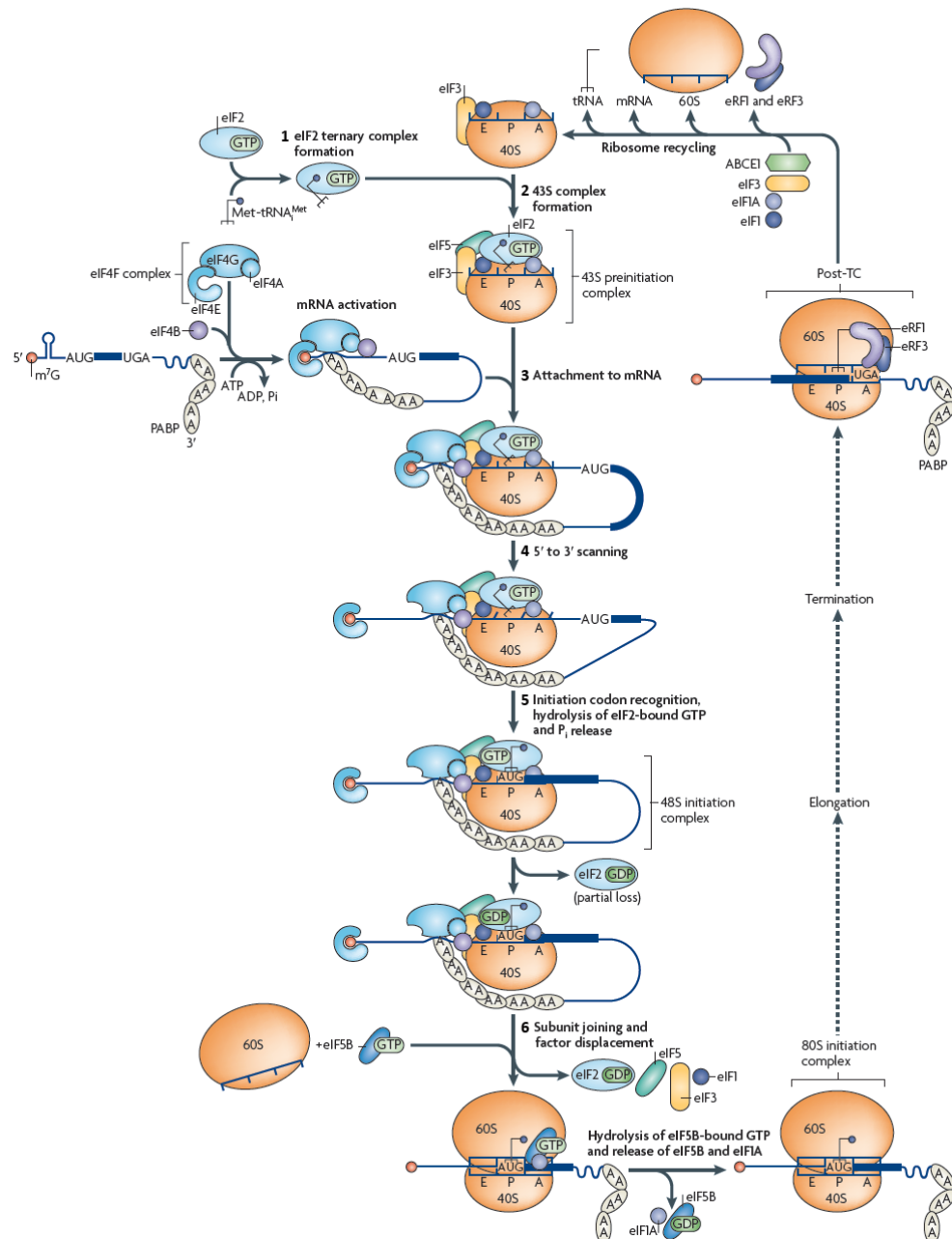


Figure 1.5 Model of the canonical pathway of eukaryotic translation initiation. Eukaryotic translation initiation divided into six stages following recycling of post-termination complexes to separate 40S and 60S ribosomal subunits. (1) eIF2-GTP-Met-tRNA^{Met} ternary complex formation; (2) 43S preinitiation complex formation comprising of a 40S subunit, eIF1, eIF1A, eIF3, eIF2-GTP-Met-tRNA^{Met} and likely eIF5; (3) recruitment of the 43S complex to the mRNA following mRNA unwinding by eIF4F with eIF4B; (4) 43S complex scanning of the 5' UTR (5' to 3'); (5) initiation codon recognition and 48S initiation complex formation leading to eIF1 displacement allowing eIF5-mediated hydrolysis of eIF2-bound GTP and P_i release; (6) 60S subunit joining to the 48S complex and displacement of eIF2-GDP and other factors (eIF3, eIF4B, eIF4F and eIF5) by eIF5B followed by GTP hydrolysis by eIF5B and release of eIF1A and eIF5B-GDP from elongation-competent 80S ribosomes. Modified from (Jackson et al., 2010).

1.2.2 Initiation factor isoforms

Several initiation factors are expressed as multiple isoforms. In mammals, eIF4A is expressed as two functionally indistinguishable isoforms, eIF4AI and eIF4AII which are expressed from different genes in a tissue-specific manner (Li et al., 1999; Nielsen and Trachsel, 1988). The EJC component eIF4AIII however, interacts differently with eIF4G and cannot substitute for eIF4AI/II in translation (Li et al., 1999).

In humans (and yeast) eIF4G is expressed as two full-length homologs, eIF4GI and eIF4GII, which maintain the same interactions with translation factors and both stimulate translation (Gradi et al., 1998a) (Imataka et al., 1998). The two forms appear to differ in their regulation however, as they are differentially sensitive to certain viral proteases (Gradi et al., 1998b; Svitkin et al., 1999) and some phosphorylated residues of eIF4GI are not conserved in eIF4GII (Raught et al., 2000). eIF4GI is also expressed as multiple isoforms which vary in length (Byrd et al., 2002, 2005) and differ in their ability to stimulate translation (Coldwell and Morley, 2006).

p97, also known as death-associated protein 5 (DAP5) is a further eIF4G family protein which is expressed in mammals but lacks the N-terminal portion of eIF4GI/II which interacts with eIF4E and PABP1 (Imataka et al., 1997). The role of p97 in cap-dependent translation is not clear since its overexpression in mammalian cells has been reported to induce both translational repression and activation (Imataka et al., 1997; Lee and McCormick, 2006). Recently p97 has been shown to be associated with polyribosomes (i.e. messages bound by more than one actively translating ribosome) and to activate translation in tether assays (Nousch et al., 2007) supporting an activator role. p97 is also important for cap-independent translation of certain messages containing internal ribosome entry sites (IRESs; see Chapter 1.3.3.1) (Hundsdoerfer et al., 2005; Lewis et al., 2008) and the caspase-cleaved form, p86 is also involved in IRES-mediated translation during certain stress conditions and apoptosis (Henis-Korenblit et al., 2002; Lewis et al., 2008). Several different cytoplasmic poly(A)-binding proteins are also expressed in higher eukaryotes which will be discussed in more detail later (Chapter 1.4.6).

1.2.3 Elongation

Translation elongation begins with a peptidyl tRNA in the peptidyl 'P' site within the 80S ribosome (Figure 1.6). A ternary complex of GTP, elongation factor 1A (eEF1A) and an aminoacyl tRNA then enters the empty aminoacyl 'A' site (Kapp and Lorsch, 2004b). If there is correct base pairing between the aminoacyl tRNA anticodon and the mRNA codon, conformational changes within small ribosomal subunit RNA and GTP hydrolysis by eEF1A result in release of the tRNA from eEF1A-GDP. The released aminoacyl tRNA is then able to form a peptide bond with the adjacent peptidyl tRNA catalyzed by the ribosomal peptidyl transferase. Translocation of the complex must then occur so that the deacylated tRNA moves to the exit 'E' site, the new peptidyl tRNA is in the P site and the next codon of mRNA is shifted to the A site. Translocation is carried out by elongation factor 2 and requires GTP hydrolysis. The ribosome continues to translocate along the message until it encounters a stop codon (Kapp and Lorsch, 2004b).

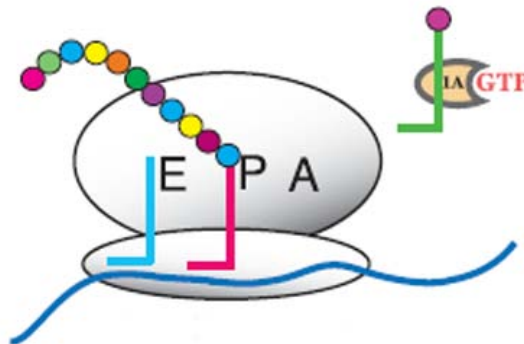


Figure 1.6 Translation Elongation. The 80S ribosome contains 3 sites through which tRNAs translocate. Aminoacyl tRNAs are recruited to the 'A' site in the 80S ribosome by eEF1A. Correct codon-anticodon pairing by the aminoacyl tRNA permits release of eEF1A and peptide bond formation with the adjacent peptidyl tRNA. The deacylated tRNA moves to the exit 'E' site and the new peptidyl-tRNA is now in the 'P' site. Adapted from (Kapp and Lorsch, 2004b).

1.2.4 Termination and ribosome recycling

Termination of translation is signalled by a termination codon (UAG, UAA or UAG) entering the ribosome A site (Ehrenberg et al., 2007). These codons are not matched with tRNA-anticodons but are bound by release factors which stimulate release of the polypeptide chain linked to the P site tRNA (Figure 1.7). Release factor 1 (eRF1) functionally and structurally mimics a tRNA and recognises the termination codon directly. A conserved GGQ motif located in the portion of eRF1 analogous to the tRNA aminoacyl group is thought to mediate nucleophilic attack on the ester bond of the peptidyl-tRNA resulting in hydrolysis and liberation of the complete polypeptide. The GTPase release factor 3 (eRF3) binds and stimulates the activity of eRF1 (Ehrenberg et al., 2007).

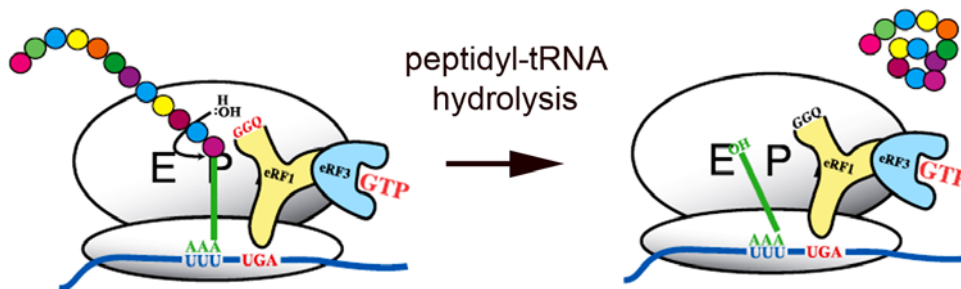


Figure 1.7 Termination of translation. The stop codon is recognised by eRF1 which mediates nucleophilic attack on the peptidyl-tRNA, releasing the polypeptide. eRF1 is bound and stimulated by eRF3. Adapted from (Kapp and Lorsch, 2004b).

Following termination the 80S ribosome remains associated with the mRNA and contains a deacylated tRNA in the P site. This post-termination complex is recycled into free 60S and tRNA and mRNA bound 40S subunits. In a mammalian *in vitro* translation system, eIF3 was recently shown to mediate this dissociation, an activity enhanced by eIF1, eIF1A and the loosely associated eIF3 subunit, eIF3j (Pisarev et al., 2007). The ATP-binding cassette family protein ABCE1 is also thought to facilitate subunit separation (Pisarev et al., 2010). Subsequent tRNA release has been shown to be promoted by eIF1 and 40S dissociation from mRNA promoted by eIF3j (Pisarev et al., 2007). How these factors are recruited to the post-termination complex however, is not yet clear. eIF6 binding to the 60S subunit is suggested to prevent re-association with the 40S subunit (Ceci et al., 2003).

1.3 Eukaryotic translational regulation

1.3.1 Importance of regulated translation

Regulation of gene expression at the level of translation produces a rapid response in the level of protein synthesis and represents an additional platform to expand genomic diversity by mechanisms such as AUG selection (reviewed in (Mathews et al., 2007)). Translational control also permits the fine tuning of the expression of genes already regulated at other levels, for example *c-myc* undergoes tightly controlled transcription, mRNA stability and translation (Marcu et al., 1992; Stoneley et al., 2000). In the absence of transcriptional control, for example in reticulocytes and oocytes, translational regulation is particularly crucial. Furthermore, spatial control of protein production within the cell can be utilised to achieve concentration gradients important in the patterning of early development and in the synaptic plasticity of neurons (reviewed in (Mathews et al., 2007)). The importance of proper translational control is emphasized by the many human diseases associated with its dysregulation, discussed later (Chapter 1.3.4)

Translational regulation mechanisms overwhelmingly target the initiation stage though there are well documented examples of control at the elongation and termination stages (Mathews et al., 2007). Regulation may be divided into two broad groups; 'specific' that targets particular mRNAs and 'global' that affects the majority of cellular mRNAs. Specific control is largely mediated by sequence elements in the 5' or 3' UTRs of messages while global control is often achieved by altering the phosphorylation status of translation factors or their regulators. Illustrative examples of each are explained below which demonstrate the breadth and variety of translation regulation mechanisms.

1.3.2 Global control of translation

1.3.2.1 Phosphorylation of eIF2

The first steps in translation initiation are the formation of the ternary complex of eIF2-GTP with Met-tRNA_i^{met} and its association with the small ribosomal subunit. eIF2B catalyses the exchange of GDP for GTP on eIF2 which is essential for eIF2 to be reused following each round of initiation. Phosphorylation of serine 51 on the eIF2 α subunit inhibits dissociation of eIF2B from eIF2, blocking GTP exchange and sequestering eIF2B (reviewed in Ron and Harding, 2007). eIF2 α phosphorylation consequently has the effect of decreasing global translation. The amount of eIF2B is limiting compared to eIF2 (~6:1 ratio of eIF2:eIF2B in RRLs) (Oldfield et al., 1994), therefore a small increase in eIF2 α phosphorylation can result in a large degree of translational inhibition. It is important to note that while eIF2 α phosphorylation results in global inhibition of translation, a small subset of mRNAs are translationally upregulated under these conditions (see Chapter 1.3.3.4).

eIF2 α is phosphorylated in response to diverse cellular stress conditions. Attenuation of protein synthesis during stress allows energy and amino acids to be redirected to other pathways where they may be critically needed for survival and relieves the load of the protein folding machinery (reviewed in (Ron and Harding, 2007). Four eIF2 α kinases have been described (reviewed in (Dever et al., 2007; Wek et al., 2006). GCN2 (general control non-repressible-2) kinase is activated by amino acid starvation. PKR (protein kinase activated by double-stranded RNA) is activated by infection with certain viruses (Dever et al., 2007). Endoplasmic reticulum (ER) stress activates the transmembrane protein PERK (PKR-like ER localised kinase) and HRI (haem-regulated inhibitor), which is highly expressed in erythroid cells, is activated by low haem levels as well as osmotic shock, heat shock and arsenite treatment (Dever et al., 2007; Lu et al., 2001; McEwen et al., 2005). Activation of eIF2 α kinases is not limited to these stresses and certain stresses may activate multiple eIF2 kinases; hypoxia and ultraviolet (UV) irradiation for example activate both PERK and GCN2 (Deng et al., 2002; Liu et al., 2010) (Jiang and Wek, 2005) (Jiang and Wek, 2005; Wu et al., 2002). There is also evidence that

dephosphorylation of eIF2 α by protein phosphatase 1 (PPP1) in a complex with GADD34 (growth arrest and DNA damage gene 34) is regulated (Garcia-Bonilla et al., 2007; Vander Mierde et al., 2007) and the stress-induced expression of GADD34 is thought to negatively feed back to relieve eIF2 α phosphorylation and promote translation recovery (Lee et al., 2009; Novoa et al., 2001).

The importance of translational regulation mediated by eIF2 α phosphorylation is demonstrated by several mouse models. PERK $-/-$ mice show early onset diabetes and exocrine pancreatic dysfunction as they are unable to protect secretory cells from protein misfolding in the ER (Harding et al., 2001). Furthermore, substitution of eIF2 α serine 51 for alanine, rendering eIF2 α non-phosphorylatable on this residue, results in the death shortly after birth of homozygous mice due to hypoglycaemia (Scheuner et al., 2001). CReP is a GADD34 homolog that, as a complex with PP1, is thought to form the constitutive cellular eIF2 α phosphatase in mammalian cells (Jousse et al., 2003). Loss of CReP leads to stunted growth and perinatal lethality and compound mutants lacking both GADD34 and CreP fail to develop beyond the preimplantation stage (Harding et al., 2009).

1.3.2.2 Phosphorylation of eIF4E binding proteins

eIF4E binding proteins (4E-BPs) are a group of translational repressors that compete for binding of eIF4E with eIF4G (reviewed in (Raught and Gingras, 2007)). Binding is dependent on hypophosphorylation of 4E-BPs and results in inhibition of 43S complex recruitment and a decrease in cap-dependent translation (Svitkin et al., 2005). Hyperphosphorylation triggers 4E-BP dissociation from eIF4E relieving the inhibition of translational initiation (Pause et al., 1994). In common with the regulation of eIF2, 4E-BP-mediated global repression is thought to help conserve energy and permit translation of specific mRNAs required for the stress response (Raught and Gingras, 2007).

Mammals express three 4E-BPs which are highly conserved, though display varied tissue distribution (Poulin et al., 1998; Tsukiyama-Kohara et al., 1996). Phosphorylation of 4E-BP1 can occur on multiple sites and is primarily controlled by

the PI3K/mTOR (phosphoinositide 3-kinase/mammalian target of rapamycin) pathway (Gingras et al., 2001; Mothe-Satney et al., 2000). This pathway processes extra-cellular cues from hormone receptors, growth factor receptors and nutrient sensors to allow global translation only when there are adequate nutrients (Raught and Gingras, 2007). Proteins which interact with eIF4E and inhibit eIF4F formation can also be recruited to specific mRNAs, a mode of specific translational regulation which is discussed later (Chapter 1.3.3.5).

1.3.2.3 Global control by other mechanisms

eIF2 and 4E-BPs are two examples of how cap-dependent translation initiation can be regulated by the phosphorylation of translation factors or their regulators. Other initiation factors have also been reported to be phosphorylated (e.g. eIF3 subunits, eIF4B, eIF4E, eIF4G) however, the effect of phosphorylation on their activity has not been elucidated (reviewed in (Raught and Gingras, 2007)). The phosphorylation status of elongation factors (eEF2) and ribosomal proteins (rpS6, rpS2) has also been suggested to result in changes in protein synthesis (Carlberg et al., 1990; Rother and Strasser, 2007; Roux et al., 2007).

eIF4E is not the only factor targeted by regulatory binding proteins. Global inhibition is also effected by programmed cell death protein 4 (Pcd4) (Yang et al., 2003). Pcd4 is a tumour suppressor protein that prevents cellular transformation (Yang et al., 2001) and is upregulated during apoptosis (Goke et al., 2002). It shares homology with eIF4G (Goke et al., 2002) and acts as an inhibitor of eIF4A helicase activity by preventing its inclusion into eIF4F and trapping it in an inactive conformation (Chang et al., 2009; Loh et al., 2009; Suzuki et al., 2008).

Proteolytic cleavage of translation factors is also known to affect global cellular translation. Apoptotic caspase-3 cleaves eIF4G (Bushell et al., 2000a), eIF4B (Bushell et al., 2000b) and eIF3j (Clemens et al., 2000) and proteolysis of translation factors is thought to contribute to the shut-down of translation during apoptosis (Bushell et al., 2004). Some viruses also use this trick and express proteases that target initiation factors that are not required for viral RNA translation (Lloyd, 2006; Mohr et al., 2007). The resulting inhibition of host translation enables increased

synthesis of viral proteins and limits the production of cellular antiviral defence components (Mohr et al., 2007).

1.3.3 Specific control of translation

The m⁷G cap and poly(A)-tail are canonical features that promote translation of messages however, elements within mRNAs can also regulate the translation of specific mRNAs (Figure 1.8)(reviewed in (Gebauer and Hentze, 2004; Hentze et al., 2007). Secondary structures such as hairpins in the 5' UTR are usually inhibitory, however complex arrangements of structures can also form internal ribosome entry sites (IRESs) that can mediate translational initiation. Short open reading frames (uORFs) upstream of the main coding ORF of a message usually inhibit translation though can result in specific upregulation in synthesis of proteins in response to certain cellular conditions. RNA binding proteins that act as translational regulators or micro RNAs may also be targeted to particular transcripts by binding to specific sequences often within the mRNA untranslated regions (Gebauer and Hentze, 2004). Often multiple regulatory elements exist on a single mRNA providing a combinatorial effect (Wilkie et al., 2003). Examples of each of these modes of specific regulation are discussed below.

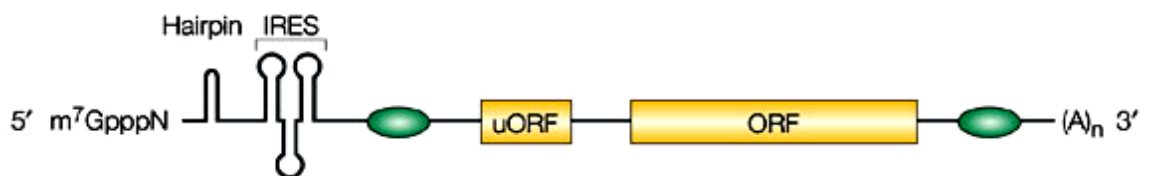


Figure 1.8 mRNA elements that influence translation. RNA secondary structure (e.g. hairpins), IRESSs, uORFs and sequence elements (green ovals) that are bound by regulatory proteins or micro RNAs can affect the translatability of an mRNA. Taken from (Gebauer and Hentze, 2004).

1.3.3.1 Internal ribosome entry sites

First identified in viral mRNAs (Jang et al., 1988; Pelletier et al., 1988), internal ribosome entry sites (IRESs) are formed by complex secondary and tertiary RNA structures (reviewed in (Doudna and Sarnow, 2007; Jackson, 2005). IRESs mediate recruitment of ribosomes to the mRNA, bypassing the requirement for the m⁷G cap and often multiple initiation factors (Jackson, 2005). The initiation factor requirements of IRESs vary greatly. For example, the cricket-paralysis virus IRES requires no eIFs or the Met-tRNA_i^{met} and binds the 40S ribosome directly (Wilson et al., 2000), whereas the encephalomyocarditis virus IRES requires eIF4G (and eIF4A) to recruit the 43S complex (Pestova et al., 1996a; Pestova et al., 1996b). Since viral mRNAs are reliant on the translational machinery of their host, by utilizing IRES-mediated initiation, cap-dependent host-cell translation can be shut off leaving viral mRNAs to appropriate the translational apparatus and attain effective protein synthesis (Doudna and Sarnow, 2007).

Many IRES-containing cellular mRNAs have now been described and frequently encode products involved in growth, differentiation and apoptosis (Elroy-Stein and Merrick, 2007). Characterised cellular IRESs are generally much weaker than viral IRESs but often support translation of products that are potent at low concentration, such as the oncogenic transcription factor c-myc (Elroy-Stein and Merrick, 2007). In certain cellular conditions such as stress, viral infection and mitosis, cap-dependent translation initiation is repressed but IRES-containing messages are able to initiate translation (Spriggs et al., 2008). The activity of cellular and some viral IRESs are dependent on IRES trans-acting factors (ITAFs) which are thought to stabilise IRES structure and regulation of ITAF abundance and sub-cellular localisation has been suggested to regulate IRES activity (Fitzgerald and Semler, 2009).

1.3.3.2 Upstream AUGs and open reading frames

During translation initiation, the small ribosomal subunit is thought to scan 5'-3' from the cap until the first initiation codon in "good context" is encountered (Chapter 1.2.1). Therefore, the presence of out of frame AUGs upstream of the coding ORF are strongly inhibitory and the strength of inhibition is dependent on the position and sequence context of the upstream start codon (Kozak, 1991). The presence of multiple in-frame AUGs may also generate multiple protein products from one message. A notable example of this mode of regulation is the transcription factor CCAAT/Enhancer β (C/EBP β). C/EBP β is an intronless gene but is expressed as two protein isoforms. The full length protein (also referred to as LAP) is an important regulator of liver development and hepatocyte proliferation but a shorter isoform (LIP) which lacks the activation domain and is thought to regulate the function of other C/EBP proteins is produced following liver damage (Luedde et al., 2004). C/EBP β AUG selection is thought to be regulated by binding of calreticulin or CUGBP (CUG repeat binding protein) to a specific site in the 5' UTR (Timchenko et al., 2005).

While upstream AUGs are generally inhibitory, upstream open reading frames (uORFs) can be less so due to the possibility of reinitiation. In certain conditions uORFS can have a positive effect on the translation of a downstream ORF. While the phosphorylation of eIF2 α results in the down-regulation of global translation (Chapter 1.3.2.1), it also results in the activation of a subset mRNAs containing multiple uORFs (Reviewed in (Gebauer and Hentze, 2004). The best characterized example of this mode of regulation is the yeast gene *GCN4* (Figure 1.9) (Gebauer and Hentze, 2004; Hinnebusch et al., 2007).

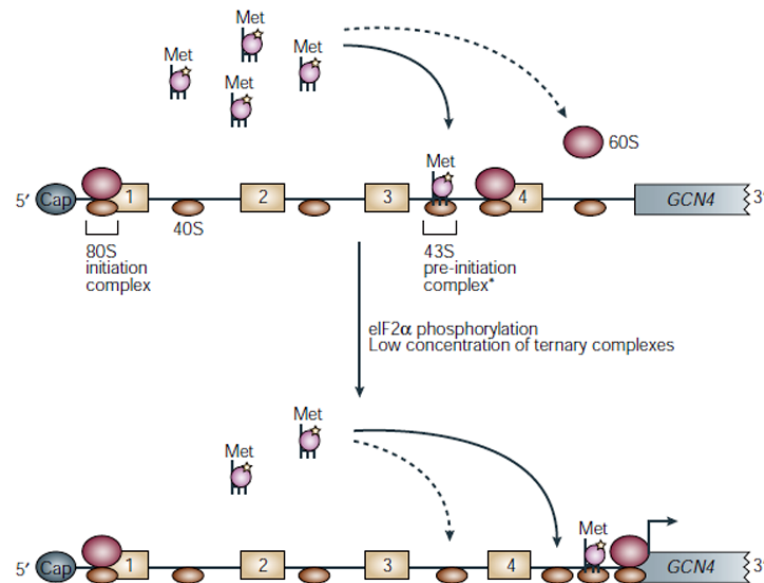


Figure 1.9 Mechanism of GCN4 translational regulation. The frequency of reinitiation at uORFs regulates the translation of *GCN4* mRNA. When amino acids are plentiful (upper panel) reinitiation at uORFs is frequent (solid arrow) and translation of *GCN4* becomes infrequent (dashed arrow). When amino acids are scarce eIF2 α phosphorylation precipitates a reduction in ternary complex availability and increases the probability of scanning 40S subunits reaching the *GCN4* ORF. Taken from (Gebauer and Hentze, 2004).

GCN4 is a transcription factor that activates genes involved in amino acid biosynthesis. Phosphorylation of eIF2 α by *GCN2* following nutrient starvation results in upregulation of *GCN4* translation allowing cells to increase amino acid production while general translation is repressed. Upstream of the *GCN4* initiation codon are four short ORFs (uORF1,2,3 and 4). Following translation of uORF1, the 60S ribosomal subunit is thought to dissociate leaving the 40S small ribosomal subunit still associated with the mRNA. Scanning can then resume but reinitiation requires the addition of a new ternary complex. When eIF2-GTP is plentiful, a new ternary complex is acquired quickly and reinitiation occurs at uORF4. This leads to full dissociation of both ribosomal subunits due to a GC-rich region adjacent to the uORF4 stop codon, inhibiting translation of *GCN4*. In the event of amino acid deprivation, eIF2 α is phosphorylated by *GCN2* kinase and the pool of ternary complexes accordingly diminished. This decreases the likelihood of reinitiation at uORF4 and leads to increased production of *GCN4* (Gebauer and Hentze, 2004; Hinnebusch et al., 2007).

The translation of the mammalian transcription factor, activating transcription factor 4 (*ATF4*) is thought to be regulated in a similar manner. Upregulation of ATF4 leads to induction of additional transcription factors important for the activation of stress responsive genes, including CHOP (C/EBP homologous protein) which activates GADD34 (Harding et al., 2000). ATF4 mRNA contains two uORFs, the second of which partially overlaps with the ATF4 coding region. Reinitiation at the second uORF inhibits translation from the ATF4 ORF; however decreased ternary complex availability caused by eIF2 α phosphorylation during cell stress results in delayed reinitiation resulting in translation of ATF4 (Vattem and Wek, 2004).

1.3.3.3 Trans-acting Factors

The targeted binding of trans-acting factors to sequence elements in specific mRNAs is a frequently used mechanism for translational repression. Such factors often, but not exclusively, inhibit recruitment of the 43S pre-initiation complex. Few factors which bind the 5' UTR have been characterised. The best studied is IRP which, in low iron conditions binds the iron responsive element (IRE) in the 5' UTR of mRNAs encoding the iron storage protein, ferritin (Gray and Hentze, 1994). IRP binding does not affect cap-binding complex formation but sterically blocks joining of the 43S complex (Gray and Hentze, 1994; Muckenthaler et al., 1998).

Several different mechanisms for repressing translation initiation have been characterised for factors which bind the 3'UTR. By binding the 3' UTR and the cap directly the *Xenopus* protein Pumilio2 prevents eIF4F recruitment and inhibits the translation of *RINGO* mRNA (Cao et al., 2010) (Figure 1.10A). The *Drosophila* 4E-homologous protein (4EHP) functions similarly, binding the 3' UTR of *caudal* mRNA indirectly via the RNA-binding protein Bicoid and directly binding the m7G cap (Cho et al., 2005). An alternative mechanism is employed by proteins which bind eIF4E and prevent its interaction with eIF4G, functioning essentially as mRNA specific 4E-BPs. An example of this is the *Xenopus* protein maskin which binds certain cytoplasmic polyadenylation element (CPE)-containing mRNAs such as cyclin B1 indirectly via cytoplasmic polyadenylation element binding protein (CPEB) (Figure 1.10B) (Stebbins-Boaz et al., 1999).

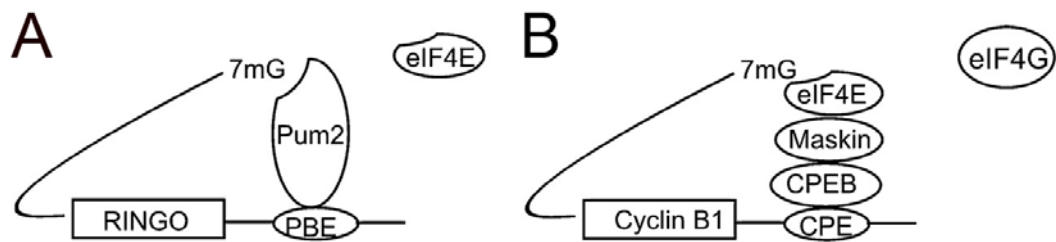


Figure 1.10 Specific inhibition of translation initiation by cap-binding proteins and eIF4E binding proteins. (A) Pumilio2 (Pum2) is recruited to RINGO mRNA by binding to the pumilio binding element (PBE) in the 3' UTR and binds the cap directly, preventing access by eIF4E. **(B)** Maskin binds the CPE via CPEB and binds eIF4E preventing recruitment of eIF4G.

Later stages of initiation may also be inhibited by trans-acting factors. Scanning of the 43S complex can be blocked by IRP if an IRE is positioned sufficiently far from the cap to allow 43S entry (Paraskeva et al., 1999). The 60S subunit joining step of initiation is prevented by heterogeneous ribonucleoproteins (hnRNPs) K and E1 bound to the differentiation control element (DICE) in the 3' UTR of *15-lipoxygenase* mRNA in erythroid precursor cells (Ostareck et al., 2001), although the mechanism of this inhibition is not known. Multiple mechanisms can also be employed to ensure efficient translational repression of an mRNA. The *Drosophila* transcript *male-specific-lethal 2* (*msl-2*) is bound by sex-lethal protein (SXL) at uridine stretches in the 5' and 3' UTRs. SXL bound at the 3' UTR recruits another RNA-binding protein, upstream of N-ras (UNR) which can interact with PABP1. This interaction is thought to interfere with PABP1-mediated stimulation of 43S pre-initiation complex recruitment, though the mechanism of this remains unclear (Duncan et al., 2009). SXL bound at the 5' UTR acts as a failsafe mechanism by preventing scanning of any 43S complexes which are able to bind the *msl-2* mRNA (Beckmann et al., 2005).

1.3.3.4 Translational repression by micro RNAs

Micro RNAs bind partially complementary sequences normally in the 3' UTR of mRNAs and the miRNA-containing ribonucleoprotein particle (miRNP) acts as a repressive translational trans-acting factor in addition to effecting mRNA degradation. miRNPs contain Argonaute (AGO) and other proteins. AGO proteins and their interaction with GW182 are required for miRNA-mediated translational repression and tethering of AGO or GW182 proteins to the 3' UTR of a reporter mRNA is sufficient to induce silencing (Eulalio et al., 2008; Pillai et al., 2004; Zipprich et al., 2009). How AGO/GW182 inhibits translation has not been established, though most studies support a model of cap-dependent repression of translation at initiation (Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Standart and Jackson, 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007). Other groups however, report translational repression post-initiation (Nottrott et al., 2006; Petersen et al., 2006) and the mode of repression (i.e. at initiation or elongation) has been suggested to reflect promoter usage (Kong et al., 2008). It is worth noting that while translational repression is a much studied consequence of miRNA binding, translational activation has also been reported for certain miRNAs during cell cycle arrest (Vasudevan et al., 2008), though this observation is yet to be reported independently.

1.3.3.5 Cytoplasmic polyadenylation

Most mRNAs are initially polyadenylated in the nucleus which, in mammals adds ~250 adenosine residues. Generally, mRNAs are gradually deadenylated in the cytoplasm but in some cases can also be readenylated which leads to their translational activation by poly(A)-binding proteins, the mechanism of which is discussed below (Chapter 1.3.4.2). Cytoplasmic polyadenylation is often mediated by binding of CPEB to CPE containing mRNAs (reviewed in (Mendez and Richter, 2001; Richter, 2007). CPEB forms a complex with several proteins including, the scaffolding protein symplekin, CPSF, the cytoplasmic poly(A)-polymerase Gld2 and the deadenylase PARN. The presence of PARN leads to poly(A)-tail shortening (Kim and Richter, 2006), and repression of translation of some CPE-containing mRNAs is further ensured by the maskin-mediated mechanism described above (Chapter 1.3.3.3). Phosphorylation of CPEB, leads to dissociation of PARN allowing polyadenylation by Gld2 to occur (Barnard et al., 2004; Kim and Richter, 2006; Mendez et al., 2000). Regulation of CPEB activity by phosphorylation therefore provides a mechanism for translational activation of specific messages in response to external signalling cues.

During oocyte maturation and early embryogenesis cytoplasmic polyadenylation is of particular importance and is thought to underlie the translational activation of messages such as cyclin B1, important for cell cycle progression during transcriptional quiescence (Mendez and Richter, 2001; Richter, 2007). Specific mRNAs are also polyadenylated in neuronal dendrites in response to stimulation at synapses and cytoplasmic polyadenylation is thought to be important for synaptic plasticity - the ability of neurons to respond differently if they have been stimulated previously which is thought to underlie memory and learning (Richter, 2007; Richter and Klann, 2009). While poly(A)-tail length changes have been known to be relevant in these specific cell types for some time, recently CPEB-mediated regulation of polyadenylation has also been shown to be important for the regulation of cellular senescence and mitotic cell division (Burns and Richter, 2008; Groisman et al., 2006; Novoa et al., 2010).

1.3.4 Translational dysregulation and disease

Mutations in both basal translation factors and mRNA specific translational regulators can result in a broad range of human disorders (reviewed in (Le Quesne et al., 2010)). For instance, mutations in eIF2B are associated with the neurological disorder vanishing white matter characterised by a progressive loss of oligodendrocytes (Leegwater et al., 2001). Homozygous mutations in the eIF2 α kinase PERK result in Wolcott-Rallison syndrome, which features infancy onset diabetes similar to the PERK $-/-$ mouse (Delepine et al., 2000; Harding et al., 2001). Both these disorders appear to reflect an inability to properly manage translation under stress in cells which require a large amount of protein synthesis. Mutations in the specific regulator Fragile-X mental retardation protein (FMRP) cause mental retardation due to loss of translation regulation of dendritic localised mRNAs (reviewed in (Bassell and Warren, 2008);(Garber et al., 2008)). Mutations in RNA sequence elements can also result in disease. Mutation of the IRES of *connexin-32* mRNA leads to Charcot-Marie-Tooth disease, a neuropathy characterised by loss of muscle and touch sensation (Hudder and Werner, 2000).

Upregulation of ribosomal proteins and several translation factors are associated with cancer progression (Reviewed in (Schneider and Sonenber, 2007);(Le Quesne et al., 2010; Silvera et al., 2010)). Of particular importance is eIF4E, the over-expression of which is sufficient to induce the malignant transformation of cultured cells (Lazaris-Karatzas et al., 1990). The explanation proposed for this is that increased levels of eIF4E lead to translational activation of specific messages encoding oncogenic products (e.g. c-myc, vascular endothelial growth factor (VEGF)). The translation of growth, proliferative and anti-apoptotic factors is often limited by long 5' UTRs or secondary structure however, excess eIF4E is thought to overcome the negative effect of these RNA features, resulting in their increased translation (Koromilas et al., 1992). Furthermore, high levels of eIF4E expression are often indicative of poor cancer prognosis (Silvera et al., 2010).

1.4 Poly(A)-binding proteins

The addition of a poly(A)-tail to an mRNA promotes its mRNA export, stability and translation, effects mediated by poly(A)-binding proteins. Two types of poly(A)-binding proteins are expressed in most eukaryotes with a different structure and function - nuclear and cytoplasmic PABPs.

1.4.1 Nuclear poly(A)-binding proteins

Mammalian nuclear PABPs (PABPNs) are ~ 33kDa and feature a glutamate rich N-terminus, a helical domain, a single RNA-recognition motif (RRM) and an arginine rich carboxy-terminal domain (Figure 1.11) (Kuhn and Wahle, 2004).



Figure 1.11 Structure of PABPN1 The N-terminus is glutamate-rich and separated from the RRM domain by a helical domain (HD). The C-terminus is arginine rich. Taken from (Kuhn and Wahle, 2004).

Nuclear PABPs are principally involved in the polyadenylation of messages in the nucleus (Chapter 1.1.3) though also shuttle to the cytoplasm (Calado et al., 2000a; Sato and Maquat, 2009). This shuttling activity has been suggested to reflect a role in mRNA export (Calado et al., 2000a) and recently knockdown of PABPN1 by RNAi in cultured mammalian cells was indeed shown to inhibit the export of poly(A)⁺ mRNA (Apponi et al., 2010). Other roles for PABPNs are also emerging. The nuclear PABP of *S. pombe* has been shown to be important for the maturation of small nucleolar RNA (snoRNA) transcripts by recruiting them to the nuclear exosome (Lemay et al., 2010) and *Drosophila* PABPN has been suggested to be involved in the poly(A) shortening of maternal mRNAs in the cytoplasm during early embryonic development (Benoit et al., 2005). A second nuclear PABP specifically

expressed during embryonic development was characterised in *Xenopus* and named ePABP2 in reference to the original name for PABPN1, PABP2 (Good et al., 2004). Surprisingly, ePABP2 was found to be mainly cytoplasmically localised (Good et al., 2004), further supporting the notion that in early development members of the PABPN family may participate in poly(A) metabolism in the cytoplasm.

Polyalanine expansion mutations in the N-terminal of human PABPN1 cause the autosomal dominant disorder oculopharyngeal muscular dystrophy (OPMD)(Brais et al., 1998). OPMD is a late-onset disease which results in muscle weakness in the eyelids, pharynx and proximal limbs. The alanine expansion causes intranuclear inclusions of PABPN1 in the muscles of OPMD patients and in mouse and cell-culture models (Calado et al., 2000b; Fan et al., 2001; Hino et al., 2004). The molecular pathology of the disorder and why it is muscle-specific when PABPN1 is widely expressed (Lee et al., 1998) is not well understood. Poly(A)-tail length is normal in OPMD tissue (Calado et al., 2000b) however, PABPN1 has been suggested to regulate the transcription of muscle-specific genes (Kim et al., 2001) and siRNA knockdown of PABPN1 shown to inhibit myoblast differentiation (Apponi et al., 2010).

While at least one PABPN has been characterised in most eukaryotic species, the budding yeast *Saccharomyces cerevisiae* is a notable exception. Though budding yeast express the prototypical cytoplasmic PABP (*pab1*), a functional nuclear PABP family protein has not been identified, though another unrelated protein Nab2p (nuclear polyadenylated RNA-binding protein 2), appears to fulfil a similar role (Anderson et al., 1993; Batisse et al., 2009; Hector et al., 2002; Viphakone et al., 2008).

1.4.2 The cytoplasmic poly(A)-binding protein family

Cytoplasmic poly(A)-binding protein 1 (PABP1 or PABPC1) is conserved among eukaryotes (Table 1.1) and is an essential gene in *Saccharomyces cerevisiae* (Sachs et al., 1987) and *Drosophila melanogaster* (Sigrist et al., 2000). Higher eukaryotes tend to express a greater number of cytoplasmic PABPs and five are present in mammals. PABP1 is the only member of the family to be characterized in great detail and is known to play important roles in mRNA translation, mRNA turnover and miRNA mediated gene regulation. Each of these functions are discussed below (Chapter 1.4.3) and the evidence for conservation of these functions and potential differences between the other mammalian PABPs are discussed later (Chapter 1.4.4).

species	identity with hs PABP1
sc	46%
sp	46%
at	43%
ce	52%
dm	58%
xl	93%
mm	99%

Table 1.1 Conservation of PABP1 between species. Table showing the percentage of amino acid identity between human PABP1 and the single PABP of *Saccharomyces cerevisiae* (sc), *Schizosaccharomyces pombe* (sp) and *Drosophila melanogaster* (dm), PABP1 of *Caenorhabditis elegans* (ce), *Xenopus laevis* (xl) and *Mus musculus* (mm) and its closest relative in *Arabidopsis thaliana* (at)(PAB8).

Most members of the cytoplasmic poly(A)-binding protein family share a conserved domain architecture and consist of four different consecutive RNA recognition motifs (RRMs) joined by a proline-rich linker region to a globular C-terminal domain - also known as the PABC or mademoiselle/MLLE domain (Figure 1.12) (Kuhn and Wahle, 2004). Full length forms typically have a molecular weight of ~70 kDa. The RNA binding activity of PABP is provided by the RRM s and the linker region has been implicated in PABP multimerization, important for ordered binding to poly(A) RNA (Chapter 1.4.3.1). The PABC domain is important for protein interactions with partners that feature a specific PABP-interacting motif, PAM2 (Kozlov et al., 2001; Roy et al., 2002). The structures of several PAM2 containing proteins complexed with the PABC domain have been solved (PAIP1, PAIP2 (Kozlov et al., 2004; Kozlov et al., 2010a), GW182, Ataxin-2 (Kozlov et al., 2010b), eRF3 (Kozlov and Gehring, 2010)) and identification of a PAM2 consensus sequence has permitted *in silico* identification of putative PABP-interacting proteins (Albrecht and Lengauer, 2004).



Figure 1.12 Structural organisation and interaction domains of cytoplasmic PABPs. RNA recognition motifs (RRMs) and the PABC domain are labelled. The regions responsible for key protein interactions with PABP1 are indicated below. Modified from (Brook et al., 2009) and (Smith and Gray, 2010).

1.4.3 PABP1 Functions

1.4.3.1 RNA Binding by PABP1

The relative contribution of each PABP1 RRM to its RNA binding affinities and specificities has been intensively investigated. PABP1 requires a minimum of 12 adenosine nucleotides to bind (Kuhn and Pieler, 1996; Sachs et al., 1987) and occupies approximately 25 nucleotides on a poly(A) tract (Sachs et al., 1987). High affinity binding of poly(A) RNA is achieved by RRMs 1+2 (Burd et al., 1991; Kuhn and Pieler, 1996) and the structure of this complex has been characterised for the human protein (Deo et al., 1999). RRMs 3+4 exhibit a lower affinity for poly(A), however they do make an independent contact with RNA (Kuhn and Pieler, 1996) and contribute to poly(A) binding of the whole protein since this is affected by amino acid substitutions in the RNP1 motif of RRMs 3 and 4 (Deardorff and Sachs, 1997).

Several PABP1 proteins are expected to bind each mRNA poly(A)-tail and in HeLa cells PABP1 is estimated to be in excess of that required to cover all poly(A)-tails in the cytoplasm (Gorlach et al., 1994). PABP1 has been shown to self-associate, an interaction that has been located the proline-rich linker region (Melo et al., 2003) and which leads to cooperative high affinity binding of multiple PABP1 protein molecules to poly(A) RNA (Kuhn and Pieler, 1996; Melo et al., 2003).

PABP1 is also able to bind sequences outside of the poly(A)-tail. It is known to bind oligo(A) regions in its own 5'UTR, known as the autoregulatory sequence, ARS, as part of a regulatory negative feedback loop to limit its own translation (see Chapter 1.4.5.1)(Bag, 2001; Bag and Wu, 1996; Wu and Bag, 1998). PABP1 binding to the ARS however is less efficient than to poly(A) RNA and formation of a heterotrimeric autoregulatory complex (ARC) with insulin-like growth factor II mRNA binding protein-1 (IMP1) and upstream of N-ras (UNR) has been suggested, though not demonstrated, to improve the affinity of PABP1 binding to the ARS (Patel et al., 2005). PABP1 also binds a 22 nucleotide poly(A) sequence in the neuronal expressed non-coding short RNA Brain Cytoplasmic 1 (BC1) (Muddashetty et al., 2002; West et al., 2002). Furthermore, PABP1 has been found to bind the 3' UTR of Y-box binding protein 1 (YB-1) mRNA which contains multiple short (3-4

nucleotide) adenosine stretches (Skabkina et al., 2003)(see Chapter 1.4.5.2) and is able to bind synthetic mRNAs containing similarly short poly(A) tracts (Gorlach et al., 1994; Kuhn and Pieler, 1996).

PABP1 is also able to bind poly(U) sequences and poly(G) *in vitro* (Kuhn and Pieler, 1996; Nietfeld et al., 1990) though poly(U) is reportedly bound by yeast pab1 with approximately 100 times lower affinity than poly(A) (Deardorff and Sachs, 1997). Human PABP1 binding to AU-rich elements has also been investigated and found to occur *in vitro* with relatively high affinity, though approximately 6-fold lower than for poly(A) RNA, with the interaction reported to be mediated by RRM3-4 (Sladic et al., 2004). Accordingly, PABP1 has been found associated with multiple AU-rich elements (Bollig et al., 2003; Nagaoka et al., 2006; Vasudevan et al., 2005; Wiklund et al., 2002) though direct RNA binding has been shown for only GM-CSF (granulocyte/macrophage-colony stimulating factor) which was demonstrated by electrophoretic mobility shift assay (EMSA) (Sagliocco et al., 2006). It is unknown how widespread PABP1 binding to endogenous AREs is and the physiological role of PABP AU-RNA binding is not fully understood (see Chapter 1.4.3.4).

1.4.3.2 Translational activation by PABP1

PABP1 stimulates the translation of mRNAs when bound to a poly(A)tail, or when artificially tethered to the 3' UTR (Gray et al., 2000; Sachs and Davis, 1989). This stimulation is thought to mainly be achieved by indirectly promoting recruitment of the 43S pre-initiation complex (Kahvejian et al., 2005; Tarun and Sachs, 1995). Interaction between PABP1 and the amino terminus of eIF4G is thought to circularize messages and promote stable 48S complex formation - a hypothesis known as 'the closed-loop model' (Figure 1.13)(Mangus et al., 2003). Circularization has been demonstrated using atomic force microscopy to visualize RNA associated with recombinant eIF4G, eIF4E and pab1 yeast proteins *in vitro* (Wells et al., 1998) and this model explains the synergistic positive effect of a cap and poly(A)-tail on mRNA translation (Michel et al., 2000; Tarun and Sachs, 1995).

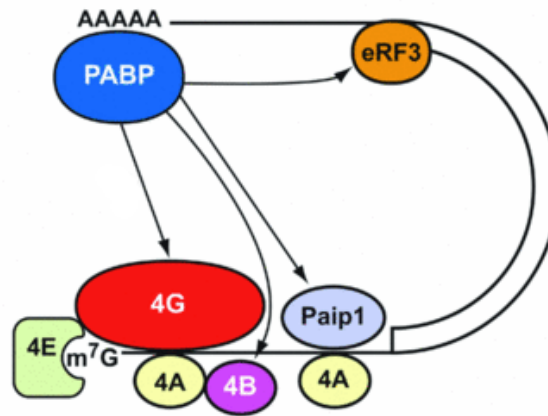


Figure 1.13 Model of stimulation of translation by PABP1. Multiple contacts with 5' bound factors eIF4G, eIF4B and Paip1 result in circularization of mRNA and enhanced 48S complex formation. Interaction with eRF3 likely increases ribosome recycling after termination. PAIP1 also interacts with eIF3 (not shown) stabilising circularization. Taken from Smith & Gray 2010.

The PABP1-eIF4G interaction is conserved in yeast (Tarun and Sachs, 1996), plants (Le et al., 1997), *Xenopus* (Gray et al., 2000; Wakiyama et al., 2000) and humans (Imataka et al., 1998) and is mediated by the RRM2 region of PABP1 (Gray et al., 2000; Groft and Burley, 2002; Imataka et al., 1998; Kessler and Sachs, 1998). eIF4G isoforms I and II are highly conserved over the N-terminal region and both forms have been demonstrated to bind PABP1 (Imataka et al., 1998; Tarun and Sachs, 1996).

The interaction of wheat germ PABP1 with eIF4G is reported to increase the affinity of PABP1 for poly(A) RNA (Le et al., 1997), increase the affinity of eIF4E for the cap approximately 40-fold (Wei et al., 1998) and increase the helicase activity of eIF4F (Bi and Goss, 2000). However, there are significant differences between the translational machinery of plants and metazoa (Gallie, 2007) and so conservation of each of these activities should not be assumed. Indeed, only the effect of PABP1 on eIF4E cap-binding has been demonstrated in other species. In rabbit reticulocyte lysates (RRL), capped mRNAs bearing a poly(A)-tail were found to be 8-10 times less susceptible to translational inhibition by a cap analogue (Borman et al., 2000). Diminished inhibition is assumed to be due to reduced binding of eIF4E to cap analogue, indicative of an increased affinity of eIF4E for the mRNA cap (Borman et al., 2000). Similarly, the ability of the poly(A) tail to confer resistance to cap analogue was recently demonstrated in yeast extracts (Amrani et al., 2008). The

contribution of PABP1 was also directly tested. Extracts from yeast strains lacking *pab1* (*pab1*Δ), but made viable by a suppressor mutation (*pbp1*Δ) were shown to be 80-fold more sensitive to cap analogue, quantified by toe-printing of 80S ribosomes at the initiation codon (Amrani et al., 2008). In an assay of cap-binding by eIF4E however, the addition of *pab1* in the presence of eIF4G increased recovery of eIF4E on an m⁷GTP column by only 50% (von Der Haar et al., 2000). Thus the magnitude of the effect of PABP1 on cap binding by eIF4F may vary between species but also appears to be influenced by the experimental method used.

Additional interactions between PABP1 bound at the poly(A) tail and proteins bound at the 5' of messages have also been suggested to further stabilize the circularised structure (Figure 1.13). PABP1 interacts with PAIP1, a protein partially homologous to eIF4G (Craig et al., 1998), through two independent binding regions in RRM1-2 and the PABC domain (Gray et al., 2000; Roy et al., 2002). PAIP1 interacts with eIF4A (Craig et al., 1998) and eIF3 (Martineau et al., 2008) and PABP1 is able to bind eIF4G and PAIP1 simultaneously (Martineau et al., 2008). PABP1 is also reported to bind eIF4B. This interaction was first identified in wheat germ extracts (Le et al., 1997) and was found to be mediated by RRM1-2 and therefore mutually exclusive with the PABP-eIF4G interaction (Cheng and Gallie, 2007). This interaction appears not to be identically conserved between species. PABP1 is not pulled down with eIF4B from *Drosophila* extracts (Duncan et al., 2009) and an interaction could not be detected between the *Xenopus* proteins (Wilkie et al., 2005). Mammalian PABP1 however, is reported to interact with the N-terminus of eIF4B (Bushell et al., 2001). Curiously, despite the identification of protein sequence similarities between human eIF4B and the N-terminus of eIF4G (Bushell et al., 2001; Groft and Burley, 2002) and in contrast to the situation in plants, the mammalian interaction was reported to be mediated by the C-terminal region of PABP1 (Bushell et al., 2001). Supporting the notion that the interactions of multiple PABPs may contribute to the stability of the closed loop structure, increased poly tail length is correlated with increased cap analogue resistance and binding of two yeast Pab1 molecules shown to be required for stable closed-loop formation (Amrani et al., 2008).

In addition to stabilisation of eIF4F, circularisation of mRNAs has been suggested to promote reloading of ribosomes (Uchida et al., 2002). PABP1 interacts with the termination factor eRF3, a PAM2 containing protein, via the PABC domain (Cosson et al., 2002a; Cosson et al., 2002b; Hoshino et al., 1999). PABP1 is able to simultaneously interact with eRF3 and eIF4G, and during translation termination this interaction, with a concomitant looping out of the 3' UTR, is proposed to increase the local concentration of recycled 40S ribosome subunits at the 5' end (Uchida et al., 2002).

The closed-loop hypothesis of PABP1-mediated translational activation is strengthened by the existence of other proteins that mediate circularisation of mRNAs to stimulate translation. Histone mRNAs are not polyadenylated but harbour a conserved stem loop structure in their 3' UTR which are bound by stem-loop binding protein (SLBP)(Wang et al., 1996). SLBP is in turn bound by SLBP-interacting protein (SLIP1) which like PABP1, interacts with eIF4G promoting end to end complex formation and translation activation (Cakmakci et al., 2008). Similarly, the rotavirus non-structural protein 3 (NSP3) binds to RNA elements in the 3' UTR of viral transcripts and interacts with eIF4G to circularise mRNAs (Piron et al., 1998). The NSP3-eIF4G interaction is several fold stronger than the PABP1-eIF4G interaction (Groft and Burley, 2002) and NSP3 evicts PABP1 from eIF4G during rotavirus infection (Piron et al., 1998).

There are likely further mechanisms by which PABP1 mediates translational activation. The interaction between PABP1 and eRF3 has been suggested to promote translation termination since over-expression of PABP1 in yeast is able to alleviate stop-codon suppression induced by the drug paromomycin (Cosson et al., 2002b). In support of this notion, human eRF3 bound by PABP1 was recently shown able to bind GTP (Kononenko et al., 2010) indicating that PABP1 binding is at least compatible with GTP-hydrolysis by eRF3 at termination. PABP1 has also been implicated in the 60S joining stage of translation initiation (Kahvejian et al., 2005; Munroe and Jacobson, 1990; Sachs and Davis, 1989). In a mammalian cell-free system, depletion of PABP1 leads to dramatic depreciation of 80S, not explained by the deficit in 48S complex formation (Kahvejian et al., 2005; Svitkin et al., 2009). Finally, the RRM3+4 domain of *Xenopus* PABP1 is able to stimulate translation to a

level equivalent to the full-length protein when tethered to reporter mRNA (Gray et al., 2000) although no translation factors have been shown to bind this region, implying that further relevant protein interactions remain undiscovered.

1.4.3.3 Translational activation by PABP1 bound indirectly to a message

It has been known for some time that tethering of PABP1 to the 3' UTR of a reporter mRNA can mimic the effect of a poly(A) tail by stimulating translation initiation (Gray et al., 2000). Similarly, translation of dengue virus RNA, which is not polyadenylated, is thought to be mediated by PABP1 binding directly to the 3' UTR (Polacek et al., 2009). Recruitment of PABP1 to messages via protein-protein interactions, rather than by RNA binding, may also serve as mechanism to enable translational activation of specific mRNAs.

One family of PABP1 interacting proteins appear to mediate just such a mode of regulation. The DAZ (Deleted in Azoospermia) family of RNA-binding proteins are PABP1-interacting proteins that stimulate translation in a manner that is dependent on PABP1 (Collier et al., 2005). The family consists of BOULE, DAZL and DAZ which are thought to be required for the translational activation of specific transcripts during gametogenesis (reviewed in (Brook et al., 2009)). Several mRNA targets of mouse DAZL have been identified which each contain conserved binding sites in their 3' UTR (Reynolds et al., 2007; Reynolds et al., 2005). PABP1 binds DAZ proteins through its C-terminus (Collier et al., 2005) leaving the RRM1-2 region available to bind eIF4G or PAIP1. Direct recruitment of PABP1 to the 3'UTR is expected to particularly benefit mRNAs with short poly(A)-tails (Collier et al., 2005), explaining the importance of DAZ proteins during gametogenesis when poly(A) tail length changes are known to occur (Gray and Wickens, 1998).

It is unknown how extensive this mechanism for the recruitment of PABP1 to mRNAs is but other known PABP-interacting proteins may function in this manner. Ataxin-2 is a PAM-2 containing protein associated with the neurodegenerative disorder spinocerebellar ataxia type 2 (Imbert et al., 1996). Ataxin-2 has been shown to interact with the PABP1 PABC domain (Kozlov et al., 2010b; Ralser et al., 2005; Satterfield and Pallanck, 2006) however its function in mammals is not clear though,

it does contain an RNA-binding domain and sediments with polyribosomes (Satterfield and Pallanck, 2006). The ability of ataxin-2 to regulate translation has not been tested and it therefore remains formally possible that ataxin-2 may also operate in a Dazl-like manner to recruit to PABP1 to mRNAs.

1.4.3.4 The role of PABPs in mRNA stability

PABP1 binding to the poly(A)-tail protects messages from deadenylation (Bernstein et al., 1989; Korner and Wahle, 1997; Wang et al., 1999) which is the first step in both 5' to 3' and 3' to 5' decay pathways (Chapter 1.1.5.1). Deadenylation intermediates are detected in mammals with ~30 nucleotides size differences reflecting protection by individual PABP1 proteins (Korner and Wahle, 1997). mRNA decay is also directly inhibited by PABP1, demonstrated by the slowed decay of a non-adenylated mRNA to which yeast *pab1* is tethered (Coller et al., 1998). Furthermore, *pab1*Δ yeast strains exhibit enhanced decapping (Caponigro and Parker, 1995). The inhibitory effect of PABP1 on mRNA decay is explained by formation of the closed-loop structure which is suggested to limit access by decapping and decay enzymes (Gorgoni and Gray, 2004; Kuhn and Wahle, 2004).

In contradiction, PABP1 also mediates the recruitment of deadenylases to mRNAs and is thought to facilitate coupling of deadenylation to translation (Funakoshi et al., 2007). The PABC domain mediates interactions between PABP and the two complexes thought to conduct most deadenylation in eukaryotic cells, Caf1-CCR4 complex and the PAN2-PAN3 complex (also called PAN) (Funakoshi et al., 2007; Siddiqui et al., 2007). These complexes bring about biphasic deadenylation of mRNAs in mammalian cells with PAN2-PAN3 initiating early deadenylation and Caf1-CCR4 mediating rapid late deadenylation (Funakoshi et al., 2007; Yamashita et al., 2005). In the PAN2-PAN3 complex, PAN2 bears the deadenylase activity while PAN3 is regulatory (Uchida et al., 2004). PAN3 contains a PAM2 motif and has been shown to directly interact with PABP1 in humans (Funakoshi et al., 2007; Siddiqui et al., 2007; Uchida et al., 2004). PABP1 interacts with Caf1-CCR4 via the anti-proliferative protein TOB (Ezzeddine et al., 2007; Okochi et al., 2005). TOB contains two PAM2 motifs (Mauxion et al., 2009) and it has been shown that it is

the most C-terminal of these PAM2 motifs which is most important for the interaction with PABP1 (Funakoshi et al., 2007). The N-terminal region of TOB mediates binding to Caf1 and simultaneous binding of TOB to PABP1 and Caf1 has been demonstrated (Funakoshi et al., 2007). Significantly, in mammalian cells binding of PABP1 to PAN2-PAN3 or Caf1-CCR4 increases the deadenylation activity of each by more than 10 fold (Funakoshi et al., 2007; Uchida et al., 2004).

TOB family proteins are conserved in metazoa (Mauxion et al., 2009) however; their interactions with PABP family proteins have not been tested beyond mammals. The interaction of PABP1 with PAN3 is conserved from human to yeast (Mangus et al., 2004; Siddiqui et al., 2007) and in yeast this interaction is also suggested to mediate trimming of poly(A)-tails in the nucleus (Dunn et al., 2005).

eRF3 is also important for deadenylation and its deletion in yeast leads to mRNAs with long poly(A) tails and a diminished rate of decay (Hosoda et al., 2003). In mammalian cells, the interactions of PABP1 with eRF3, PAN2-PAN3 and Caf1-CCR4 are mutually exclusive and the exchange of PABP1 binding between these protein partners is hypothesised to mediate translation-coupled deadenylation (Funakoshi et al., 2007). In support of this notion the ratio of PAN3 and eRF3 bound by PABP1 was shown to be dependent on translation (Funakoshi et al., 2007). How the transfer of PABP1 from eRF3 to PAN3 is regulated is as yet unknown.

The TOB-mediated interaction of PABP1 with Caf1-CCR4 is also implicated in miRNA mediated deadenylation (Fabian et al., 2009; Zekri et al., 2009). PABP1 interacts with the miRNP-associated protein GW182 and is suggested to recruit the Caf1-CCR4 deadenylase to miRNA targets (Fabian et al., 2009; Zekri et al., 2009). However, like TOB, GW182 also contains a PAM2 motif through which it is able to bind PABP (Fabian et al., 2009; Kozlov et al., 2010b). It is therefore difficult to understand how PABP1 bound to this motif in GW182 via the PABC domain could recruit Caf1-CCR4, since this interaction is also mediated by PABC domain binding to the PAM2 motif of TOB. To explain this problem, it has been proposed that GW182 proteins may, similar to PAIP1, interact with the RRM regions, in addition to the PABC domain of PABP1 (Tritschler et al., 2010). In support of this idea, mutation or deletion of the GW182 PAM2 motif in *drosophila* or human respectively reduces but does not abolish binding to PABP1 (Jinek et al., 2010; Zekri et al.,

2009). While PABP1 is emerging as a key player in regulation by miRNAs, we are evidently far from fully understanding its role, or indeed the molecular mechanisms of miRNA-mediated silencing.

As discussed earlier, PABP1 is able to bind AU-rich elements (Sagliocco et al., 2006; Sladic et al., 2004) and it has also been shown to interact with the mammalian ARE-binding proteins HuR (Nagaoka et al., 2006), TTP (Kedar et al., 2010; Rowlett et al., 2008) and AUF1 (Laroia et al., 1999; Lu et al., 2006; Sagliocco et al., 2006) implying that it may be recruited to some AREs via protein-protein interactions. Reported effects of PABP1 association with AREs vary. PABP1 was reported to be inhibitory of TTP-induced deadenylation of tumour necrosis factor (TNF) ARE-containing mRNA (Rowlett et al., 2008) and similarly was suggested to have a stabilising effect on the β -casein ARE which is bound by HuR (Nagaoka et al., 2006). In contrast, AUF1 has been suggested to displace PABP1 from the poly(A)-tail, leaving it vulnerable to deadenylation (Grosset et al., 2004; Lu et al., 2006; Sagliocco et al., 2006). However, there has been no in depth mechanistic analysis of the role of PABPs in the regulation of ARE-containing mRNAs, nor of the possibility that PABP1 might promote their decay by recruitment of deadenylases.

1.4.4 PABP1 sub-cellular localisation

PABP1 has a mainly cytoplasmic localisation (Gorlach et al., 1994) but has been shown by heterokaryon assay to shuttle between the cytoplasm and nucleus (Afonina et al., 1998). While yeast Pab1 contains a classical leucine rich NES and has been shown to be exported by exportin-1 (Brune et al., 2005), no classical import or export signals have been identified in mammalian PABPs. Nevertheless, nuclear export of mammalian PABP1 has been reported to be sensitive to leptomycin B (LMB) (Woods et al., 2002), a specific inhibitor of CRM1 (the mammalian homolog of exportin-1) responsible for the export of NES-containing proteins (Fornerod et al., 1997). Contradictory results have also been published however, reporting that human PABP1 is not sensitive to LMB (Khacho et al., 2008b).

PABP1 nuclear export also appears to be dependent on ongoing transcription since the transcriptional inhibitors actinomycin D and 5,6-dichlororibofuranosylbenzimidazole (DRB) both result in a nuclear accumulation of PABP1 (Afonina et al., 1998; Khacho et al., 2008b). Recently, this effect has been attributed to a ‘transcription dependent nuclear export motif’ (TD-NEM) in RRM4 (Khacho et al., 2008b); the experimental evidence supporting this is discussed in more detail in Chapter 6.1. Overexpression of PABP1 in human cells also leads to its nuclear accumulation implying that its nuclear export, but not import is limiting and nuclear import of PABP1 has been shown to be energy dependent (Afonina et al., 1998).

Nuclear export of budding yeast Pab1 has been linked to mRNA export (Brune et al., 2005). In this organism, however, Pab1 has characterised nuclear roles in polyadenylation and 3’ end processing (Dunn et al., 2005; Minvielle-Sebastia et al., 1997; Viphakone et al., 2008) and shows significant sequence divergence from mammalian PABP1 (Table 1.1). Mammalian PABP1 has been shown to bind pre-mRNAs in the nucleus leading to a suggested role in pre-mRNA processing and mRNA trafficking (Hosoda et al., 2006). As yet, there has been no investigation of role of metazoan cytoplasmic PABPs in mRNA export though it seems likely that at least a portion of PABP1 exits the nucleus bound to mRNAs.

PABP1 has been shown to localise to the dendrites of neurons (Muddashetty et al., 2002; Wang et al., 2002a) and the leading edges of migrating fibroblasts (Woods et al., 2002). Localisation of PABP1 to leading edges and also its nuclear export has been suggested to be regulated by the focal adhesion associated protein paxillin, with which PABP1 is reported to interact via RRM4 (Woods et al., 2005; Woods et al., 2002).

In normal conditions PABP1 is diffusely cytoplasmic, however in conditions of cell stress such as osmotic shock, PABP1 localises to cytoplasmic stress granules (SGs) (Kedersha et al., 1999). SGs contain stalled initiation complexes and are composed of mRNA, small ribosomal subunits, some initiation factors and numerous RNA binding proteins and are thought to facilitate storage and remodelling of mRNPs (discussed in more detail in Chapter 4.1) (Anderson and Kedersha, 2009; Buchan and Parker, 2009). Localisation of PABP1 to stress granules may be a

consequence of mRNA binding and/or protein interactions and its function at stress granules has not been investigated.

1.4.5 Regulation of PABP1

1.4.5.1 Regulation of PABP1 expression and availability

PABP1 is generally described as being ubiquitously expressed, however northern blotting of human tissues (Yang et al., 1995) and western blotting of *Xenopus* tissues (Cosson et al., 2002c) indicates that its expression is in fact highly variable. Indeed, expression of PABP1 in mammals is translationally regulated by two independent RNA elements in the 5' UTR, a terminal oligopyrimidine tract (TOP) motif (Hornstein et al., 1999a) and an A-rich auto-regulatory sequence (ARS) (de Melo Neto et al., 1995).

TOP motifs confer growth-dependent translational control on numerous vertebrate mRNAs involved in protein synthesis such as ribosomal proteins, eEF1 and eEF2 (Avni et al., 1997; Avni et al., 1994; Hamilton et al., 2006; Iadevaia et al., 2008). While most eukaryotic mRNAs have an A residue at their most 5' end, TOP containing mRNAs begin with a C residue that is followed by 4-14 polypyrimidine tract (Meyuhas, 2000). As phosphorylation of ribosomal protein S6 is induced by mitogenic stimuli, initially the S6-kinase pathway was implicated in the regulation of TOP mRNAs (Caldarola et al., 2004; Meyuhas, 2000). Subsequently however, a role for S6 phosphorylation was resolutely ruled out (Barth-Baus et al., 2002; Stolovich et al., 2002; Tang et al., 2001) and genetic evidence now indicates that the mTOR signalling pathway affects translational activation of TOP mRNAs, but independently of the Raptor containing complex (mTORC1) that signals to S6-kinase and 4E-BP1 (Patursky-Polischuk et al., 2009). Specificity of the regulation of TOP mRNAs is thought to be mediated by an RNA-binding protein, since repression of a TOP mRNA in low-growth conditions can be relieved with addition of an excess of synthetic TOP (Biberman and Meyuhas, 1999). The autoantigen La has been shown to bind TOP sequences and suggested to act as this repressor (Intine et al., 2003; Schwartz et al., 2004; Zhu et al., 2001) however, the mechanism of TOP translational

repression is not clear and the involvement of La is controversial (Hamilton et al., 2006).

The PABP1 autoregulatory sequence is a ~60 nucleotide element containing multiple short (6-8) adenosine tracts that is conserved from yeast to human (de Melo Neto et al., 1995) and has been shown to confer down-regulation of PABP1 mRNA translation with increasing concentration of PABP1 protein (Bag and Wu, 1996). The ARS is bound by a heterotrimeric autoregulatory complex (ARC) consisting of PABP1, UNR and IMP1 (Patel et al., 2005). PABP1 is thought to simultaneously bind UNR and IMP (Patel et al., 2005). Interaction with IMP1 is mediated by the PABP1 C-terminus (Patel and Bag, 2006) and binding to UNR occurs through PABP1 RRM2-3 (Chang et al., 2004; Duncan et al., 2009). Binding of this complex to the ARS is proposed to inhibit translation initiation by obstructing ribosome scanning as it is located in a cap-distal position (Bag, 2001). It is not known whether other cytoplasmic PABPs are able to bind the PABP1 ARS or UNR and IMP, however, such abilities could serve to maintain a constant level of total PABPs in the cytoplasm. A further mode of PABP1 autoregulation may also exist since ectopic overexpression of PABP1 leads to reduced detection of the endogenous mRNA implying either a reduction in transcription or stability of PABP1 mRNA (Hornstein et al., 1999b).

The amount of PABP1 protein available to participate in the control of mRNA translation and stability may also be regulated. PABP1 has been shown to bind A-rich sequences in the rodent non-coding RNA BC1 and its human counterpart BC200 (Muddashetty et al., 2002). BC1 has an inhibitory effect on translation *in vitro* (Wang et al., 2002a) which is thought to be at least partially due to sequestration of PABP1, since the effect can be reversed by the addition of PABP1 (Kondrashov et al., 2005). BC1 is expressed in neuronal cells (Tiedge et al., 1991) where the local concentration of PABP1 available to bind poly(A)-tails would be expected to be of particular relevance, since poly(A)-tail length changes are thought to be important for the translational control of some dendritically localised transcripts (Chapter 1.3.3.5) (Richter and Klann, 2009).

The availability PABP1 is often the target of viruses (Reviewed in (Smith and Gray, 2010)). Viruses frequently inhibit cellular translation in order to usurp the

translation machinery for viral protein production. To this end, PABP1 is subject to cleavage by several viral proteases. Cleavage of PABP1 between the RRM and C-terminal regions in infected cells has been reported for picorna-, calici- and retroviruses (Alvarez et al., 2006; Joachims et al., 1999; Kerekatte et al., 1999; Kuyumcu-Martinez et al., 2004a; Kuyumcu-Martinez et al., 2002; Kuyumcu-Martinez et al., 2004b). As intact N-terminal cleavage products retain the ability to bind RNA and eIF4G it has been suggested that loss of eRF3 and eIF4B binding or loss of multimerization of PABP1 leading to destabilisation of poly(A) binding, could contribute to the translational repression observed in cells infected with these viruses (Lloyd, 2006; Smith and Gray, 2010). Supporting this notion, cleavage of PABP1 by enterovirus 3C protease or HIV-1 protease has been shown to inhibit cap- and poly(A)-dependent translation in a manner that can be rescued by addition of full length PABP1 (Castello et al., 2009; Kuyumcu-Martinez et al., 2004b). Degradation of PABP1 is also observed in apoptotic cells (Marissen et al., 2004); however the protease responsible and site of cleavage have not been identified.

Infection with certain viruses has also been reported to cause the redistribution of PABP1 from the cytoplasm to the nucleus (Smith and Gray, 2010). Infection with the herpes family virus Kaposi's sarcoma associated virus (KSHV) relocalises PABP1 to the nucleus where it was shown to colocalise with the viral protein K10/10.1 (Kanno et al., 2006). Infection with rotavirus has also been shown to relocalise PABP1 to the nucleus and this relocalisation was found to be dependent on the viral protein NSP3 (Harb et al., 2008; Montero et al., 2008). NSP3 evicts PABP1 from initiation complexes (Piron et al., 1998) and is responsible for the inhibition of host cell translation (Montero et al., 2006). NSP3 binds eIF4G and functions as a PABP analogue to circularise messages when bound to an element in the rotavirus 3'UTR (Piron et al., 1998). However, this interaction is insufficient for maximal PABP1 relocalisation, which also requires interaction of NSP3 with the cellular protein RoXaN (rotavirus X protein associated with NSP3) (Harb et al., 2008). Neither NSP3 nor RoXaN bind PABP1 directly, and the cellular function of RoXaN is unknown (Harb et al., 2008). How RoXaN might contribute to rotaviral relocalisation of PABP1 or indeed the regulation of PABP1 localisation in uninfected

cells, is unknown. Sequestration of PABP1 to the nucleus would be expected to be inhibitory of poly(A)-dependent translation, though this has not been demonstrated.

1.4.5.2 Regulation of PABP1 activity

The activity of PABP1 in translation is regulated by PAIP2 protein. PAIP2 (or PAIP2A) is a small (~14 kDa) cytoplasmic protein which binds PABP1 via independent interactions in the RRM region and PABC domain (Khaleghpour et al., 2001a; Khaleghpour et al., 2001b). Accordingly, PAIP2 contains a PAM2 motif which is reported to be the highest affinity PABC ligand tested (Kozlov et al., 2010a). PAIP2 binding to PABP1 inhibits translation by competing with eIF4G binding and interfering with the association of PABP1 with poly(A) RNA (Karim et al., 2006; Khaleghpour et al., 2001a; Khaleghpour et al., 2001b). PAIP2 was first identified in human cells, however it is conserved in mouse, *Xenopus* and *Drosophila*, (Berlanga et al., 2006), and was shown to similarly regulate the activity of PABP1 in *Drosophila* cells (Roy et al., 2004). A homolog of PAIP2 has been described in mammals, PAIP2B which is highly similar to PAIP2 (59% identity) but exhibits a different tissue specific expression pattern in mice and humans (Berlanga et al., 2006). PAIP2B also binds PABP1 and is thought to repress translation in an analogous manner to PAIP2; it has been suggested that while the two proteins appear functionally equivalent, they could be regulated differently (Berlanga et al., 2006). Regulated expression and turnover of PAIP2 and PAIP2B has been proposed to modulate the activity of PABP1 (Berlanga et al., 2006).

Interaction of PABP1 with PAIP2 also regulates the levels of PAIP2, creating a feedback system (Yoshida et al., 2006). PAIP2 is targeted for degradation by the E3 ubiquitin ligase, EDD (Yoshida et al., 2006) which is one of the few non-PABP proteins that contain a PABC domain (Albrecht and Lengauer, 2004; Deo et al., 2001). When levels of PABP1 are reduced by RNAi, more EDD is able to interact with PAIP2 and it is degraded faster, implying that when PABP1 is less abundant, inhibition of its activity in translation by PAIP2 is relieved (Yoshida et al., 2006). Consistent with this model, siRNA knockdown of PABP1 in HeLa cells yielded only a modest effect on translation of a reporter mRNA, ~10% inhibition; however this

increased to 50% inhibition in the presence of a mutant PAIP2 that could not be ubiquitinated (Yoshida et al., 2006).

The general RNA-binding protein Y-box 1 (YB-1) has also been implicated in the regulation of PABP1 activity in translation (Svitkin et al., 2009). YB-1 at high concentrations is inhibitory to translation (Pisarev et al., 2002). By coating mRNAs it is suggested to inhibit the binding of eIF4G to RNA adjacent to the cap (Nekrasov et al., 2003) an interaction which is suggested to stabilise the eIF4F-cap interaction (Yanagiya et al., 2009). Addition of YB-1 to *in vitro* extracts rendered the system more PABP1 dependent, that is, that translation of a reporter benefited from PABP1 to a greater degree and it is suggested that PABP1 supports eIF4F resistance to YB-1 inhibition (Svitkin et al., 2009). Therefore, the abundance of YB-1 in cells and tissues is likely to affect the efficiency with which PABP1 stimulates mRNA translation.

YB-1 translation is autoregulated; binding of YB-1 to sequences in the 3' UTR of its own mRNA inhibits translation initiation (Skabkina et al., 2005; Skabkina et al., 2003). PABP1 levels however also influence *YB-1* translation since PABP1 can bind an overlapping site in the *YB-1* 3' UTR, displacing YB-1 protein and relieving the translational repression it imposes (Skabkina et al., 2005). Therefore, high levels of PABP1 increases YB-1 translation, and increased levels of YB-1 acts to inhibit the activity of PABP1, thus maintaining translation homeostasis.

Post-translational modifications may also regulate the activity of PABP1. In plants, phosphorylation of PABP has been correlated to changes in the affinity of poly(A) RNA, eIF4G and eIF4B binding (Le et al., 2000). Human PABP1 has been reported to be a substrate of the mitogen-activated protein kinase-kinase 1 (MKK1) (Ma et al., 2006) and p38 MAP kinase, which was shown to phosphorylate RRM3-4 in an *in vitro* kinase assay (Rowlett et al., 2008); however no correlation with PABP1 function has been demonstrated and specific sites phosphorylated by these kinases have not been mapped, though high throughput screens have identified several PABP1 phosphorylation sites (Choudhary et al., 2009; Dephoure et al., 2008; Gauci et al., 2009). Human PABP1 is also methylated on arginine residues in its linker region by the coactivator-associated arginine methyltransferase (CARM1) (Lee and Bedford, 2002) though the effect of this modification is also unknown.

The existence of multiple levels of regulation of PABP1 expression and multiple feedback systems to regulate its activity implies that maintaining appropriate levels of PABP1 is of great importance to the cell.

1.4.6 Other members of the mammalian PABP family

Higher eukaryotes express a greater number of cytoplasmic PABPs. Mammals are known to express four structurally similar and closely related cytoplasmic PABPs – PABP1 (HGNC symbol: PABPC1), testes specific PABP (tPABP; HGNC symbol: PABPC3 in human, Pabpc2 in mouse), PABP4 (also known as inducible or iPABP; HGNC symbol: PABPC4) and embryonic PABP (ePABP; HGNC symbol: PABPC1L) (Table 1.2). A shorter PABP, homologous only to the RRM of PABP1, with which it shares 62% identity, has also been described and is named PABP5 (HGNC symbol: PABPC5).

	hs PABP4	hs tPABP	hs ePABP
hs PABP1	75%	92%	69%
hs PABP4		70%	65%
hs tPABP			65%

Table 1.2 Conservation between human PABPs Table showing percentage amino acid identity between human PABP1, PABP4 (PABPC4-002), tPABP and ePABP (PABPC1L-001).

With the exception of PABP5, each PABP has been shown to bind poly(A) RNA in at least one vertebrate species, implying all could potentially function analogously to PABP1. Study of mammalian PABP expression is largely limited to the mRNA level; however, expression of different PABPs appears to be regulated in a developmental and tissue specific manner. Our understanding of the expression and function of each of the other mammalian PABP family members is discussed in turn below.

1.4.6.1 ePABP

Embryonic PABP is so named due to its initial discovery in *Xenopus* oocytes and early embryos (Voeltz et al., 2001). ePABP was subsequently found to be conserved throughout vertebrate species, and expression of ePABP mRNA was later detected in adult ovary and testis in *Xenopus* and mouse supporting a role in gametogenesis (Seli et al., 2005; Wilkie et al., 2005). In addition to ovary and testes, human ePABP mRNA expression has also been detected in other tissues, such as pancreas, kidney and liver (Guzeloglu-Kayisli et al., 2008; Sakugawa et al., 2008), suggesting that it may be more widely expressed in humans. There has however been no assessment of ePABP protein expression in humans and presence of ePABP mRNA is not necessarily synonymous with presence of ePABP protein. During mouse and *Xenopus* development, early expression of ePABP mRNA is replaced by later expression of PABP1 in what appears to be an exquisitely regulated and conserved strategy (Seli et al., 2005; Voeltz et al., 2001).

ePABP is the next best-studied member of the family after PABP1, however, all functional characterisation of ePABP has focused on the *Xenopus* protein. ePABP was first identified as an AU-rich RNA binding protein (Voeltz et al., 2001) and poly(A)-binding by ePABP has since been demonstrated using a poly(A) column (Cosson et al., 2002c) and EMSA (Kim and Richter, 2007) but has not been quantitatively compared to that of PABP1. ePABP was shown to interact with eIF4G (Kim and Richter, 2007; Wilkie et al., 2005), PAIP1 (Wilkie et al., 2005) and eRF3 (Cosson et al., 2002c) and accordingly was shown to be associated with cap complexes in oocytes (Cosson et al., 2002c). The ability of ePABP to stimulate translation was also directly tested in oocytes by tethering to a reporter mRNA and shown to be similar to that of PABP1, i.e. 8-10 fold. Stimulation of translation by ePABP is increased upon oocyte maturation (to ~23 fold) and to a greater degree than PABP1 (~ 17 fold) (Wilkie et al., 2005). How ePABP stimulates translation more efficiently in matured oocytes is unknown but may explain its preferential use in early development.

Importantly, ePABP has been shown to protect messages from deadenylation (Kim and Richter, 2007; Voeltz et al., 2001) and activate the translation of messages

that are polyadenylated in the cytoplasm during oocyte maturation (Kim and Richter, 2007; Wilkie et al., 2005). Moreover, recent work has revealed that prior to oocyte maturation ePABP transiently interacts with the polyadenylation complex but following polyadenylation leaves the complex and binds the nascent poly(A) tail (Kim and Richter, 2007). A direct interaction between ePABP and CPEB mediates association with the polyadenylation complex and this interaction is abrogated by the phosphorylation of CPEB which occurs at maturation (Kim and Richter, 2007). PABP1 ectopically expressed in oocytes is also able to interact with CPEB (Kim and Richter, 2007) suggesting that PABP1 may similarly bind cytoplasmic polyadenylation complexes in somatic tissues.

Some other features of PABP1 are also conserved by ePABP. *Xenopus* ePABP binds PAIP2 and this interaction inhibits its association with poly(A) RNA (Kim and Richter, 2007). ePABP has also been shown to self-associate and interact with PABP1 (Wilkie et al., 2005) suggesting that in cells in which both proteins are expressed they could multimerize along a poly(A)-tail. It has also been shown to interact with Dazl (Collier et al., 2005) indicating that ePABP may be recruited to specific messages by protein interactions and could be important for Dazl function. While it is known that ePABP can bind AU-rich sequences, like PABP1 its function in their regulation is not clear (Voeltz et al., 2001).

1.4.6.2 tPABP

tPABP arose from a retrotransposition of PABP1 and shares with it 92% amino acid identity in humans (Feral et al., 2001; Kleene et al., 1998). tPABP RNA expression has been reported to be restricted to the testes by northern blot (Feral et al., 2001) and *in situ* hybridization revealed cell-type restricted expression of both PABP1 and tPABP within the testes (Feral et al., 2001). Human tPABP appears to bind poly(A) with a comparable affinity to PABP1 (Feral et al., 2001; Kimura et al., 2009) and was recently demonstrated to have a mainly cytoplasmic sub-cellular localisation (Kimura et al., 2009). Given the very close similarity between tPABP and PABP1 proteins it seems likely that many protein partners would also be shared with tPABP. Accordingly, tPABP has been shown to interact with PABP1 in testes

extract and to interact with eIF4G, PAIP1 and PAIP2 *in vitro* (Kimura et al., 2009). Finally, when tethered to a non-adenylated reporter mRNA, tPABP stimulated translation to a similar level to PABP1 in RRL (~1.6 fold) (Kimura et al., 2009) and so it appears that tPABP likely does function similarly to PABP1 to stimulate translation in testes.

1.4.6.3 PABP4

PABP4 was originally identified as an induced mRNA in activated human T-cells and hence is also known as inducible PABP or iPABP (Yang et al., 1995). It has also however, been shown by northern blot to be expressed at the mRNA level to different degrees in a wide range of human tissues, with highest levels in testis, ovary, heart and muscle (Yang et al., 1995). PABP4 was also identified as a protein expressed on the surface of activated platelets and consequently has also been referred to as activated platelet protein 1 (APP-1) (Houng et al., 1997).

Poly(A) binding by human PABP4 has been demonstrated (Houng et al., 1997) and its RNA binding affinities have also been directly compared with those of PABP1 by EMSA (Sladic et al., 2004). PABP4 has an affinity for poly(A) slightly lower than that of PABP1 (K_d of 1.1nM compared to 0.67), though it is less discriminatory in its RNA binding as it has a higher affinity for AU-rich sequences (2.4nM compared to 3.9nM binding by PABP1) and mixed RNA sequences (5.3nM compared to 7.7nM binding by PABP1) (Sladic et al., 2004). This suggests that PABP4 may also participate in the poly(A) and AU-RNA mediated activities described for PABP1.

Recent work elucidating the crystal structure of the human PABP1 PABC domain bound to the PAM2 domain of PAIP2 identified a sequence (LGE-LF) essential for generating a hydrophobic pocket that binds a phenylalanine residue in PAM2 (Kozlov et al., 2010a). The phenylalanine residue at position 567 in human PABP1 was determined to be the most important residue for PABC/PAM2 binding (Kozlov et al., 2010a). This residue and the entire LGE-LF sequence are identically conserved in PABP4 suggesting that PABP4 may maintain interactions with other

PAM2 containing proteins. Consistent with this notion, human PABP4 has been shown to interact with PAM2 proteins eRF3, by yeast 2-hybrid (Cosson et al., 2002a), and TOB, by co-immunoprecipitation (Okochi et al., 2005) implying that the characterised roles of PABP1 in translation and turnover may also be conserved by PABP4.

Addition of human PABP4 to an *in vitro* translation system resulted in a small increase in the protein expression from a polyadenylated luciferase reporter mRNA and a larger increase in protein expression from a polyadenylated *interleukin-2* (IL-2) mRNA (Okochi et al., 2005). This experiment was not controlled for mRNA abundance and so may reflect the effect of PABP4 on both translation and stability. Translation in *in vitro* extracts is known to exhibit only limited sensitivity to mRNA polyadenylation and PABP1 unless they are depleted of ribosomes (Michel et al., 2000), endogenous PABPs (Kahvejian et al., 2005) or supplemented with YB-1 (Svitkin et al., 2009) and so the modest effect of PABP4 on expression of a polyadenylated reporter is not necessarily indicative of a reduced ability to stimulate translation by binding to poly(A) tails. These data also implicate PABP4 in the regulation of specific messages via binding to non-poly(A) sequences, since stimulation of IL-2 expression was partly mediated by its 3'UTR (Okochi et al., 2005). The effect of PABP4 on IL-2 expression was reversed by the addition of TOB (Okochi et al., 2005), suggesting that PABP4 interaction with TOB may also stimulate the Caf1-CCR4 deadenylase, though this has not been tested.

Overexpression of PABP1 leads to down regulation of the abundance of its own mRNA (Hornstein et al., 1999b). Interestingly, it also leads to reduced abundance of PABP4 mRNA (Hornstein et al., 1999b) indicative of crosstalk in the regulation of both proteins and the implication that the total level of PABPs in a cell may be regulated. The ability of PABP4 (or any other cytoplasmic PABP) to cross-regulate the translation of PABP1 mRNA has not been tested.

1.4.6.4 PABP5

PABP5 is predicted to be expressed as a ~43kDa protein and lacks the proline-rich and globular domains that mediate many PABP1 protein interactions. It

is the member of the PABP family about which the least is known. A low level of RNA expression has been reported in various human tissues and to a higher level in the ovary which was determined by RT-PCR (Blanco et al., 2001) but the protein expression pattern has not been demonstrated. RNA binding by PABP5 has not been investigated however, the high degree of peptide sequence similarity with PABP1 hints that this ability is likely shared. The ability of PABP5 to bind RNA and its function in translation is the subject of current investigation in the Gray lab.

1.4.7 Why express multiple PABPs?

No clear explanation of the necessity for several highly similar PABPs in mammals has emerged and firm conclusions are restricted by the limited investigation into the functional characteristics of ePABP, tPABP, PABP4 and PABP5. While the absence of any deleterious effects of PABP gene duplication would be sufficient for the evolution of five cytoplasmic PABPs in mammals, the family members may also have acquired specialized roles. The metabolism of specific transcripts could be differentially regulated in a PABP-specific manner or differences in the regulation of their expression or activity could underlie the conservation of multiple PABPs.

In *Caenorhabditis elegans*, two PABPs are expressed (pab-1 and pab-2). Depletion by RNAi of pab-1 leads to sterility (Ceron et al., 2007; Ciosk et al., 2004; Kamath et al., 2003; Maciejowski et al., 2005) but RNAi of pab-2 does not cause a detectable phenotype (Ceron et al., 2007; Maciejowski et al., 2005). Knockdown of both pab-1 and pab-2 however, results in somatic developmental defects (Ciosk et al., 2004) suggesting that pab-1 and -2 have redundant functions in somatic cells in *c. elegans* in addition to the essential role of pab-1 in the germline. In *Arabidopsis* eight PABP genes have been identified (Belostotsky, 2003). Mutations in two of these (PAB2 and PAB5) have been generated and both mutants were non-viable, though the expression of PAB5 is restricted to the plant reproductive organs (Bravo et al., 2005) demonstrating the importance of multiple PABPs in this system. Deletion of the single *Drosophila* PABP is lethal (Sigrist et al., 2000) and as yet there have been no published reports of PABP knockout animals in higher eukaryotes.

Thesis outline

At the outset of my PhD little was known about the function of PABP4 in translation and there had been no investigation of PABP4 protein expression or localisation in any metazoan species. Therefore, I set out to characterise the expression pattern of PABP4 in mammals and test its potential to regulate translation (Chapter 3). As recent work has shown that the localisation of PABP1 and other eIFs is highly regulated, I examined the sub-cellular localisation of PABP4 in non-stressed and stressed conditions and investigated the requirement of PABP1 and PABP4 for stress granule assembly (Chapter 4). Interestingly, PABP1 and PABP4 were found to relocalise to the nucleus following UV irradiation and the consequences of this altered localisation were explored (Chapter 5). In order to dissect the mechanism for UV-induced PABP relocalisation, investigation of the regulation of PABP1 and PABP4 subcellular localisation was undertaken (Chapter 6).

Chapter 2: Materials and Methods

2.1 General Buffers and solutions

TAE

90 mM Tris-HCl pH 8.3, 90mM acetic acid, 2mM EDTA

Tris-buffered saline (TBS)

10mM Tris, 137mM NaCl; pH 7.4

TBST

TBS + 0.1% Tween-20

Phosphate buffered saline (PBS)

137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4 , 6.5mM Na_2HPO_4 ; pH 7.4

20x Sodium Chloride / Sodium Citrate buffer (SSC)

3M NaCl, 0.3M $\text{Na}_3\text{citrate}\cdot 2\text{H}_2\text{O}$; pH7.0

4% Paraformaldehyde (PFA)

4% weight/volume (w/v) paraformaldehyde in PBS, heated to 60°C with stirring in fumehood until solution clears.

Luria Broth (LB) (supplied by MRC HGU/HRSU technical services)

10g/l NaCl, 10g/l Bacto-tryptone, 5g/l Yeast extract

LB agar (supplied by MRC HGU/HRSU technical services)

10g/l NaCl, 10g/l Bacto-tryptone, 5g/l Yeast extract, 15g/l Difco Agar

All chemicals were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Oligonucleotides were purchased from Eurogentec (Seraing, Belgium).

2.2 Plasmids

His-PABP1

His-tagged PABP1 was created by M. Brook by PCR amplification of the human PABP1 ORF using primers 5'-GGATCCTTATGAACCCCAGTGCCC-3' and 5'-GTCGACTCAAACAGTTGGAACACCGGTGG-3' and IMAGE clone #6816124 as a template. This fragment was cloned into ZEROblunt (Invitrogen, Paisley, UK) following the manufacturer's instructions. The PABP1 fragment was then released using BamHI and SalI restriction enzymes and ligated into pET28c (Novagen/Merck, Nottingham, UK) linearised by BamHI and SalI.

His-PABP4

His-tagged PABP4 was created by PCR amplification of the full length human PABP4 ORF using primers 5'-AGGCTTCTATGAACGCTGCGGCCAGC-3' and 5'-CTCGAGAGAGGTAGCAGCAGCAACAGCG-3' and IMAGE clone #30331896 as a template. This fragment was cloned into ZEROblunt (Invitrogen, Paisley, UK) following the manufacturer's instructions. The PABP4 fragment was then released using XhoI and HindIII restriction enzymes and ligated into pET28c linearised by XhoI and HindIII.

ICP27-GFP

Kind gift of Dr P. Malik.

pSUPER and pSUPERTAP

Kind gift of S. Wilson (Williams et al., 2005).

2.3 Antibodies

2.3.1 In-house antibodies

PABP1 and PABP4 antibodies were generated by CovalAB (Cambridge, UK) using their 88 day protocol and peptides of our design. A single peptide from human/mouse PABP1 (AQKAVNSATGVPTV) and two peptides from human/mouse PABP4 (VRSPHPAIQPLQA and KKEAAQKVGTVAAATS) were used to raise polyclonal antibodies in rabbits. ELISA testing by CovalAB indicated that the majority of the immune-reactivity of the anti-PABP4 sera was provided by the more C-terminal peptide (KKEAAQKVGTVAAATS). Polyclonal sera were affinity purified by CovalAB or in-house before use. Affinity purified stocks were stored in 20% glycerol in PBS at -20°C in aliquots to avoid freeze thawing.

2.3.2 Commercial antibodies

Commercial antibodies were purchased from Abcam (Cambridge, UK), BD (Oxford, UK), Santa Cruz (Santa Cruz, CA, USA), Sigma, Bethyl Labs (Montgomery, TX, USA), Pierce (ThermoFisher, Loughborough, UK), Invitrogen (Paisley, UK) and Vector Labs (Peterborough, UK) as indicated in Tables 2.1 and 2.2, which also detail the dilutions each was used at in western blot (WB), immunofluorescence (IF) and immunohistochemistry (IHC).

2.4 Mouse tissues

Mice used were adult CD-1 strain (Charles River, Edinburgh, UK). Dissection and fixation for immunohistochemistry was performed by L. McCracken.

Table 2.1 Primary Antibodies

Antibody	Species	Supplier/cat no	WB	IF	IHC
Ataxin-2	mouse	BD (611378)	-	-	1/500
eIF4GI	rabbit	S.J. Morley	1/5000	-	-
GFP	mouse	Abcam (Ab38689)	1/1000	-	-
GAPDH	mouse	Abcam (Ab9484)	1/1000	-	-
G3BP	mouse	BD (611126)	-	1/200	-
HuR	mouse	Santa cruz (sc-56709)	-	1/1000	-
PABP1 (117ng/μl)	rabbit	Gray group	1/10000	1/500	1/1000
PABP4 (900ng/μl)	rabbit	Gray group	1/5000	1/500	1/500
Paip1	rabbit	N. Sonenberg	1/2000	-	-
Paxillin	mouse	BD (610051)	1/5000	1/1000	-
Poly-Histidine	mouse	Sigma (H1029)	1/1000	-	-
RNAPII CTD pS5	rabbit	Abcam (Ab5131)	1/2000	-	-
SC-35	mouse	Abcam (Ab11826)	-	1/500	-
TAP/NXF1	mouse	Abcam (Ab50609)	1/2000	1/500	-
α-tubulin	mouse	Sigma (6074)	1/10000	-	-
IgG control	mouse	Sigma (I5381)	-	-	-
IgG control	Rabbit	Sigma (I5006)	-	-	-

Table 2.2 Secondary antibodies

Anti-species	Species raised in	conjugate	Supplier/cat no	WB	IF	IHC
rabbit	goat	HRP	Sigma (A0545)	1/100,000	-	-
mouse	goat	HRP	Pierce (31444)	1/20,000	-	-
rabbit	donkey	Alexa-488	Invitrogen (A21206)	-	1/500	-
mouse	donkey	Alexa-488	Invitrogen (A21202)	-	1/200	-
rabbit	goat	Alexa-546	Invitrogen (A11035)	-	1/200	-
mouse	donkey	Alexa-546	Invitrogen (A10036)	-	1/500	-
rabbit	goat	biotin	Vector (E0432)	-	-	1/500
mouse	goat	biotin	Vector (E0433)	-	-	1/500

2.5 General Molecular Biology Methods

2.5.1 Agarose gel electrophoresis

0.8-1.5% agarose gels were made by dissolving appropriate amounts of agarose in TAE by heating in a microwave. For visualisation of bands, ethidium bromide (BDH Electran, Lutterworth, UK) was added to a concentration of 0.5µg/ml or Gelred (Biotium, Hayward, CA, USA) was added to a 1x concentration. 6x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was added to DNA samples to aid loading. Gels were run at 50-150 volts in 1 x TAE buffer for 30-90 minutes and visualised using a Geneflash transilluminator. Benchtop 1kb or 100bp ladders (Promega, Southampton, UK) were used to determine DNA band sizes.

2.5.2 Polymerase chain reaction (PCR)

Unless otherwise stated, 50µl PCRs were conducted with the following reaction mixture: 50ng of template plasmid DNA (or 5µl of reverse transcription reaction), 1.5mM MgCl₂, 1x reaction buffer (Bioline, London, UK), 0.2mM dNTPs, 3 units Bio-X-Act Long High-Fidelity polymerase (Bioline), 1µM each of forward and reverse primers. Reactions were incubated in a Dyad PCR machine (Biorad, Hemel Hempstead, UK) with the following program:

- 1) 5 minutes at 95°C
- 2) 1 minute at 55°C
- 3) 2 minutes at 72°C
- 4) 30 seconds at 95°C
- 5) 30 seconds at 55°C
- 6) Repeat steps 3-5 29 times
- 7) 10 minutes at 72°C.

2.5.3 RT-PCR

cDNA was prepared from RNA using a First Strand cDNA Synthesis AMV Kit (Roche, Welwyn Garden City, UK) according to the manufacturer's instructions. Poly(dT)₁₅ and random primers were used together in the reaction to amplify adenylated and non-adenylated RNAs. Intron-spanning gene specific primers were then used in a standard PCR reaction to detect specific transcripts (Table 2.3).

Table 2.3 RT-PCR primers

Detects	Primer direction	Sequence (5'-3')
hPABP1	Forward	GAGCTGTTCCCAACCCTGTA
	Reverse	CCAACATGGAAGCAGTCAAA
hPABP4	Forward	ACACGAGGATGCCAATAAGG
	Reverse	GGCGTATAGCACTTGGCATT
hPABP4 isoforms	Forward	GGCTACTTTGTGCCAGCAGT
	Reverse	TATTTGTAGGGGGCAACAGC

2.5.4 PCR purification by column

PCR products were purified before restriction digest or ligation using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) according to manufacturer's instructions.

2.5.5 PCR purification by gel extraction

DNA was run on 1-2% agarose gel containing ethidium bromide or Gelred. The appropriate band was identified by UV light and a gel slice extracted using a sterile scalpel. DNA was extracted using QIAquick Gel Extraction Kit (Qiagen, Crawley, UK) according to manufacturer's instructions.

2.5.6 Isolation of plasmid DNA

Plasmid DNA was extracted from bacterial cultures using Qiagen (Crawley, UK) mini-prep, midi-prep or maxi-prep kits according to the manufacturer's instructions.

2.5.7 RNA isolation

RNA was isolated from cultured cell pellets using TRI reagent (Ambion, Austin, TX, USA). Pellets of approximately 10^6 cells were lysed in 100 μ l volume of TRI reagent then incubated at room temperature for 5 minutes. Samples were then centrifuged at 12,000 *g* at 4°C for 10 minutes and the supernatant transferred to a new tube. 1/10th the TRI reagent volume of chloroform was then added before vortexing and incubation at room temperature for 5-15 minutes. The samples were then centrifuged for 15 minutes at 12,000 *g* at 4°C and the aqueous phase collected for RNA precipitation. ½ volume of TRI reagent of isopropanol was added to each of the samples which were then vortexed for 5-10 seconds and incubated at room temperature for 5-10 minutes. Samples were then centrifuged at 12,000 *g* for 8 minutes at 4°C. The supernatant was discarded and the pellets washed by adding 1ml of 75% ethanol and centrifuging again for 5 minutes at 12,000 *g*. Pellets were briefly air dried and resuspended in dH₂O.

2.5.8 Nucleic acid quantification

DNA and RNA was quantified by measuring absorbance at 260nm using a Nanodrop ND-1000 (Nanodrop Technologies Inc., Wilmington, DE, USA) spectrophotometer.

2.5.9 Restriction enzyme digests

Restriction digests were carried out using Roche (Welwyn City, UK) restriction enzymes according to manufacturer's instructions and using the recommended supplied buffers.

2.5.10 DNA dephosphorylation

Restriction enzyme cut plasmid DNA for ligation was dephosphorylated using shrimp alkaline phosphatase (SAP; Roche, Welwyn Garden City, UK) to prevent self-ligation. The enzyme was used according to manufacturer's instructions and deactivated by heating to 65°C for 10 minutes after the reaction.

2.5.11 DNA ligation

Ligations of dephosphorylated cut plasmid and DNA inserts were conducted using T4 DNA ligase (New England Biolabs, Hitchin, UK) overnight at room temperature and reactions set up according to the manufacturer's instructions. In general, a molar ratio of vector:insert of 1:3 was used.

2.5.12 pGEM-T Easy cloning

For cloning into pGEM-T Easy plasmid the PGEM-T Easy vector system kit (Promega, Southampton, UK) was used according to manufacturer's instructions.

2.5.13 DNA sequencing

Sequencing to verify the content of all constructed plasmids was performed using the sequencing service at the MRC Human Genetics Unit, Edinburgh, UK. Sequences were analysed using Vector-NTI 11 software (Invitrogen, Paisley, UK).

2.6 Bacterial techniques

2.6.1 Bacterial transformations with plasmids

Chemically competent *E. coli* cells were thawed on ice. Approximately 100ng-1µg of plasmid was added to 6µl of cells. Cells were incubated on ice for 2 minutes then heat shocked at 42°C for 2 minutes and then incubated on ice for another 2 minutes. 100µl of LB media was added to the cells which were then incubated for 30 minutes at 37°C. The cell suspension was then plated on LB agar containing the appropriate selective antibiotic. Cultures were grown overnight at 37°C.

2.6.2 Bacterial plasmid propagation

All plasmids were propagated in XL1-Blue *E. Coli* (Stratagene, Cedar Creek, TX, USA), genotype: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)]. Bacteria were maintained on Luria Bertani (LB) agar

plates or in LB medium containing Kanamycin (Melford, Ipswich, UK) at 40µg/ml or Ampicillin (Melford) at 100µg/ml.

2.6.3 Bacterial transformations with ligation solutions

Chemically competent XL1-Blue *E. Coli* cells were thawed on ice. 5µl (1-2ng) of a 10µl ligation solution was added to 50µl cells. Cells were incubated on ice for 30 minutes, heat shocked at 42°C for 45 seconds and then incubated on ice for 2 minutes. 450µl of LB media was added to the cells which were then incubated for 60 minutes at 37°C. The cell suspension was then plated on LB agar containing the appropriate selective antibiotic. If blue/white selection was required 50µl of 20mg/ml X-gal (Melford, Ipswich, UK) and 100µl of 100mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Melford) were spread over the plates before the transformed bacteria were plated. Cultures were grown overnight at 37°C.

2.6.4 Recombinant protein expression in bacteria

Protein-expression constructs were transformed into BL21 *E. Coli* (GE Healthcare, Amersham, UK), genotype: B F⁻, ompT, hsdS (r_B⁻, m_B⁻), gal, dcm. A single colony was used to inoculate a 5ml LB culture containing the appropriate antibiotic which was then incubated overnight (approximately 16 hours) at 37°C. This culture was used to inoculate a 100ml LB which was grown at 37°C until it reached an optical density (OD) of 0.4 at 600nm absorbance. OD was measured using a Genequant Pro spectrophotometer (GE Healthcare). Recombinant protein expression was induced by the addition of IPTG to a final concentration of 1mM. The culture was then incubated for 30 hours at 16°C before the bacteria were collected by centrifugation at 12,000 g for 15 minutes. Bacteria were then resuspended in bug lysis buffer (0.6M KCl, 0.25M NaCl, 20mM Tris.HCl (pH8.0), 0.5% NP-40, 20mM imidazole with EDTA-free Complete protease inhibitor tablets (Roche, Welwyn Garden City, UK)) and disrupted by five 15 second exposures to sonication using a microtip sonicator (MSE Soniprep 150). The sonicated extract was then clarified by centrifugation at 8000 g for 10 minutes at 4°C.

2.7 Protein techniques

2.7.1 Protein quantification by Bradford Assay

1 µl of protein sample was added to 99µl of dH₂O in a cuvette and a further 900µl of 1x Bradford reagent (Biorad, Hemel Hempstead, UK) then added. A standard curve of bovine serum albumin from 0 to 10µg was prepared in the same manner. A Genequant Pro spectrophotometer (GE Healthcare, Amersham, UK) was used to read the absorbance at 595nm and to calculate the sample concentration from the standard curve.

2.7.2 SDS polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted either with NuPAGE 4-12% MOPS gels (Invitrogen Paisley, UK) or 10% acrylamide gels detailed below. All samples were mixed with the appropriate volume of 4x SDS-PAGE loading buffer (20% Glycerol, 200mM 2-mercaptoethanol, 4% SDS, 0.2% Bromophenol blue and 100mM Tris-HCl pH 6.8) and heated for 5 minutes at 95°C before loading onto the gel alongside either pre-stained or unstained Benchmark marker (Invitrogen, Paisley, UK).

10% acrylamide gels:

Stacking gels were made with 4% polyacrylamide (29:1 acrylamide:bis-acrylamide; Biorad, Hemel Hempstead, UK), 115mM Tris-HCl pH6.8 and 0.1% SDS. Separating gels were made with 10% polyacrylamide, 375 mM Tris-HCL pH8.8 and 0.1% SDS. Gels were polymerised with 1% ammonium persulphate (APS) and 0.1% tetramethylethylenediamine (TEMED). 10% gels were run at 160V in SDS-PAGE running buffer (25mM Tris, 250mM Glycine, 0.1% SDS; pH 8.8) until the dye front ran off the gel.

NuPAGE system:

4-12% NuPAGE MOPS gels (Invitrogen, Paisley, UK) were run in MOPS running buffer (50mM MOPS, 50mM Tris, 0.1% SDS, 1mM EDTA; pH7.7) at 160 or 200 volts until the dye front ran off the gel. For RNAPII and eIF4G western blots, gels were run for 2 hours at 160V.

2.7.3 Western blotting

Transfer

Acrylamide gels were transferred to polyvinylidene fluoride (PVDF) membranes using semi-dry transfer apparatus. NuPAGE gels were transferred onto Immobilon membrane (Thermo Fisher, Loughborough, UK) sandwiched between 3 sheets of blotting paper soaked in NuPAGE transfer buffer (Invitrogen, Paisley, UK). 10% gels were transferred in the same manner in transfer buffer (25mM Tris, 192mM Glycine, 20% Methanol; pH 7.4). Proteins were transferred using a fixed current of 100 mA per gel for 1 hour. Following transfer, total protein was stained using Gelcode Blue (Thermo Fisher) according to the manufacturer's instructions and unstained molecular weight markers marked on the membrane.

Blocking, antibody incubations and detection

Membranes were blocked in 5% (w/v) skimmed milk powder in TBST. Primary antibodies were diluted in blocking solution and incubated with membranes either for 1 hour at room temperature or at 4°C overnight on a rocking platform. Membranes were then washed for 5 minutes in TBST three times before incubation with an appropriate secondary antibody conjugated to HRP in blocking solution for 1 hour at room temperature. Membranes were then washed 3 more times with TBST before the application of enhanced chemiluminescence (ECL) solution (GE Healthcare, Amersham, UK) which was left on the membrane for 1 minute. Membranes were blotted dry with filter paper and wrapped in saran wrap before being exposed onto X-ray film (Hyperfilm; GE Healthcare, Amersham, UK) and processed using an Exograph compact X4 X-ray processor. Dilutions of all primary and secondary antibodies used are detailed in tables 2.1 and 2.2 respectively.

2.8 Cell culture techniques

2.8.1 Cell Culture

HeLa (human cervical carcinoma; strain A in (Viegas et al., 2007)), HEK293 (human embryonic kidney), Vero (African green monkey kidney epithelial) and 3T3 (mouse fibroblast) cells were maintained at 37°C in 5% CO₂ in Dulbecco's minimal essential medium (DMEM; Invitrogen, Paisley, UK) containing 10% fetal calf serum (Invitrogen). To remove cells from flask surface, cells were washed twice with PBS and incubated with 10% trypsin in PBS for 3 minutes at room temperature. Hakat (Human keratinocyte), HT1080 (human fibrosarcoma), RPE (human retinal pigmented epithelium) and SW480 (human colon adenocarcinoma) cells were kind gifts of Wendy Bickmore, David Fitzpatrick, Alan Wright and Malcolm Dunlop respectively at MRC HGU Edinburgh, UK.

2.8.2 Cell counting

Cells harvested by trypsin were resuspended in a large volume of DMEM (typically 12ml per T75 flask) and a 10µl aliquot of this suspension applied to an improved Neubauer haemocytometer (Hawksley Crystallite BS748; 0.1mm depth, 1/400 mm²). Cells in four 1mm² squares were counted and the average of these multiplied by 10⁴ to give the concentration of cells per ml.

2.8.3 DNA Transfection

HeLa cells were grown to 60-80% and transfected with plasmid DNA using Attractene (Qiagen, Crawley, UK) diluted in OPTIMEM (Invitrogen, Paisley, UK) and following the manufacturer's instructions.

2.8.4 siRNA Transfection

HeLa cells were transfected with siRNAs (Qiagen, Crawley, UK) using Hiperfect (Qiagen) diluted in OPTIMEM (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Typically 2 x 10⁵ cells were seeded per 24-well plate well and transfected the following day using 3µl of Hiperfect. Cells were then

incubated for a further 48h before lysis, fixation or trypsinisation, during which time siRNA complexes were not removed.

2.8.5 Lysis of cultured cells in phospho-RIPA buffer

Adherent cultured cells were washed twice in ice-cold PBS on ice and collected in PBS using a cell scraper. Cells were pelleted by centrifugation at 1000 g for 5 minutes at 4°C and the cell pellet resuspended in phospho-RIPA buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.2% SDS, 10mM Na₄P₂O₇ (sodium pyrophosphate), 25mM glycerol-2-phosphate, 0.5% C₂₄H₃₉O₄.Na (sodium deoxycholate), 100mM Na₃OV₄ (sodium orthovanadate), 5mM NaF, 2mM DTT, 10nM calyculin A (Calbiochem/Merck, Nottingham, UK) with Complete protease inhibitor tablets (Roche, Welwyn Garden City, UK); pH 7.4). Cells were lysed for 10 minutes on ice. The extract was then centrifuged at 12,000 g for 10 minutes to pellet the cell debris and the supernatant retained for further analysis.

2.8.6 Trypan Blue cell viability assay

HeLa cells grown in a 24 well plate were trypsinised, diluted 1:1 with DMEM and transferred to an eppendorf. This cell suspension was mixed 1:1 with trypan blue stain (0.4%) and incubated for 5 minutes at room temperature. Cells were then examined in an improved Neubauer haemocytometer (Hawksley Crystallite BS748; 0.1mm depth, 1/400mm²) under a light microscope and approximately 300 cells scored for blue staining, which indicates non-viability.

2.9 Experimental procedures

2.9.1 Cell fractionation

Adherent cultured cells were washed twice in ice-cold PBS and collected in PBS using a cell scraper. Cells were pelleted by centrifugation at 1000 g for 5 minutes at 4°C. The pellet was then resuspended in cytoplasmic lysis buffer (10mM HEPES pH7.6, 3mM MgCl₂, 40mM KCl, 50mM glycerol-2-phosphate, 5% glycerol, 0.5% Igepal CA-630, 2mM NaF, 1mM Na₃OV₄, 2mM DTT, 10nM calyculin A

(Calbiochem/Merck, Nottingham, UK) with Complete protease inhibitor tablets (Roche, Welwyn Garden City, UK)). Cells were incubated on ice for 5 minutes and the nuclei separated from the soluble cytoplasmic fraction by centrifugation at 1000 *g* for 5 minutes at 4°C. The supernatant was collected as the cytoplasmic fraction. The nuclei were then resuspended in 8 volumes of nuclei wash buffer (10mM HEPES pH7.6, 1.5mM MgCl₂, 10mM KCl, 50mM glycerol-2-phosphate, 2mM NaF, 1mM Na₃VO₄, 1mM DTT, 10nM calyculin A with Complete protease inhibitor tablets (Roche, Welwyn Garden City, UK)) and centrifuged again at 1000 *g* for 5 minutes at 4°C. Wash buffer was aspirated from the nuclei which were then lysed in an appropriate volume of 2x SDS-PAGE loading buffer to achieve a 1x final concentration. To shear the DNA the lysates were then passed through a Qias shredder column (Qiagen, Crawley, UK) following manufacturer's instructions, until the lysate was no longer viscous, typically 3 times. Cell equivalent volumes of nuclear and cytoplasmic extracts were then analysed by western blotting.

2.9.2 Immuno-precipitation (IP)

0.5mg of RIPA cell lysate was incubated overnight on a rotating wheel at 4°C with 1µg of anti-PABP1, anti-PABP4 or rabbit IgG control antibodies. Where appropriate, the mixture was supplemented with 200 units of RNaseI (Ambion, Austin, TX, USA). 100µl of 50% protein G sepharose beads (GE Healthcare, Amersham, UK) were washed in high salt (0.3M NaCl) phospho-RIPA buffer and then added to each IP. The beads were mixed for a further 4 hours at 4°C. Beads were then collected in Pierce spin cups (ThermoFisher, Loughborough, UK) by gentle centrifugation and washed eight times with high salt phospho-RIPA. Beads were then resuspended in 50µl 1x SDS-PAGE loading buffer and heated to 75°C for 10 minutes before collection of immunoprecipitated proteins by centrifugation at 12,000 *g* for 1 minute.

2.9.3 Dithiobis [succinimidyl propionate] (DSP) cross-linking and immunoprecipitation

To crosslink intracellular proteins, DSP was solubilised in DMSO to a 10mM stock solution. Cells were collected by scraping and the cell pellet resuspended in PBS containing DSP at a final concentration of 0.1mM. Cells were then incubated on a rotating wheel at 4°C for 10 minutes. The DSP was then quenched by the addition of Tris pH7.5 to a final concentration of 20mM and incubated on the rotating wheel at 4°C for a further 10 minutes. The cells were then pelleted by centrifugation at 1000 *g* for 5 minutes and lysed in phospho-RIPA buffer without DTT for 10 minutes on ice before centrifugation at 1000 *g* for 10 minutes. The remainder of the immunoprecipitation was carried out as described above, with the exception that buffers before the elution step did not contain DTT, which acts to reverse the cross-linking.

2.9.4 Sucrose gradient polysome analysis

To separate translation complexes, cell lysates were centrifuged through a sucrose density gradient which was then fractionated, and the proteins in each fraction subject to western blotting.

Gradient preparation

10 and 50% (w/v) sucrose solutions were prepared in gradient buffer (20mM HEPES pH7.2, 250mM KCl, 2mM MgCl₂, 2.5mM DTT, 0.5µg/ml Heparin, 0.5% NP-40). 10-50% gradient were poured by mixing equal volumes of 10 and 50% solutions using a dual chamber manual gradient pourer attached to a peristaltic pump. Gradients were weighed and made up to equal mass and left to stand overnight at 4°C prior to centrifugation.

Cell lysis and centrifugation

Cultured cells were removed from their culture dish by trypsinisation, washed once with ice cold PBS and collected by centrifugation at 1000 *g* for 5 minutes. One T-75

of sub-confluent (70-80%) cells was used per gradient. The cell pellet was then resuspended in gradient lysis buffer (10mM Tris-HCl pH7.2, 150mM KCl, 10mM MgCl₂, 20mM DTT, 0.5% NP-40, 100u/ml RNasin (Promega, Southampton, UK) with Complete protease inhibitor tablets (Roche, Welwyn Garden City, UK)) which was supplemented with either 150µg/ml cycloheximide or 20mM EDTA, and incubated on ice for 10 minutes. 1ml of lysis buffer was used per T-75 of cells. Lysates were then centrifuged for 1 minute at 12,000 *g* at 4°C and the KCl concentration of the supernatant increase to 250mM. Lysates were then gently loaded onto the gradients which were centrifuged for 2 hours at 38000 RPM at 4°C in a Sorval TH-641 rotor.

Fraction collection

Gradients were then divided into fractions using Superfrac fraction collector (Pharmacia, Uppsala, Sweden) at 4°C. Absorbance of RNA at 254nm was recorded by an inline UV monitor (Pharmacia) to assign the location of polysomal, 80s, 40s and mRNP peaks to the fractions collected.

TCA Precipitation of proteins

Proteins were isolated from the sucrose fractions by trichloroacetic acid (TCA) precipitation. An equal volume of 20% TCA was mixed with each sucrose fraction and incubated on ice for 30 minutes. Samples were then centrifuged for 15 minutes at 12,000 *g* at 4°C. The protein pellets were then washed twice with ice cold acetone which was in each case left on the pellet for 30 minutes. After the second acetone wash the pellet was air dried and resuspended in 1x SDS-PAGE loading buffer.

2.9.5 Metabolic Labelling

To assay protein synthesis rate, newly synthesized proteins in HeLa cells were pulse-radiolabelled. HeLa cells were grown in 6 well plates and each experimental point conducted in triplicate within each independent experiment. Immediately prior to labelling, cells were washed twice with PBS. Cells were then incubated for 15 minutes in methionine-free DMEM (MP Biomedicals, Cambridge, UK) supplemented with 2mM L-glutamine (Invitrogen, Paisley, UK), 10% FCS and 50 μ Ci/ml ³⁵S-methionine (from stock with specific activity of 10 μ Ci/ μ l; MP Biomedicals - cell culture grade). Following labelling, cells were washed twice on ice with ice cold PBS. Cells were then lysed in RIPA buffer and protein concentration determined by Bradford assay.

To count only ³⁵S-methionine incorporated into proteins, 10 μ g of lysate was TCA precipitated. Samples were first mixed with 100 μ g BSA and then 1ml ice cold 10% TCA and incubated on ice for 30 minutes. Samples were then vacuum filtered through glass microfibre filters (Whatman GF-C ϕ 2.4mM; Whatman Ltd, Kent, UK) prewetted with 10% TCA. Filters were washed twice with 2ml ice cold 10% TCA and then twice with 2ml ice cold 100% ethanol. Filters were counted in 3ml scintillation liquid (OPTIPHASE HISAFE2; Perkin Elmer, Beaconsfield, UK) using a 1450 microbeta Wallac trilux liquid scintillation counter.

2.9.6 Lysis and homogenization of mouse tissues in phospho-RIPA buffer

Mouse organs and tissues were snap frozen in cryovials in liquid nitrogen immediately following collection. They were then homogenized in phospho-RIPA buffer (see Chapter 2.7.5) using an electric tissue homogenizer and lysis allowed to proceed for 10 minutes on ice. The extracts were then centrifuged at 12,000 *g* for 10 minutes to pellet the cell debris and the supernatant retained for western blot analysis.

2.9.7 Immunohistochemistry (IHC)

Immunohistochemistry was used to identify protein expression patterns within mouse tissues. The detection system used involved incubation with a primary antibody to detect the protein of interest and a secondary antibody conjugated to biotin (Figure 2.1). Detection relied upon biotin binding to streptavidin conjugated to horseradish peroxidase (HRP) which catalyses the oxidation of 3,3'-diaminobenzidinetetrahydrochloride (DAB) to an insoluble brown coloured product.

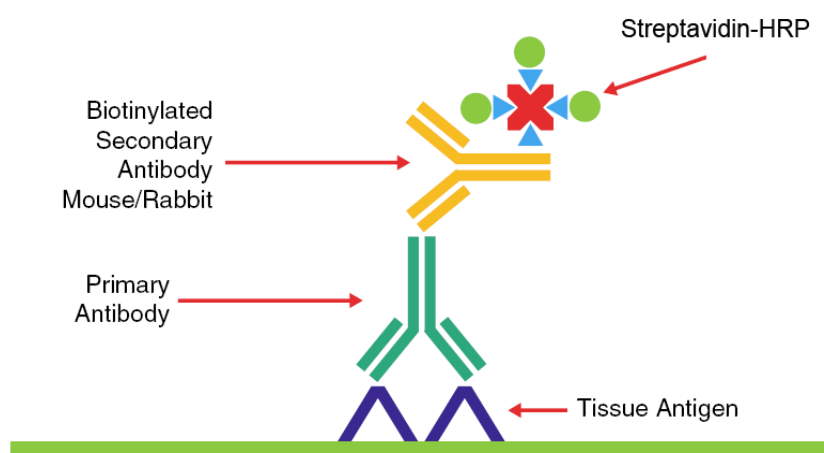


Figure 2.1 Schematic of immunohistochemistry detection method. Taken from Kumar and Rudbeck, 2009.

Tissue fixation and processing

Tissue samples were fixed overnight in Bouins fluid (Clintech, Guilford, UK). Samples were then transferred to 70% ethanol and alcohol rehydrated in increasing concentrations of alcohol, followed by xylene. They were then embedded in hot paraffin wax in block holders. Five micron sections were cut using a microtome and mounted on glass slides. Slides were incubated at 55°C overnight to dry.

Dewaxing and rehydrating

Paraffin embedded sections on slides were dewaxed and rehydrated by the following sequence of incubations: 2 x 5 minutes in xylene, 20 seconds in 100% ethanol, 20 seconds in 95% ethanol, 20 seconds in 75% ethanol, wash in tap water. All steps were conducted at room temperature.

Blocking

Following rehydration, samples were first blocked in methanol peroxide (3% v/v hydrogen peroxide (VWR, Lutterworth, UK) in methanol) for 30 minutes. This step minimises the effect of endogenous peroxidases in the tissue on DAB substrate. Slides were then washed once in water and once in TBS. Samples were then incubated with IHC blocking solution (5% v/v goat serum, 5% w/v BSA in TBS) for one hour at room temperature. Samples were washed in TBS once and then treated using a Streptavidin-Biotin Blocking Kit (Vector Labs, Peterborough, UK) according to manufacturer's instructions, to minimise the background effect of endogenous biotin in the tissue. This involved adding a streptavidin solution to the samples and incubating for 15 minutes, two TBS washes, incubation with a biotin solution for 15 minutes and two more TBS washes. All steps were conducted at room temperature.

Antibody incubations

Primary antibodies were diluted in IHC blocking solution and added to the samples which were incubated overnight at 4°C. Slides were then washed twice in TBS. Biotin-conjugated secondary antibodies were diluted in IHC blocking solution, added to the samples and incubated for one hour at room temperature. Slides were then washed twice in TBS and incubated with streptavidin-HRP (Vector Labs #SA5004) diluted 1/1000 in TBS for 15 minutes at room temperature. Slides were then washed two final times in TBS.

DAB development

DAB working solution was made up from the ImmPACT™ DAB kit (Vector Labs #SK-4105) and added to slides. Colour development was monitored by light microscopy and the reaction stopped by washing in water.

Counterstaining, Dehydration and Mounting

Nuclei were counterstained in Harris's haematoxylin with the following series of incubations: 1 minute haematoxylin (Triangle Biomedical Sciences; Durham, NC USA), wash in water, 10 seconds acid alcohol (1% concentrated HCl in 70% ethanol), wash in water, 30 seconds Scott's Tap Water (20mM KHCO₃, 81mM

MgSO₄·7H₂O), wash in water. Slides were then rehydrated in increasing concentrations of ethanol (70%, 95%, 100% x2) for 20 seconds each and bathed in xylene for 5 minutes twice consecutively. Slides were then mounted in Pertex (Cell Path, Hemel Hemstead, UK).

2.9.8 Immunofluorescence

Cells grown on coverslips or chamberslides (NUNC Labtek II; Thermo Fisher) were washed 3 times with PBS and fixed for 15 minutes with 4% (w/v) paraformaldehyde in PBS. Cells were then permeabilised with ice cold methanol for 10 minutes, washed with PBS and then stored in PBS at 4°C. Slides/coverslips were blocked in 4% horse serum, 0.02% sodium azide in PBS for a minimum of 30 minutes. Primary antibodies were applied in blocking solution for a minimum of 1 hour. Slides/coverslips were washed 3 times with PBS before secondary antibodies were applied in blocking solution for a minimum of 1 hour. Slides/coverslips were washed again 3 times in PBS. Cells grown on chamberslides were mounted in the liquid mounting medium “Vectashield with DAPI” (Vector Labs, Peterborough, UK), covered with coverslips and sealed with nail varnish. Cells grown on 22 x 22mm square coverslips in 6-well plates were also mounted in “Vectashield with DAPI”, dropped on to glass slides and sealed with nail varnish. Cells grown on 13mm diameter circular coverslips in 24-well plates were stained with DAPI at 1µg/ml during secondary antibody incubation, mounted in Permafluor (Thermo Fisher) and dropped onto glass slides. These coverslips did not require sealing as Permafluor sets to become a solid mountant.

2.9.9 Poly(A)+ RNA In Situ

Protocol was slightly modified from that at http://openwetware.org/wiki/Poly_A_RNA_in_situ_protocol. Cells grown on coverslips or chamberslides were washed 3 times with PBS and fixed for 15 minutes with 4% (w/v) paraformaldehyde. Cells were then permeabilised with ice cold methanol for 10 minutes, and then incubated in 70% ethanol for at least 10 minutes. When pre-hybridisation RNase treatment was applied, 0.1µg/µl RNase A in PBS was added to the cells which were then incubated for 30 minutes at 37°C. Cells were then incubated in 1M Tris pH8 for 5 minutes. A Cy3 conjugated oligo dT₄₀ probe (Eurogentec, Seraing, Belgium) was added to hybridization buffer (0.005% BSA, 1mg/ml yeast RNA, 10% dextran sulphate, 25% formamide in 2x SSC) to a final concentration of 1.3ng/µl. The probe was added to the slides which were then placed in a humidity chamber and incubated for 1 hour at 37°C. Following hybridization, cells were washed once with 4x SSC and then 2x SSC. For immediate visualisation cells were washed once with 2x SSC +0.1% triton X-100 and the slides mounted in vectashield containing DAPI (Vector Labs, Peterborough, UK). For additional immunofluorescence after *in situ*, primary antibodies were diluted in 2x SSC +0.1% triton X-100 (with the exception of anti-PABP1 which was diluted in 1x SSC +0.1% triton X-100) and incubated for an hour at room temperature. The cells were then washed in 2x SSC (or 1xSSC+0.1% triton X-100 after anti-PABP1 staining) three times and the appropriate secondary antibody diluted in 2x SSC +0.1% triton X-100 applied. The cells were incubated for 1 hour at room temperature again before three final washes in 2x SSC (1x SSC +0.1% Triton X-100 after anti-PABP1 staining) and mounted as for immunofluorescence.

2.9.10 Cell treatments

For each cell stress experiment approximately 2×10^5 cells were seeded on 22mm x 22mm coverslips in 6-well plates or chamberslides with 2ml media and allowed to grow overnight before the treatment was administered.

Leptomycin B treatment

Cells were treated with 5ng/ml leptomycin B, which was added directly to media, for 3 hours at 37°C. Control cells were treated with an equivalent volume of 70% methanol. If cells were left to recover after leptomycin B treatment they were washed 3 times with PBS and incubated with fresh media for a further 3 hours at 37°C.

Actinomycin D treatment

Cells were treated with 5µg/ml actinomycin D which was added directly to the media from a 1µg/µl stock in 5% DMSO in PBS. Cells were treated for 3-15 hours as indicated; control cells were treated with an equivalent volume of 5% DMSO in PBS.

Cycloheximide treatment

Cycloheximide was added directly to cell media to a final concentration of 100µg/ml from a 50mg/ml stock solution in ethanol for 30 minutes prior to and during leptomycin/actinomycin D treatment. Control cells were treated with an equivalent volume of ethanol.

Arsenite treatment

Cells were treated for 1 hour at 37°C with 0.5mM sodium arsenite from a 0.05M stock solution in PBS. Control cells were treated with an equivalent volume of PBS.

Sorbitol treatment

Cells were treated for 3 hours at 37°C with 0.6M sorbitol from a 3M stock solution in PBS. Control cells were treated with an equivalent volume of PBS.

UV

Immediately prior to treatment cells were washed three times with PBS. 50µl of PBS was added to the cells which were then treated with 0-100 Joules/m² UV-C as indicated using a CL-1000 ultraviolet crosslinker (Camlab, Cambridge UK). Fresh media was then added to the cells which were then incubated for the indicated time at 37°C.

Heatshock

Cells were moved to a 44°C incubator (5% CO₂) for the indicated time.

2.10 Microscopy

2.10.1 Brightfield Microscopy

Brightfield images were taken using an Axiocam camera (Zeiss, Welwyn Garden City, UK) on an Olympus Ax70 Provis microscope with UPlanFl objectives. Image capture was performed using Axiovision software (Zeiss) and images were analysed using Adobe Photoshop CS2.

2.10.2 Fluorescence microscopy

Fluorescence images were taken using a Coolsnap HQ CCD camera (Photometrics Ltd, Tucson, AZ, USA) on a Zeiss Axioplan II fluorescence microscope with Plan-neofluar objectives, a 100W Hg source (Zeiss, Welwyn Garden City, UK) with Chroma #83000 triple band pass filter set and Chroma #83700 emission filters (Chroma Technology Corp, Rockingham, VT, USA). Image capture was performed using IPLab Spectrum (Scanalytics Corp, Fairfax, VA, USA) and images were analysed using Adobe Photoshop CS2. All pixels in an image and each image within an experiment were processed equally.

2.10.3 Confocal microscopy

Laser confocal fluorescence microscopy was used to visualize optical sections of representative cells. Images were captured using a LSM710 (Zeiss, Welwyn Garden City, UK) with a Plan-neofluar 40x/1.3 oil ph3 M27 or Plan-apochromat 60x/1.3 oil ph3 M27 objective and Zen 2009 software (Zeiss). DAPI signals were obtained with excitation at 405nm and emission at 409-480nm. Alexa-488 signals were obtained with excitation at 488nm and emission at 490-539nm. Alexa-546 signals were obtained with excitation at 546nm and emission at 549-670nm. Images were recorded at a resolution of 1024 x 1024 pixels. Except where stated, capture

parameters of all images were individually optimized according to manufacturer's recommendations. Images were analysed using Adobe Photoshop CS2.

2.11 Statistical Analysis

Paired Student's *t*-test was used to analyse significance of differences in continuous data between groups. Fisher's two-tailed test was used to analyse significance of categorical data between groups. Statistical significance was considered as $P < 0.05$. Statistical analyses were performed using GraphPad software (GraphPad Software Inc, La Jolla, CA USA).

Chapter 3: Expression and function of PABP4

3.1 Introduction

PABP4 was first described in 1995 however, there has been very little work to date characterising its expression and function (Chapter 1.4.6.3). PABP4 is highly similar to PABP1 with 75% protein identity shared between the human proteins, suggesting that it may well share the ability of PABP1 to stimulate translation. Poly(A)-binding by human PABP4 has been proven (Houng et al., 1997), and shown to be of a similar affinity to that of PABP1 (Sladic et al., 2004); however protein interactions with eIF4G and PAIP1, thought to lend PABP1 its translational activation faculty, have not been reported for PABP4. Any ability of PABP4 to self associate, or to interact with other PABP family proteins has also yet to be investigated.

Characterization of PABP4 expression across tissues is limited to northern blotting of a human tissue panel which indicated elevated expression in heart, muscle, testes and ovary (Yang et al., 1995). At the cellular level PABP4 mRNA has been reported to be expressed in T-cells, platelets and several myeloid cell culture lines (Houng et al., 1997; Yang et al., 1995). The relative levels of PABP4 RNA however, may not necessarily be directly indicative of its protein levels. PABP4 contains an A-rich sequence in the 5' UTR which may function similarly to the auto-regulatory sequence of PABP1, but it remains untested whether PABP4 is subject to translational control.

Surprisingly, there has been relatively little work investigating the expression of PABP1 in metazoa. Indeed, it is widely regarded as ubiquitously expressed, which may be due to its essential role in budding yeast (Sachs et al., 1987) or its abundance in cultured cell lines such as HeLa (Gorlach et al., 1994). Western blotting for PABP1 across selected *Xenopus laevis* tissues (Cosson et al., 2002c; Wilkie et al., 2005) suggests that PABP1 is not expressed equally across vertebrate adult tissues. One report from the Sonenberg lab showed western blotting for PABP1 across mouse tissues, however the antibody used was raised against the entire C-terminal portion of PABP1 and specificity with regard to other PABP proteins was not demonstrated (Berlanga et al., 2006). Nevertheless, the levels of PABP appeared to vary across tissues with highest levels in the testes (Berlanga et al., 2006).

Furthermore, some early immunohistochemical data showing PABP1 expression in primary spermatocytes and round spermatids but not spermatogonia or late spermatids of rat testes (Gu et al., 1995) hints that PABP1 expression may vary among different cell types and may not be quite so ubiquitous after all.

In this chapter I determine the expression pattern of PABP4 in mammals and challenge the notion that PABP1 is ubiquitous. The interactions and characteristics of PABP4 which would support a role as a translational activator are also examined.

3.2 Results

3.2.1 PABP4 mRNA expression in cultured cell lines

In order to investigate the properties of PABP4, a cell line expressing PABP4 was required. To this end, intron-spanning primers were designed to specifically detect PABP1 and PABP4 (see Chapter 2.2.3) and were verified as specific when tested in a PCR reaction against plasmids containing human PABP1 or PABP4 cDNA (Figure 3.1A). A variety of available human cell lines were screened for PABP1 and PABP4 mRNA expression by RT-PCR - Hakat (keratinocyte), HEK293 (embryonic kidney), HeLa (cervical carcinoma), HT1080 (fibrosarcoma), RPE (retinal pigmented epithelium) and SW480 (colon adenocarcinoma) (Figure 3.1B). PABP1 and PABP4 mRNA was detected in every cell-type tested (Figure 3.1B) suggesting that many mammalian cell lines contain multiple PABP proteins. In each case a single band of the predicted size was detected excluding the possibility of genomic DNA contamination.

3.2.1.1 PABP4 isoforms

Human PABP4 is predicted by Ensembl (<http://www.ensembl.org>)(Hubbard et al., 2009) to be expressed as four isoforms. These are created by inclusion or exclusion of a portion of exon 10 and the whole of exon 11. A schematic of the splicing pattern annotated with the Ensembl transcript names is shown in Figure 3.1C. The predicted transcript variants (PABP4-2, PABP4-1, PABP4-201, PABP4-3) when translated would give rise to proteins of 660, 644, 631, 615 amino acids respectively. The variable portion corresponds to the proline-rich linker region of the protein. All four isoforms are also predicted by Ensembl to be conserved in mouse, suggestive of functional relevance.

To discover whether these isoforms are expressed in cells, I designed PCR primers that flank the variable region of the PABP4 transcripts. RT-PCR from HeLa cell RNA produced four distinct bands (Figure 3.1D). The observed bands were consistent with the Ensembl predicted transcripts. To verify that these bands did in fact correspond to the predicted transcripts, each was cloned and sequenced. This

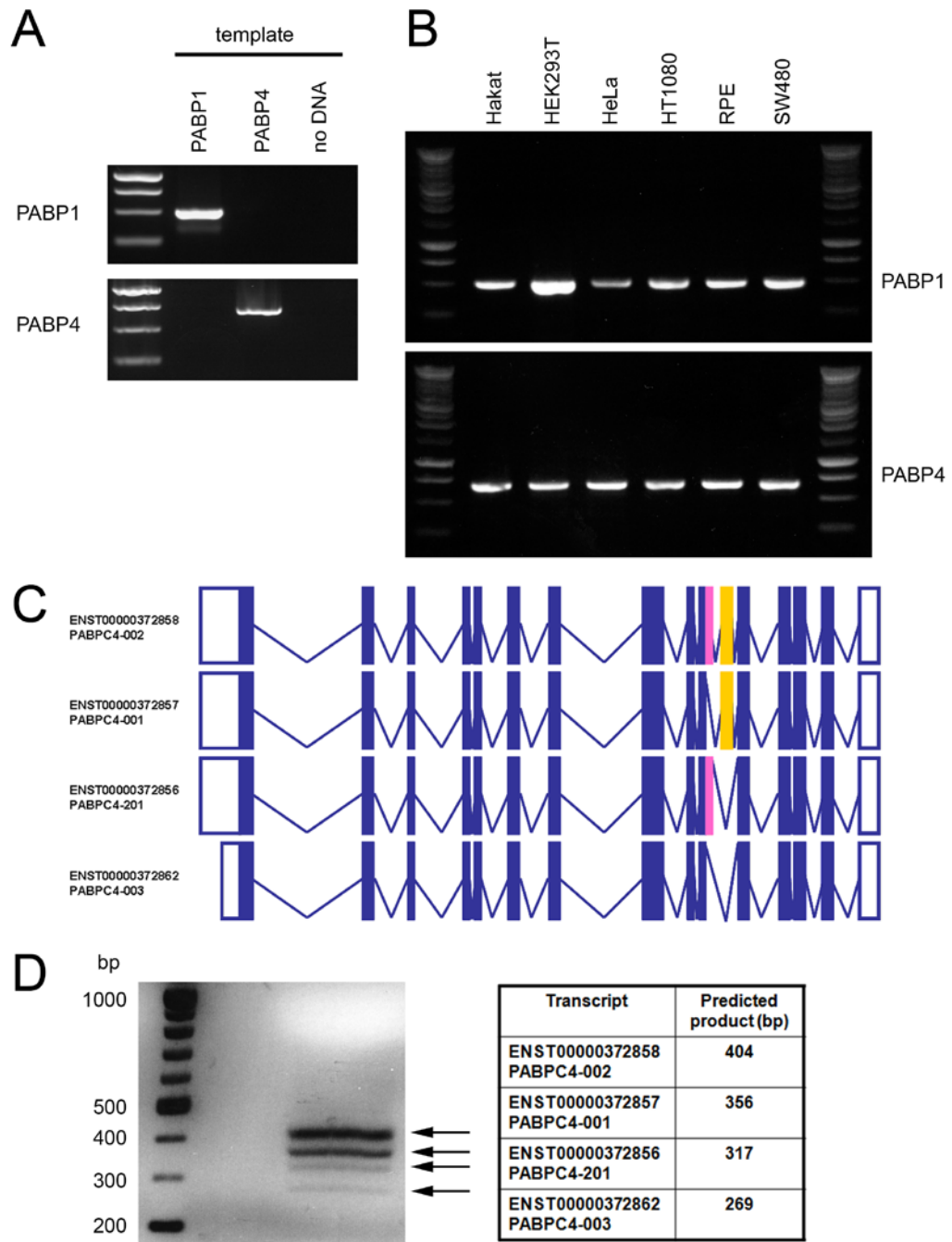


Figure 3.1 RT-PCR analysis of PABP1 and PABP4 in human cell lines. A. PCR from PABP1 or PABP4 containing plasmids was used to confirm the specificity of PABP1 and PABP4 primers. **B.** cDNA derived from the indicated cell lines was subject to PCR for PABP1 and PABP4. **C.** Four human PABP4 isoforms varying in coding exon inclusion and non-coding sequences are predicted by Ensembl. A schematic of each isoform transcript in descending size order is shown. Constitutive exons are shown in blue, alternative exons in pink (variable portion of exon 10) and yellow (exon 11) and UTRs as unfilled. Each transcript is labelled with the appropriate Ensembl transcript ID and transcript name. **D.** Primers which flank the predicted variable coding region of human PABP4 were used to conduct PCR from HeLa cDNA. Table shows expected products for each isoform. Each PCR product obtained was cloned into pGEM-T Easy vector and sequenced.

revealed that the Ensembl predictions were accurate and confirmed that PABP4 is expressed, at least at the mRNA level, as four isoforms in HeLa cells.

3.2.2 PABP1 and PABP4 specific antibodies

To examine the expression and function of PABP4 protein I took advantage of novel antibodies generated in the lab. Antibodies designed to be specific for PABP1 and PABP4 were raised in rabbits using a single peptide unique to human PABP1 (AQKAVNSATGVPTV) and two peptides unique to human PABP4 (VRSPHPAIQPLQA and KKEAAQKVGTVAAATS) (Figure 3.2A). These peptides are also conserved in mouse. Polyclonal sera were affinity purified before use and tested by western blot against *E. coli* expressing his-tagged recombinant proteins expressed in and HeLa cell extract (Figure 3.2B). The PABP4 sequence used for his-tagged expression was that of the full length human protein (660 amino acids) encoded by transcript PABPC4-002 (ENST00000372858).

As shown in Figure 3.2B, PABP1 and PABP4 antibodies were found to be specific and displayed no cross-reactivity to the other PABP protein although each was abundantly expressed. These antibodies were therefore used for further analysis of PABP1 and PABP4 protein expression and function.

Consistent with the RT-PCR data (Figure 3.1B), both PABP1 and PABP4 proteins were found to be expressed in HeLa cells (Figure 3.2B) although the endogenous proteins migrated slightly above their respective predicted molecular masses of 70.3kDa and 72.4kDa (Feral et al., 1999). Both PABP1 and PABP4 (PABPC4-002) are predicted by the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (<http://www.expasy.org>) to have basic isoelectric points (9.52 and 9.31 respectively). Basic proteins with a high mass to charge ratio tend to migrate slower on SDS-PAGE which may explain the slight discrepancy between the predicted and observed migration of PABP1 and PABP4. Another explanation could be post-translational modifications, several of which have been reported for PABP1 (Chapter 1.4.5.2).

A

hPABP1	MNPSAPSYPMASLYVGDLDHPDVTEAMLYEKFSFAGPILSIRVCRDMITRRSLGYAVVNFQ	60
hPABP4	MNAAASSYPMASLYVGDLDHSDVTEAMLYEKFSFAGPVLSIRVCRDMITRRSLGYAVVNFQ	60
hPABP1	QPADAERALDTMNFVDVIKGPVIRIMWSQRDPSLRKSGVGNIFIKNLDKSIDNKALYDTFS	120
hPABP4	QPADAERALDTMNFVDVIKGPVIRIMWSQRDPSLRKSGVGNVFIKNLDKSIDNKALYDTFS	120
hPABP1	AFGNILSCKVVDENSGSKGYGFVHFETQEAARAIEKMGMLLNDKRVFVGRFVKSRKERE	180
hPABP4	AFGNILSCKVVDENSGSKGYAFVHFETQEAADKAIEKMGMLLNDKRVFVGRFVKSRKERE	180
hPABP1	AELGARAKEFTNVYIKNFGEDMDDERLKDLEFGKFGPALSVKVMTDESQKSGFGFVSFER	240
hPABP4	AELGAKAKEFTNVYIKNFGEEVDDESLEKELFSQFGKTLVSKVMRDPNGKSGFGFVSYEK	240
hPABP1	HEDAQKAVDEMNGKELNGKQIYVGRAQKKVERQTELKRRKFEQMKQDRITRYQGVNLYVKN	300
hPABP4	HEDANKAVEEMNGKEISGKIIFVGRAQKKVERQAELEKRRKFEQLKQERISRYQGVNLYIKN	300
hPABP1	LDDGIDDERLRKEFSFPGTITSKVMMEGGRSKGFGFVCFSSPEEATKAVTEMNGRIVAT	360
hPABP4	LDDTIDDEKLRKEFSFPGSITSKVMLEDEGRSKGFGFVCFSSPEEATKAVTEMNGRIVGS	360
hPABP1	KPLYVALAQRKEERQAHLTNQYMQRMASVRAVP-NPVINPYQPAPPSGYFMAAIPQTQNR	419
hPABP4	KPLYVALAQRKEERKAHLTNQYMQRVAGMRALPANAILNQFQPAAG-GYFVPAVPAQAGR	419
hPABP1	AAAYPPSQIAQLRPSRWTAQGGRPHPFQNMPGAIRPAAPRPPFSTMRPAS-SQVPR--V	476
hPABP4	PPYYTPNQLAQMARNRPRWQ-QGGRPQGFQGMPSAIRQSGRPTLRHLAPTGNAPASRGLP	478
hPABP1	MSTQRVANTS-----TQTMGPRPAAAAAATPAVRTVPQ	510
hPABP4	TTTQRVGSECPDRLAMDFGAGAAQGLTDSQSGGVPTAVQNLAPRAAVAAAAPRAVAP	538
hPABP1	YKYAAGVRNPOOHLNAQPOVTMQQPAVHVQGGQEPLTASMLASAPPQEQKQMLGERLFPFI	570
hPABP4	YKYASSVRSP--HPAIQP-LQAPQPAVHVQGGQEPLTASMLAAAPPQEQKQMLGERLFPFI	595
hPABP1	QAMHPTLAGKITGMLEIDNSELHLMLESPELSRSKVDEAVAVLQAHQAKEAAQKAVNSA	630
hPABP4	QTMHSNLAGKITGMLEIDNSELHLMLESPELSRSKVDEAVAVLQAHHAKKEAAQKVGAV	655
hPABP1	TGVPTV	636
hPABP4	AAATS-	660

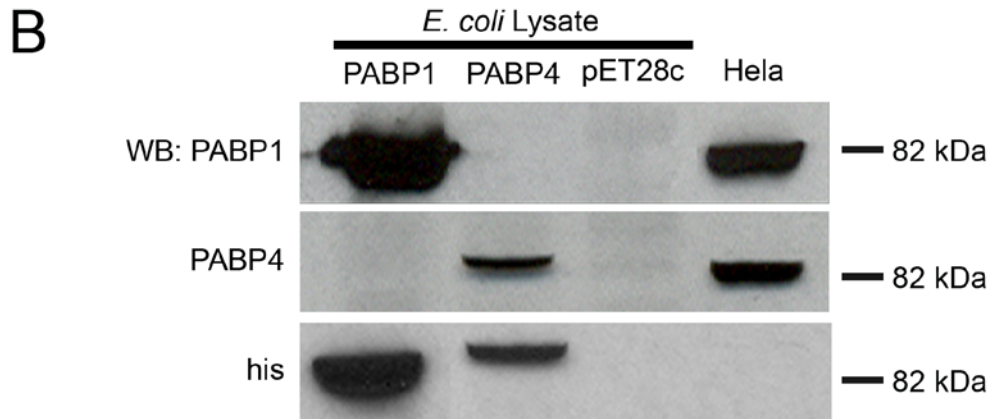


Figure 3.2 Generation and testing of PABP1 and PABP4 specific antibodies
A ClustalW2 (Larkin et al., 2007) alignment of human PABP1 (PABPC1-201) and PABP4 (PABPC4-002) protein sequences. Peptide sequences used for PABP1 (yellow) and PABP4 (blue) antibody generation are indicated. **B.** Western blotting demonstrating the specificity of PABP1 and PABP4 antibodies. His-tagged human PABP1, PABP4 or a vector control (pET28c) were expressed in *E. coli*. 10µg of each *E. coli* lysate and 10µg HeLa extract were western blotted for PABP1, PABP4 and poly-histidine, as indicated.

Interestingly, PABP4 is detected by western blotting as a single band in HeLa cell extract (Figure 3.2B). Four PABP4 mRNA isoforms were previously detected in HeLa cells (Figure 3.1D) which would be expected to produce proteins that varied in size by up to 45 amino acids. The detection of only a single band by western blotting implies that either only a single isoform is expressed at the protein level or that despite the size differences, multiple protein isoforms are present but not separated by the 10% SDS polyacrylamide gel used here. Figures 3.3 and 3.5 show western blotting of HeLa extract resolved on 4-12% acrylamide gradient gels and a single band is again detected. Furthermore, western blotting of whole mouse tissue lysates also run on a 10% acrylamide gel (Figure 3.6) leads to detection of two closely migrating bands in some tissues suggesting that these electrophoresis conditions are sufficient to separate at least two of the PABP4 protein isoforms.

3.2.3 PABP4 sub-cellular localisation

If the main function of PABP4 is translational regulation it would be expected to be a predominantly cytoplasmic protein. However, the sub-cellular localisation of endogenous PABP4 has not previously been tested. Yang *et al* examined the localisation of haemagglutinin (HA) tagged PABP4 transfected into COS-7 (African green monkey kidney) cells and found it to be cytoplasmic by indirect immunofluorescence (Yang *et al.*, 1995). PABP1 is mainly cytoplasmic but has also been found in nuclear extracts and bound to unspliced pre-mRNA (Hosoda *et al.*, 2006).

Availability of a PABP4 specific antibody permitted examination of the sub-cellular localisation of endogenous PABP4 protein by biochemical fractionation and indirect immunofluorescence (Figure 3.3). HeLa cells were chosen for this and future functional analyses as they are an easily available and easily cultivated cell type and were found to express both PABP1 and PABP4 at the RNA (Figure 3.1A) and protein (Figure 3.2B) level. Furthermore, there is much published work examining the characteristics of PABP1 in HeLa cells (Funakoshi *et al.*, 2007; Imataka *et al.*, 1998; Michel *et al.*, 2000).

Western blotting of nuclear and cytoplasmic extracts showed that PABP4 was predominantly cytoplasmic with no PABP4 detected in nuclear extracts (Figure 3.3A). The integrity of fractionation was demonstrated by western blotting for cytoplasmic and nuclear proteins, tubulin and RNA polymerase II (RNAPII) respectively. Indirect immunofluorescence using anti-PABP4 gave a clear cytoplasmic signal in HeLa cells (Figure 3.3B) consistent with the results of the biochemical fractionation. Taken together, these data show that PABP4 is a predominantly cytoplasmic protein, consistent with a role in translation regulation. PABP1 was also only detectable in cytoplasmic extracts in contrast to the results of Hosoda and colleagues who found approximately 25% of PABP1 in HeLa extracts in the nuclear fraction (Hosoda et al., 2006). Detection of PABP1 exclusively in the cytoplasm by immunofluorescence (Figure 4.1) however, supports my fractionation results and the PABP1 nuclear signal detected by Hosoda and colleagues could be due to ER contamination of nuclear extracts.

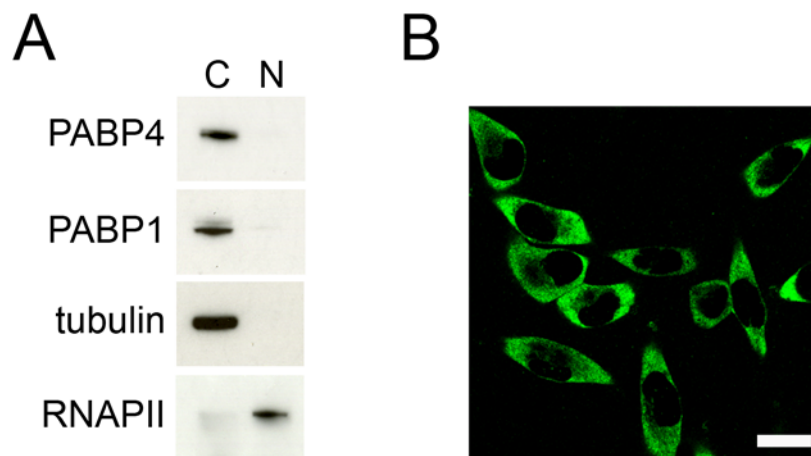


Figure 3.3 Cell-fractionation and immunofluorescence analysis of PABP4 subcellular localisation. **A.** HeLa cytoplasmic (C) and nuclear (N) extracts were obtained by biochemical fractionation and western blotted for PABP4, PABP1, α -tubulin as a cytoplasmic marker and RNA polymerase II (Ser5p) as a nuclear marker. **B.** HeLa cells were subject to indirect immunofluorescence for PABP4 using an alexa-488 labelled secondary antibody (green) and detection by confocal microscopy using a Zeiss LSM710. Scale bar represents 20 μ m.

3.2.4 PABP4 association with polysomes

The role of PABP1 in translation is evidenced by its association with actively translating polysomes, demonstrated in several mammalian cell types including HeLa cells (Gu et al., 1995; Hinton et al., 2007; Rivera and Lloyd, 2008). Though it has been previously established that PABP4 has a similar affinity to bind poly(A)-RNA as PABP1 (Sladic et al., 2004), association of PABP4 with polysomes has not been investigated. To address this, HeLa cells were lysed in the presence of cycloheximide, which acts to block peptidyl transfer and prevent ribosome run off, or EDTA, which chelates Mg^{2+} ions causing ribosomes to dissociate (Munroe and Jacobson, 1990). Lysates were then centrifuged through 10-50% sucrose gradients and absorbance at 254nm was used to establish the position of polysomes, 80S ribosomes and mRNPs. Fractions from the gradient were TCA precipitated and western blotted for PABP1 and PABP4.

PABP4 was detected in polysomal fractions in the presence of cycloheximide indicating that it is indeed associated with messages that are being actively translated (Figure 3.4). With the addition of EDTA, PABP4 was found in lighter fractions, providing further support that its sedimentation into the heavier fractions with cycloheximide treatment is dependent on the presence of polysomes. PABP1 was detected throughout the gradient, including the polysomal fractions in the presence of cycloheximide treatment and relocalised to lighter fractions with EDTA treatment consistent with previously published results (Gu et al., 1995; Proweller and Butler, 1996).

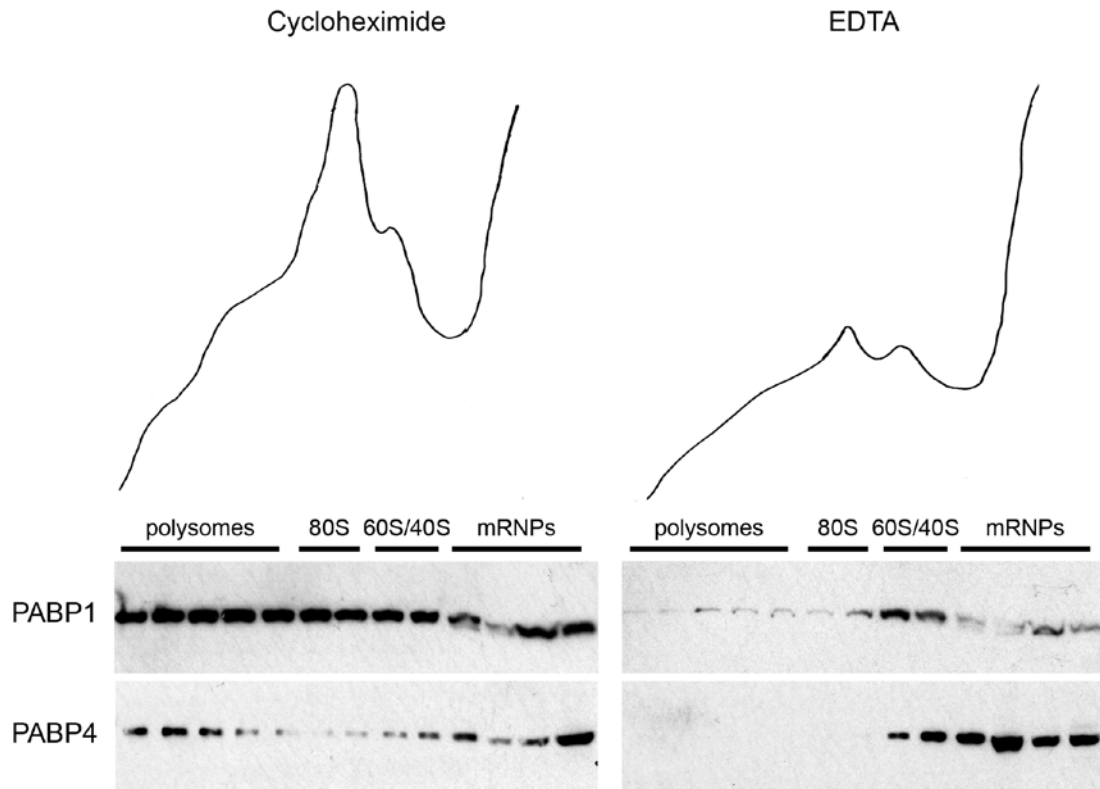


Figure 3.4 Sucrose gradient analysis of PABP4 in HeLa cell extract. HeLa extract, lysed in the presence of either cycloheximide or EDTA, was centrifuged through a 10-50% sucrose gradient, fractionated and TCA precipitated protein samples from each fraction western blotted for PABP1 and PABP4. Absorbance traces at 254nm are shown above.

3.2.5 PABP4 protein interactions

The interaction of PABP1 with eIF4G is thought to be of key importance for its role as a translational activator. The interaction is known to increase the affinity of eIF4E for the cap (Borman et al., 2000) and stimulate translation (Imataka et al., 1998; Wakiyama et al., 2000). Similarly, *Xenopus* ePABP has also been shown to interact with eIF4G and stimulate translation (Wilkie et al., 2005). A potential interaction between PABP4 and eIF4G has not been investigated. To this end, I immunoprecipitated PABP4 from HeLa cell extracts and western blotted for eIF4G. Initial experiments failed to co-immunoprecipitate eIF4G (data not shown), however the PABP1-eIF4G interaction is known to be technically challenging to isolate with only a small percentage of eIF4G in mammalian cells thought to be associated with PABP1 (Imataka et al., 1998).

In order to stabilise protein interactions between PABP4 and eIF4G I treated cells with a chemical cross-linker dithiobis (succinimidyl propionate) (DSP) prior to lysis and immunoprecipitation (IP). DSP was chosen as a crosslinking agent as it is reversible and membrane permeable, allowing it to enter intact cells and stabilise only endogenous interactions before extract preparation. DSP reacts with primary amine groups forming covalent bonds with adjacent proteins. Excess DSP is quenched before cell lysis by Tris which provides an excess of amines, preventing stabilisation of any interactions that may occur once cells are lysed. Elution of the immunoprecipitated proteins with a buffer (SDS-loading buffer) that contains dithiothreitol (DTT) cleaves the disulfide bond within DSP releasing cross-linked proteins, allowing their resolution by SDS-PAGE.

Following optimisation of DSP cross-linking conditions (not shown), immunoprecipitations were conducted using anti-PABP4 and a rabbit immunoglobulin G to control for non-specific interactions. Immunoprecipitations were also conducted in the presence and absence of RNaseI, which cleaves after all 4 bases of single stranded RNA, to ensure co-immunoprecipitating proteins detected were not simply bound to the same RNA as PABP4.

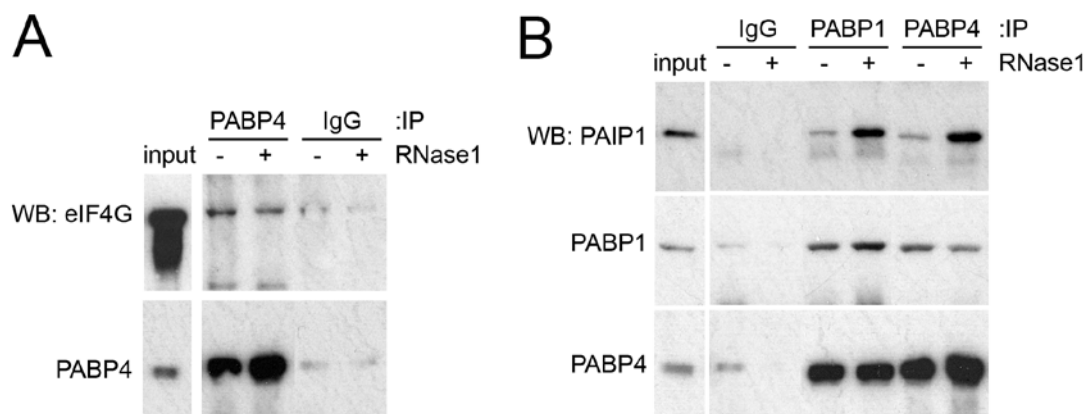


Figure 3.5 Co-immunoprecipitation of eIF4GI, PABP1 and PAIP1 with PABP4.

A. Immunoprecipitations with anti-PABP4 or rabbit IgG control from DSP-treated HeLa extract +/- RNaseI treatment were western blotted for eIF4GI and PABP4. **B.** Immunoprecipitations with rabbit IgG control, anti-PABP1 or anti-PABP4 from HeLa extract +/- RNaseI treatment were western blotted for PAIP1, PABP1 and PABP4. 10µg is shown as input, corresponding to 2% of material used for each IP.

Western blotting for PABP4 demonstrated that our PABP4 antibody efficiently immunoprecipitates PABP4, with a signal which appears several fold stronger in the IP lanes compared to the 2% input lane (Figure 3.5A). Western blotting for eIF4G revealed that it is efficiently co-immunoprecipitated with PABP4, even in the presence of RNase (Figure 3.5A). Weak eIF4GI bands caused by non-specific binding were observed in the IgG control lanes; however the bands detected in the PABP4 IP lanes were much stronger, indicative of specific binding. By comparing the western blot signal for eIF4GI from input (equivalent to 2% of total IP input) and PABP4 IP lanes it appears that less than 2% of the eIF4GI in the extract has been co-immunoprecipitated with PABP4.

PABP1 is also known to interact with PAIP1, a protein with homology to eIF4G (Craig et al., 1998) (Chapter 1.4.3.2). PAIP1 also binds eIF4A bound to the 5' end of the message and eIF3 (p44 subunit) which also binds eIF4G (Martineau et al., 2008). It is thought that the PABP1-PAIP1 interaction stabilises the interaction between PABP1 and eIF4G and thus circularisation of mRNAs, enhancing translation activation by PABP1 (Martineau et al., 2008; Roy et al., 2002).

In the absence of DSP crosslinking, PAIP1 co-immunoprecipitated with PABP4 (Figure 3.5B). PAIP1 was detected by western blotting above background levels in the presence of RNase indicating the interaction with PABP4 is direct and not bridged by RNA. A similar amount of PAIP1 was co-immunoprecipitated from HeLa extract with anti-PABP4 as with anti-PABP1; however, as the comparative amounts of PABP1 and PABP4 immunoprecipitated are unknown, definitive conclusions regarding the relative affinity of PAIP1 for binding PABP1 and PABP4 cannot be made. Unfortunately sufficient PAIP1 antibody was not available to perform the reciprocal IP. Interestingly, more PAIP1 appears to co-IP with PABP1 and PABP4 when RNase is added to the IP mixture. A possible explanation for this may be that release of PAIP1 from other RNA-protein complexes could increase its availability for interactions with PABPs.

PABP1 self-associates (Sachs et al., 1987) via protein-protein interactions within the proline-rich linker of the C-terminal region (Melo et al., 2003). This interaction is thought to lead to cooperative binding of multiple PABP1 molecules along a poly(A) tail (Kuhn and Pieler, 1996; Sachs et al., 1987)(Chapter 1.4.3.1).

Western blotting for PABP1 on PABP4 immunoprecipitates revealed that PABP1 co-immunoprecipitates with PABP4 even in the presence of RNase (Figure 3.5B). The PABP1-PABP4 interaction was also tested in the reverse direction, by immunoprecipitation with anti-PABP1 and western blotting with anti-PABP4. Likewise, PABP4 was clearly detected above background levels in the absence or presence of RNase in PABP1 immunoprecipitates.

Localisation of PABP4 to polysomes (Figure 3.4) and its interaction with eIF4G and PAIP1 (Figure 3.5) strongly suggests that PABP4 acts as a translational activator in a similar manner to PABP1. That is, PABP4 may bind to the poly(A)-tails of mRNAs and facilitate their circularisation via interactions with eIF4G and PAIP1, stimulating translation initiation. The interaction of PABP4 with PABP1 hints that binding of PABP4 to poly(A)-tails could be stabilised via this interaction, and vice versa. This further intimates that mRNAs in cells where both PABP1 and PABP4 are expressed may not necessarily be bound by only PABP1 or PABP4 but by a combination of the two proteins. The functional significance of heteromultimerisation of poly(A)-binding proteins on poly(A)-tails remains to be determined.

3.2.6 PABP4 protein expression in mouse tissues

To investigate the tissue distribution of PABP1 and PABP4 proteins in mammals, tissue lysates from adult mice were prepared and subject to western blotting for PABP1, PABP4 and tubulin as a loading control (Figure 3.6). The concentration of total protein was determined by Bradford assay so that an equal amount of total protein from each tissue was analysed.

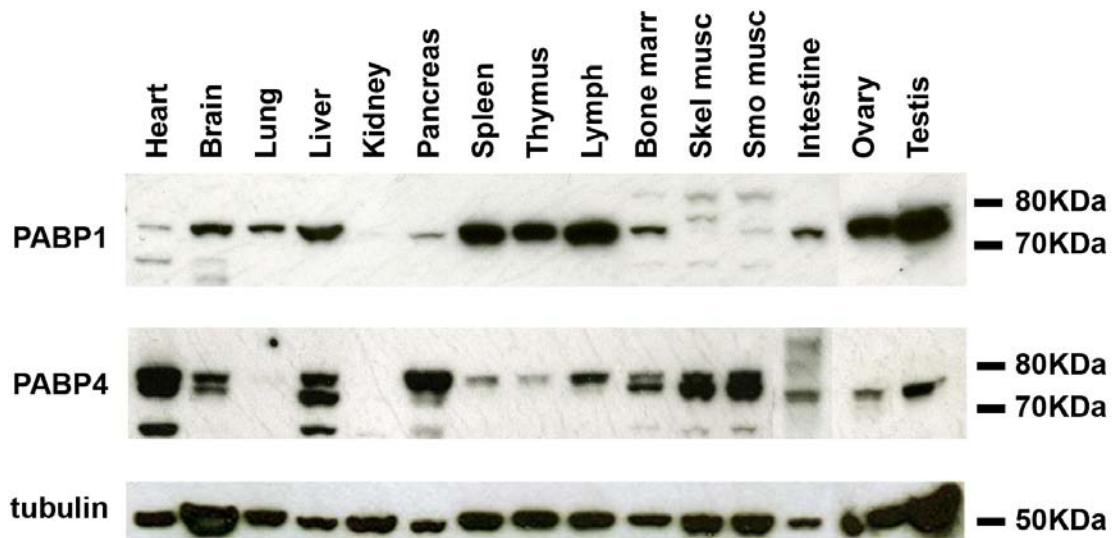


Figure 3.6 Expression analysis of PABP1 and PABP4 in adult mouse tissues. 10 μ g of mouse tissue lysates were western blotted for PABP1, PABP4 and α -tubulin as a loading control. A representative blot from the results of two individual mice is shown.

PABP1, PABP4 and tubulin showed great variation in their abundance relative to total protein across the tissues tested. Tubulin is known to vary in its expression over mouse tissues and the higher expression of tubulin in brain and testes observed here is consistent with published reports (Lewis et al., 1985). PABP1 protein appears most abundant in spleen, thymus, lymph glands, ovary and testis with moderate abundance in brain, lung, liver, bone marrow and intestine. Expression of PABP1 in heart, pancreas, skeletal and smooth muscle appears relatively low, with expression in kidney below the sensitivity threshold of the western blot. By contrast the highest levels of PABP4 are found in the heart, liver, pancreas, skeletal and smooth muscle, with lower expression in brain, spleen, thymus, lymph, bone marrow, intestine, ovary and testis. Expression of PABP4 in lung and kidney were below the sensitivity threshold of the western blot. Though both PABP1 and PABP4 are not detected in kidney, a strong tubulin signal here demonstrates that this is not due to an absence of protein.

These results show that PABP1 and PABP4 are by no means ubiquitously expressed across mouse tissues. It is particularly interesting that while some tissues appear to display an almost reciprocal relationship between PABP1 and PABP4 expression (e.g. lung and skeletal muscle) in others both PABP1 and PABP4 are

expressed (e.g. brain and pancreas). This implies that certain tissues may have a requirement for a particular PABP while others require a combination of PABP1 and PABP4.

The observed patterns of expression are broadly similar to those detected for PABP1 and PABP4 mRNA in human tissues (Yang et al., 1995). Yang and colleagues observed highest expression of PABP1 mRNA in testis and ovary and lowest in brain, skeletal muscle and kidney, very similar to the expression pattern of PABP1 protein observed in mouse. PABP4 mRNA is reported to be expressed highly in human heart and skeletal muscle and at low levels in kidney and lung similar to the expression of PABP4 proteins in mouse. However, human PABP4 mRNA was also found to be abundant in ovary and at moderate levels in spleen, and thymus (Yang et al., 1995), tissues in which relatively little PABP4 protein was detected in mouse. These differences may reflect species specific differences in the expression of PABP4 or translational regulation of PABP4 mRNA.

Interestingly, PABP4 was detected as two closely migrating bands in several tissues and the abundance of each of the bands appeared to vary. For example, in pancreas the higher molecular weight band is most abundant where as in skeletal and smooth muscle the lower band is predominant. These bands may represent alternative splice forms of PABP4 indicating that alternative splicing of PABP4 may be regulated in a tissue-specific manner. Four PABP4 splice forms were characterised in HeLa cells (see Chapter 3.2.1.1) and are predicted by Ensembl to be conserved mice encoded by transcripts Pabpc4-001(660 amino acid product), Papbc4-002 (615 amino acids), Pabpc4-003 (631 amino acids) and Pabpc4-004 (644 amino acids). Like the human isoforms, the predicted mouse isoforms vary only in their proline-rich central linker region and all would be expected to be detected by our PABP4 antibody. Tissue-specific regulation of PABP4 splicing suggests that the different splice forms may display differences in their function important in the tissues in which they are expressed.

3.2.7 Cell type-specific expression of PABP4 and PABP1 in mouse

Western blotting of tissue extracts revealed that in several tissues both PABP1 and PABP4 proteins were both expressed (Figure 3.6). I was interested to discover whether within these tissues expression of PABP1 and PABP4 may be restricted to certain cell types, as has been suggested for PABP1 in testes (Kleene et al., 1994). To address this question I used immunohistochemistry to examine PABP1 and PABP4 protein expression within selected adult mouse tissues – brain, pancreas, intestine and testis, which contain multiple cell types with distinct roles.

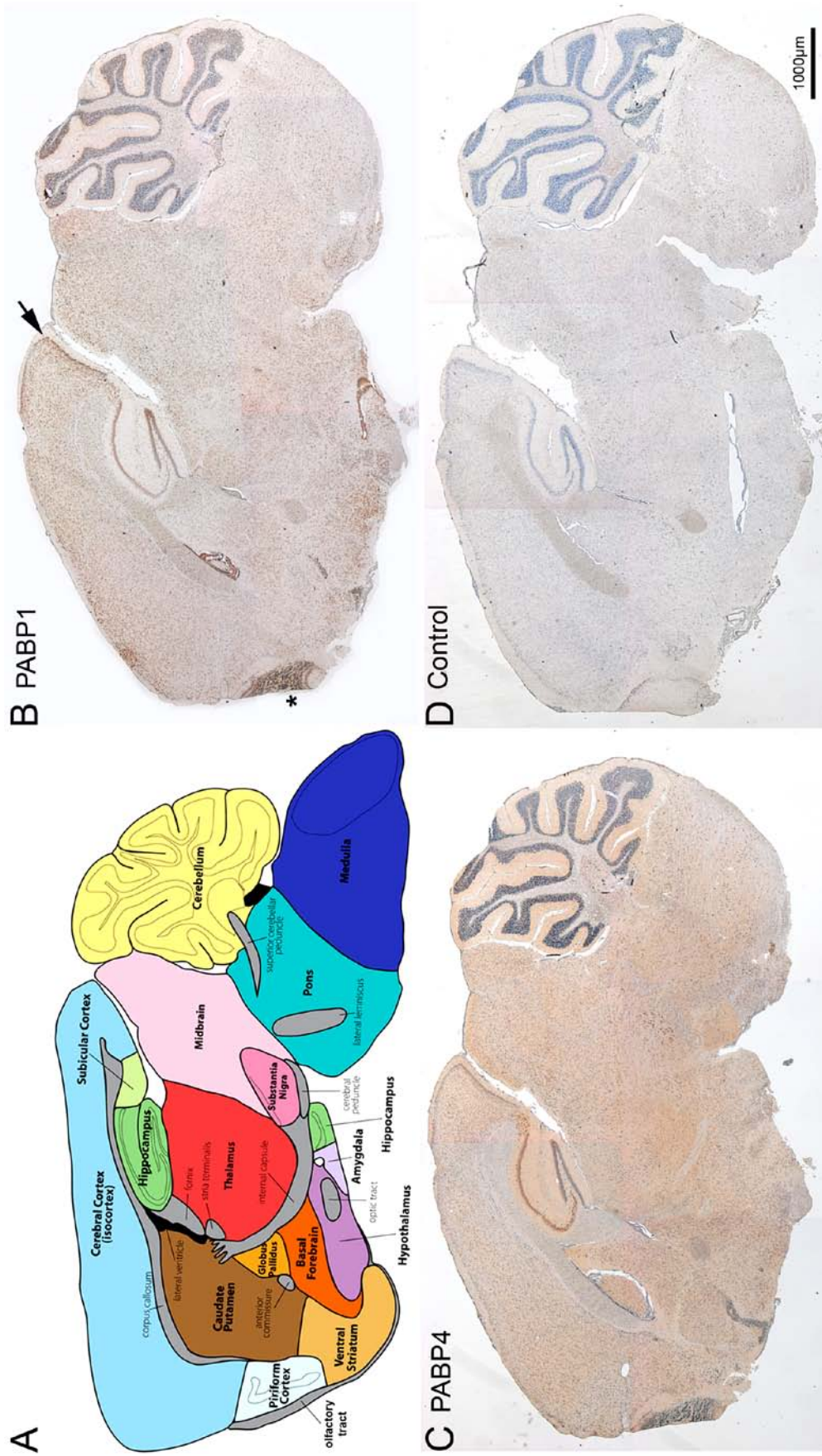
Brain and testes were each fixed in Bouins solution and neutral buffered formalin (NBF), however in both cases Bouins fixation maintained better tissue structure and provided better staining and was used for fixation of each tissue type shown. PABP1 and PABP4 antibodies were optimized for use in each tissue by serial dilution, though in each case dilutions of 1/500 and 1/1000 (representing final concentrations of 1.8ng/μl and 0.1ng/μl respectively) were found to be optimal. Rabbit immunoglobulin G (IgG) was used as a control at a final concentration of 1.8ng/μl, equivalent to the higher concentration of PABP antibody used. The detection system, detailed in Chapter 2.9.7, uses DAB to produce brown staining wherever primary antibodies are bound to their antigen and each slide was counterstained with haematoxylin which stains nuclei dark blue.

3.2.7.1 Brain

Expression of PABP1 and PABP4 in the whole brain

Low magnification images were taken of anti-PABP1 and anti-PABP4 staining of brain sections (Figure 3.7). PABP1 appears not to be widely expressed at significant levels but appears enriched in regions of the hippocampus, cerebral cortex (specifically the retrosplenial cortex) and olfactory tract (Figure 3.7B). By contrast, PABP4 appears to be widely expressed though the whole brain, with expression enriched in the hippocampus but is not particularly enriched in any areas of the cerebral cortex or the olfactory tract (Figure 3.7C).

These images of the whole brain demonstrate that although in some regions PABP1 and PABP4 show strong overlapping expression (e.g. hippocampus) they also show differences in their expression in other areas. For example, only PABP1 is enriched in the retrosplenial region of the cerebral cortex, an area important for learning and memory, specifically spatial and episodic memory (Figure 3.7B).



Expression of PABP1 and PABP4 in the hippocampus

It is particularly interesting that both PABP1 and PABP4 are highly expressed in the hippocampus (Figure 3.7). The hippocampus again plays important roles in learning and memory with specific importance for spatial navigation. Several other RNA-binding proteins have been reported to be specifically expressed in the hippocampus (e.g. HuD (Clayton et al., 1998), FMRP (Hergersberg et al., 1995) and local translation at the dendrites of hippocampal neurons is thought to be important for synaptic plasticity (Costa-Mattioli et al., 2009; Richter and Klann, 2009).

The hippocampus consists of two interlocking C-shaped structures the *Cornu Ammonis* (CA) and the dentate gyrus (Figure 3.8) (Andersen et al., 2007). The *Cornu Ammonis* is divided histologically into three regions in rodents (four in humans) termed CA1, 2 and 3 (Andersen et al., 2007) and these regions have also been found to be defined by expression of specific genes (Lein et al., 2007). The *Cornu Ammonis* is continuous with the subiculum which merges with the entorhinal complex (EC) of the parahippocampus. Hippocampal neurons are interconnected to form a trisynaptic loop (Figure 3.8). The granule cells of the dentate gyrus receive input from the entorhinal complex (EC). The dentate gyrus projects to CA3 pyramidal cell neurons through mossy fiber (MF) axons. CA3 in turn projects to CA1 through Schaffer Collateral (SC) axons. The CA1 projects out of the hippocampus back to the EC (Andersen et al., 2007).

The *Cornu Ammonis* consists of a layer of pyramidal cells flanked by the *stratum oriens* externally and *stratum radiatum* internally (Lopes da Silva et al., 1990) (Figure 3.8). Pyramidal cells are the principal excitatory neurons of the hippocampus. The *stratum oriens* contains pyramidal cell dendrites, septal fibres (afferent fibres from the septal nuclei), commissural fibres (fibres connecting brain hemispheres) and cell bodies of inhibitory basket cells and other interneurons. The *stratum radiatum* also contains septal and commissural fibres, basket cells and other interneurons as well as Schaffer collateral axons extending from CA3 pyramidal cells to CA1 dendrites (Andersen et al., 2007).

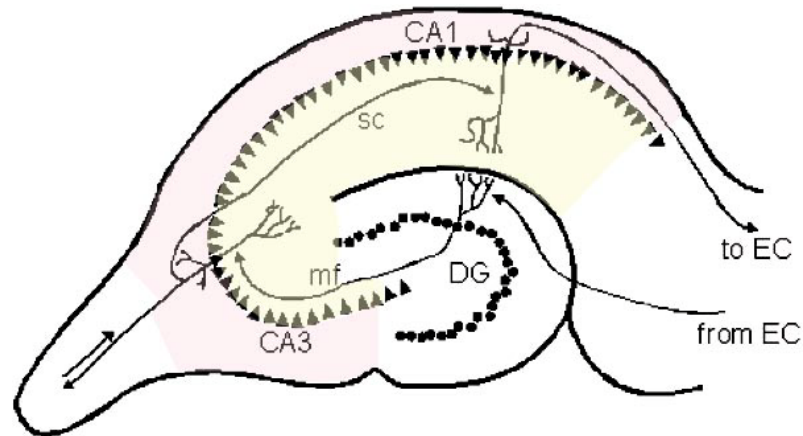


Figure 3.8 Histological regions and the trisynaptic loop of the hippocampus. Triangles represent the pyramidal cell layer of the CA and circles represent the granular cell layer of the dentate gyrus (DG). *Stratum oriens* of the CA is highlighted in pink and the *stratum radiatum* in yellow. Entorhinal cortex (EC), mossy fibers (MF) and Schaffer collaterals (SC) are labelled. Modified from (Ikonen, 2001).

Higher magnification images of PABP1 and PABP4 immunostaining of the hippocampus reveal subtle differences in their expression (Figure 3.9). PABP1 is expressed in the cell bodies of granule cells of the dentate gyrus and the pyramidal cells of the *Cornu Ammonis* with higher expression in CA1 than in CA2 or CA3 (Figure 3.9A, B). While PABP4 is also observed in the granule cell bodies in the dentate gyrus, it is also expressed throughout the CA and staining is observed not only in the pyramidal cell layer but also the stratum oriens and stratum radiatum layers either side (Figure 3.8C, D). PABP1 staining is also noticeable in the cytoplasm of oligodendrocytes of the alveus layer (Figure 3.9B – indicated by arrow) while no staining for PABP4 is observed in these cells (Figure 3.9D). The alveus layer consists of myelinated axons arising from cell bodies in the subiculum and hippocampus. Oligodendrocytes are non-neuronal and are a type of glial cell that serve to myelinate nerve axons in the brain.

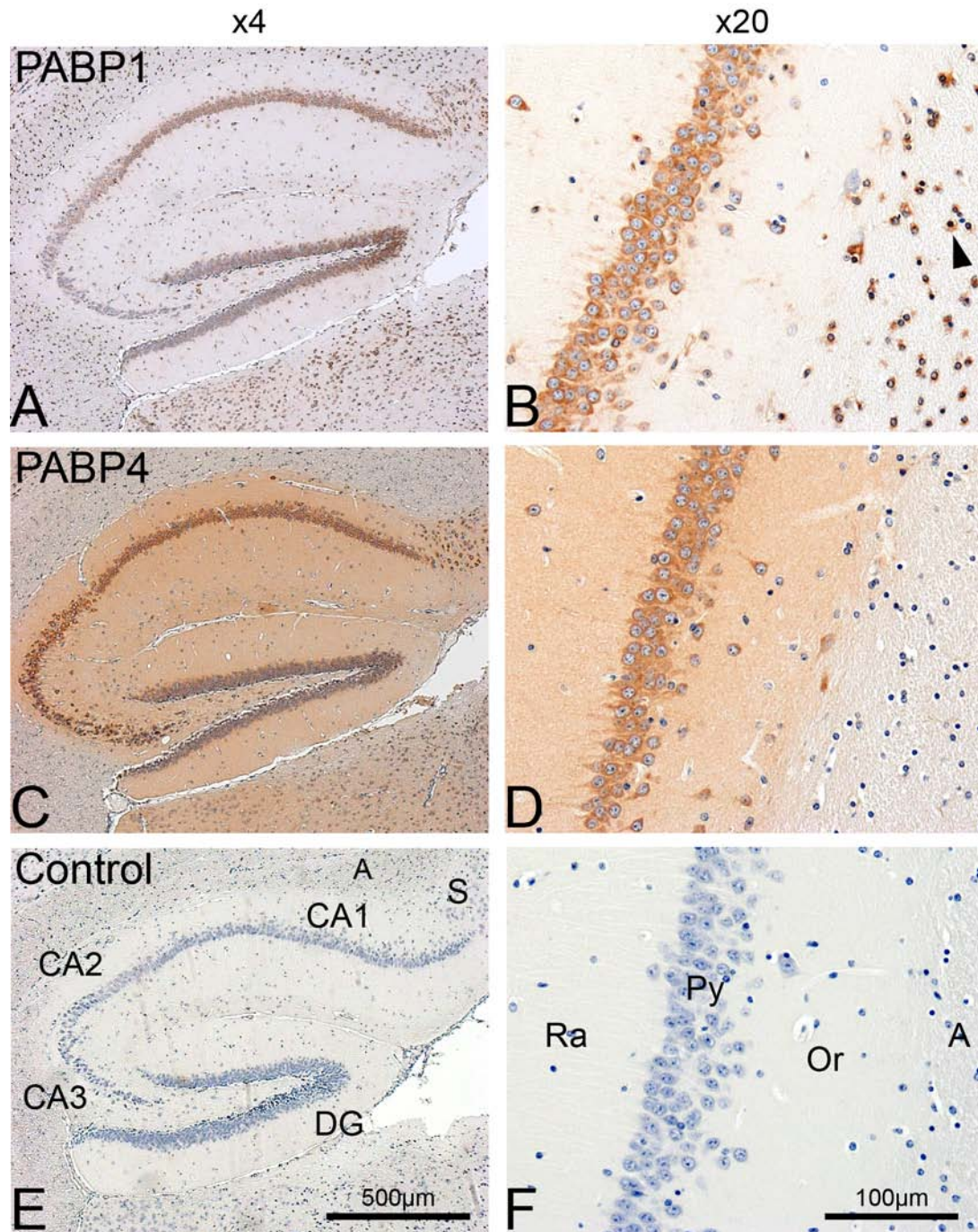


Figure 3.9 Expression analysis of PABP1 and PABP4 in mouse hippocampus. Sagittal sections of mouse brains were stained by immunohistochemistry for PABP1 (**A,B**), PABP4 (**C, D**) or rabbit IgG control (**E,F**). x4 magnification images of the hippocampus (**A,C,E**) and higher magnification (x20) images of CA1 are shown (**B,D,F**). Image **E** is annotated for the subiculum (S), alveus (A), CA1, CA2 and CA3 zones and the dentate gyrus (DG). Image **F** is annotated for *stratum radiatum* (Ra), pyramidal cell layer (Py) and *stratum oriens* (Or). A PABP1 expressing oligodendrocyte is indicated by the arrow in **B**.

The observed staining pattern demonstrates that both PABP1 and PABP4 are expressed in the cell bodies of multiple neuronal cell types (i.e. granule cells and pyramidal cells). PABP4 also appears to be expressed in the axonal fibres that constitute the *stratum oriens* and *stratum radiatum*, though PABP1 staining is not observed in these layers. Local translation is known to occur in axons and is required for axonal guidance (Lin and Holt, 2008); in the hippocampus at least, axonal translation may therefore be mediated by PABP4. In contrast, PABP1 is detected in oligodendrocytes while PABP4 is not. While expression of PABP4 in glial cells within the *stratum oriens* and *stratum radiatum* cannot be ruled out, it is indeed a possibility that PABP4 expression in the hippocampus may be restricted to neurons, while PABP1 is certainly expressed in both neuronal and glial cells.

Expression of PABP1 and PABP4 in the cerebellum

The cerebellum is important for the proper control of motor functions. While not involved in the initiation of movement, it is crucial for the proper coordination and precision of movement. Disorders of the cerebellum (e.g. spinocerebellar ataxia) lead not to paralysis, but impaired coordination, posture and motor learning (Barlow, 2002).

The cerebellum consists of a layer of tightly folded grey matter (cortex) with white matter underneath. The cerebellar cortex itself consists of three layers (see Figure 3.10I). Innermost is the granular layer consisting of densely packed granule cells and some interspersed interneurons. In the middle is a layer of Purkinje cell bodies. Outermost is the molecular layer containing the expansive dendrites of Purkinje cells, and at right angle to these, parallel fibres. This layer is also interspersed with inhibitory interneurons (stellate and basket cells) which act on Purkinje cell dendrites. Purkinje cells process excitatory signals from granule cells via parallel fibres and inferior olive cells in the pons via climbing fibres and deliver their output to cells in the deep cerebellar nuclei (Barlow, 2002).

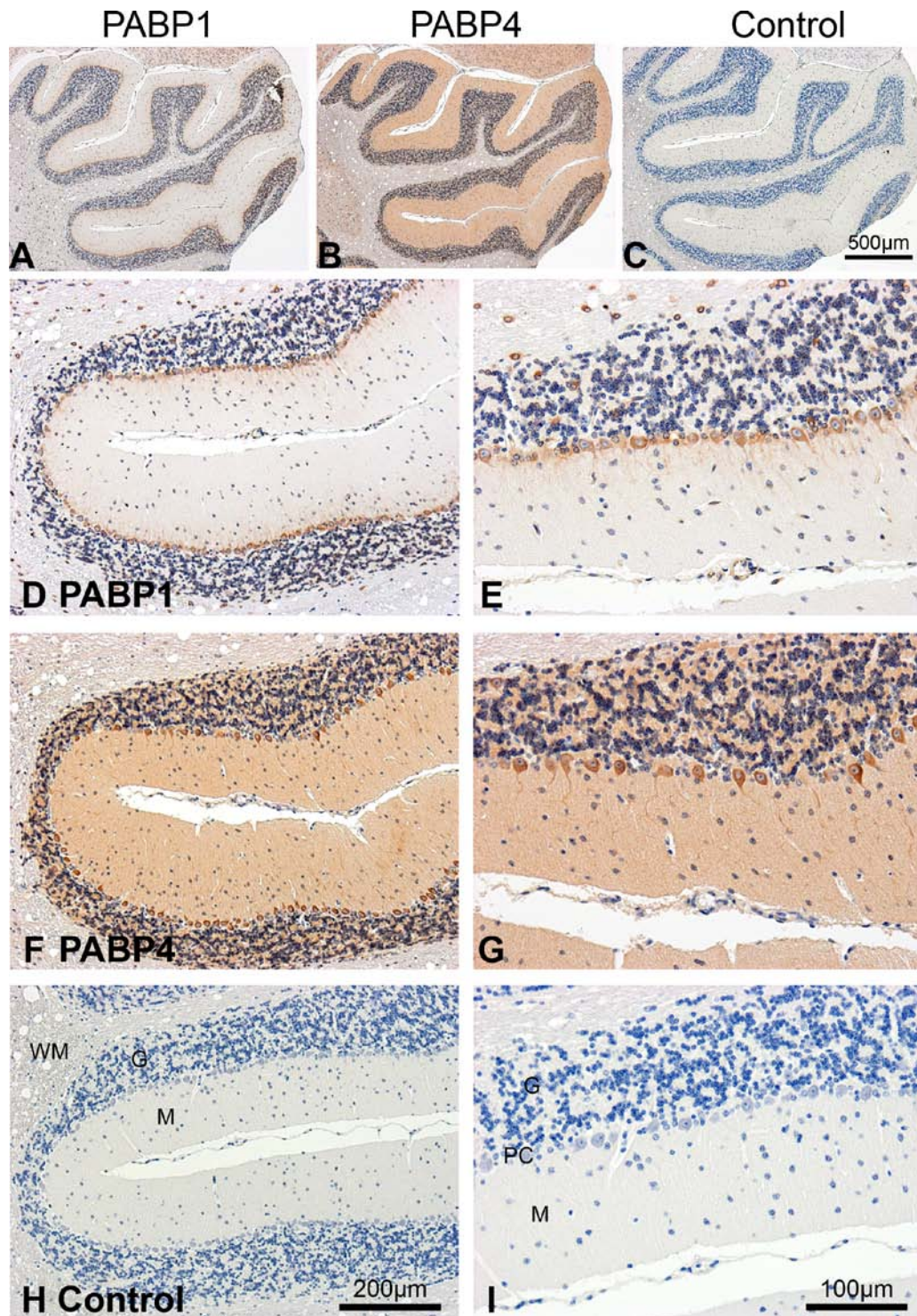


Figure 3.10 Expression analysis of PABP1 and PABP4 in mouse cerebellum. Saggital sections of mouse brains were stained by immunohistochemistry for PABP1 (A,D,E), PABP4 (B,F,G) or rabbit IgG control (C,H,I). x4 images of the cerebellum (A,B,C), x10 images of a single cerebellar fold (D,F,H) and x20 magnification images (E,G,I) revealing specific staining in Purkinje cells are shown. White matter (WM), the granule cell layer (G), the molecular layer (M) and Purkinje cell layer (PC) are labelled and oligodendrocytes indicated by arrows.

A closer examination of PABP1 and PABP4 expression within the cerebellum again reveals interesting differences between the two proteins (Figure 3.10). Both PABP1 and PABP4 are found in the cytoplasm of Purkinje cells, with staining observed not only in the cell bodies but extending within dendrites visible within the molecular layer (Figure 3.10 E, G). Again, PABP4 expression appears more widespread with staining observed in the molecular layer and granule layer where staining for PABP1 is largely absent (Figure 3.10 D, F). However, within the white matter in the centre of the cerebellum which contains myelinated axons, PABP1, and not PABP4, is again seen to be expressed in oligodendrocytes (Figure 3.10 D, F).

Expression of Ataxin-2 in the cerebellum

Ataxin-2 is a PAM-2 containing protein that has been shown to interact with PABP1 in *Drosophila* (Satterfield and Pallanck, 2006) by co-immunoprecipitation of endogenous proteins and in humans by yeast 2-hybrid and co-immunoprecipitation of overexpressed tagged proteins (Ralser et al., 2005). Interaction of ataxin-2 with PABP4 has not been tested.

Poly-glutamine expansion mutations in the human gene for ataxin-2 (*ATXN2*) cause the inherited neurodegenerative disorder spinocerebellar ataxia 2 (SCA2)(Imbert et al., 1996). It is unclear whether the pathophysiology of the disease is entirely due to dominant gain of function by the mutation or whether loss of function may also contribute, though ataxin-2 knockout mice are viable and have no neurological phenotype (Kiehl et al., 2006). Ataxin-2 is reported to be expressed in various adult mouse tissues (Nechiporuk et al., 1998), in brainstem and cortical neurons (Huynh et al., 1999) and Purkinje cells of the cerebellum (Fusco et al., 2001; Huynh et al., 2000; Kiehl et al., 2006). Purkinje cell loss is the primary target of SCA2 (Lastres-Becker et al., 2008).

The function of ataxin-2 is unknown but a role in some aspect of RNA metabolism is suggested by the presence of LSM (likeSM) and LSMad (LSM-associated) domains which confer RNA-binding in other proteins involved in splicing and mRNA decay (Beggs, 2005; Satterfield and Pallanck, 2006). Furthermore, both the *Drosophila* and human ataxin-2 proteins have been shown to be associated with polysomes and, in the case of the *Drosophila* protein, this association is at least partly dependent on the PAM2 domain (Satterfield and Pallanck, 2006). Taken together, this suggests that ataxin-2 may have a role in translational regulation in which an interaction with PABPs may play an important role (also discussed in Chapter 1.4.3.3).

To address whether ataxin-2 is present in the same mouse Purkinje cells that were positively stained for PABP1 and PABP4 I optimised use of a commercial ataxin-2 antibody for immunohistochemistry by serial dilution (not shown). Immunohistochemistry for ataxin-2 in the brain demonstrated strong expression in Purkinje cell bodies and dendrites (Figure 3.11C) consistent with published reports (Kiehl et al., 2006). Furthermore, similar to the results of Fusco et al (2001) in rat, I found that only about 50% of Purkinje cells were stained for ataxin-2. This is in contrast to PABP1 and PABP4 staining which was observed in almost all Purkinje cells (Figure 3.10E, G). Ataxin-2, PABP1 and PABP4 are therefore likely to be present together in some cerebellar Purkinje cells implying that the interaction with PABP1, or potentially an interaction with PABP4, could be involved in the SCA2 pathogenesis and normal function of ataxin-2 in these cells.

Ataxin-2, PABP1 and PABP4 antibodies each produce clear staining of Purkinje cells. As the ataxin-2 antibody used here is mouse-raised, dual-colour immunofluorescence would be perfectly suited to continue this work and clearly characterise colocalisation of PABP1 and PABP4 with ataxin-2 in mouse cerebellar Purkinje cells.

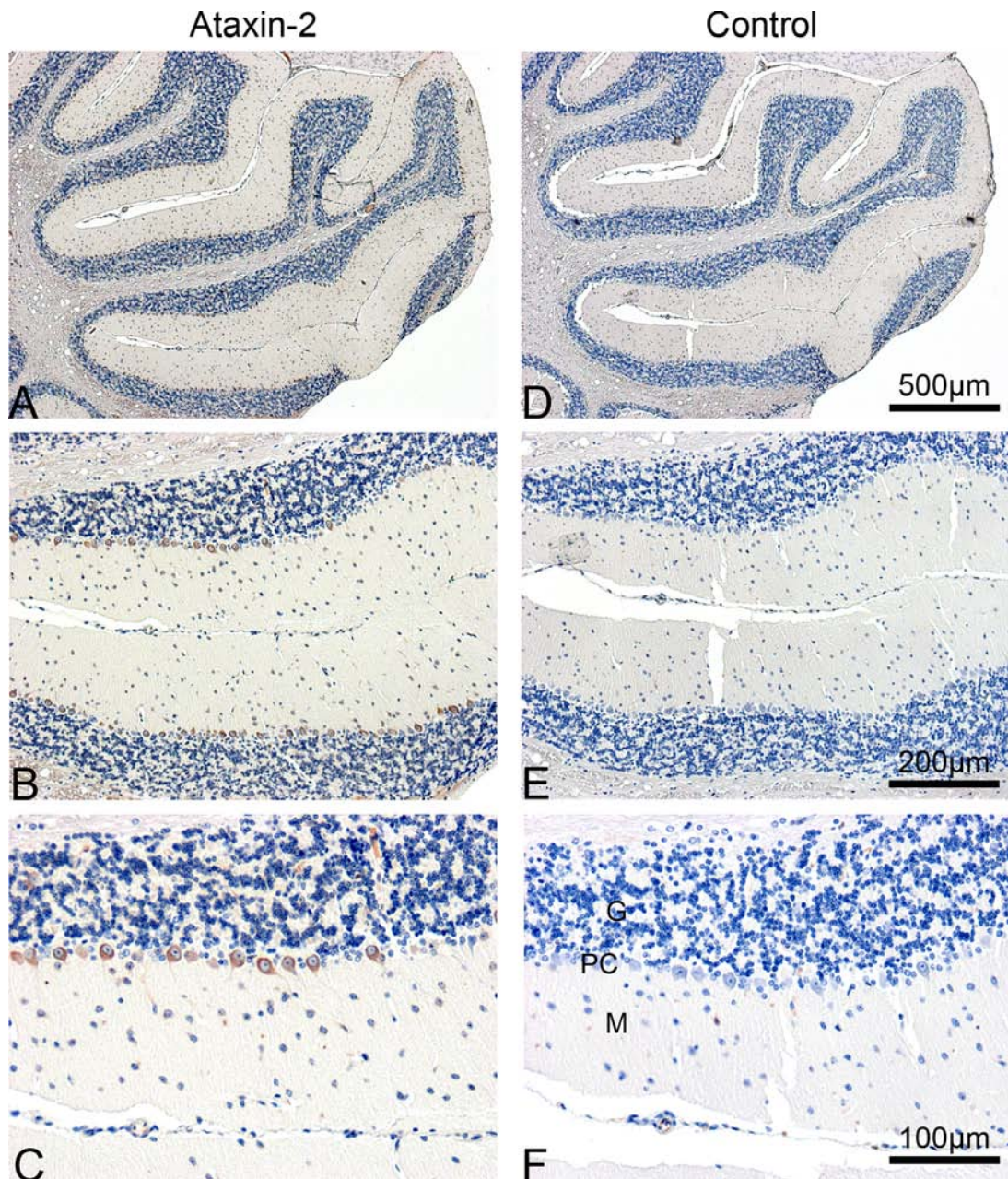


Figure 3.11 Expression analysis of Ataxin-2 in mouse cerebellum. Saggital sections of mouse brains were stained by immunohistochemistry for ataxin-2 (**A,B,C**) or mouse IgG control (**D, E,F**). x4 magnification images of the cerebellum (**A,C**) x10 magnification images of a single cerebellar fold (**B,E**) and x20 magnification images (**C,F**) revealing specific staining in a subset of Purkinje cells are shown. White matter (WM), the granule cell layer (G), the molecular layer (M) and Purkinje cell layer (PC) are labelled.

3.2.7.2 Pancreas

Western blotting of tissue extracts established that both PABP1 and PABP4 are expressed in the pancreas (Figure 3.6). The pancreas has two very different roles. As an exocrine gland it secretes digestive enzymes such as trypsin and lipase into the small intestine. As an endocrine gland it produces and secretes into the blood stream peptide hormones such as insulin and glucagon that are important for carbohydrate metabolism. Endocrine secretions are produced from the islets of Langerhans while exocrine secretions are produced by acinar cells. These regions are easily identifiable by haematoxylin staining as acinar cells stain very darkly blue compared to lighter staining islet cells (Figure 3.12E, F) (Lowe and Stevens, 2002).

Immunostaining for PABP1 and PABP4 in the pancreas revealed a striking inverse pattern of expression between the two proteins (Figure 3.12). PABP1 is observed in the endocrine tissue at islets of Langerhans but not in the acinar cells of the exocrine tissue. Islets are heterogeneous and consist of multiple cell types including glucagon producing alpha cells and insulin producing beta cells (Lowe and Stevens, 2002). PABP1 expression however does not appear to be restricted to any particular cell type within the islets, with staining observed throughout. PABP1 is also seen to be expressed in the cuboidal epithelial cells lining the ducts which collect exocrine secretions. In stark contrast, PABP4 staining is strongly detected in exocrine acinar cells but is absent or weak in islet cells. Additionally, no PABP4 staining is observed in duct epithelial cells.

Islets of Langerhans make up only a small proportion of the total mass of the pancreas with the bulk consisting of exocrine tissue (Lowe and Stevens, 2002). Thus, the expression patterns identified by immunohistochemistry in the pancreas are entirely consistent with the western blot in Figure 3.6 which shows comparatively high levels of PABP4 in the pancreas and low levels of PABP1.

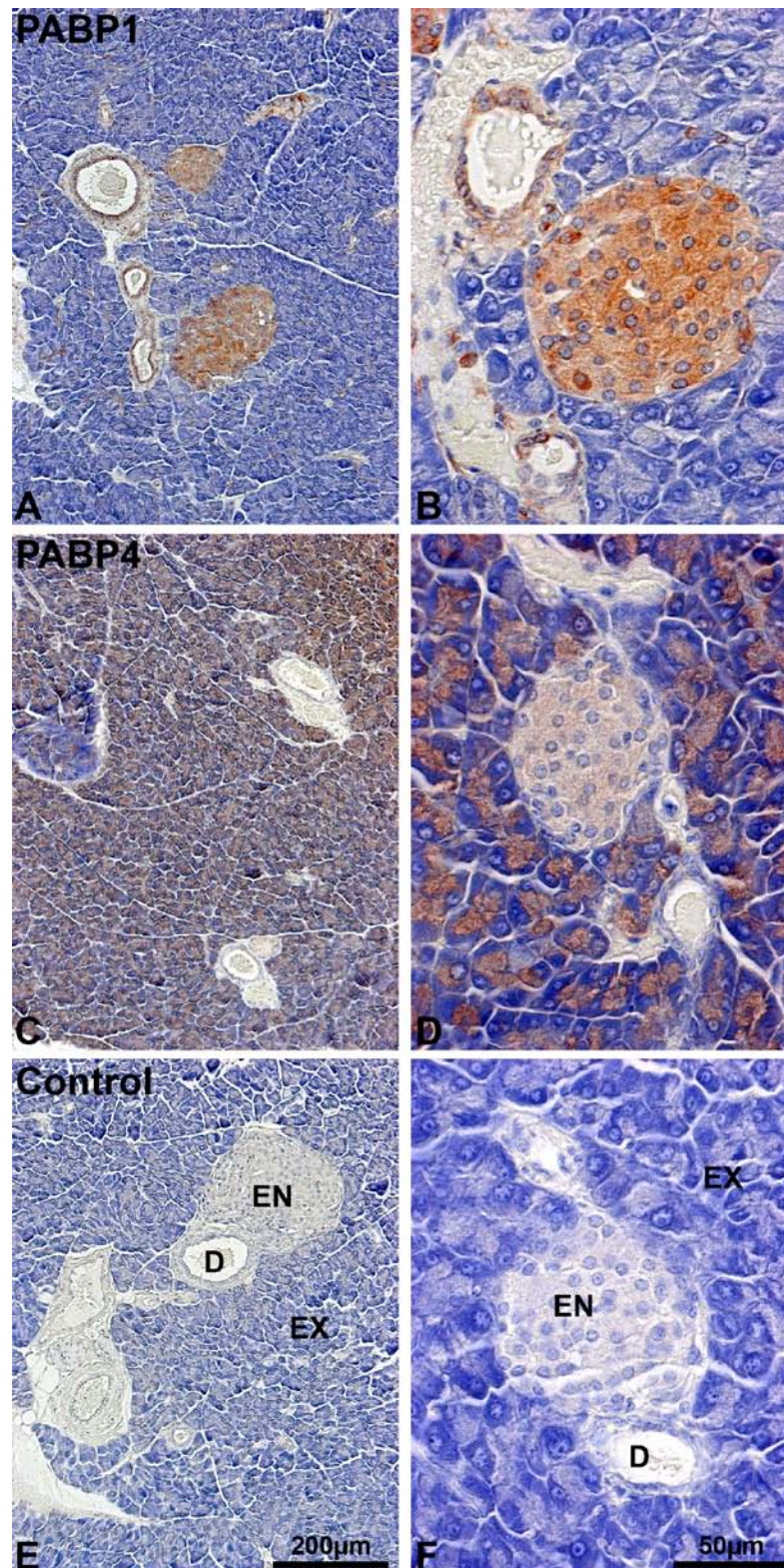


Figure 3.12 Expression analysis of PABP1 and PABP4 in mouse pancreas. Sections of mouse pancreas were stained by immunohistochemistry for PABP1 (A,B), PABP4 (C,D) or rabbit IgG control (E,F). x10 magnification images (A,C,E) and x40 magnification images (B,D,F) are shown with annotation of exocrine tissue (EX), ducts (D) and endocrine tissue (EN) within islets of Langerhans.

3.2.7.3 Small intestine

The exocrine products of the pancreas are secreted via the pancreatic duct into the duodenum, a part of the small intestine. The duodenum is the first portion of the small intestine beyond the stomach. It is the site of most food breakdown within the small intestine and also contains Brunner's glands which secrete alkaline mucous that neutralises stomach acid and supports digestive enzymes. In common with the jejunum and ileum (the subsequent portions of the small intestine), the duodenum is lined with villi, small finger like projections of the intestinal wall which serve to increase the absorptive area available (Young and Heath, 2002).

Sectioning of the pancreas permitted examination of the attached duodenum. Staining for PABP1 and PABP4 again revealed differences in their expression patterns (Figure 3.13). PABP4 staining was observed in the columnar epithelial cells which line the duodenal villi (Figure 3.13B). These cells function to absorb nutrients and are also known as enterocytes. PABP1 staining was observed in some of the enterocytes at the bottom of each villus but expression appears to diminish in the epithelial layer toward the apex of each villus (Figure 3.13A). Both PABP1 and PABP4 are expressed in the intestinal crypts between the villi (Figure 3.13D, E - black arrows) which are populated by enterocytes, paneth cells and stem cells. Paneth cells have a defensive function and crypt stem cells divide to replenish enterocytes. Between the crypts and extending into the core of each villus is the lamina propria. This layer contains blood and lymphatic vessels. PABP1 and PABP4 also appear to be expressed in some but not all cells in the lamina propria within villi but neither is strongly detected in the surrounding submucosa and muscle layers (Figure 3.13D, E).

The epithelial cells that line intestinal villi are constantly renewed. They are generated by division of stem cells in the crypts and then move up to the tip of each villus where they are sloughed off (Young and Heath, 2002). It is interesting that as enterocytes move up the villus, expression of PABP1 appears to cease implying that PABP1 is not required for their absorptive role but is required during their differentiation in the crypts.

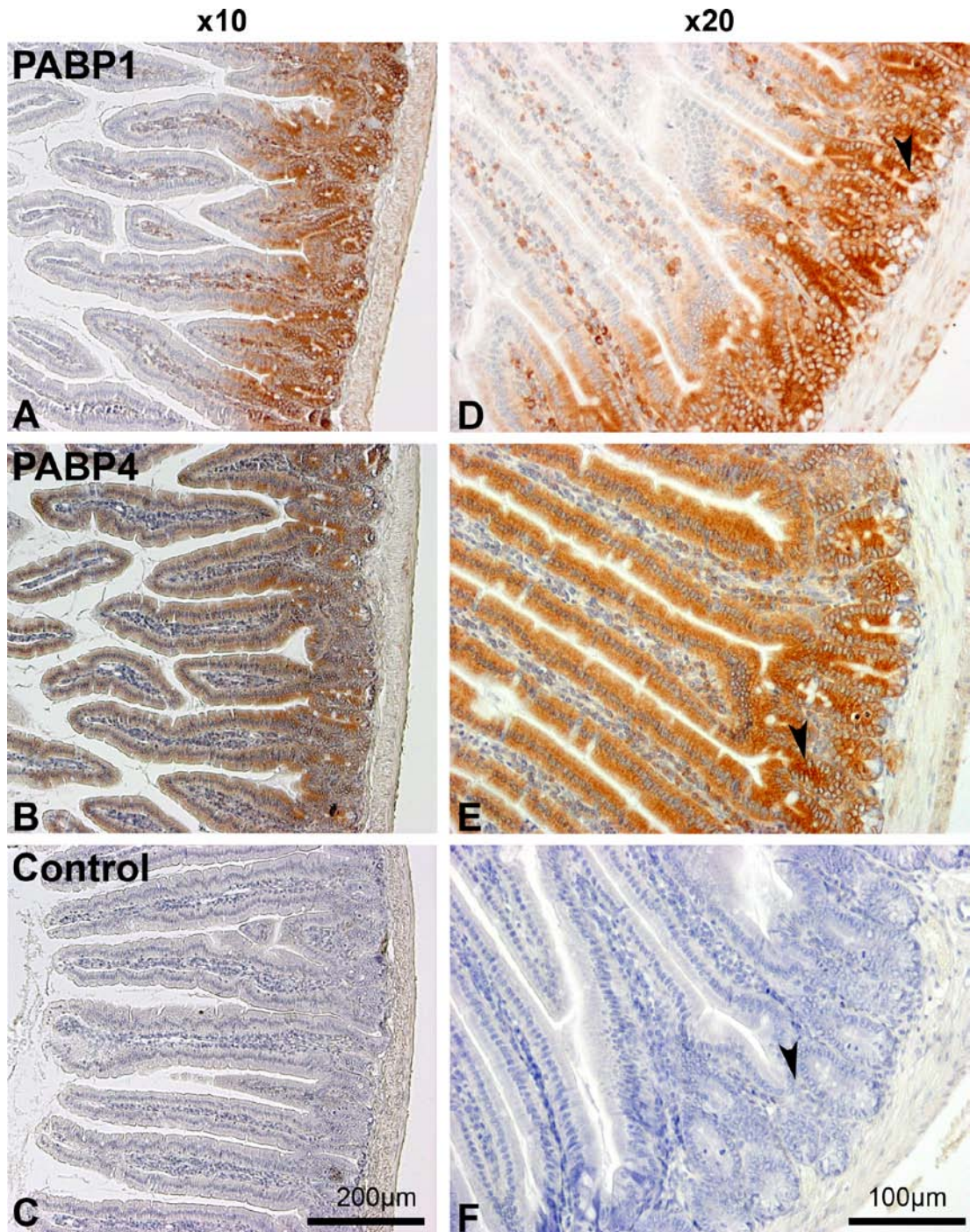


Figure 3.13 Expression analysis of PABP1 and PABP4 in mouse duodenum. Sections of mouse small intestine were stained by immunohistochemistry for PABP1 (A,D), PABP4 (B,E) or rabbit IgG control (C,F). x10 magnification images of villi extending into the intestinal lumen (A,B,C) and x20 magnification images (D,E,F) are shown. Intestinal crypts are indicated by black arrowheads.

3.2.7.4 Testes

Within mammalian testes gametogenesis occurs in seminiferous tubules. The gamete precursor cells are called spermatogonia and are found adjacent to the basement membrane (see Figure 3.14) (Borg et al., 2009). These divide by mitosis, to ensure a constant supply of spermatogonia. Some spermatogonia develop into primary spermatocytes which then undergo meiosis. The first meiotic division gives rise to diploid secondary spermatocytes. These cells then undergo the second meiotic division to form haploid spermatids which mature into spermatozoa. During spermatogenesis the developing spermatocytes move towards the tubule lumen before progressing to the epididymis for final maturation and storage. Also present within the seminiferous tubules are non-spermatogenic Sertoli cells that support the developing germ cells and intercalate between them (Borg et al., 2009).

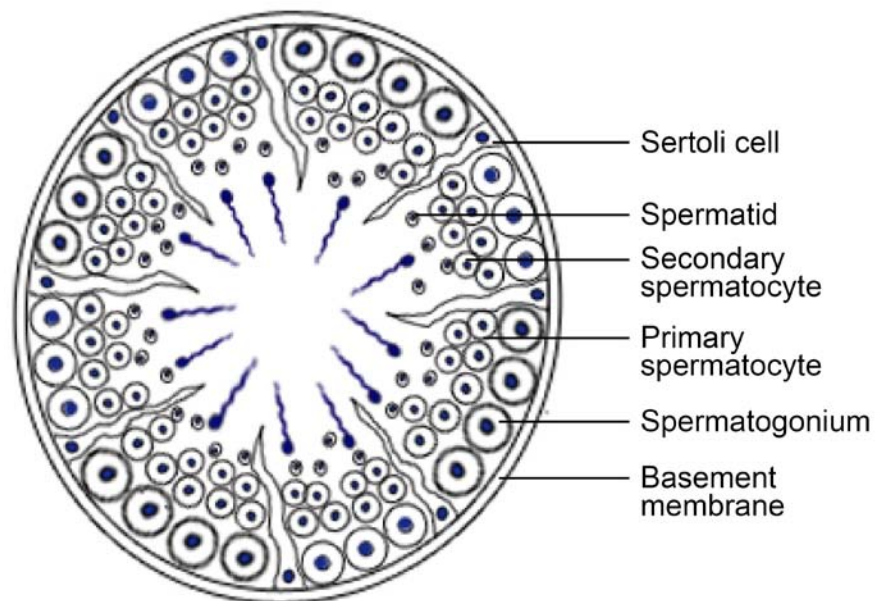


Figure 3.14 Simplified schematic of cell types within a seminiferous tubule

Expression of PABP1 and PABP4 protein was examined in adult mouse testes by immunohistochemistry. PABP1 staining was observed at a low level in spermatocytes and highest in round spermatids. Low level staining was also observed in elongating spermatids but was not detected in spermatogonia (Figure 3.15A). These results are consistent with published analyses of PABP1 mRNA expression in mouse and human (Kleene et al., 1994) (Feral et al., 2001) and PABP1 protein expression in rat (Gu et al., 1995). These results have the caveat that the peptide used to generate the PABP1 antibody used is, with one amino acid exception, perfectly conserved in tPABP. Thus, our PABP1 antibody may cross-react with tPABP. As tPABP mRNA is expressed in round spermatids and spermatocytes in mice (Kleene et al., 1994) the expression I observed for PABP1 may have been artificially enhanced by cross-reaction with tPABP protein in these cell types.

Interestingly, the pattern of staining obtained using anti-PABP4 was very different to that using anti-PABP1. PABP4 expression was seen in all cell types (Figure 3.15B) with higher levels of expression noticeable in spermatogonia and Sertoli cells (indicated by arrow) which lack PABP1. Like the intestine however, despite apparent widespread expression of PABP4 across testes cell types, western blotting indicates that total expression of PABP4 in testes is relatively low compared to PABP1 (Figure 3.6).

Thus, examination of PABP1 and PABP4 expression by immunohistochemistry in each of the four tissues studied here has revealed cell-type specific expression of both proteins.

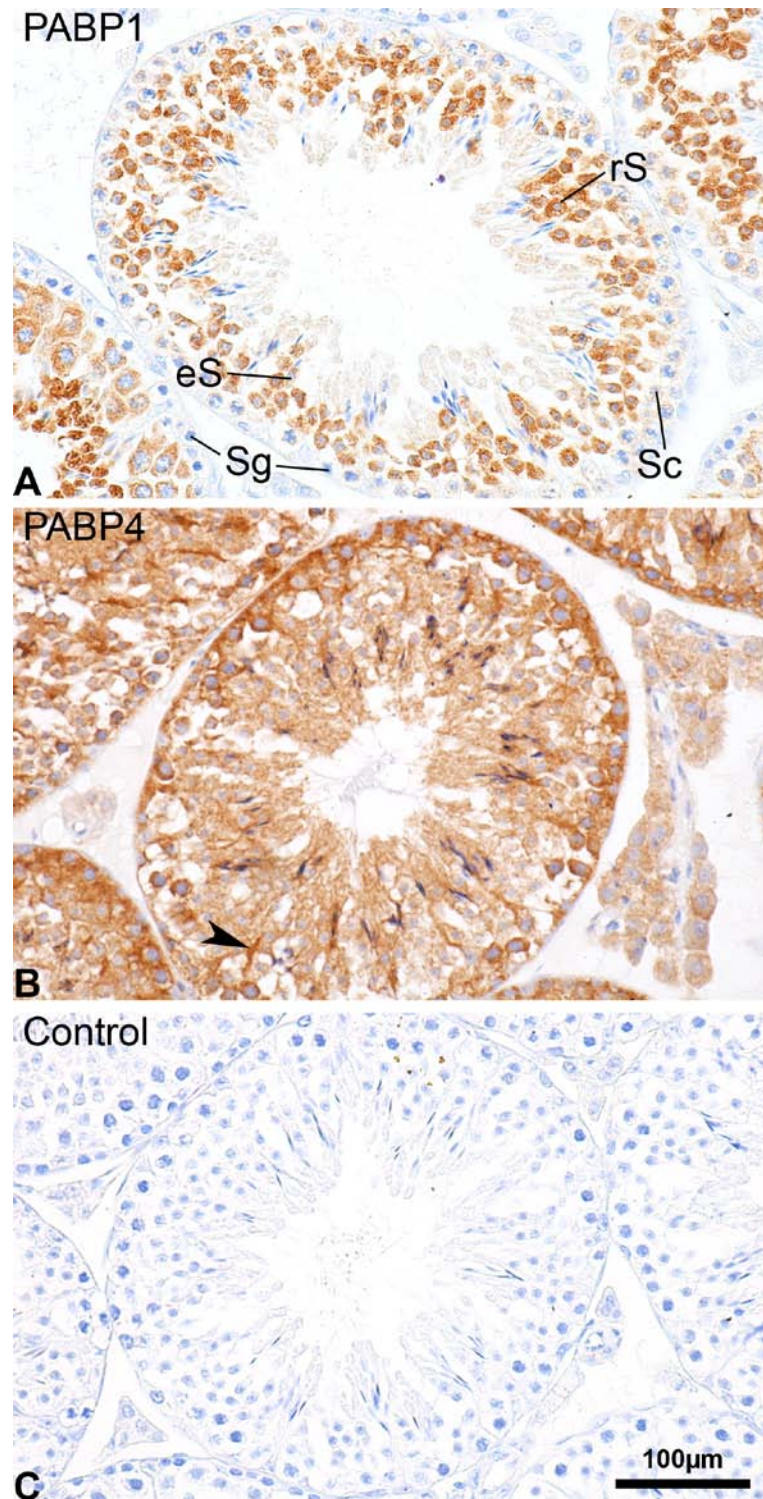


Figure 3.15 Expression analysis of PABP1 and PABP4 in mouse testes. Sections of mouse testes were stained by immunohistochemistry for PABP1 (A), PABP4 (B) or rabbit IgG control (C). Images taken at 40x magnification showing a cross section through a single representative seminiferous tubule are shown. In A spermatogonia (Sg), primary spermatocytes (Sc), round spermatids (rS) and elongating spermatids (eS) are indicated. Black arrowhead indicates a Sertoli cell stained for PABP4.

3.3 Discussion

In this chapter I have characterised expression of PABP4 in human cell lines and mouse tissues. I have compared the expression of PABP1 and PABP4 protein in mouse tissues and examined the cell-type differences in the expression of both proteins. I have also presented evidence to support a role for PABP4 as a translation factor.

Expression

The expression patterns of PABP1 and PABP4 protein in mouse were investigated using two approaches– western blotting of whole tissue lysates (Figure 3.6) and immunohistochemical analysis of tissue sections (Figures 3.7-3.15). The relative levels of expression of PABP1 and PABP4 proteins were shown to greatly vary across mouse tissues (Figure 3.6) demonstrating that the expression of each protein is neither ubiquitous nor consistent. This is in keeping with reports showing that expression of PABP1 and ePABP proteins vary in *Xenopus* tissues (Cosson et al., 2002c) (Wilkie et al., 2005). However, the pattern of PABP1 protein expression reported in *Xenopus* tissues (high expression in kidney, heart, muscle by Wilkie *et al*, and high expression in brain, testis and heart by Cosson *et al*) is quite dissimilar to the results obtained in mouse, where the highest expression was observed in spleen, ovary and testis, a medium level of expression in brain, lung and liver, and lowest expression in heart, kidney and muscle. These results (Figure 3.6) are however consistent with the expression of human PABP1 and PABP4 mRNA (Yang et al., 1995) suggesting that while the expression patterns of poly(A)-binding proteins are not be conserved among all vertebrates they be similar among mammals.

My results, with respect to PABP1 expression are largely consistent with a recent study by Kimura and colleagues (2009) who also examined expression of PABP1 (and tPABP) across mouse tissues by western blotting. They found the highest levels of PABP1 expression in testes and ovary, with medium level of expression in brain, liver and kidney, and lowest levels detected in lung, spleen, and heart (Kimura et al., 2009). Levels of PABP1 in kidney and spleen differed between

my results and theirs. Moderate levels of PABP1 were detected in the kidney and low levels in the spleen by Kimura *et al* whereas I did not detect PABP1 in the kidney and found spleen to be among the tissues in which PABP1 was expressed at a highest level. These differences may be explained by natural variation in PABP1 expression between individual mice or strains or could be a result of the post-translational modifications of PABP1 to which the alternate antibodies used bind with differing affinities.

In some tissues PABP1 and PABP4 appeared to show an almost reciprocal pattern of expression (Figure 3.6) reminiscent of the reciprocal pattern of PABP1 and ePABP expression during early development (Cosson *et al.*, 2002c; Seli *et al.*, 2005). As the detection limits of our PABP specific antibodies are unknown, it is unclear to what absolute levels each protein is expressed in mouse though it would be interesting to know whether tissues have differing requirements for total PABP levels. Quantification of PABP1 and PABP4 levels could be achieved by comparing the western blot signal obtained from mouse tissues with quantified amounts of purified recombinant proteins, however in testes tPABP protein is also present (Kimura *et al.*, 2009) and the expression of ePABP or PABP5 proteins in adult mammalian tissues has not been addressed. While one study detected ePABP mRNA expression in mouse only in ovary and testes (Seli *et al.*, 2005) in human ePABP mRNA was also detected in liver, kidney, pancreas, spleen and thymus (Guzeloglu-Kayisli *et al.*, 2008). Human PABP5 RNA expression has also been detected in various tissues with the highest levels found in ovary (Blanco *et al.*, 2001). Therefore, availability of tPABP, ePABP and PABP5 antibodies would allow not only conclusive characterisation of the expression of these proteins in mice but would also allow us to determine the tissue-specific requirements for total levels of poly(A)-binding proteins.

Several tissues expressed both PABP1 and PABP4 and examination of their cellular expression by immunohistochemistry revealed differences in the expression of both proteins in every tissue examined (Chapter 3.2.7) conclusively showing that expression of either protein is by no means uniform or ubiquitous. The most striking pattern of expression was identified in the pancreas where PABP1 and PABP4 expression appear segregated according to the secretory function of cells

(Figure 3.12). PABP1 expression also seemed enriched in the epithelial cells lining pancreatic ducts (Figure 3.12) however; it was seen to vary in strength over the epithelium of a single intestinal villus (Figure 3.13) indicating PABP1 is not universally highly expressed in epithelium. Also, while both PABP1 and PABP4 were found to be expressed in the intestinal crypts where stem cells reside, only PABP4 was highly expressed in the spermatogonial stem cells indicating that co-expression of PABP1 and PABP4 is not associated with multipotency. Thus, while both PABP1 and PABP4 were detected in multiple types of neurons and PABP1 detected in glial cells (oligodendrocytes) in two different regions of the brain (Figures 3.9 and 3.10), no further themes of expression were identified.

PABP4 mRNA was shown by RT-PCR to be expressed in HeLa cells as four different isoforms (Figure 3.1A) though multiple protein bands were not detected by western blotting (Figure 3.2B). It is however, detected as two closely migrating bands in several mouse tissues which vary in abundance (Figure 3.6) suggesting that expression of alternative PABP4 protein isoforms may be tissue regulated and these isoforms might differ functionally. Mass spectroscopy or siRNA knockdown of individual isoforms in organ culture could be used in the future to identify which isoforms the observed bands represent. It remains possible however, that the two PABP4 bands detected mouse tissues could be due to post-translational modifications rather than splicing though differently modified PABP4 or alternative PABP4 isoforms, would represent a novel level of tissue specific regulation of PABP4 and could alter its function.

Expression of each of the predicted PABP4 mRNA isoforms has also been detected in our lab in mouse testes (P. Martins – personal communication) though only a single faint band was detected by western blotting (Figure 3.6). This raises the possibility of a regulatory mechanism down-stream of pre-mRNA splicing and it would be interesting to investigate whether expression of PABP4 isoforms is regulated at the translational level. Interestingly, while Ensembl predicted sequences for the 5' UTRs of three of the human PABP4 isoforms contain A-rich stretches, the predicted transcript for the shortest PABP4 isoform (PABPC4-003) does not. Furthermore, this pattern is conserved in the mouse isoforms. If these stretches function analogously to the PABP1 ARS, a transcript lacking such an autoregulatory

sequence would be expected to be immune to auto-regulation perhaps providing a route to increase PABP expression without translational inhibition.

Relevance of PABP1 and PABP4 expression patterns to PABP-interacting proteins

Analysis of PABP1 and PABP4 in the brain showed expression of PABP1 and PABP4 in Purkinje cells of the cerebellum where PABP1-interacting protein Ataxin-2 is also expressed. Purkinje cells are the site of neurodegeneration in the disease spinocerebellar ataxia 2, resulting from polyglutamine expansion mutations in the *ATXN2* gene. The molecular pathogenesis of this disease is not well understood and so the possibility of a functional interaction between PABP1/PABP4 and ataxin-2 in Purkinje cells is incredibly interesting. This work opens the door for more investigation of this potentially functional relationship. In addition to co-localisation studies, immunoprecipitation from brain lysates could also be employed to identify if PABP1-ataxin-2 and PABP4-ataxin-2 interactions occur *in vivo*.

Immunohistochemical analysis of the testes revealed cell-type specific expression of PABP1, mainly in round spermatids but also detectable to a lesser degree in spermatocytes and elongating spermatids. The recent study by Kimura *et al* generally supports my findings of PABP1 localisation in testes and implies that any cross-reaction of our PABP1 antibody with tPABP is likely only to have artificially increased the signal in round spermatids. Expression of PABP4 in addition to PABP1 and tPABP protein in the testis means that there are at least three poly(A)-binding proteins expressed in this tissue with overlapping expression patterns. Furthermore, as ePABP mRNA has also been detected in mouse testes (Seli *et al.*, 2005; Wilkie *et al.*, 2005) it is possible that ePABP protein is also present.

The distribution of PABPs in testes is of relevance to the study of a PABP1-interacting protein – DAZL (deleted in azoospermia-like). DAZL is a DAZ family protein whose function in translation is not fully understood. DAZL is expressed specifically in the germ cells of the gonads and DAZL homozygote knock-out mice are infertile, a condition thought to be caused by defects in germ-cell differentiation (Ruggiu *et al.*, 1997). DAZL contains an RNA binding domain (Yen *et al.*, 1997) and is able to stimulate translation dependent on binding of PABP1 (Collier *et al.*, 2005)

It is thought that DAZL binds and stimulates translation of specific messages, the products of which are essential for spermiogenesis and oogenesis (Chapter 1.4.3.3). DAZL expression in testes is mainly in spermatogonia (Reijo et al., 2000) and PABPs that are expressed in these cells would be most likely to participate in DAZL-mediated stimulation of translation. My data suggests that only PABP4 appears to be co-expressed with DAZL in the spermatogonia, however an interaction between PABP4 and DAZL has not been investigated. Ongoing research in our lab is directed toward characterizing this putative interaction and defining co-expression of PABP and DAZ family members in mammalian gonads.

PABP4 function

A number of observations support a role for PABP4 in translation. PABP4 was found to be a predominantly cytoplasmic protein using two independent methods, immunofluorescence and biochemical fractionation of HeLa cells (Figure 3.3). Sucrose gradient analysis revealed that PABP4 is associated with polysomes (Figure 3.4). This technique is commonly used to characterise the involvement of proteins in translation, however, some proteins not involved in translational activation have been isolated from polysomal fractions such as the decapping enzyme hDCP2 (Wang et al., 2002b) and the 5'-3' exonuclease Xrn1p (Brenques et al., 2002). Nevertheless, most characterised translation factors including PABP1 (Gu et al., 1995; Proweller and Butler, 1996) and ePABP (Wilkie et al., 2005) associate with polysomes in an Mg^{2+} -dependent manner and association of PABP4 with polysomes is consistent with a role in translation.

PABP4 also interacts with proteins which mediate the functions of PABP1 in translation (Figure 3.5). Interaction of PABP4 with eIF4G and PAIP1 suggest that like PABP1, PABP4 could stimulate translation when bound to a poly(A)-tail by circularizing mRNA. As PABP4 and PABP1 were found to interact however, it remains possible that co-immunoprecipitation of PAIP1 and eIF4G with PABP4 may have been bridged by PABP1. Further characterisation of the interactions of PABP4 with eIF4G and PAIP1 using purified proteins would not only allow us to rule out

this possibility but would permit comparison of the relative affinity of PABP1 and PABP4 for these proteins.

These results are consistent with our analysis of PABP4 function in *Xenopus laevis* (*Xl*) (Gorgoni et al., Submitted). We found that xIPABP4 was able to stimulate translation to a similar degree to PABP1 and ePABP in a tether-function assay. Furthermore, xIPABP4 was shown by targeted yeast 2-hybrid assay to interact with *Xenopus* eIF4G, PAIP1, eRF3, PABP1 and ePABP proteins and an epitope-tagged xIPABP4 was shown to associate with polysomes in oocytes. Moreover, morpholino knock-down of PABP4 in developing *Xl* embryos resulted in a global translation defect, evidenced by a reduction in polysomes (Gorgoni et al., Submitted).

The demonstrated interaction between PABP1 and PABP4 (Figure 3.5B) is particularly interesting as it suggests both PABPs may function cooperatively on poly(A)-tails in cells in which they are both expressed. Indeed, PABP1 and PABP4 were shown to be co-expressed in HeLa cells and many cell types in different mouse tissues (eg, Purkinje cells in the cerebellum). Recently, murine PABP1 and tPABP were shown to associate by co-immunoprecipitation from testes and GST-pulldown (Kimura et al., 2009). This raises the possibility that in testes, mRNAs may be bound by combinations of PABP1, PABP4 and tPABP - though a direct interaction between PABP4 with tPABP is yet to be tested. As discussed above, the mammalian pattern of ePABP protein expression is yet uncharacterised however, an interaction between *Xl* PABP1 and ePABP has been demonstrated by yeast 2-hybrid and co-immunoprecipitation (Wilkie et al., 2005). This suggests that if ePABP protein is co-expressed with PABP1 (or PABP4 or tPABP) it too may participate in cooperative binding of poly(A)-tails.

PAIP1 interacts with PABP1 via two regions, RRM1 and 2 and the PABC domain (Gray et al., 2000; Roy et al., 2002). The demonstrated interaction between PABP4 and PAIP1 further supports the notion that the PABC domain of PABP4 maintains interactions with other PAM2 containing proteins (e.g. ataxin-2, PAIP2, GW182) as suggested by close similarity of PABP4 to PABP1 over the PABC domain and the published interactions of PABP4 with eRF3 (Cosson et al., 2002a) and TOB (Okochi et al., 2005). While my results suggest that PABP4 functions similarly to PABP1 in translation, full characterisation of which PABP1-interactions

are maintained by PABP4 in mammals may provide insight into possible differences between the function or regulation of PABP1 and PABP4.

Functional significance of differing expression of PABP1 and PABP4

Results presented in this chapter have shown that while PABP1 and PABP4 appear to have different tissue and cell-type expression patterns they may function similarly in translation, giving no indication of why multiple PABPs are required. Recent work in our group investigating PABP-dependency during *Xenopus laevis* development however, establishes that PABPs are not functionally equivalent (Gorgoni et al., Submitted). Morpholino knock-down of PABP1, ePABP and PABP4 in *Xenopus* each lead to different abnormalities and lethality at different stages of development. While knock-down of PABP1 resulted in death by stage 30/31, ePABP knock-down embryos survived until stage 35 and PABP4 knock-down embryos survived until stage 50. Interestingly, whilst PABP1 morphants were rescued with ectopic expression of PABP1, expression of PABP4 or ePABP did not fully rescue, implying that PABPs have non-redundant roles in vertebrate development (Gorgoni et al., Submitted). How the roles of PABPs might differ is discussed further in Chapter 7.

In conclusion, I have demonstrated that PABP4 maintains protein interactions that, given its proven ability to bind poly(A)-RNA, make a role as a translational activator likely. I have challenged the dogma that PABP1 is ubiquitous, and described the different tissue and distinct yet overlapping cell-type expression patterns shown by PABP1 and PABP4. As yet we have not identified functional differences between PABP1 and PABP4, which may be necessary to fully comprehend why multiple and different poly(A) binding proteins are expressed in different mammalian tissues.

Chapter 4: Localisation of poly(A)-binding proteins under stress

4.1 Introduction

The previous chapter explored the expression of PABP1 and PABP4 in tissues, showing them to have quite different patterns of expression. PABP4 was found to be diffusely cytoplasmic in cultured cells (Figure 3.3) and multiple cell types within a variety of tissues (Chapter 3.2.7). Under conditions of cell stress however, PABP1 localises to cytoplasmic stress granules (SGs) (Kedersha et al., 1999). Stress granules form when translation initiation is inhibited by cell stresses (Holcik and Sonenberg, 2005) (Yamasaki and Anderson, 2008), drug treatment (Dang et al., 2006; Mazroui et al., 2006) or over-expression of RNA-binding proteins that repress translation (e.g. Fragile X mental retardation protein (FMRP) (Mazroui et al., 2002); T-cell intracellular antigen-1 (TIA-1) (Gilks et al., 2004)) (reviewed in (Anderson and Kedersha, 2009; Buchan and Parker, 2009)). They contain stalled initiation complexes and are typically composed of poly(A)+ RNA, 40S ribosomal subunits, eIF4E, eIF4G, eIF4A, eIF3, eIF2 α , eIF2B as well as PABP1 (Kedersha et al., 2002; Kedersha et al., 1999; Kimball et al., 2003; Mazroui et al., 2006).

Numerous other proteins have also been found to localise to stress granules including the PABP1 interacting protein ataxin-2 (Nonhoff et al., 2007). 60S ribosomal subunits however, are not found at SGs (Kedersha et al., 2002; Kimball et al., 2003) and it is unclear whether mRNAs in SGs represent pre-initiation complexes i.e. are bound by 40S ribosomes. Following stress recovery and correlating with recovery of protein synthesis rates, stress granules disassemble (Mazroui et al., 2007).

How SGs assemble is not entirely clear. Phosphorylation of eIF2 α is involved in effecting the inhibition of global translation associated with many SG-inducing cell stresses, but it is not a requirement for SG formation (Mazroui et al., 2006) (Dang et al., 2006; Mokaš et al., 2009). RNAi studies have identified many proteins whose knockdown impairs SG formation including, GTP-ase activating protein SH3 domain-binding protein (G3BP), ubiquitin specific protease 10 (USP10), the translational silencer TIA-1, small ribosomal subunits and eIF3 subunits, implying that these proteins are important for SG assembly (Ohn et al., 2008). Furthermore, TIA-1 and G3BP are capable of self-aggregation and overexpression of either protein

leads to SG formation in the absence of stress (Kedersha et al., 1999; Tourriere et al., 2003). Assembly of SGs may be further regulated by post-translational modifications of constituent proteins; phosphorylation of G3BP affects its incorporation into SGs (Tourriere et al., 2003) and the ubiquitin and O-Glc-NAc modification pathways have been implicated in the regulation of SG formation (Ohn et al., 2008).

Live imaging studies have shown that stress granules are dynamic, move within the cytoplasm and sometimes divide or merge with one another over time (Kedersha et al., 2005; Nadezhdina et al., 2009). The movement of GFP tagged PABP1 in and out of SGs has been examined by fluorescence recovery after photobleaching (FRAP) by three independent groups with differing results (Guil et al., 2006; Kedersha et al., 2000; Kedersha et al., 2005; Nadezhdina et al., 2009). While Guil and colleagues found a relative slow rate of exchange within SGs with approximately 9% of the bleached signal restored after 30 seconds, Kedersha and colleagues found a faster rate with approximately 40% of the signal restored after 30 seconds. Nadezhdina and colleagues reported a very similar exchange time to Kedersha and further showed that this was not affected by the disruption of microtubules with nocodazole (Nadezhdina et al., 2009). Both Guil and Kedersha found PABP1 at SGs to be exchanged more slowly than other SG proteins tested in parallel (hnRNPA1, TIA-1, G3BP), and all groups reported a slower exchange rate for PABP1 than that reported for mRNA (63% recovery within 30 seconds (Mollet et al., 2008)). This suggests that PABP1 may shuttle in and out of SGs independent of RNA, however as the movement of mRNA and PABP1 have not been examined together this cannot be stated with confidence.

Stress granules have been observed to transiently contact or 'dock' with processing bodies (P-bodies, PBs), another type of dynamic mRNP containing cytoplasmic granule (Kedersha et al., 2005). P-bodies contain mRNAs associated with the mRNA decay machinery (decapping enzymes, 5-3' exonucleases and their co-factors) and also share some protein components with stress granules e.g. eIF4E (reviewed in Anderson and Kedersha, 2006; Franks and Lykke-Andersen, 2008). P-bodies exist in the cytoplasm of unstressed cells though they increase in number and size when translation is repressed under stress conditions (Kedersha et al., 2005). mRNA decapping and decay occurs in the cytosol but it is thought that formation of

PBs may further facilitate decay by concentrating enzymes that might otherwise be rate limiting (Kulkarni et al., 2010).

The interaction between stress granules and P-bodies, and their dynamic nature, has led to a popular view that mRNPs may cycle between P-bodies, stress granules and polysomes, their protein composition being remodelled along the way (Anderson and Kedersha, 2009; Balagopal and Parker, 2009; Buchan and Parker, 2009). mRNAs have been shown to move between polysomes and P-bodies (Brenques et al., 2005) (Bhattacharyya et al., 2006), though there is disagreement whether mRNPs can move bidirectionally between P-bodies and stress granules (Buchan and Parker, 2009; Anderson and Kedersha, 2009).

The function of stress granules is not entirely clear. They are not required for global inhibition of translation (Buchan et al., 2008) (Mokas et al., 2009) and in yeast at least, do not affect the global stabilization of mRNAs observed under some stress conditions (Buchan et al., 2008). However, the popular hypothesis remains that sequestering mRNAs in SGs protects them from degradation and increased local concentrations of SG components facilitates mRNP remodelling (Anderson and Kedersha, 2009; Buchan and Parker, 2009).

Recently an additional role for SGs protecting against apoptosis has been described (Arimoto et al., 2008) (Tsai and Wei, 2010). Receptor for activated kinase 1 (RACK1) is a component of the 40S ribosome which regulates translation via recruitment of protein kinase C (PKC) to the ribosome (Nilsson et al., 2004). RACK1 is also an important protein partner of the apoptosis mediator MTK1 which enhances its function. Under moderate stress, RACK1 is incorporated into SGs and is unavailable to bind and enhance the function of MTK1, preventing apoptosis (Arimoto et al., 2008). Several other pro-apoptotic factors are recruited to SGs (Kolobova et al., 2009; Tsai and Wei, 2010) and rates of cell survival following stress are reduced when SG formation is impaired (Baguet et al., 2007; Eisinger-Mathason et al., 2008). These data suggest that sequestration of apoptosis regulatory factors into stress granules prevents cells from entering apoptosis when exposed to a moderate stress.

In this chapter, I investigate the localisation of PABP1 and PABP4 under stress conditions. Localisation of PABP4 under stress has not been studied, however, given that it is likely to have a role in translation initiation (Chapter 3), I would hypothesise that PABP4 is also a stress granule component. Here, I have directly tested this hypothesis and further explored the involvement of PABPs in stress granule assembly.

4.2 Results

4.2.1 PABP4 localisation to stress granules

Many stresses are reported to incite formation of stress granules including metabolic stress, osmotic shock, heat shock, UV irradiation, glucose starvation and hypoxia (Kedersha and Anderson, 2007). To test whether PABP4 localises to stress granules, sodium arsenite was used to stress HeLa cells. Sodium arsenite is one of the most commonly used stress granule stimuli and is reported to produce large readily detectable SGs in treated cells (Kedersha and Anderson, 2007). Arsenite toxicity is thought to be due to modification of cysteine residues of cellular proteins resulting in protein misfolding and oxidative stress and results in eIF2 phosphorylation by HRI (McEwen et al., 2005).

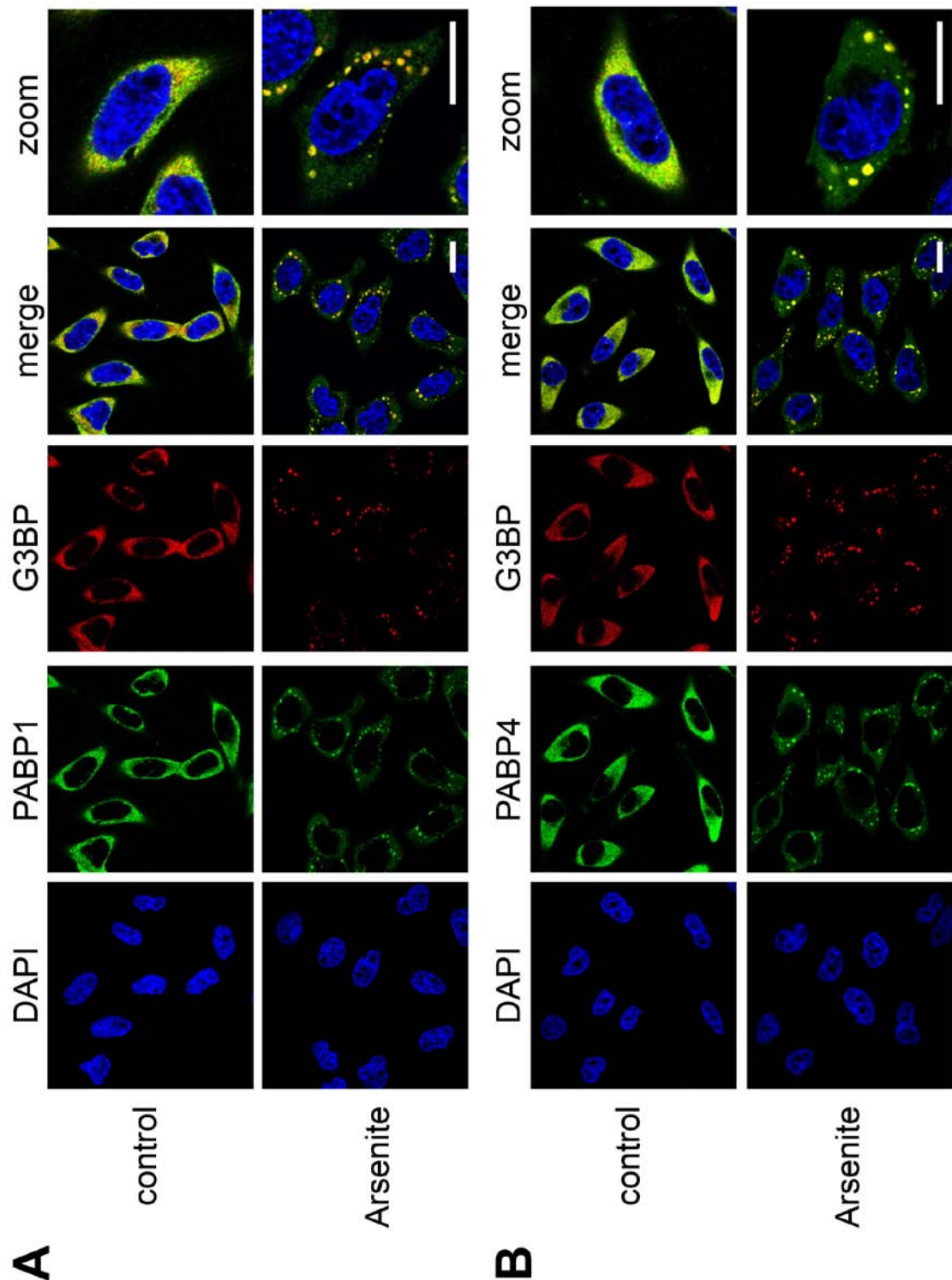


Figure 4.1 PABP4 localises to stress granules. Confocal images (x63) of HeLa cells stained by immunofluorescence for PABP1 (green), PABP4 (green) and G3BP (red) and counterstained with DAPI (blue). PABP1 (**A**) and PABP4 (**B**) localise to G3BP-containing stress granules after sodium arsenite treatment (1 hour 0.5mM). Single colour, merged images and magnification of merge images (zoom) are shown, scale bars represent 20µm.

Treatment of HeLa cells with arsenite effectively induced stress granules. Staining by indirect immunofluorescence for endogenous PABP1 and stress granule marker G3BP revealed large cytoplasmic SGs in all treated cells examined (Figure 4.1A). Immunostaining for PABP4 revealed that it also localises to cytoplasmic granules following arsenite treatment (Figure 4.1B). Localisation of PABP4 perfectly matched that of G3BP. As confocal microscopy provides images of optical sections this pattern implies that the two proteins are not simply superimposed but do indeed colocalise at stress granules. As G3BP localises to SGs and not P-bodies (Tourriere et al., 2001), this data implies that like PABP1, PABP4 is a stress granule component and does not localise to P-bodies.

While PABP1 is frequently described as a stress granule marker its incorporation into SGs under different stresses has also not been methodically tested. Some proteins are only found in SGs formed under certain stresses. For example, HSP27 is reportedly found in heat-shock induced SGs and not those induced by arsenite (Kedersha et al., 1999). To investigate whether this might be the case for PABP1 or PABP4 I tested other cell stresses known to result in SG formation – osmotic shock, heat shock and UV irradiation (Kedersha and Anderson, 2007)

Consistent with published reports, SGs formed following UV were much smaller than those induced by arsenite (Kedersha and Anderson, 2007) and were only observed in approximately 5% of cells (Figure 4.2 and 4.3). Heat shock effectively induced stress granules in most cells (Figure 4.2 and 4.3), which were observed to round up, a known effect of heat-shock induced cytoskeletal changes (Thomas et al., 1982). Following each stress condition PABP1 (Figure 4.2) and PABP4 (Figure 4.3) localised to G3BP-containing stress granules. Additionally, no SGs were observed that stained for G3BP and not PABP1/PABP4 or vice versa. These data suggest that PABP1 and PABP4 are not a selective constituent of SGs but are indiscriminating components of all SGs.

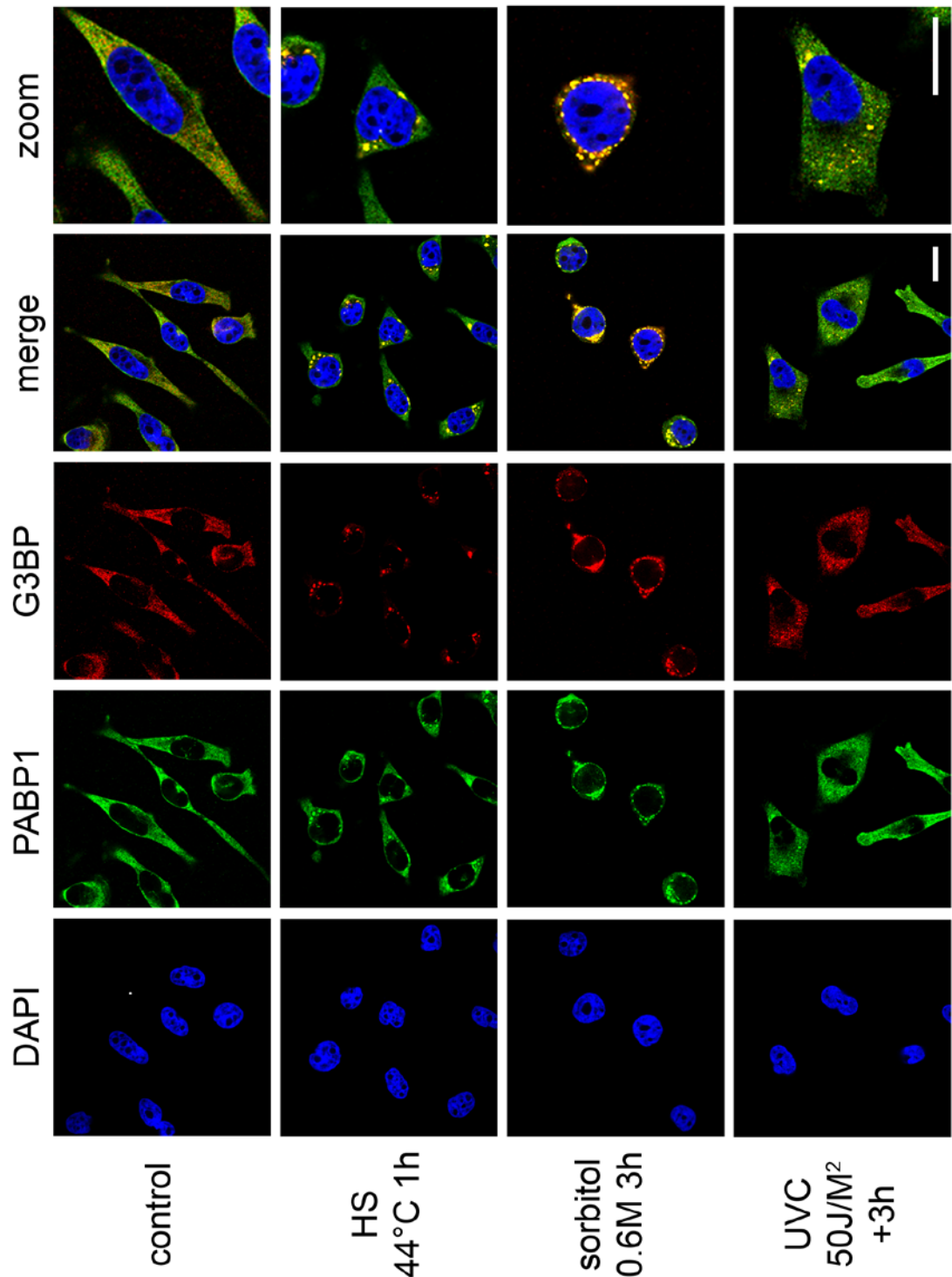


Figure 4.2 PABP1 localises to SGs after multiple stress types. Confocal images (x63) of HeLa cells stained by indirect immunofluorescence for PABP1 (green) and G3BP (red) and counterstained with DAPI (blue). HeLa cells were treated with heat shock (44°C), osmotic shock (3 hours 0.6M sorbitol), UV (50J/m² UVC with 3 hours subsequent incubation) or not treated (control). Single colour, merged images and greater magnification of merge images (zoom) are shown, scale bars represent 20µm.

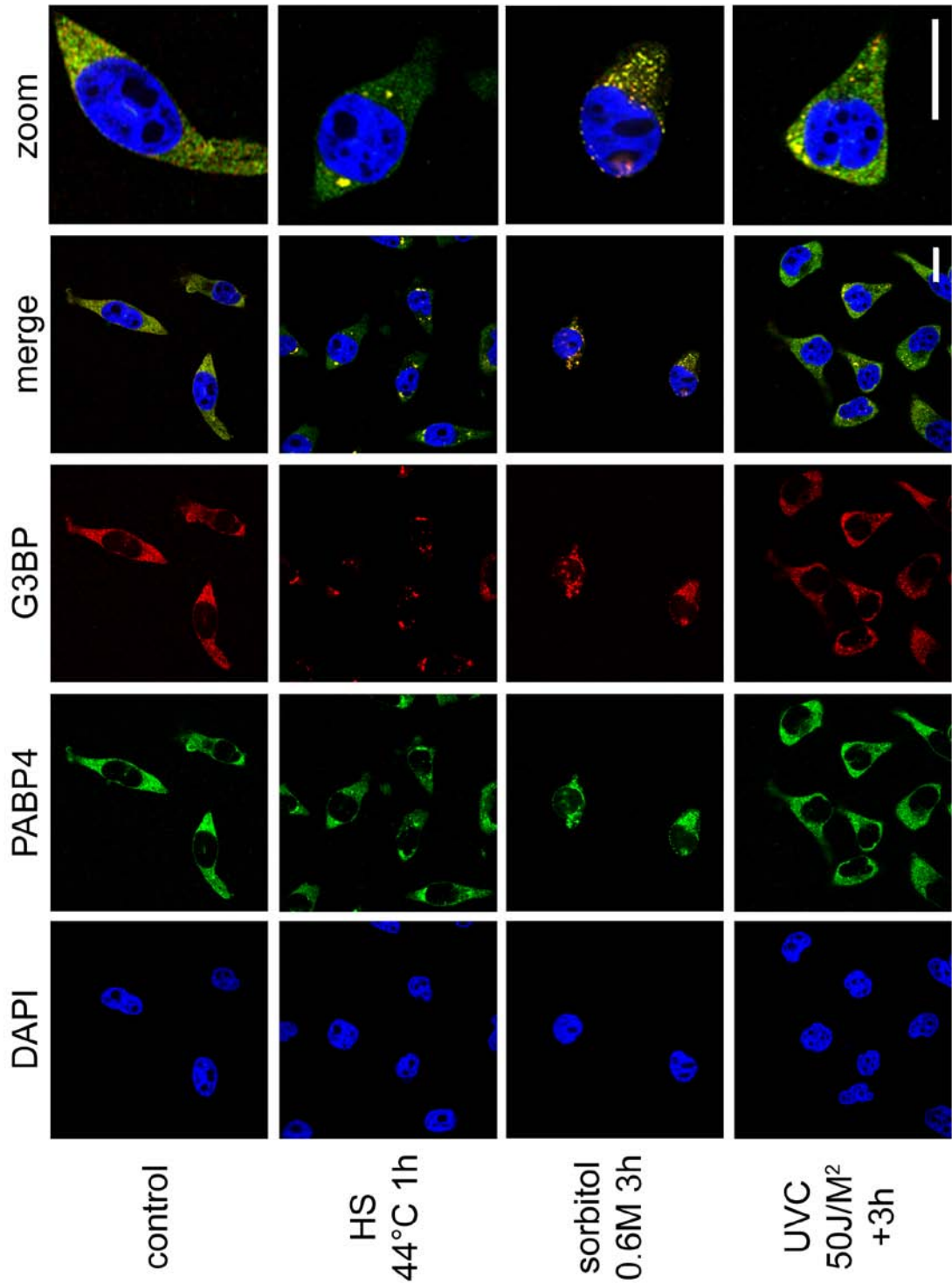


Figure 4.3 PABP4 localises to SGs after multiple stress types. Confocal images (x63) of HeLa cells stained by indirect immunofluorescence for PABP4 (green) and G3BP (red) and counterstained with DAPI (blue). HeLa cells were treated with heat shock (44°C), osmotic shock (3 hours 0.6M sorbitol), UV (50J/m² UVC with 3 hours recovery) or not treated (control). Single colour, merged images and greater magnification of merge images (zoom) are shown, scale bars represent 20µm.

4.2.2 Effect of PABP1 and PABP4 siRNA knockdown on stress granule formation

Recently, Mokas *et al* published work investigating the effect of siRNA knockdown of translation factors on stress granule assembly. They found that knockdown of certain factors involved in translation initiation, eIF2 α , eIF4B, eIF4H and PABP1, resulted in spontaneous formation of stress granules in HeLa cells (Mokas et al., 2009). By contrast, knockdown of factors involved in the 60S joining step of translation, large ribosomal subunit protein L28 and eIF5B, did not effect SG formation. To test whether knockdown of PABP4 induces stress granules, or whether knockdown of PABP1 and PABP4 together might further increase formation of stress granules, I optimized use of siRNAs directed against these proteins.

4.2.2.1 Optimisation of siRNA knockdown of PABP1 and PABP4

Four individual predesigned siRNAs (Qiagen) were used to direct knockdown of PABP1 and PABP4. Sequences for each siRNA and the position of their target sequence within the PABP1 and PABP4 mRNA are shown in Figures 4.5 (A, B) and 4.6 (A, B). Initial 24 hour transfection trials using siRNAs at 5nM produced no appreciable knockdown of PABP1 or PABP4 by western blot (Figure 4.4). This is perhaps not surprising since PABP1 is known to be a very stable protein, with no noticeable decrease in PABP1 immunoprecipitated from pulse-labelled HeLa cells detected over 8 hours (Gorlach et al., 1994).

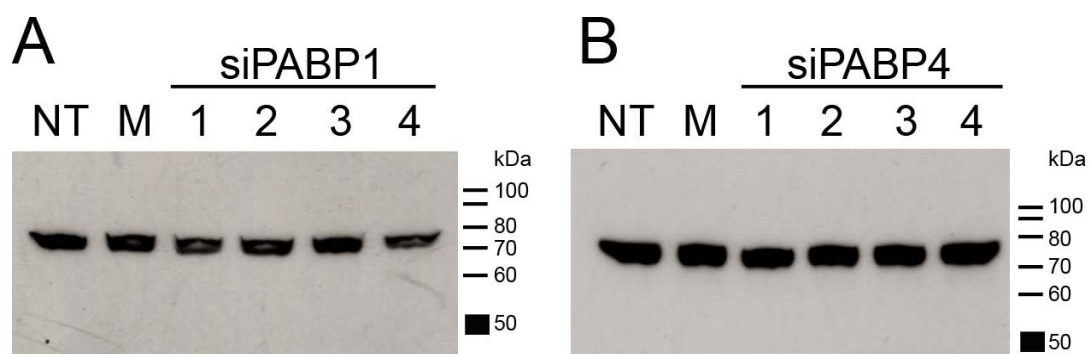


Figure 4.4 Optimization of PABP1 and PABP4 siRNA knockdown. HeLa cells were transfected with 5nM of the indicated siRNAs (Qiagen) directed against PABP1 and PABP4, lysed at 24 hours post-transfection and western blotted for PABP1 (A) and PABP4 (B). Control cells were not transfected (NT) or mock transfected (M).

Incubation time post-transfection was extended to 48 hours and each siRNA for PABP1 and PABP4 was transfected at 5, 10 and 20nM. Knockdown was assessed by western blotting and compared to a tubulin loading control (Figures 4.5C and 4.6C). Incubation for 48 hours post siRNA transfection produced a much more effective knockdown of PABP1 and PABP4. PABP1 siRNAs #3 and #4 failed to produce any appreciable protein knockdown, even at the highest concentration tested (20nM) (Figure 4.5C). PABP1 siRNA #1 effected PABP1 protein knockdown at 20nM but not at lower concentrations; however, PABP1 siRNA #2 produced an effective knockdown of PABP1 protein even at the lowest concentration used (5nM) (Figure 4.5C).

Each of the PABP4 siRNAs produced a detectable degree of knockdown of PABP4 protein (Figure 4.6C). PABP4 siRNAs #2 and #4 were the least effective and required a 20nM concentration to elicit a moderate knockdown. PABP4 siRNAs #1 and #3 were most effective, though siRNA #3 produced a slightly more effective knockdown at 5nM (Figure 4.6C).

As PABP1 siRNA #2 and PABP4 siRNA #3 produced the most potent effects at the lowest doses, they used hereafter and are subsequently referred to as siPABP1 and siPABP4 respectively.

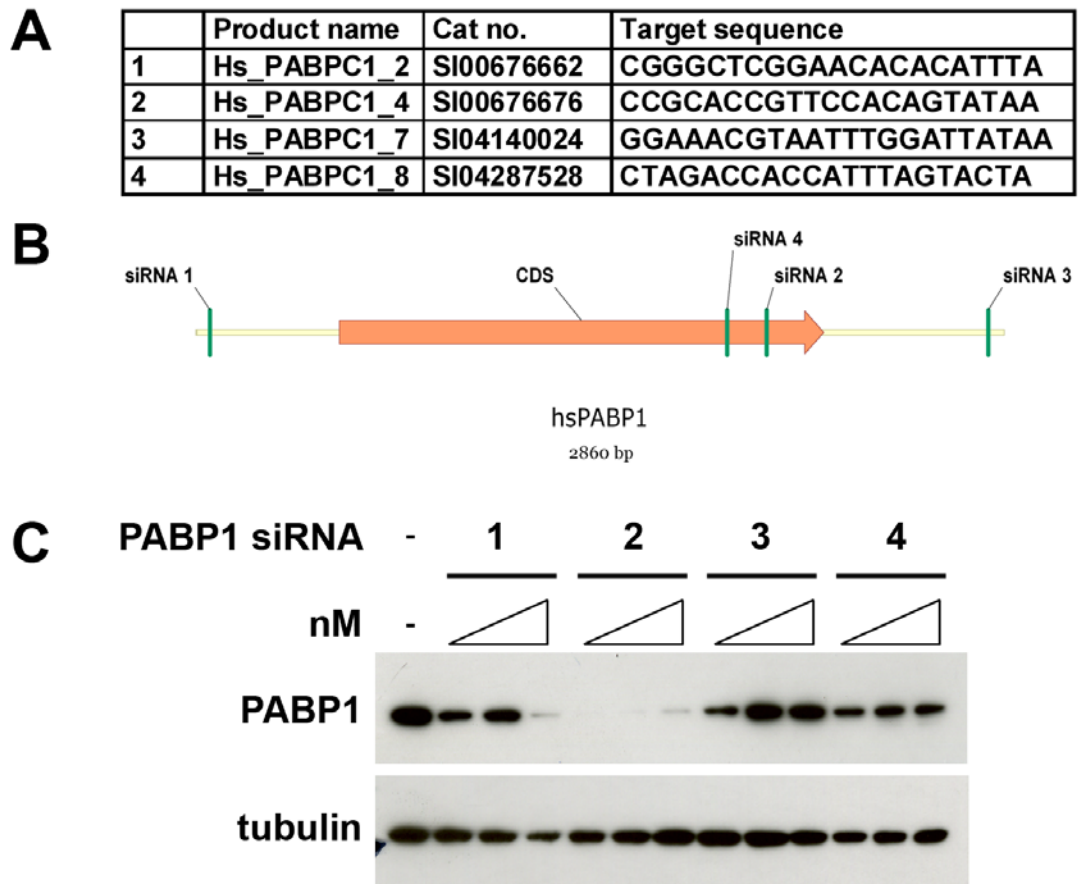


Figure 4.5 Optimization of PABP1 siRNA knockdown. **A.** Table showing sequences of human PABP1 siRNAs (Qiagen). **B.** Location of siRNA target sequences (green bars) within PABP1 mRNA. Coding sequence (CDS) is shown by orange arrow and untranslated regions by a yellow line. Image generated by Vector NTI (Invitrogen). **C.** Western blot for PABP1 and α -tubulin on lysates (10 μ g) of HeLa cells transfected for 48 hours with increasing concentrations (5, 10, 20nM) of the indicated PABP1 siRNAs. Control cells were mock transfected.

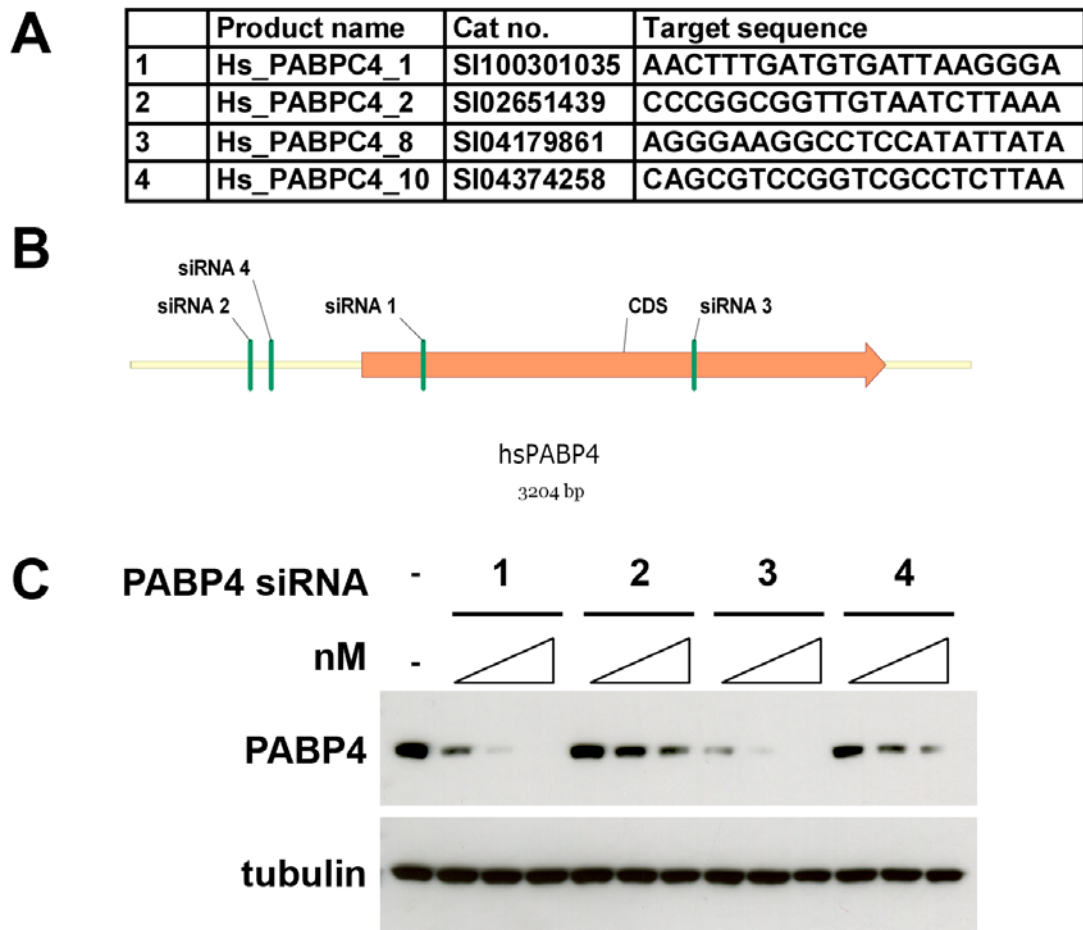


Figure 4.6 Optimization of PABP4 siRNA knockdown. **A.** Table showing sequences of human PABP4 siRNAs (Qiagen). **B.** Location of siRNA target sequences (green bars) within PABP4 mRNA. Coding sequence (CDS) is shown by orange arrow and untranslated regions by a yellow line. Image generated by Vector NTI (Invitrogen). **C.** Western blot for PABP4 and α -tubulin on lysates of HeLa cells transfected for 48 hours with increasing concentrations (5, 10, 20nM) of the indicated PABP4 siRNAs. Control cells were mock transfected.

4.2.2.2 Effect of siRNA knockdown of PABP1 and PABP4 on cell viability

Knockdown of another translation factor, eIF4E has been shown to affect cell survival (Yamagiwa et al., 2004). Therefore, before testing the effect of PABP knockdown under stress conditions, the effect of PABP1 and/or PABP4 knockdown on cell viability was tested. A trypan blue exclusion assay was used for this purpose which is based on the assumption that the trypan blue stain will enter only those cells which are non-viable and which do not have intact cell membranes.

Western blotting confirmed efficient PABP1/4 knockdown and showed that knockdown of PABP1 protein was specific and did not negatively effect the levels of PABP4 and vice versa (Figure 4.7A). Levels of the stress granule marker G3BP, used here as a loading control, were also not notably affected by knockdown of PABP1 and/or PABP4. No significant difference was observed in the viability of cells following knockdown with siPABP1 (97.15%), siPABP4 (95.00%) or siPABP1 and siPABP4 together (97.66%) compared to mock transfected control (96.03%) (Figure 4.7B). This implies that knockdown of PABP1 and/or PABP4 does not affect cell viability of normally growing HeLa cells.

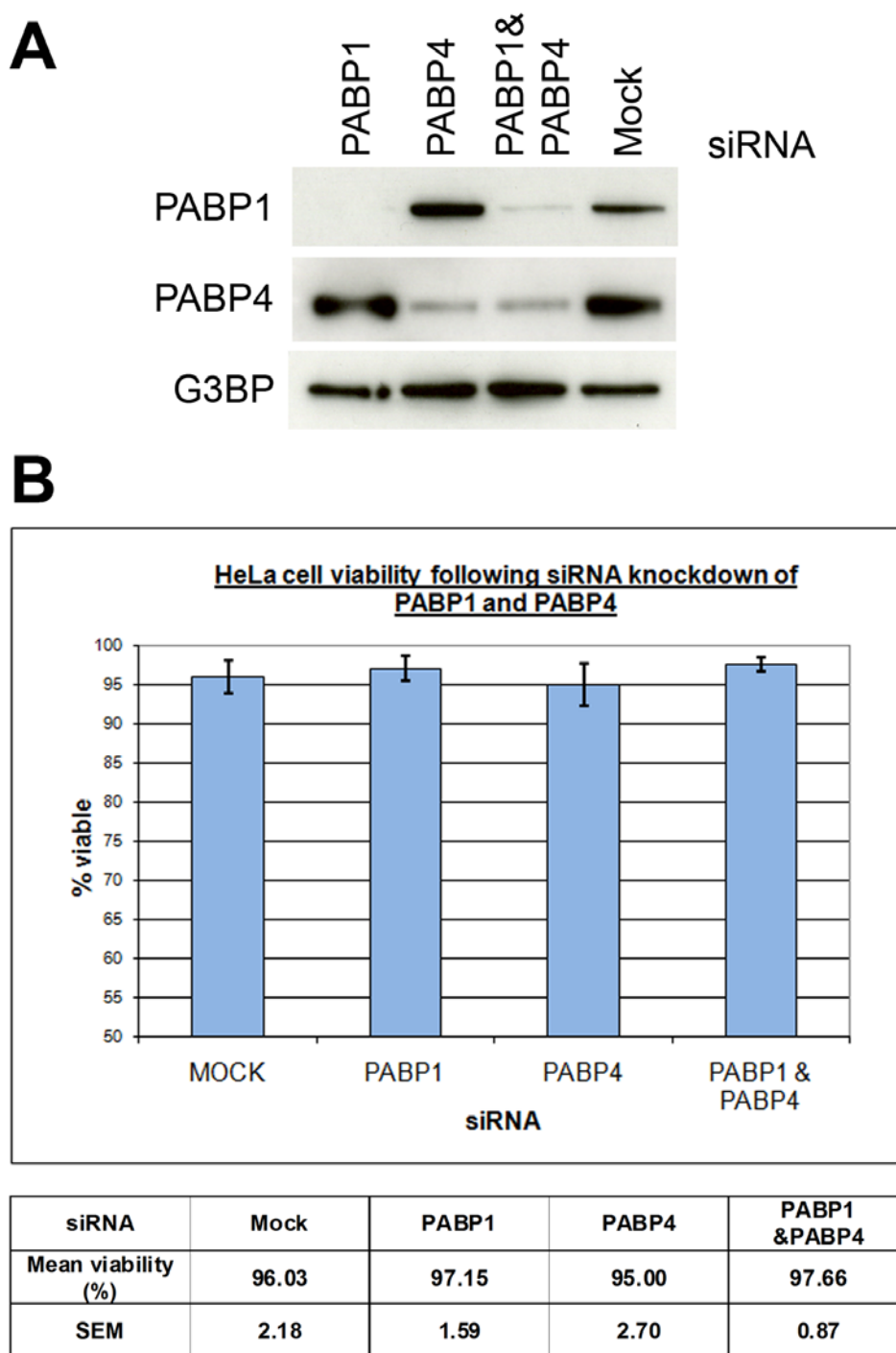


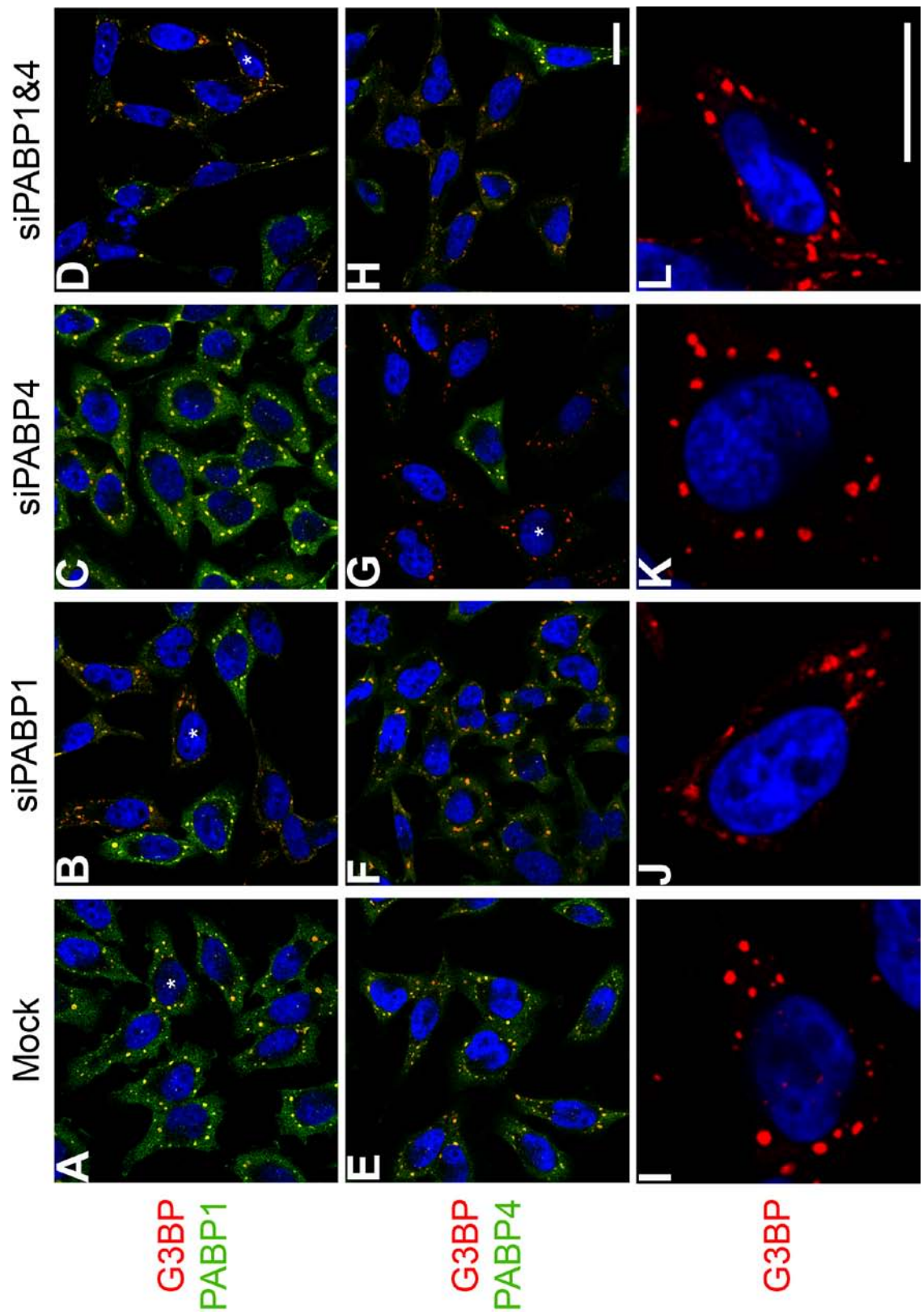
Figure 4.7 Effect of PABP1 and PABP4 siRNA knockdown on HeLa cell viability. HeLa cells were transfected with 5nM siPABP1, siPABP4, siPABP1 and siPABP4 or mock transfected and incubated for 48 hours. **A.** Efficiency of protein knockdown was monitored by western blotting for PABP1, PABP4 and G3BP as a loading control. **B.** Cell viability following siRNA knockdown was assayed by trypan blue exclusion. Results shown are the mean \pm SEM (standard error of the mean) of 3 independent experiments wherein more than 300 cells were examined per point.

4.2.2.3 Does siRNA knockdown of PABP1 and PABP4 impair stress granule formation?

It is not known whether PABPs play a role in stress granule formation. PABP1 is known to self associate and I have shown that PABP1 and PABP4 can interact (Figure 3.4B). It is therefore conceivable that interaction between PABPs bound to different mRNAs may contribute to nucleation of stress granules.

To test whether knockdown of PABP1 and PABP4 affects the ability of cells to form stress granules, siRNA-treated cells were further treated with arsenite to induce stress granules. Cells were then stained for G3BP and PABP1 or PABP4 and analysed by confocal microscopy (Figure 4.8). Stress granules were clearly visible in cells treated with each siRNA with no obvious difference in the number or size of granules formed. Staining for PABP1 and PABP4 permitted clear identification of siRNA transfected cells in which individual PABP proteins were successfully knocked down. Double knockdown cells were stained for either PABP1 or PABP4 and cells with diminished levels of either PABP were assumed to have been successfully transfected with both siRNAs. This experiment revealed that neither PABP1 nor PABP4 is required for stress granule assembly.

These results are consistent with those of Ohn and colleagues who did not identify PABP1 or PABP4 in their RNAi based screen to identify proteins required for SG formation, though it was not reported whether these genes were tested (Ohn et al., 2008). These data further show that simultaneous knockdown of both PABP1 and PABP4 also does not impair SG assembly.



4.2.2.4 Does siRNA knockdown of PABP1 and PABP4 cause spontaneous formation of stress granules?

To test whether siRNA knockdown of PABP4 is sufficient to induce spontaneous stress granules, as has been reported for eIF2 α , eIF4B, eIF4H and PABP1 (Mokas et al., 2009), HeLa cells were treated with siPABP1, siPABP4, or siPABP1 and siPABP4 and then stained by immunofluorescence for G3BP (Figure 4.9). In mock and siRNA treated cells G3BP was diffusely distributed throughout the cytoplasm. More than 1000 cells were examined per treatment and no stress granules were identified. Staining of siRNA treated cells for a different SG marker, poly(A)+ RNA also failed to identify any spontaneously formed SGs in knockdown cells (Chapter 6, Figure 6.14).

The absence of SGs after PABP knockdown in untreated cells conflicts with the results of Mokas *et al* who observed SGs in 6-8% of PABP1-knockdown cells, compared to less than 0.1% of cells treated with a negative control siRNA. The authors reported PABP1 protein knockdown in HeLa cells (to approximately 30% of control) resulted in a reduction in protein synthesis of approximately 70%, as measured by metabolic labelling and spontaneous SG formation was attributed to this perceived inhibition of translation (Mokas et al., 2009).

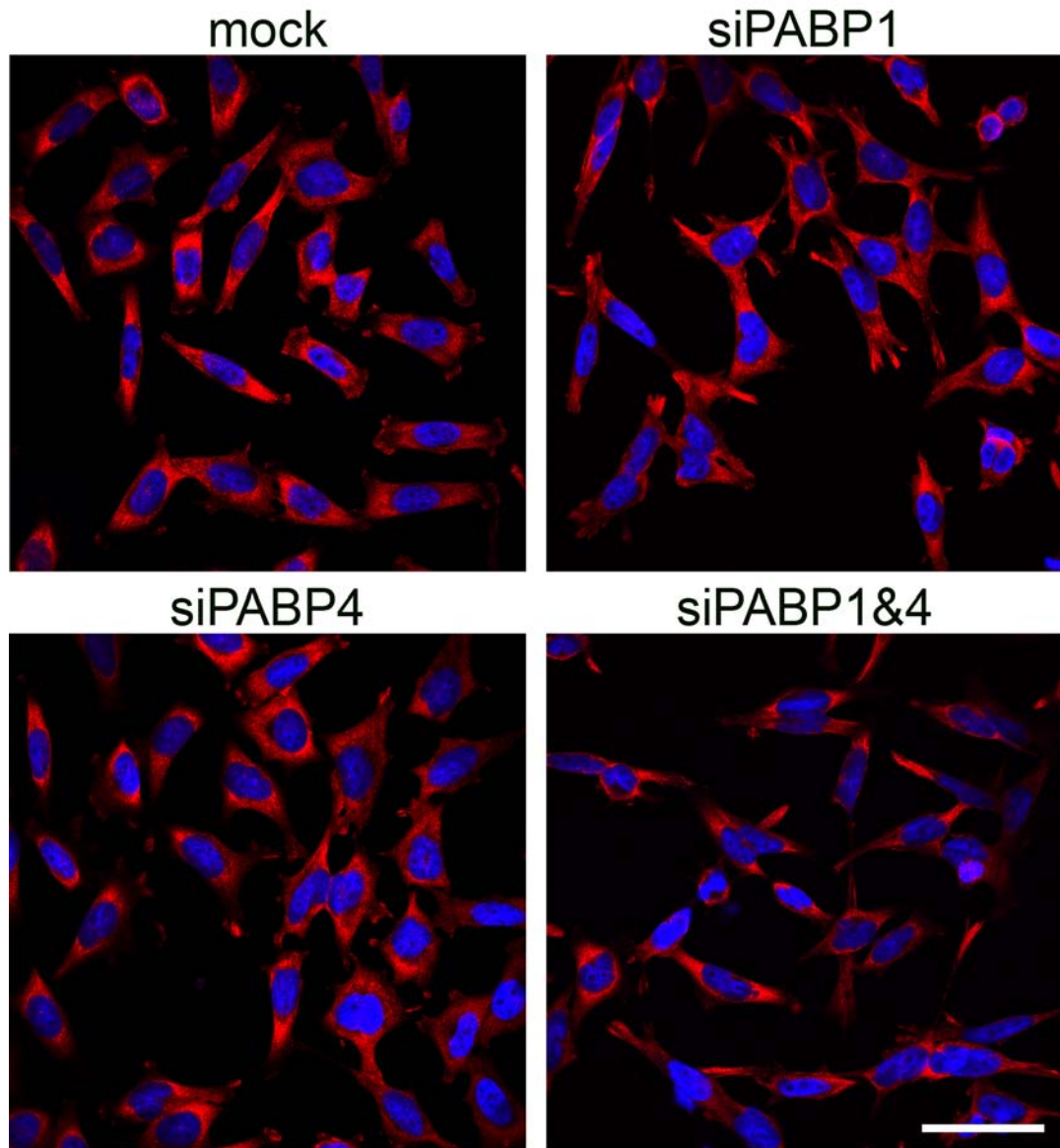


Figure 4.9 Effect of PABP1 and PABP4 siRNA knockdown on spontaneous stress granule formation. HeLa cells were transfected with 5nM siPABP1, siPABP4, siPABP1 and siPABP4 or mock transfected as indicated, incubated for 48 hours and stained by immunofluorescence for G3BP (red) and counterstained with DAPI (blue). Merged confocal images (x40) are shown. Scale bar represents 50 μ m.

4.2.2.5 Effect of siRNA knockdown of PABP1 and PABP4 on protein synthesis

To test whether siRNA knockdown of PABP proteins here resulted in a similar reduction of protein synthesis rate, siRNA treated cells were labelled with ^{35}S -methionine and incorporation into newly synthesized proteins measured by liquid scintillation of TCA precipitated protein (Figure 4.10).

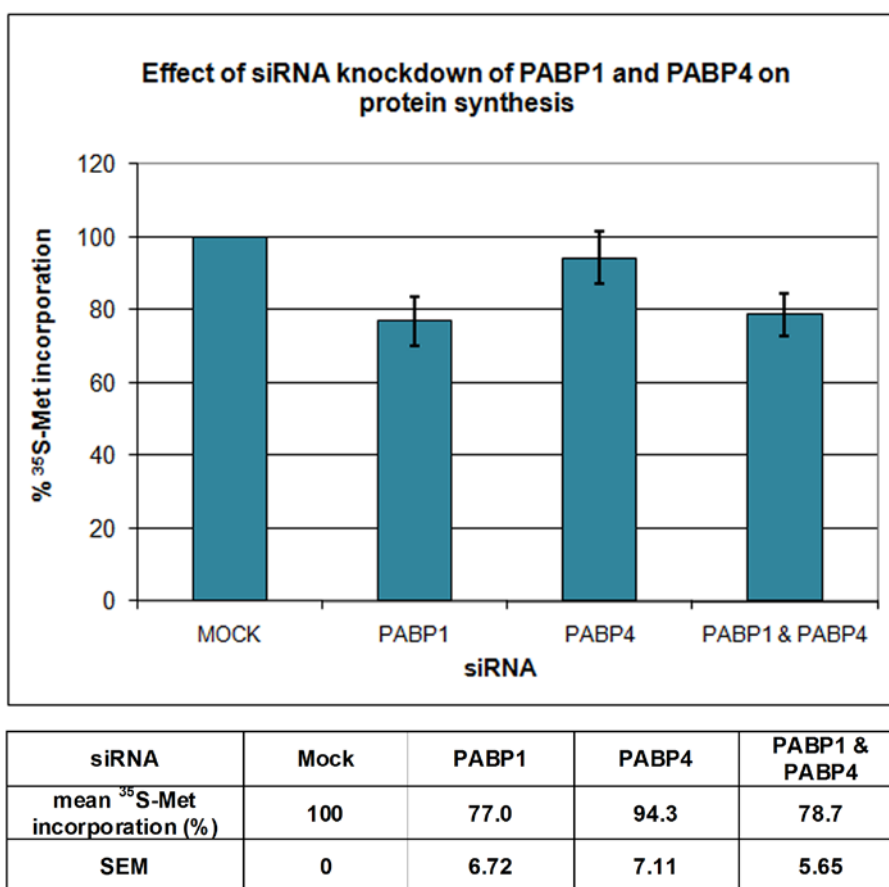


Figure 4.10 Effect of PABP1 and PABP4 siRNA knockdown on protein synthesis. HeLa cells were transfected with siPABP1, siPABP4, siPABP1 and siPABP4 or mock transfected for 48 hours. Protein synthesis rate was measured as ^{35}S -methionine incorporation into protein by liquid scintillation following labelling for 15 minutes. Each experiment was performed in duplicate and means normalised to mock treated cells as a percentage. The mean of 3 independent experiments are shown \pm SEM.

Knockdown of PABP1 alone produced a modest reduction in mean protein synthesis rate (23%). The effect of treatment with siPABP1 compared to mock treatment by paired T-test had a low p value (0.076) but was not quite significant (i.e. $p = <0.05$). Knockdown of PABP4 alone produced only a slight reduction in protein synthesis (6%). The effect of treatment with siPABP4 compared to mock treatment by paired T-test had a p value of 0.509 which was not significant. Knockdown of PABP1 and PABP4 resulted in a reduction in mean protein synthesis rate similar to knockdown of PABP1 alone (21%). Compared to mock treatment by paired T-test, the effect of siPABP1+4 had a low p value (0.063) but was again not quite significant. PABP4 knockdown therefore did not increase the effect of PABP1 knockdown on protein synthesis when both siRNAs were co-transfected.

These results are markedly different from those of Mokaš *et al* and may explain the difference in the frequency of SG formation seen in siRNA treated cells. Though translational inhibition is not sufficient for SG formation, it is thought to be necessary (Anderson and Kedersha, 2009 ; Buchan and Parker, 2009).

4.3 Discussion

In this chapter I have shown that PABP4 is a stress granule component (Figure 4.1) and explored the role of PABPs 1 and 4 in stress granule formation. Localisation of PABP1 or PABP4 to stress granules was not restricted to those formed under a specific stress type and PABP1 and PABP4 -positive SGs were observed under all stress conditions tested (Figure 4.3).

The role of PABPs at stress granules is unknown. Knockdown of PABP1 and/or PABP4 did not compromise the ability of cells to form stress granules (Figure 4.8). The self-aggregating proteins TIA-1 and G3BP are thought to act as scaffolds for SG assembly and are essential for SG formation (Kedersha et al., 1999; Tourriere et al., 2003). As SGs can form in the absence of PABP1 and/or PABP4 this suggests that PABPs do not play a scaffolding role essential for SG formation. PABPs may therefore be passively incorporated into SGs bound to mRNAs. This is consistent with recently published work in yeast (Swisher and Parker, 2010). Stress granules were able to form in a *pab1*Δ *sbp2*Δ strain following glucose deprivation, though, fewer *pab1*Δ *sbp2*Δ cells formed SGs than the *sbp2*Δ strain (7.3% compared to 20.6%) (Swisher and Parker, 2010). These results indicate that *pab1* is not required for SG formation in budding yeast but does promote SG formation.

Cells transfected with PABP1 and/or PABP4 directed siRNAs did not form stress granules in the absence of external stress stimuli (Figure 4.9), consistent with observations in yeast (Swisher and Parker, 2010). However, my results differ from published data in mammalian cells (Mokas et al., 2009) which reported that siRNA knockdown of PABP1, eIF2 α , eIF4A, eIF4B or eIF4H induces formation of SGs in a small percentage of HeLa cells (Mazroui et al., 2006) (Mokas et al., 2009). In each case inhibition of translation was thought to be causative, though whether the few cells exhibiting SGs were those in which the siRNA targeted proteins were most reduced was not examined.

This difference in the observed effect on spontaneous SG formation may be explained by a differing efficiency of PABP1 knockdown. Efficiency of knockdown here could not be determined and compared to that of Mokas et al (70%) as a method

to quantify western blot detection of proteins was unavailable. Analysis of the effect of siRNAs on PABP RNA by quantitative RT-PCR, though feasible, is unlikely to be informative of protein levels as PABP1 mRNA is known to be translationally regulated (Wu and Bag, 1998) and PABP1 protein is very stable (Gorlach et al., 1994). The siRNA transfection conditions used by Mokas and colleagues (PABP1 siRNA #1 at 20nM + 48 hours incubation – R. Mazroui, personal communication) and those used here (PABP1 siRNA #2 at 5nM + 48 hours incubation) (Figure 4.5) can however be directly compared in Figure 4.5. Greater reduction in PABP1 signal was achieved using siRNA #2 at 5nM than siRNA #1 at 20nM implying that the efficiency of PABP1 protein knockdown achieved here is likely to be as good if not better than that of Mokas et al – though this interpretation assumes that responsiveness to these siRNAs is equivalent between HeLa strains.

Another explanation for the discordance of my results and those of Mokas et al could be the apparent difference in the translation inhibition resulting from siRNA knockdown of PABP1. Indeed, differences in the effect of PABP1 siRNA knockdown on translation exist in the published literature even when considering only studies carried out on HeLa cells (e.g. 15% by (Yoshida et al., 2006), 50% by (Blakqori et al., 2009)) implying there is variation in this response even between strains. This could be due to variation between the cells used in the amount of PABP-regulatory factors present (i.e. PAIP2 or YB-1) or co-factors for PABP-mediated translational activation (e.g. eIF4G, PAIP1). Mokas and colleagues observed a 70% reduction in protein synthesis rate following PABP1 knockdown (Mokas et al., 2009) while I found only a 22% reduction compared to control cells (Figure 4.10). In Mokas' study, the minimal reduction in protein synthesis which induced spontaneous SG formation was 35% caused by siRNA knockdown of eIF4B - significantly greater than the reduction in protein synthesis I obtained by PABP1 siRNA knockdown. This implies that while translation inhibition alone is not sufficient for SG formation, in situations when SGs are formed there may be a threshold below which the translation rate must fall to precipitate SG assembly.

A modest reduction in protein synthesis following PABP1 siRNA knockdown is not particularly unexpected since with decreased PABP1 protein levels autoregulation ensures increased translational efficiency of PABP1 mRNA and

feedback systems involving YB-1 and PAIP2 facilitate an increase in PABP1 activity (see Chapter 1.4.5). No significant reduction in protein synthesis was detected following siRNA knockdown of PABP4 (Figure 4.10). This could be taken to imply that PABP4 is not required for efficient global translation in HeLa cells. This would be surprising since morpholino knockdown of PABP4 in developing *Xenopus* embryos has been shown to result in a significant effect on translation evidenced by diminished polysome formation (Gorgoni et al., Submitted). However, in PABP4 knockdown HeLa cells, PABP1 protein appeared to be more abundant than mock treated cells (Figure 4.7) suggesting that expression of PABP1 is upregulated in response to decreased PABP4 levels. Additionally, if PABP4 interacts with PAIP2 or regulates YB-1 translation analogously to PABP1, loss of PABP4 could lead to an increase in PABP1 activity.

I have shown that the expression of PABP4 varies in different mammalian tissues and cell types (Chapter 3) which may reflect a differing specific requirement for PABP4 mediated translation in different cell types. It would be interesting to test whether translation in primary cell culture lines derived from other cell types might be more sensitive to PABP4 depletion. For example, since PABP4 appears to be expressed relatively highly compared to PABP1 in smooth and skeletal muscle (Figure 3.6), global translation in a myocyte cell line may be more PABP4-dependent than in HeLa cells. It is also possible however, that PABP4 is important for the translation of only a specific subset of messages which analysis of global protein synthesis is unlikely to reveal. Indeed, the only published analysis of mammalian PABP4-mediated translational activation showed a potent effect of PABP4 on a specific message (*IL-2*) even without depletion of endogenous PABPs from the cell-free extract used (Okochi et al., 2005). Translational profiling would be a useful tool to identify any mRNAs whose translation is specifically affected by PABP4 knockdown and this technique has been effectively used in the past to identify mRNAs sensitive to eIF4G depletion (Ramirez-Valle et al., 2008).

There is considerable overlap between the constituents of SGs with other types of RNA granules (Anderson and Kedersha, 2006). Neuronal granules are heterogenous and consist of mRNAs packaged with RNA binding proteins, translation factors and ribosomes for transport to dendrites (reviewed in (Kiebler and

Bassell, 2006). Many SG components are also found in neuronal granules (Anderson and Kedersha, 2006) including PABP1 (Shiina et al., 2005). As PABP4 is a SG component (Figure 4.1) and widely expressed in neurons (Chapter 3) this raises the possibility that PABP4 may also be a component of neuronal granules.

tPABP was recently found to be enriched in cytoplasmic foci within the round spermatids of mice that resembled a chromatoid body (Kimura et al., 2009). Chromatoid bodies contain many P-body proteins such as GW182, Ago2, DCP1 (Kotaja et al., 2006) and though their function is as yet uncharacterised they are speculated to function similarly to P-bodies in mRNA degradation and translational repression (Chuma et al., 2009). PABP1 was shown by Kimura et al (2009) not to localise to chromatoid bodies and similarly no cytoplasmic foci were observed in my analyses of PABP1 localisation in testes (Figure 3.15). This suggests that though PABP4, like PABP1 is a SG protein, all PABP family proteins may not necessarily behave identically in their localisation to RNA granules.

The physiological relevance of stress granule formation is not fully understood. SGs have however been shown to form in ischemia-affected pyramidal cells in the rat hippocampus, a model for stroke (Kayali et al., 2005), implying that SG formation is a *bone fide* response *in vivo* and not a cell culture artefact. As PABP1 and PABP4 were found to be localised to SGs formed under every SG stimulus tested (Figure 4.2 and 4.3) they are likely to be constituents of SGs induced in tissues in which they are expressed – including hippocampal neurons following stroke.

Chapter 5: Localisation changes of poly(A)- binding proteins after UV irradiation

5.1 Introduction

Although PABP1 is predominantly cytoplasmic, it is a nuclear-cytoplasmic shuttling protein and has been reported to relocalise to the nucleus following treatment with transcriptional inhibitors or upon over-expression (Afonina et al., 1998). The function of PABP1 nuclear localisation following transcriptional inhibition is unknown but during viral infection relocalisation of PABP1 is speculated to restrict it from other components of the translational machinery and contribute to host translation shut off (Smith and Gray, 2010). PABP1 has been reported to relocalise to the nucleus following infection with several viruses – rotavirus (Montero et al., 2006), bunyamwera virus (Blakqori et al., 2009), Kaposi Sarcoma-associated herpes virus (Kanno et al., 2006) (Harb et al., 2008) and herpes simplex virus type 1 (HSV-1) (Dobrikova et al., 2010) (Salaun et al., Submitted). During investigation of PABP1 relocalisation in HSV-1 infection it was noted that certain cell stress conditions including UVB irradiation may also relocalise PABP1 to the nucleus (Salaun et al., Submitted; C. Salaun, unpublished).

UVB (280-315nm) and UVC (200-280nm) cause direct DNA damage resulting in two major types of mutagenic lesions, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (Tornaletti and Pfeifer, 1996). Complex genomic surveillance and DNA damage checkpoints ensure cell cycle arrest until lesions are repaired. CPDs and (6-4) photoproducts are repaired by the two types of nuclear excision repair (NER): global genomic repair (GGR) and transcription coupled repair (TCR). GGR involves repair of DNA lesions across the genome. In TCR the NER machinery is recruited to sites where RNA polymerases are stalled at DNA lesions (Fousteri and Mullenders, 2008; Shuck et al., 2008).

During DNA damage repair (DDR) there are major changes in gene expression. Transcription is generally inhibited and RNA Polymerase II (RNAPII) is ubiquitinated and degraded by the proteasome (Bregman et al., 1996; Lee et al., 2002; Ratner et al., 1998) and polyadenylation and mRNA 3' processing are also generally inhibited (Mirkin et al., 2008). However, the transcription of many genes important for DDR and stress recovery are selectively increased (Tyrrell, 1996) (Sesto et al., 2002) and UV irradiation also results in regulated changes in alternative

splicing (Munoz et al., 2009) and miRNA expression (Guo et al., 2009; Pothof et al., 2009).

UV irradiation also inhibits global translation via phosphorylation of eIF2 α by GCN2 (Deng et al., 2002) (Jiang and Wek, 2005) and PERK (Jiang and Wek, 2005; Wu et al., 2002). The importance of translational regulation in the cellular response to UV has been demonstrated by the massive increase in apoptosis in GCN2 $^{-/-}$ (80%) compared to wildtype cells (<10%) (Jiang and Wek, 2005).

Recent work has shed light on the upstream signalling pathways of the eIF2 α kinases GCN2 and PERK after UV irradiation. Activation of nitric oxide-synthase is proposed to induce GCN2 and PERK activation by causing L-arginine starvation and oxidative stress respectively (Lu et al., 2009; Wang et al., 2010) while the DNA damage checkpoint kinase DNA-PKcs (DNA dependent protein kinase, catalytic subunit) has also been identified as an upstream regulator of GCN2 after UV exposure (Powley et al., 2009).

In stressed cells, global depression of translation permits translational upregulation of specific transcripts encoding proteins important for stress recovery (Blais et al., 2004; Johannes et al., 1999; Thomas and Johannes, 2007). Several transcripts upregulated in their translation after UV have recently been identified by translational profiling (Powley et al., 2009). These included mRNAs encoding DNA repair enzymes as well as enzymes involved in metabolism and cell signalling proteins. Many of these messages were found to contain uORFs in their 5' UTR a feature shown to effect their escape from global inhibition of translation (Powley et al., 2009).

As translational control is an important means to control gene expression and ensure cell survival after UV, changes in PABP localisation may influence this process. In this chapter I have characterised a relocalisation of PABP1 and PABP4 late after UVC irradiation and investigated potential functions of this relocalisation.

5.2 Results

5.2.1 Nuclear relocalisation of PABP1 and PABP4 after UV

To test whether the UVB-induced relocalisation of PABP1 observed in preliminary experiments in our collaborator's (Dr SV Graham) lab is also induced by UVC, HeLa cells were treated with 50J/m² UVC (254nm) and incubated for 15 hours. Localisation of PABP1 and PABP4 was then examined by indirect immunofluorescence and confocal microscopy (Figure 5.1).

In untreated cells PABP1 was detected as wholly cytoplasmic. After UVC treatment of HeLa cells PABP1 was detected strongly in the nucleus with a weaker cytoplasmic signal also detected (Figure 5.1). PABP4 also appeared in the nucleus after UVC treatment but showed stronger staining intensity in the cytoplasm after UV compared to PABP1 (Figure 5.1). Relocalisation to the nucleus of PABP1 and PABP4 was observed in more than 90% of cells.

To test if this response is conserved in a different species and cell type, the same UV treatment was applied to 3T3 cells, a mouse fibroblast cell line. A change in localisation of both PABP1 and PABP4 to the nucleus from the cytoplasm was observed (Figure 5.1). As in HeLa cells, a more complete nuclear relocalisation of PABP1 was observed after UV in 3T3 cells compared to PABP4.

The IF signal detected by PABP1 and PABP4 antibodies in the nucleus after UV could feasibly result from cross-reaction of these antibodies with a protein which is expressed only after UV irradiation. Alternatively, UV may induce cleavage of PABP1 and PABP4 yielding C-terminal fragments detected by our antibodies which are nuclear localised. Indeed, while the translation factor eIF4G is cytoplasmic, the short N-terminal product of caspase-3 cleavage of eIF4G, N-FAG localises to the nucleus (Coldwell et al., 2004).

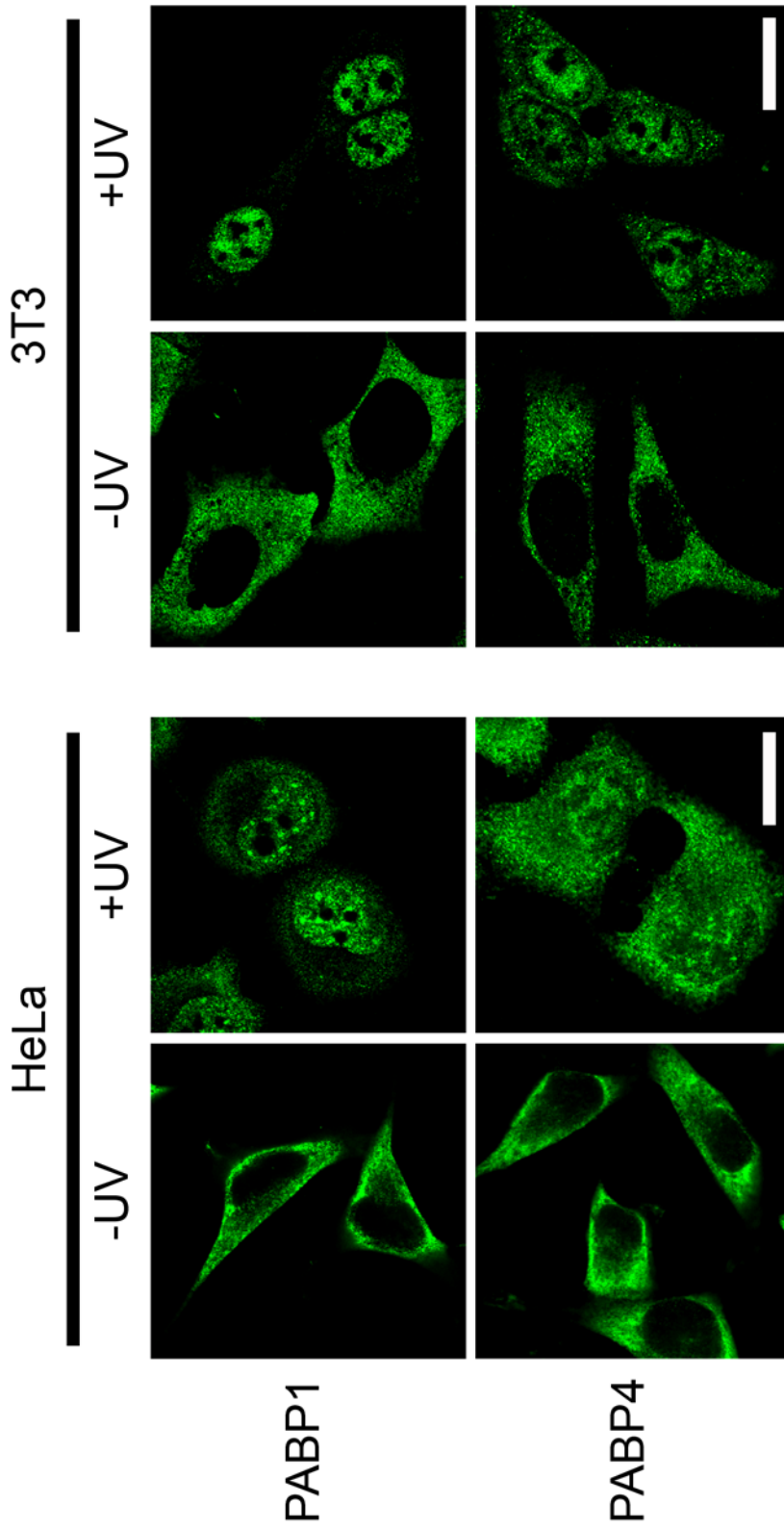


Figure 5.1 PABP1 and PABP4 relocate to the nucleus after UVC irradiation. HeLa and 3T3 cells were treated with 50J/m^2 UVC or mock treated and allowed to recover for 15 hours. They were then stained by immunofluorescence for PABP1 or PABP4 as indicated. Confocal images ($\times 63$) are shown; scale bar represents $20\mu\text{m}$.

To exclude these possibilities, UVC irradiated and mock irradiated cells were subject to western blotting for PABP1 and PABP4 (Figure 5.2). No additional bands were detected by anti-PABP1 or anti-PABP4 after UV treatment (Figure 5.2). This implies that the nuclear signal observed in UV-treated cells by immunofluorescence is not due to non-specific detection of another protein, or detection of PABP cleavage products. Furthermore, total levels of PABP1 and PABP4 were not observed to increase, excluding an increase in PABP levels as an explanation for nuclear accumulation of PABP1 and PABP4 after UV.

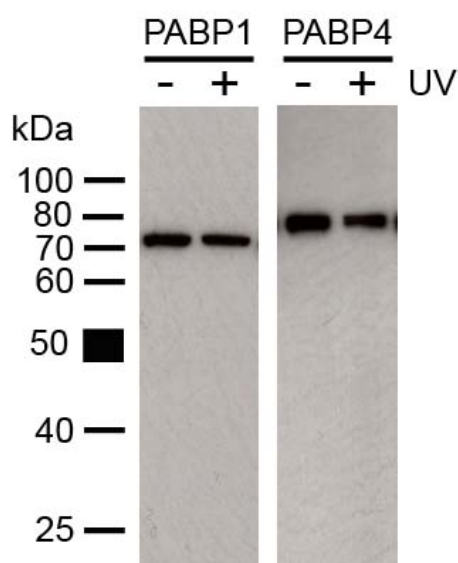


Figure 5.2 PABP1 and PABP4 antibodies do not detect non-specific proteins after UVC treatment. HeLa cells were treated with 50J/m² UVC or mock treated as indicated and incubated for 24 hours before lysis and western blotting for PABP1 or PABP4 as indicated.

Therefore, PABP1 and PABP4 relocalise to the nucleus in different species and cell types following UVC treatment. Conservation of this cellular response between different species and cell types suggests that it may be important for cell recovery from UV stress.

5.2.2 Characterisation of the timing of nuclear relocalisation of PABP1 and PABP4 after UV

Staining for PABP1 and PABP4 revealed robust relocalisation of a portion of both proteins from the cytoplasm to the nucleus in HeLa cells at 15 hours after 50J/m² UV-C treatment (Figure 5.1). However, neither PABP1 nor PABP4 were detected in the nucleus at 3 hours after treatment, when stress granules were observed in a small percentage of cells (Chapter 4; Figures 4.2 and 4.3). To determine when the change in PABP localisation occurs after UV treatment, HeLa cells were treated with 50J/m² UVC and fixed at 3 hour time points between 0 and 15 hours before staining for PABP1 (Figure 5.3) and PABP4 (Figure 5.4).

Stress granules were observed in some cells at early time points after UV treatment being most apparent at 3 and 6 hours post-treatment in approximately 5% of cells. This observation is consistent with the recent results of Pothof et al who found SGs induced in HeLa cells most frequently at 4 hours after 20J/m² UVC treatment (Pothof et al., 2009).

Staining for PABP1 was visible within nuclei at low levels from 9 hours post-treatment with staining intensity increasing at 12 and 15 hours post-treatment consistent with a steady accumulation of PABP1 in the nucleus over time. Staining for PABP4 was barely visible in nuclei at 9 hours but was clearly detectable at 12 hours post-treatment and was more intense at 15 hours post-treatment. PABP1 exhibited a more complete nuclear relocalisation than PABP4 at 9, 12 and 15 hour time points (Figures 5.3 and 5.4), consistent with previous observations in HeLa and 3T3 cells (Figure 5.1)

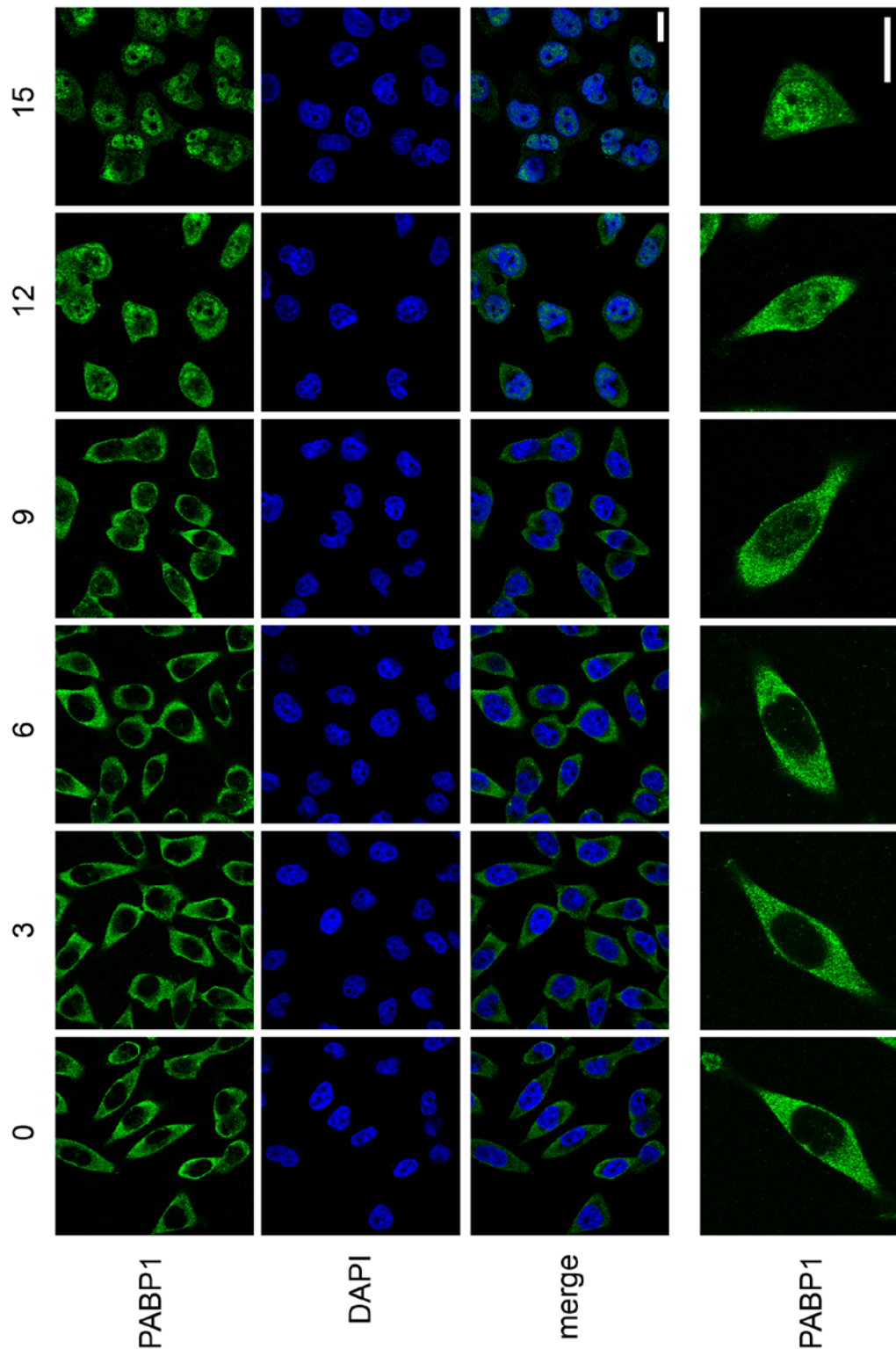


Figure 5.3 PABP1 relocalises to the nucleus from 9h after treatment with 50J/m^2 UVC. HeLa cells were treated with 50J/m^2 UVC and then incubated for the indicated number of hours. They were then stained by immunofluorescence for PABP1. Single colour and merged x63 confocal microscopy images are shown with additional higher magnification images of single cells stained for PABP1; scale bars represent $20\mu\text{m}$.

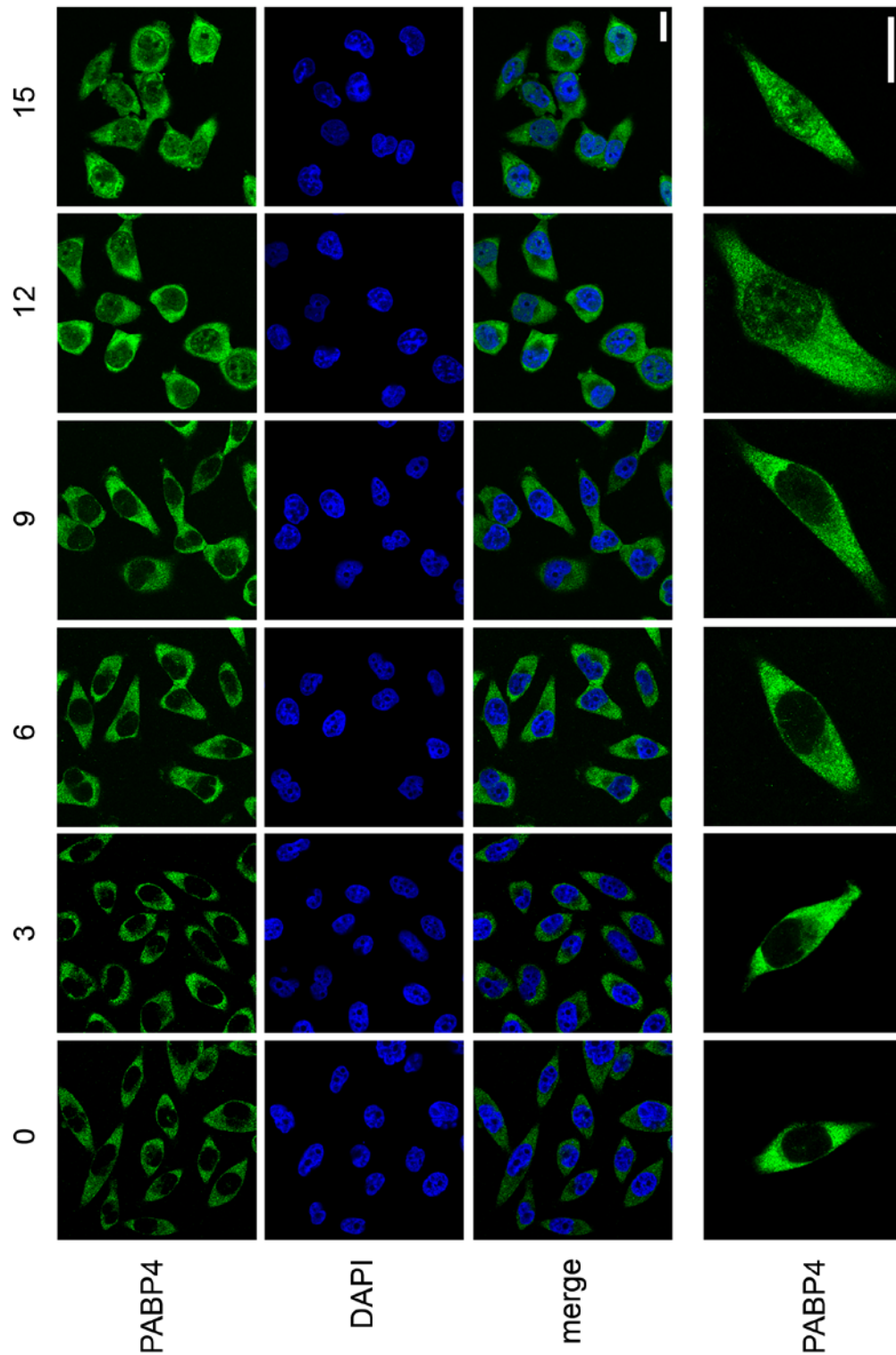


Figure 5.4 PABP4 relocates to the nucleus from 12h after treatment with 50J/m^2 UVC. HeLa cells were treated with 50J/m^2 UVC and incubated for the indicated number of hours. They were then stained by immunofluorescence for PABP4. Single colour and merged x63 confocal microscopy images are shown with additional higher magnification images of single cells stained for PABP4; scale bars represent $20\mu\text{m}$.

5.2.3 Characterisation of minimal UVC dose able to induce relocalisation of PABP1 and PABP4

I was interested in determining the minimal treatment requirement to elicit UV-induced relocalisation of PABPs. This would also permit evaluation of published data on the effect of UV irradiation on translation which has used varied UVC doses (e.g. (Wu et al., 2002)). HeLa cells were treated with increasing doses of UVC from 0 to 50J/m², incubated for 15 hours and then stained by immunofluorescence for PABP1 and PABP4 (Figure 5.5).

In cells that were mock treated (0J/m²) or treated with 10J/m² PABP1 and PABP4 were diffusely cytoplasmic. Treatment with 20J/m² UVC resulted in relocalisation of PABP1 and PABP4 to the nucleus in more than 80% of cells. Treatment with 30J/m², 40J/m² and 50J/m² UVC resulted in relocalisation of PABP1 and PABP4 to the nucleus in more than 95% of cells.

The minimum dose required to relocalise PABP1 and PABP4 was 20J/m². No differences were observed between the treatment requirements to induce relocalisation of PABP1 and PABP4, although PABP4 remained more cytoplasmic than PABP1 at each dose, as noticed previously as various time points after 50J/m² UVC treatment (Figures 5.3 and 5.4).

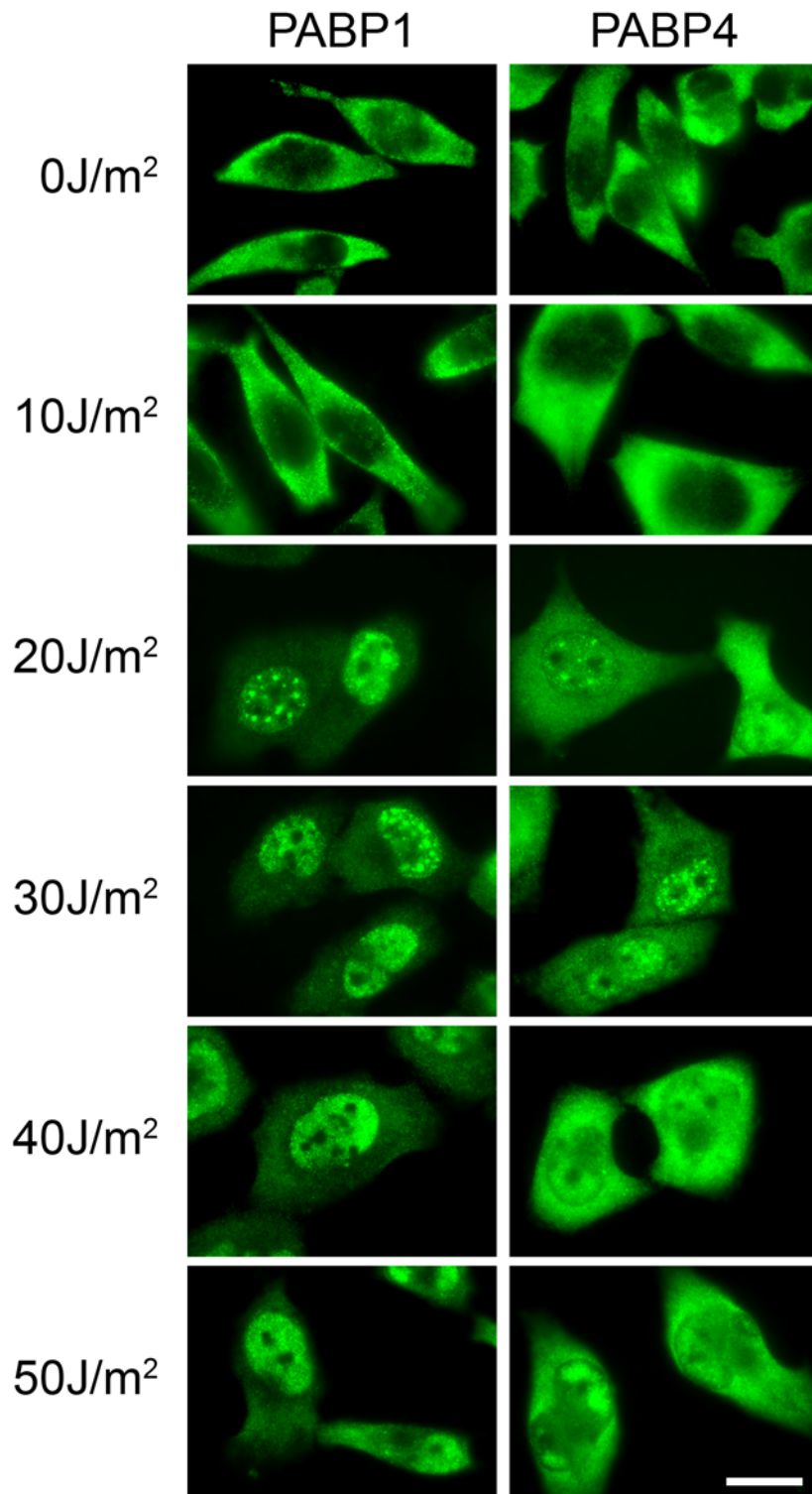


Figure 5.5 PABP1 and PABP4 localise to the nucleus 15h after treatment with 20-50J/m² UVC. HeLa cells were treated with the indicated dose of UVC and incubated for 15 hours. They were then stained by immunofluorescence for PABP1 or PABP4. Fluorescence microscopy (x100) images are shown; scale bar represents 20µm.

5.2.4 Intranuclear localisation of PABP1 and PABP4

In many UV-treated HeLa cells, both PABP1 and PABP4 were observed as small foci in the nucleus (Figures 5.1B, 5.3, 5.4, 5.5). Over-expressed GFP-tagged PABP has also been reported to localise in the nucleus as foci which co-stained for the nuclear speckle marker SC-35 (Afonina et al., 1998). To ascertain whether endogenous PABP1 and PABP4 localise to splicing speckles after UV treatment, cells were co-stained for PABP1 and PABP4 with SC-35 by immunofluorescence (Figure 5.6).

In untreated cells, while PABP1 and PABP4 were cytoplasmic, SC-35 staining was exclusively nuclear and showed a punctate distribution typical of nuclear speckles. After UV treatment speckles detected by SC-35 appeared enlarged consistent with the enlargement of speckles reported after transcriptional inhibition (Carmo-Fonseca et al., 1992) (Spector, 1993). After UVC treatment, nuclear PABP1 and PABP4 was enriched at punctate foci in many cells and these foci perfectly colocalised with SC-35 (Figure 5.6). This data suggests that nuclear-localised PABP1 and PABP4 are enriched in nuclear speckles after UVC treatment.

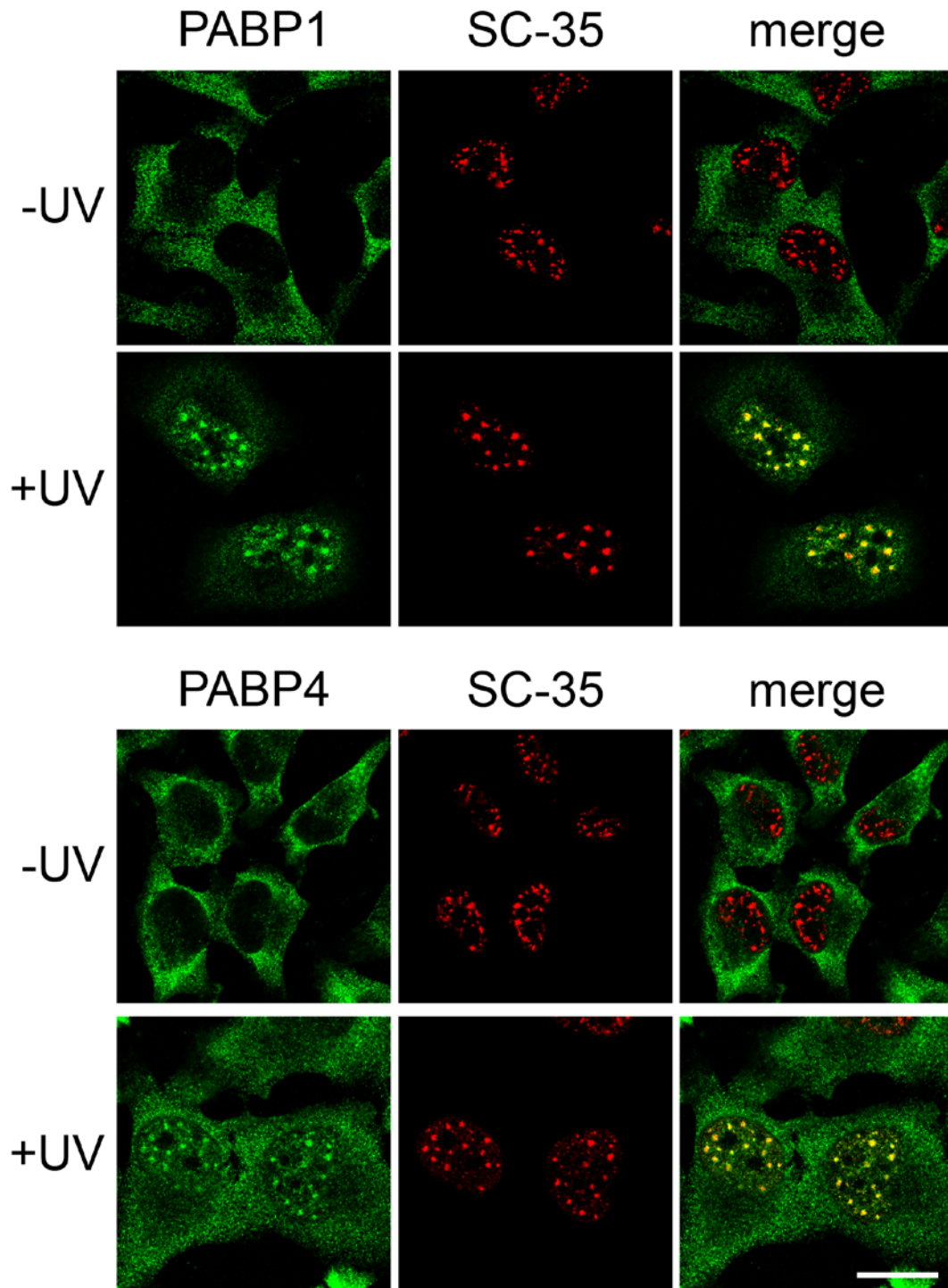


Figure 5.6 PABP1 and PABP4 colocalise with SC-35 in the nucleus. HeLa cells were treated with UV ($50\text{J}/\text{M}^2$ UV-C and 15 hours incubation) or mock treated then stained by immunofluorescence for PABP1 (green), PABP4 (green) and nuclear speckle marker SC-35 (red). Confocal images (x63) are shown; scale bar represents $20\mu\text{m}$.

5.2.5 Investigating the function of PABP1 and PABP4 nuclear relocalisation

Relocalisation of PABP1 and PABP4 may reflect a nuclear requirement for these proteins during recovery from UV or a requirement to exclude them from the cytoplasm. There is no characterised nuclear function of metazoan cytoplasmic poly(A) binding proteins. In *S. Cerevisiae* a single poly(A)-binding protein (Pab1) performs nuclear and cytoplasmic roles. In addition to cytoplasmic regulation of mRNA stability and translation, Pab1 interacts with the cleavage stimulation factor complex (CF1) and participates in 3' mRNA processing (Amrani et al., 1997; Minvielle-Sebastia et al., 1997). In mammals however, PABPN1 fulfils this nuclear role and interacts with both cleavage and polyadenylation specificity factor (CPSF) and poly(A)-polymerase (PAP). Exchange between PABPN1 and cytoplasmic PABPs is thought to occur soon after mRNAs enter into the cytoplasm (Bear et al., 2003; Sato and Maquat, 2009) however, PABP1 has been found to bind unspliced pre-mRNA and has been reported to interact with poly(A)polymerase (PAP) (Hosoda et al., 2006) suggesting that it may, like PABPN1 participate in the stimulation of polyadenylation.

The cleavage stimulatory factor (CstF) subunit CstF-50 has recently been found to be important for transcription coupled repair following UV irradiation and cells in which CstF is knocked down by RNAi display reduced viability after UV treatment (Mirkin et al., 2008). Since PABP1 may play a role in 3' processing in the nucleus, nuclear PABP1 may also be important for TCR, and hence cell survival after UV.

5.2.5.1 Requirement of PABPs for cell viability after UV

To directly test whether PABP1 and PABP4 have a nuclear role important for cell survival following UV irradiation, HeLa cells were transfected with siRNAs directed against these proteins, as previously described (Chapter 4.2.2), and UV irradiated. Trypan blue exclusion was used to assay whether cells in which PABP1 and/or PABP4 were depleted displayed diminished viability 15 hours after UV treatment. PABP1 and PABP4 are known to be relocalised at this time point after UV treatment of non-transfected cells (Figures 5.3 and 5.4).

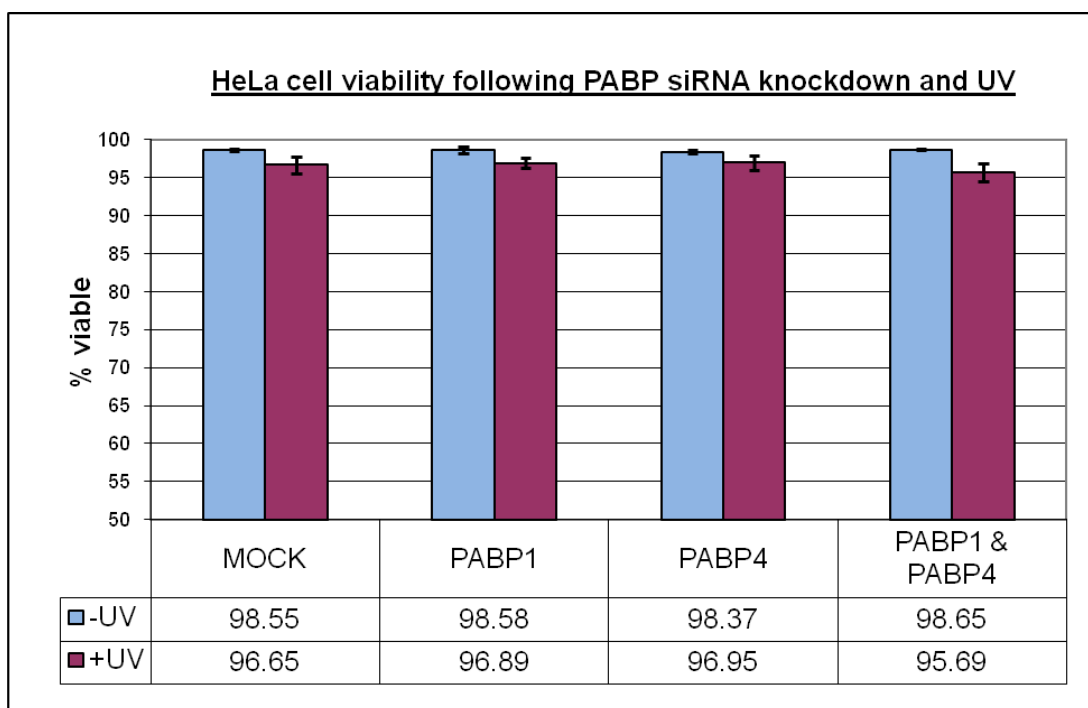


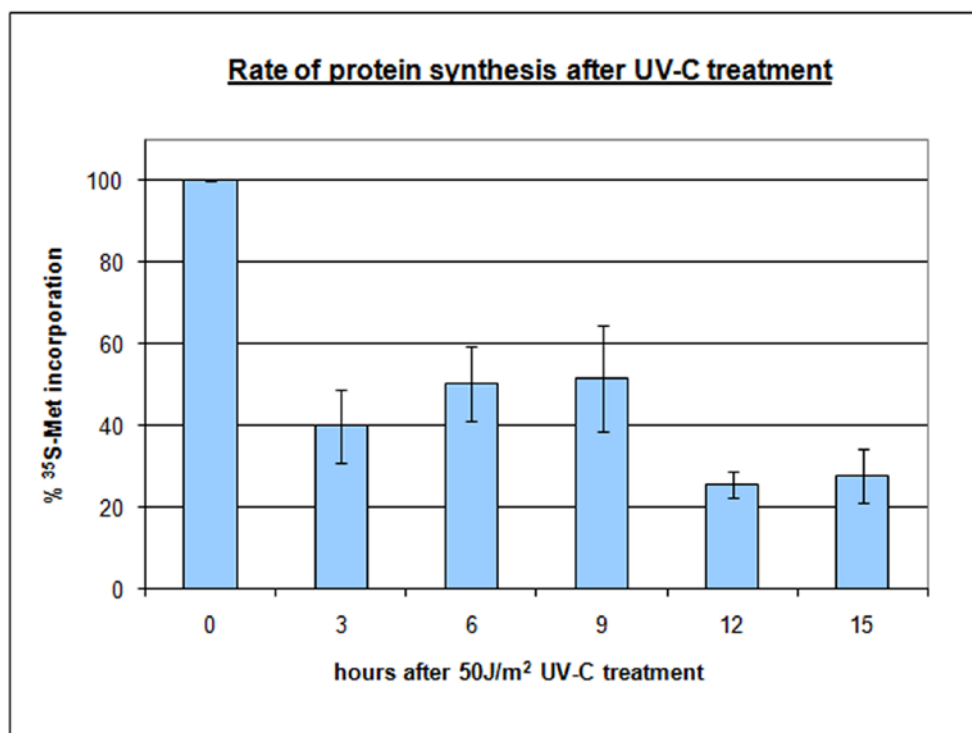
Figure 5.7 Effect of PABP1 and PABP4 siRNA knockdown on HeLa cell viability after UV. HeLa cells were transfected with siPABP1, siPABP4, siPABP1 and siPABP4 or mock transfected for 48 hours as indicated, treated with 50J/m² UVC or mock treated and incubated for a further 15 hours. Cell viability was then assayed by trypan blue exclusion. Results shown are the mean of 3 independent experiments wherein more than 300 cells were examined per point. Error bars show standard error of the mean (SEM).

As shown previously (Chapter 4; Figure 4.7), no difference in viability was detected between cells that were transfected with PABP1 and /or PABP4 directed siRNAs and those mock transfected in the absence of UV treatment (Figure 5.7). A small but consistent decrease in cell viability (2%) was observed in UV treated cells compared to mock-treated cells (mean viability of 96.54% versus 98.54%), which was found to be statistically significant by paired *t*-test (n=12 p=0.0012). However, no difference in viability was detected between cells transfected with siRNAs (siPABP1:96.89%, siPABP4: 96.95%, siPABP1+4: 96.69%) or mock transfected (96.65%) following UV treatment.

Depletion of PABP1 and PABP4 by siRNA therefore indicates that the presence of PABP1 and PABP4 in the nucleus is not required for cell survival following UV. This suggests that the function of relocalisation of PABPs to the nucleus after UV may be to sequester them away from the cytoplasm, perhaps as part of the reprogramming of translation.

5.2.5.2 Inhibition of protein synthesis after UV treatment

To investigate how protein synthesis rates correlate with changes in PABP localisation, HeLa cells were labelled with ³⁵S-methionine at 3 hour intervals after UV treatment and incorporation measured as previously described (Chapter 4; Figure 4.10). Protein synthesis was significantly inhibited within 3 hours of UV irradiation and reduced to 40% of untreated cells (Figure 5.8). Synthesis rates remained at about 50% of normal at 6-9 hours after treatment but further decreased at 12 and 15 hours to approximately 25% compared to untreated control (0 hours). The reduction in protein synthesis rates at each time-point compared to the control was found to be significant (p = <0.05) by paired *t*-test in each case with the exception of 9 hours. Data obtained at 9 hours showed a slightly larger variation between experimental replicates resulting in a p value of 0.064 when compared to control cells which does not quite reach significance. These results are consistent with those of Wu et al who reported a 50% reduction in protein synthesis at 4 hours after treatment of MCF7 cells (a human breast cancer line) with 50J/m² UVC (Wu et al., 2002).



Hours after UV	0	3	6	9	12	15
Mean incorporation (%)	100	39.91	50.18	51.47	25.57	27.86
SEM	0	8.96	9.14	12.95	3.14	6.60
Significant dif. compared to 0h	-	*	*	ns	*	*

Figure 5.8 Inhibition of protein synthesis after UV treatment. Each experiment was performed in triplicate and means normalised to untreated cells (0 hour) as a percentage. The mean of 3 independent experiments are shown. Error bars represent SEM. Table shows chart data and results of paired *t*-tests performed between 0 hours and each other time points with significant difference indicated by an asterisk (*) and non significance indicated by ns.

It is clear that relocalisation of PABP proteins do not play a significant role in the early phase of inhibition but may contribute to the further inhibition of protein synthesis at later times (12 and 15 hours), as these correspond to the time points when PABP1 and PABP4 are most robustly relocalised. It is worth noting however that these results reflect protein synthesis and not translation rates. Global transcription is inhibited by UV and it is unknown to what extent transcriptional and translational inhibition each contributes to the observed reduction in protein synthesis.

5.3 Discussion

In this chapter I have characterised a novel cellular response to UV – relocalisation of cytoplasmic poly(A) binding proteins 1 and 4 to the nucleus. The dose requirements and timing of this response were characterised and the relevance of nuclear PABP1 and PABP4 to cell viability and translational inhibition after UV treatment investigated.

UV-induced relocalisation of PABP1 and PABP4 was observed in HeLa cells however, this response was also observed in 3T3 cells (Figure 5.1), providing confidence that it is not a HeLa cell specific response. It would be interesting to test if this effect is conserved in a keratinocyte cell line which would more reliably indicate whether this response occurs in mammalian skin epidermis following sun exposure.

Relocalisation of PABP4 to the nucleus has not been previously reported under any treatment or stress condition and conservation of this response between PABP1 and PABP4 implies a shared mechanism of regulation, perhaps by a protein partner. Nuclear localisation of endogenous PABP1 has been reported in HeLa cells following transcriptional inhibition and prolonged heat shock (2 hours at 44°C) (Ma et al., 2009). Relocalisation of PABP1 during heat shock was accompanied by a 2-fold increase in protein expression (Ma et al., 2009) which may explain this observation as over-expressed GFP-tagged PABP1 also accumulates in the nucleus (Afonina et al., 1998). Unusually however, in this report stress granules were not observed at any time points during heat shock and no other cell lines were tested (Ma et al., 2009) suggesting that the observed relocalisation of PABP1 to the nucleus during heat shock may be specific to the HeLa strain used. Supporting this notion, I was unable to reproduce this response, finding instead that PABP1 localised to stress granules at early time points (Chapter 4; Figure 4.2) and after 2 hours PABP1 remained cytoplasmic (Appendix 1).

Knockdown of PABP1 and/or PABP4 by siRNAs did not affect cell viability following UV-irradiation. This suggests that PABP1 and PABP4 do not play a role in the nucleus that is essential for cell survival, such as DNA repair. Many proteins

important for DNA repair are specifically translocated to the nucleus following DNA damage/UV (Knudsen et al., 2009) and PABP1 is known to interact with the tumour suppressor protein BRCA1 (Dizin et al., 2006) which is involved in DNA repair and localises to DNA lesions following DNA damage (Wang et al., 2000). However, nuclear PABP1 and PABP4 staining did not correlate with DAPI staining of DNA (Appendix 2).

Whether the UV-treated cells in which PABP1/4 were nuclear localised were apoptotic or pre-apoptotic was not formally tested and recovery from UV was not verified. It is therefore possible that the change in PABP1/4 localisation could be involved in UV-induced apoptosis. To test whether cells are recovering from UV treatment the amount of cyclo-pyrimidine dimers present could be assayed using an antibody that specifically detects these lesions (Powley et al., 2009). To test whether treated cells are apoptotic lysates could be western blotted for an early apoptosis marker such as TNF-related apoptosis inducing ligand (TRAIL).

Nuclear PABP1 and PABP4 localised to splicing speckles (Figure 5.6). Nuclear speckles are dynamic interchromatin nuclear domains enriched in splicing components (e.g. snRNPs, SR proteins) (Reviewed in (Handwerger and Gall, 2006; Lamond and Spector, 2003). Speckles are thought to function as a reservoir of splicing factors and as sites of post-transcriptional splicing (Lamond and Spector, 2003), but are not thought to represent sites of active transcription (Xie et al., 2006). They are sensitive to transcriptional inhibition becoming enlarged upon treatment with transcriptional inhibitors (Lamond and Spector, 2003). This effect was also detected following UV treatment by SC-35 staining (Figure 5.6) consistent with the effect of UV on transcription.

Poly(A)+ pre-mRNA is enriched at nuclear speckles and these transcripts have been shown to persist in speckles following transcriptional inhibition (Shopland et al., 2002). PABP1-binding of nuclear pre-mRNA has been demonstrated (Hosoda et al., 2006) and localisation of PABP1 and PABP4 after UV to nuclear speckles may therefore be a result of poly(A)+ RNA binding in the nucleus.

Relocalisation of PABP1 and PABP4 to the nucleus after UV may represent a strategy to reprogramme translation in the cytoplasm. Several viruses are thought to interfere with the availability of cytoplasmic PABP1 as a tactic to modulate host

translation (Chapter 1.4.5;(Smith and Gray, 2010)). However, PABP1 nuclear relocalisation during viral infection has not been demonstrated to directly result in decreased host cell translation. Following 50J/m² UVC irradiation, inhibition of protein synthesis in HeLa cells appeared roughly biphasic initially dropping to 40-50% of untreated cells at 3-9 hours post-treatment and subsequently dropping further to ~25% at 12 and 15 hours post-treatment (Figure 5.8). Sequestration of PABP1 and PABP4 to the nucleus at 12 and 15 hours after UV treatment may therefore play a role in this later further inhibition of protein synthesis.

Global inhibition of translation after UV treatment is thought to be mainly mediated by phosphorylation of eIF2 α (Deng et al., 2002) as expression of only non-phosphorylatable eIF2 α (S51A) or deletion of the eIF2 α kinase GCN2 almost completely prevents the early attenuation of protein synthesis after UVC irradiation (Deng et al., 2002; Jiang and Wek, 2005). Phosphorylation of eIF2 α occurs very rapidly after UV treatment and is detectable within 15 minutes of treatment of MEFs with 30J/m² UVC (Jiang and Wek, 2005). Despite an initial rapid increase, the ratio of phosphorylated eIF2 α to total eIF2 α however is reported to be relatively stable at later time points after UVC treatment (Wu et al., 2002). It is possible therefore, that phosphorylation of eIF2 α is responsible for the early inhibition of protein synthesis after UV which is augmented at later time points by relocalisation of PABPs from the cytoplasm. An assay of eIF2 α phosphorylation levels in my treated HeLa cells would be useful to directly compare with the timing of PABP1 and PABP4 localisation and it would also be interesting to test whether relocalisation of PABPs is dependent on prior phospho-eIF2 α mediated translational inhibition by using recently described small molecule inhibitors of GCN2 (Robert et al., 2009).

The contribution of PABP1 and PABP4 localisation changes to protein synthesis could also be directly assessed by knocking down these proteins using siRNAs. Knockdown of PABP1 and PABP4 would be expected to reduce protein synthesis rates in unirradiated cells (see Chapter 4.2.2) however comparison of the relative response of protein synthesis rates to UV treatment in PABP1/4 knockdown versus wildtype cells would reveal the role of PABP1/4.

Lastly, the contribution of transcriptional inhibition and cytoplasmic mRNA abundance to UV-induced inhibition of protein synthesis has not been investigated.

Rather than metabolic labeling, monitoring of the synthesis of individual proteins which could be normalized to their mRNA abundance would provide an indication of translation rates after UV treatment independent of these effects.

The results presented in this chapter characterise novel localisation changes of PABP proteins in response to stress and raise the possibility that the late relocalisation of PABPs from the cytoplasm to the nucleus after UV irradiation may lead to a reduction in global translation rates and contribute to translational reprogramming. A reduction of available cytoplasmic PABPs may of course have implications for other cytoplasmic processes. Following depletion of PABP1 from the cytoplasm, mRNAs may be more readily deadenylated and turned over, an effect which has been demonstrated *in vitro* (Bernstein et al., 1989) and microRNA mediated gene regulation has recently also been shown to be sensitive to levels of available PABP1 (Walters et al., 2010).

As relocalisation to the nucleus may represent a way to modulate the cytoplasmic functions of PABP1 and PABP4, it is important to understand the mechanism behind this change which will also help us to understand how normal PABP localisation and nucleocytoplasmic shuttling is controlled. This mechanism is investigated in Chapter 6.

Chapter 6: Mechanism of nuclear relocalisation of PABPs

6.1 Introduction

Accumulation of PABP1 and PABP4 in the nucleus following UV irradiation may be the result of an acceleration of nuclear import or a block in nuclear export of PABPs. PABPs do not contain canonical nuclear import or export signals and the control of PABP1 localisation and nuclear import/export is not fully understood (Chapter 1.4.4). There has been no investigation of the regulation of PABP4 localisation to date.

There is no characterised pathway of nuclear import of PABPs in metazoa. In *S. cerevisiae* an interaction between RRM4 of Pab1 and the import receptor protein Kap108/Sxm1 has been described (Brune et al., 2005). Kap108/Sxm1 shares similarity with human importins 7 and 8 (Pemberton and Paschal, 2005), though the possibility of their involvement in mammalian PABP1 nuclear import has not been investigated. If the importin binding site is conserved, it is unlikely to be the only mechanism for nuclear import of mammalian PABP1 as GFP-tagged N-terminal (i.e. RRM-containing) and C-terminal fragments are both able to enter the nucleus (Afonina et al., 1998). Nuclear import of PABP1 has however, been shown to be energy dependent, since incubation of cells at 4°C or in the presence of presence of 2-deoxyglucose, which depletes ATP pools, prevents nuclear accumulation induced by the transcriptional inhibitor 5,6-dichlororibofuranosylbenzimidazole (DRB) (Afonina et al., 1998).

Multiple different modes of regulation of mammalian PABP1 nuclear export have been suggested. Nuclear export of PABP1 has been proposed to be mediated by CRM1. CRM1 is a nuclear export protein involved in the export of proteins containing a classical leucine-rich nuclear export signal (NES) and is specifically inhibited by the drug leptomycin B (LMB) (Fornerod et al., 1997). Nuclear export of Pab1 in *S. cerevisiae* is dependent on exportin-1, (the yeast CRM1 homolog) and accumulates in the nucleus following LMB treatment and in exportin-1 (*Xpo1*) defective strains (Brune et al., 2005). In mammals however, while PABP1 was initially reported to be sensitive to LMB (Woods et al., 2002) a recent publication reports LMB insensitivity (Khacho et al., 2008b).

An interaction with paxillin has also been reported to be important for PABP1 nuclear export (Woods et al., 2005). Paxillin is a scaffold protein that recruits numerous regulatory and structural proteins to the intracellular sites of cell adhesion, termed focal adhesions (Deakin and Turner, 2008). Knockdown of paxillin by RNAi lead to nuclear accumulation of transfected HA-tagged PABP1 and a mutation at a putative paxillin interaction site (termed PBS2) in RRM4 also localised HA-PABP1 to the nucleus (Woods et al., 2005). Any other effects of mutation of PBS2 on PABP1 activities (such as RNA-binding) were not tested. Furthermore, as paxillin is an NES containing protein, the apparent sensitivity of PABP1 to LMB was attributed to its dependence on paxillin for nuclear export (Woods et al., 2002).

Transcriptional inhibition by two different drugs, actinomycin D and DRB has been reported to lead to nuclear accumulation of PABP1 (Afonina et al., 1998). Recently, this effect has been ascribed to a ‘transcription dependent nuclear export motif’ (TD-NEM) in RRM4 of PABP1, identified by sequence similarity to the TD-NEM in the von Hippel-Lindau (VHL) tumor suppressor protein (Khacho et al., 2008b). Deletion of this motif was reported to lead to nuclear accumulation of GFP-tagged PABP, though again any other effects of this deletion on PABP1 activities were not tested (Khacho et al., 2008b). An interaction between the TD-NEM (in PABP1 and VHL) and eEF1A was also suggested to be important for this mode of transcription regulated export, however, co-immunoprecipitations were conducted in the absence of RNase and siRNA knockdown of eEF1A did not result in convincing nuclear accumulation of endogenous PABP1 (Khacho et al., 2008a). The mechanism by which transcriptional inhibition results in a block to PABP1 export has not been fully explained.

A role for the export of mRNA in export of Pab1 has also been implied using mutants of *mex67*, the yeast homolog of TAP. While inhibition of mRNA export in a *mex67* deletion strain is insufficient on its own to cause nuclear accumulation of Pab1, *mex67 Xpo1* double mutants show a greater number of cells with nuclear Pab1 than single *Xpo1* mutants, implying that both export pathways are used in yeast and are partially redundant (Brune et al., 2005). Furthermore, in *S. pombe* GFP-PABP accumulates in the nucleus when mRNA export is blocked by a mutation in the

export factor *Rae1* (Thakurta et al., 2002). A role of mRNA export has not been investigated in the nuclear export of PABPs in mammals.

In order to understand how the UV-induced change in PABP1 and PABP4 localisation is regulated, I have investigated each of the proposed mechanisms for PABP1 nuclear export and examined how they might be changed following UV.

6.2 Results

6.2.1 Is PABP nuclear export dependent on the CRM1 export pathway?

To test whether PABP1 and PABP4 are exported from the nucleus via the CRM1 protein export pathway, HeLa cells were treated with leptomycin B and stained by immunofluorescence for PABP1 (Figure 6.1) or PABP4 (Figure 6.2) and paxillin. Paxillin contains a classical nuclear export signal (NES) and is known to be sensitive to LMB treatment (Woods et al., 2002) (Dong et al., 2009) and so is a positive control for CRM1 mediated export. As expected, following LMB treatment a nuclear accumulation of paxillin was observed which was relieved after withdrawal of LMB (Figure 6.1 and 6.2). The localisation of PABP1 and PABP4 however, was not altered by leptomycin B treatment and both proteins remained diffusely cytoplasmic following LMB treatment and at 18 hours after treatment (Figures 6.1 and 6.2). This indicates that neither PABP1 nor PABP4 are exported from the nucleus by the CRM1 protein export pathway, though since relocalisation of paxillin was partial and not absolute under these conditions, implying that CRM1-mediated export was not completely abrogated, the possibility of a minor role of CRM1 in the export of PABP1 and PABP4 cannot be resolutely ruled out. These data do however suggest that inhibition of paxillin nuclear export is not sufficient to inhibit nuclear export of PABP1 or PABP4 in contradiction of Woods et al (2002).

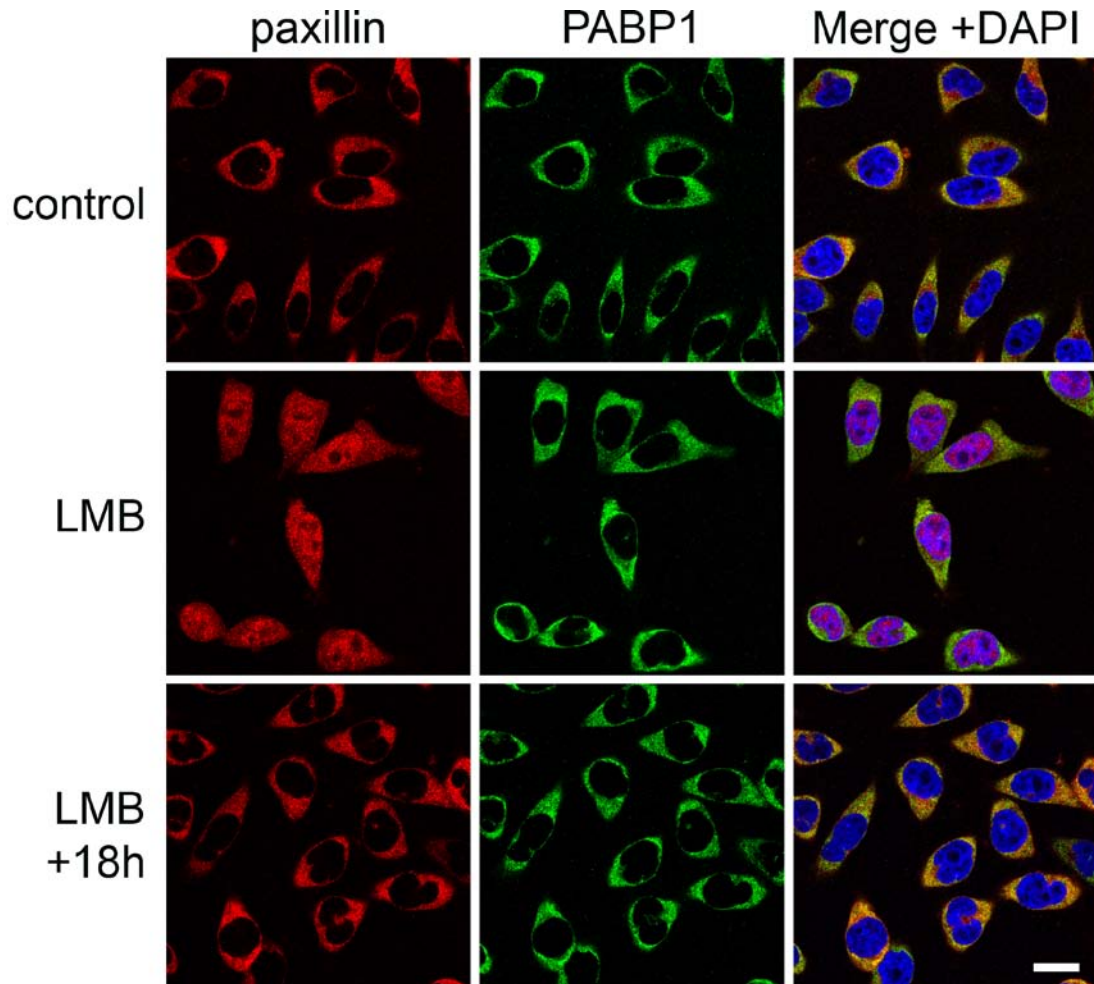


Figure 6.1 PABP1 nuclear export is not dependent on the CRM1 pathway. HeLa cells were treated with vehicle control or 5ng/ml leptomycin B (LMB) for 3 hours. LMB treated cells were also allowed to recover overnight where indicated (LMB+18h). Cells were stained by immunofluorescence for paxillin (red) or PABP1 (green) and counterstained with DAPI. Confocal images (x63) are shown, scale bar represents 20 μ m.

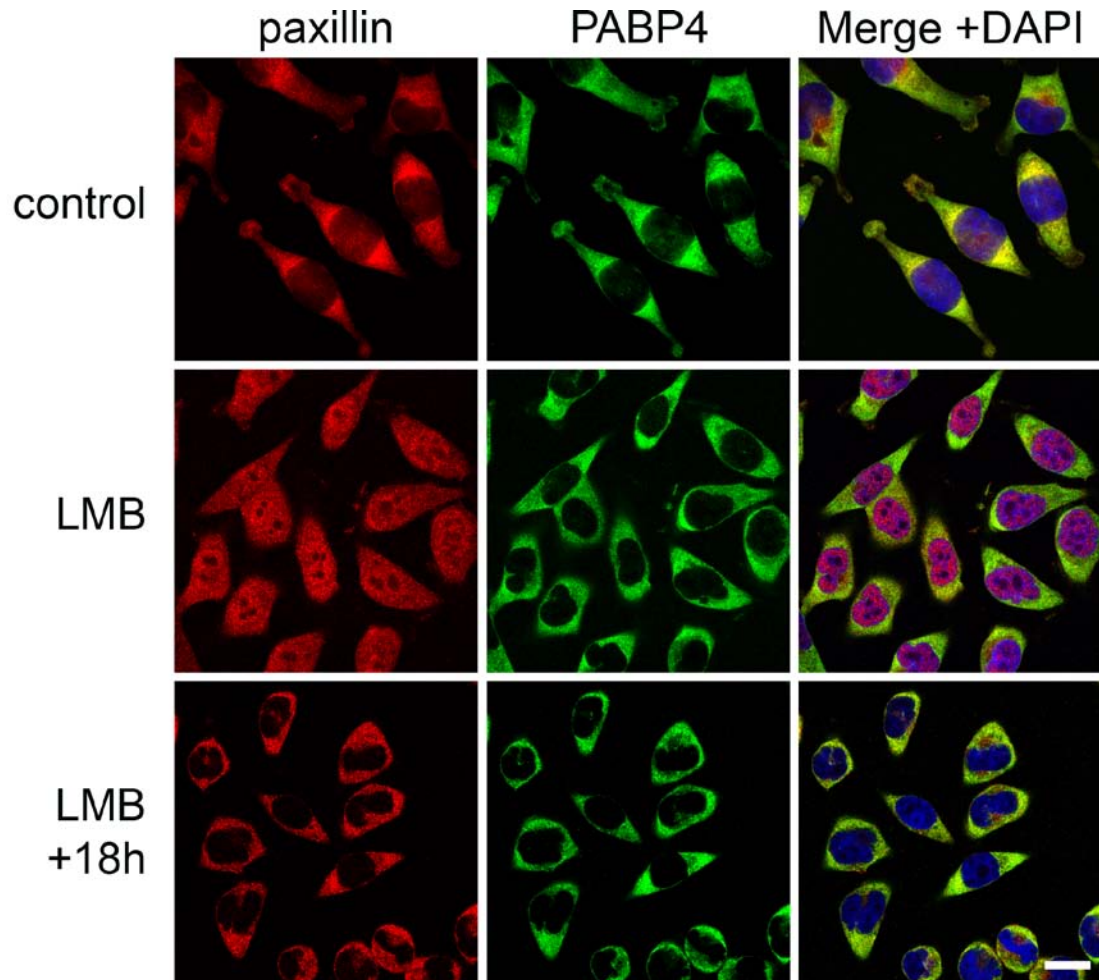


Figure 6.2 PABP4 nuclear export is not dependent on the CRM1 pathway. HeLa cells were treated with vehicle control or 5ng/ml leptomycin B (LMB) for 3 hours. LMB treated cells were also allowed to recover overnight where indicated (LMB+18h). Cells were stained by immunofluorescence for paxillin (red) or PABP4 (green) and counterstained with DAPI. Confocal images (x63) are shown, scale bar represents 20 μ m.

6.2.2 Does UV affect the interaction of PABP1 with paxillin?

It has been previously reported that upon leptomycin B treatment PABP1 and paxillin both accumulate in the nucleus, and it was suggested that this is indicative of a dependence of PABP1 on paxillin for its nuclear export (Woods et al., 2002). This explanation was based on an interaction between PABP1 and paxillin determined by GST-pulldown and co-immunoprecipitation of the endogenous proteins in 3T3 cells (Woods et al., 2002). However, my results appear to argue against this notion (Figure 6.1). Nonetheless, I sought determine whether paxillin localisation is affected by UV to further rule out a role of paxillin. HeLa cells were UV-irradiated and stained by immunofluorescence for paxillin and PABP1 (Figure 6.3A). No change in paxillin localisation was observed. This suggests that the CRM1 protein export pathway is not affected by UV treatment and that nuclear relocalisation of PABP1 is not accompanied by a change in the localisation of paxillin.

Although paxillin does not relocalise to the nucleus, a loss of interaction between PABP1 and paxillin in UV-treated cells could result in nuclear accumulation of PABP1. To address this, I used the published experimental conditions used for PABP1-paxillin co-immunoprecipitation (Woods et al., 2002) in UV-treated or untreated HeLa cells (Figure 6.3B). I was unable to detect any paxillin above background level in PABP1-immunoprecipitates in UV-treated or untreated cells. Western blotting for PABP1 demonstrated effective immunoprecipitation and western blotting for PAIP1 demonstrated that protein interactions with PABP1 are maintained in these conditions (Figure 6.3B). I was also unable to detect an interaction between PABP1 and paxillin by co-immunoprecipitation from HeLa cells treated with the chemical crosslinker DSP or 3T3 cells (Appendix 3), in which the interaction was first reported (Woods et al., 2002). Paxillin is known to be highly modified and its localisation and interaction with several protein partners are regulated by phosphorylation (Brown and Turner, 2004; Dong et al., 2009). Consistent with the absence of any change in paxillin localisation, no change in paxillin mobility was detected after UV (see input lanes, Figure 6.3A).

While I cannot formally rule out the involvement of paxillin in the regulation of PABP localisation after UV, the available data and lack of reproducible interaction between these proteins makes an important role for paxillin unlikely.

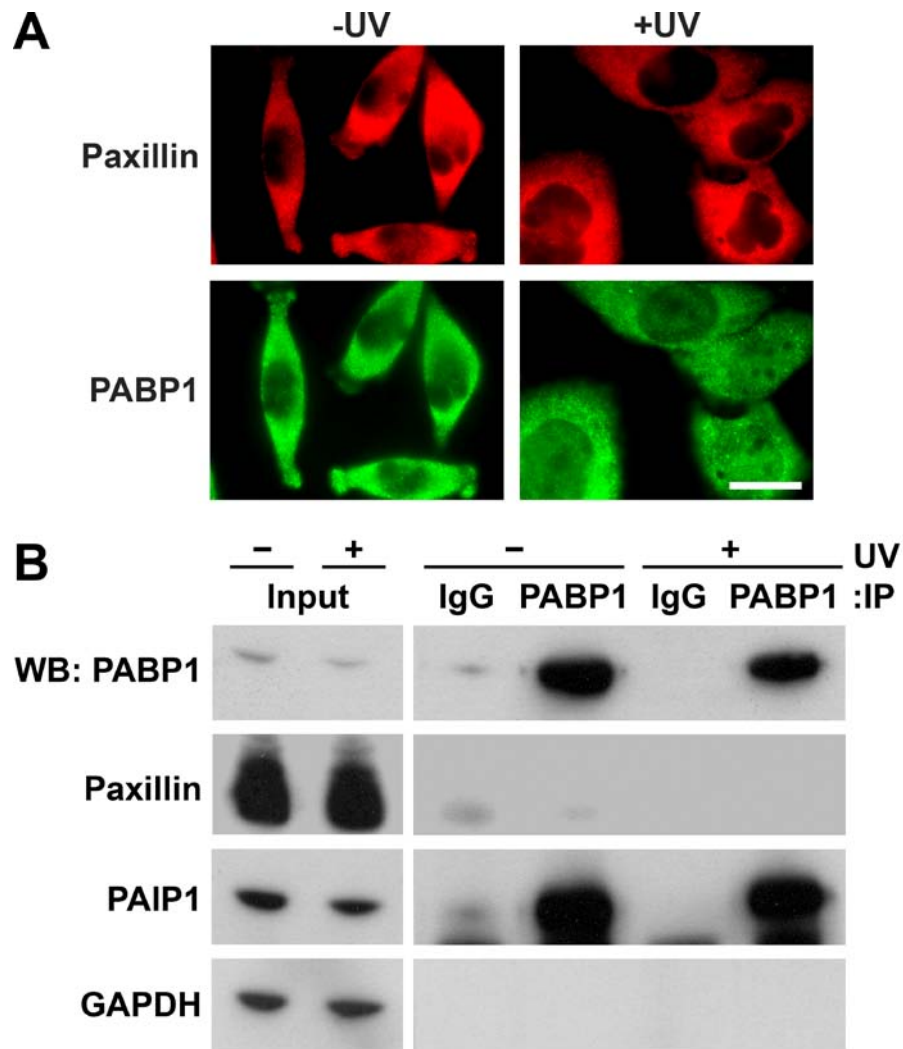


Figure 6.3 Effect of UV on paxillin subcellular localisation and interaction with PABP1. **A.** HeLa cells were treated with UV (50J/M² UV-C and 14 hours incubation) or mock treated and then stained by immunofluorescence for PABP1 (green) and paxillin (red). Fluorescence microscopy images (x100) are shown; scale bar represents 20µm. **B.** HeLa cells were treated with UV (50J/M² UV-C and 15 hours incubation) or mock treated. Immunoprecipitations with anti-PABP1 or rabbit IgG control from HeLa extracts were performed as in (Woods et al., 2002) in the presence of RNase1 and western blotted for PABP1, Paxillin and PAIP1. 10µg is shown as input, corresponding to 1% of material used for each IP.

6.2.3 Does UV-irradiation affect post-translational modification of PABP1 and PABP4?

The regulation of the sub-cellular localisation of many proteins is regulated by post-translational modifications (e.g. paxillin (Dong et al., 2009)). PABP1 has been reported to be modified by arginine-methylation (Lee and Bedford, 2002) and phosphorylation (Ma et al., 2006; Rowlett et al., 2008) and in plants the RNA and protein binding activities of PABP have been suggested to be regulated by its phosphorylation state (Le et al., 2000).

The migration of PABP1 and PABP4 on SDS-PAGE was not affected by UV treatment (Figure 5.2) however post-translation modifications (PTMs) may not necessarily effect a change in electrophoretic mobility in one dimension. Therefore, whether the PTM status of PABP1 and PABP4 might be affected by UV was further tested by separating nuclear and cytoplasmic extracts of UV-treated or untreated HeLa cells by 2D electrophoresis. Effective protein focusing and separation was confirmed by protein staining of the membrane (Appendix 4). Western blotting revealed that PABP1 and PABP4 migrated as a single spot in nuclear and cytoplasmic fractions of untreated cells (Figure 6.4A and B). The mobility of PABP1 and PABP4 was unchanged following UV treatment. This implies that PABP1 and PABP4 are not differently modified following UV-irradiation; however, it is formally possible that the pattern of PABP1 and PABP4 modification may alter so as to produce no net effect on pI (isoelectric point) or mass.

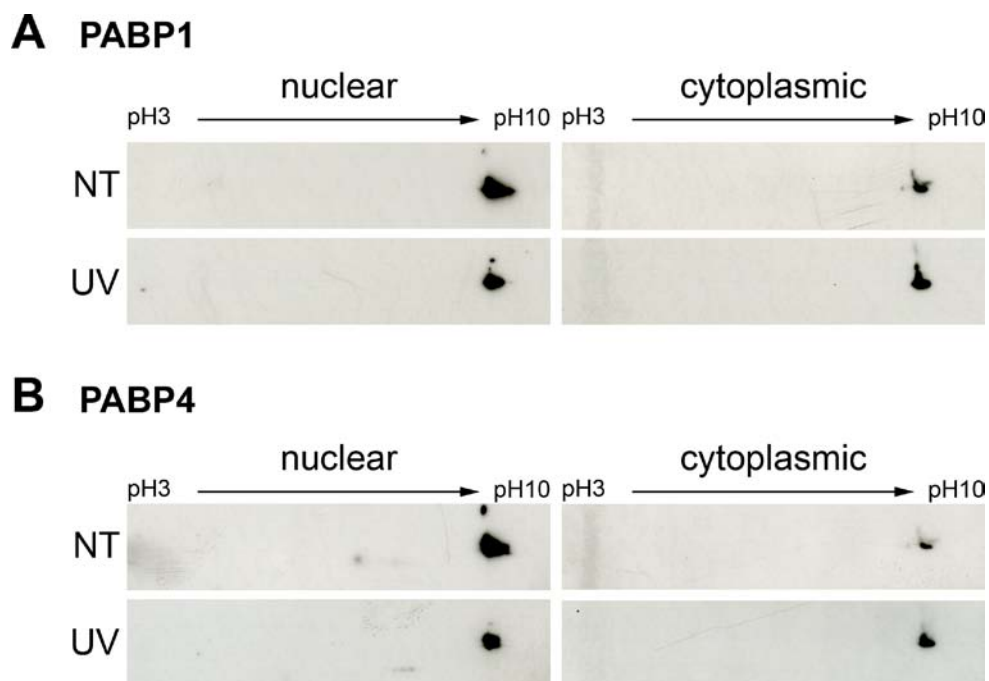


Figure 6.4 PABP1 and PABP4 do not appear to be post-translationally modified in response to UV treatment. HeLa cells were treated with UV (50J/M² UVC) and 15 hours incubation or not treated (NT). Cytoplasmic and nuclear extracts were prepared and separated on non-linear pH3-10 IPG strips in the first dimension and on 4-12% SDS-PAGE in the second dimension then western blotted for PABP1 (A) and PABP4 (B). Isoelectric focusing sample preparation and focusing were conducted by M. Brook.

6.2.4 Is nuclear relocalisation of PABP1 and PABP4 after UV explained by transcriptional inhibition?

6.2.4.1 Is transcription inhibited after UV?

UV-irradiation is known to cause an inhibition of transcription (Heine et al., 2008) and drug induced transcriptional inhibition has been suggested to affect PABP1 nuclear export (Khacho et al., 2008b). The COOH-terminal domain (CTD) of RNAPII undergoes dynamic changes in its phosphorylation during the transcription cycle. Serine 5 phosphorylation is associated with initiation of transcription at promoters and serine 2 phosphorylation is associated with transcription elongation in coding regions (Komarnitsky et al., 2000). RNAPII Serine 5 phosphorylation has been shown to be diminished following transcriptional

inhibition by DRB (Bregman et al., 1995) and UVC irradiation (Luo et al., 2001). To test whether transcriptional inhibition temporally correlates with the change in PABP1 and PABP4 localisation, extracts from UV-treated HeLa cells were western blotted using an antibody that detects RNA polymerase II (RNAPII) phosphorylated on serine 5 of the CTD (Figure 6.5).

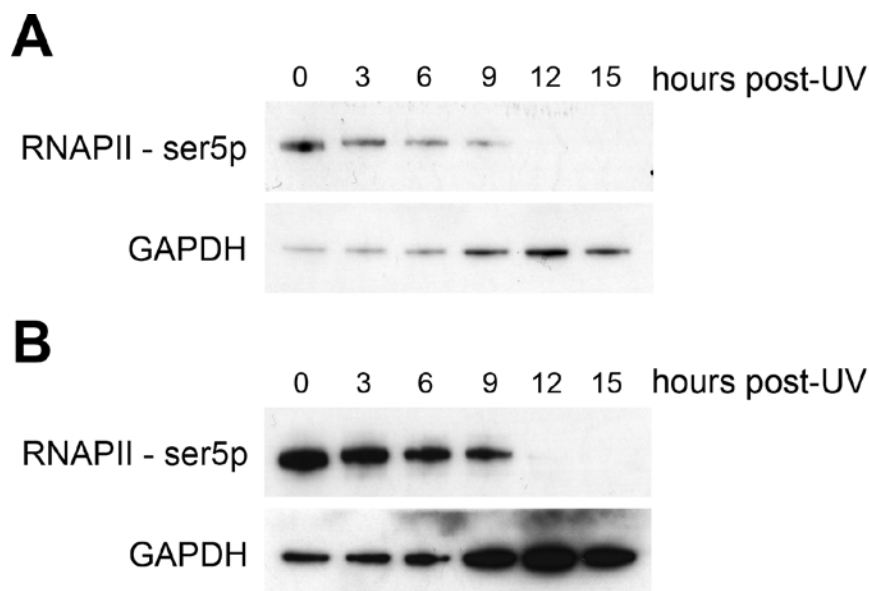


Figure 6.5 Transcriptional inhibition after UV coincides with PABP relocalisation. HeLa cells were treated with UV (50J/M² UVC) and incubated for the indicated times. Extracts (10µg) were western blotted for phosphorylated RNAPII (ser5) and GAPDH; short (A) and long (B) exposures of the same blots are shown.

The level of RNAPII-ser5p was visibly reduced by 3 hours after UV treatment and continued to decrease over time (Figure 6.5). At 12 and 15 hours post-treatment levels of RNAPII-ser5p were below the detection threshold of the western blot, though blotting for GAPDH demonstrated this was not due to an absence of total protein. GAPDH levels appeared to increase with time post-treatment. This was not due to unequal loading of protein since equivalence was ensured by Bradford assay of lysates and Gelcode blue staining of total proteins on the membrane prior to western blotting, implying that the observed increase in GAPDH levels may represent a regulated increase in its expression in response to UV. Since global translation is markedly reduced at these time points, this could reflect a specific upregulation of its translation similar to the uORF-containing transcripts encoding

DNA damage repair enzymes which have been previously identified (Powley et al., 2009).

The decrease in detected RNAPII-ser5p likely represents both a change in RNAPII phosphorylation and a reduction in total RNAPII protein as it is known undergo proteasome-dependent degradation after UV-irradiation (Luo et al., 2001). This time-dependent reduction in RNAPII-ser5p is consistent with the pattern observed following treatment of human fibroblasts with 10J/m² UVC (Luo et al., 2001). Thus transcriptional activity of UV- irradiated HeLa cells decreases over time with maximal reduction reached between 9 and 12 hours. This is coincident with PABP1 and PABP4 localisation to the nucleus, suggesting that transcriptional inhibition after UV may be important for this relocalisation. It further suggests that the reduction in protein synthesis rates observed between 9 and 12 hours post treatment (Chapter 5; Figure 5.8) may be the result of transcriptional inhibition in addition to PABP nuclear relocalisation.

6.2.4.2 Does PABP4 relocalise to the nucleus following transcriptional inhibition?

PABP1 has been shown to accumulate in the nucleus following inhibition of transcription by DRB and actinomycin D (Afonina et al., 1998; Khacho et al., 2008b). Sensitivity of PABP4 to transcriptional inhibition has not been tested; however the transcription-dependent nuclear export motif described in PABP1 is closely conserved in PABP4. To test whether transcriptional inhibition relocalises PABP4 to the nucleus, HeLa cells were treated with 5µg/ml actinomycin D and stained by immunofluorescence for PABP1 as a positive control and PABP4. Cells were co-stained for HuR as a further positive control. HuR is an ARE-specific RNA binding protein, the sub-cellular localisation of which is also sensitive to transcriptional inhibition. Usually predominantly nuclear, upon actinomycin D treatment, HuR relocalises to the cytoplasm (Fan and Steitz, 1998). After 3 hours actinomycin D treatment HuR was clearly detected in the cytoplasm, however PABP1 remained cytoplasmic (Figure 6.6). After 6 hours treatment however, PABP1 became visible in the nucleus and further treatment lead to more PABP1 in the

nucleus at 9 hours (Figure 6.6). Similarly, 3 hours actinomycin D treatment did not alter the cytoplasmic localisation of PABP4 (Figure 6.7). After 6 hours treatment PABP4 was just visible in the nucleus at low levels but was more obvious at 9 hours (Figure 6.7). Therefore PABP4 also relocalises to the nucleus following inhibition of transcription with actinomycin D.

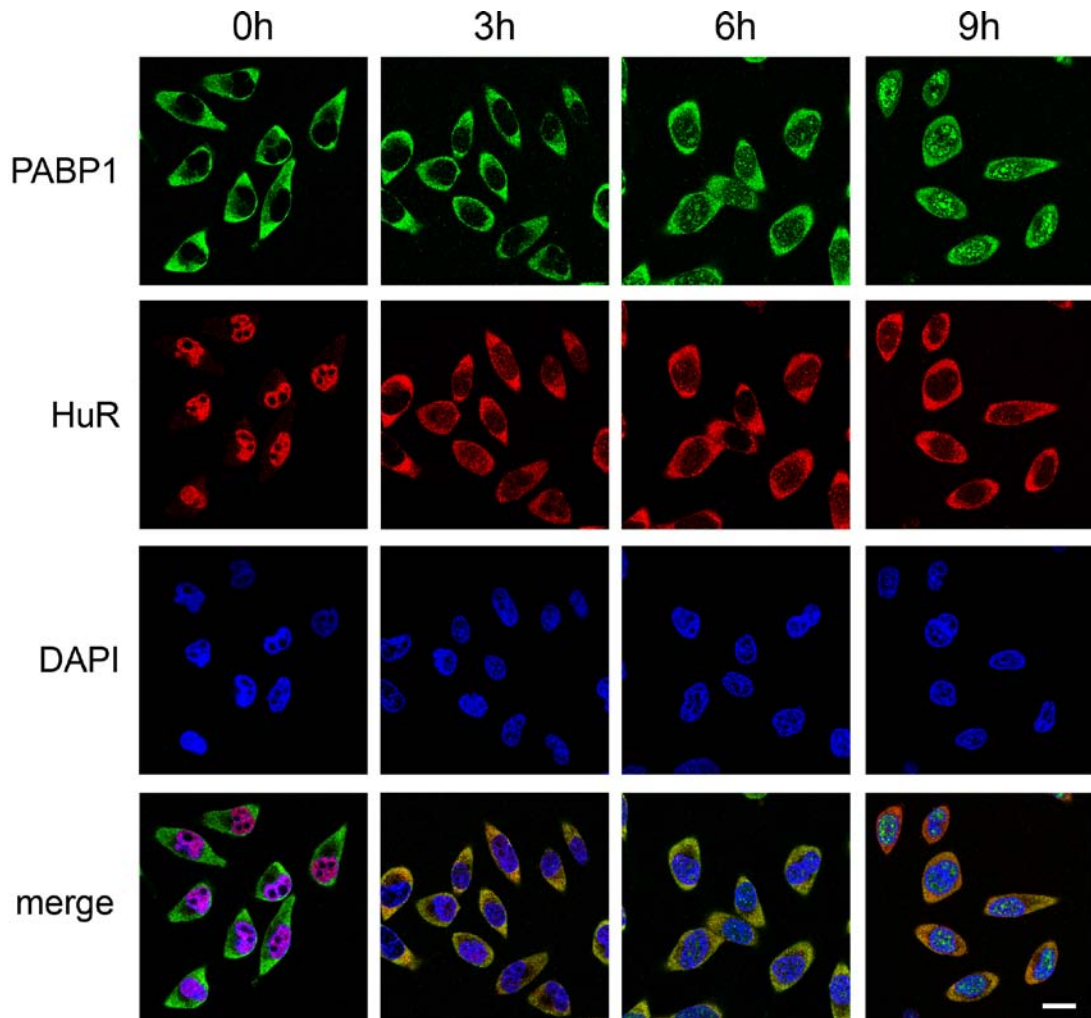


Figure 6.6 PABP1 relocalises to the nucleus following prolonged actinomycin D treatment. HeLa cells were treated with 5 μ g/ml actinomycin D for the indicated time (hours). Cells were stained by immunofluorescence for HuR (red) or PABP1 (green) and counterstained with DAPI. Confocal images (x63) are shown; scale bar represents 20 μ m.

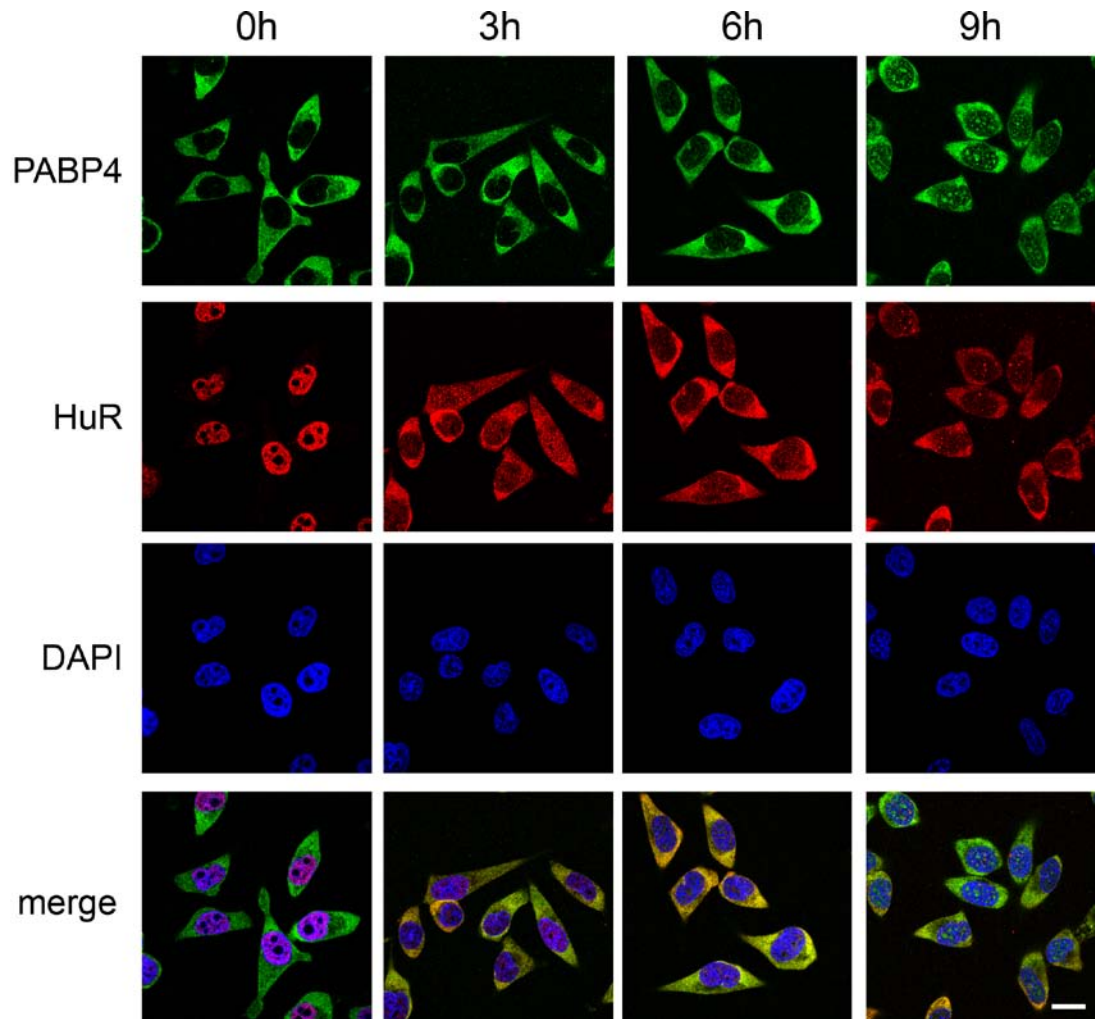


Figure 6.7 PABP4 relocalises to the nucleus following prolonged actinomycin D treatment. HeLa cells were treated with 5 μ g/ml actinomycin D for the indicated time (hours). Cells were stained by immunofluorescence for HuR (red) or PABP4 (green) and counterstained with DAPI. Confocal images (x63) are shown; scale bar represents 20 μ m.

It is surprising that PABP relocalisation to the nucleus required prolonged actinomycin D treatment. This drug is known to act very rapidly to inhibit transcription, with more than 90% reduction in RNA synthesis reported within 10 minutes (Sawicki and Godman, 1971) and HuR was relocalised to the cytoplasm within 3 hours treatment (Figure 6.6 and 6.7). To directly ascertain whether transcription is rapidly and effectively inhibited in actinomycin D treated cells, extracts were western blotted for RNAPII-ser5p.

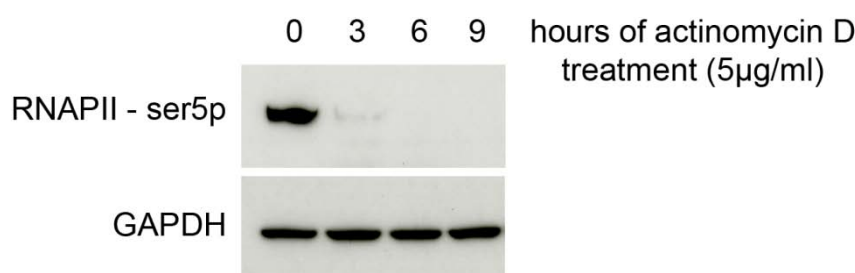


Figure 6.8 Transcription is inhibited within 3 hours actinomycin D treatment. HeLa cells were treated with 5µg/ml actinomycin D for the indicated time. Extracts (10µg) were western blotted for phosphorylated RNAPII (ser5) and GAPDH.

While GAPDH levels remained relatively constant throughout treatment, RNAPII-ser5p was massively reduced within 3 hours (Figure 6.8). This shows that at 3 hours of actinomycin D treatment, transcription is inhibited but PABP1 and PABP4 remain cytoplasmic (Figures 6.6 and 6.7). Though other studies report nuclear relocalisation of GFP-PABP1 within 3 hours, GFP-tagged PABP1 is known to be prone to nuclear localisation when over-expressed (Afonina et al., 1998; Khacho et al., 2008b) which perhaps explains the temporal difference in results.

These results suggest a time-dependent response of PABP1 and PABP4 localisation to transcriptional inhibition, an effect which has not previously been reported. A delayed response to actinomycin D suggests that PABP1 and PABP4 may accumulate very slowly in the nucleus or that the block to PABP nuclear export may require an event downstream of transcriptional inhibition.

6.2.5 Is PABP localisation dependent on mRNA?

The mechanism for transcriptional dependence of PABP1 nuclear export is not understood and is likely to rely on events downstream of inhibition of RNA polymerase activity. Interestingly, active transcription has been demonstrated to be required for mRNA export since a fluorescently-labelled mRNA injected into the nucleus fails to be exported to the cytoplasm in the presence of transcriptional inhibitors actinomycin D, DRB and α -amantin (Tokunaga et al., 2006). After UV treatment PABP1 and PABP4 in the nucleus were observed to localise to splicing speckles (Chapter 5; Figure 5.6), which are known to contain poly(A)⁺ RNA (Lamond and Spector, 2003). PABP1 and PABP4 also localise to stress granules (Chapter 4; Figure 4.1) which contain poly(A)⁺ RNA (Kedersha et al., 1999). This raises the question of whether the localisation of mammalian PABPs is linked to mRNA localisation? And whether nuclear export of mammalian PABPs is dependent on mRNA export, as has been demonstrated in budding and fission yeast (Brune et al., 2005; Thakurta et al., 2002)?

6.2.5.1 Using fluorescent *in situ* hybridisation to detect Poly(A)⁺ RNA

To investigate these possibilities, I used fluorescent *in situ* hybridisation (FISH) to examine the distribution of poly(A)⁺ RNA. Poly(A)⁺ RNA *in situ* hybridisation has been used previously to monitor the localisation of mRNAs to stress granules and investigate mRNA export (Chakraborty et al., 2006; Kedersha et al., 1999). FISH was carried out according to the method of N. Gilks in the laboratory of Paul Anderson (http://openwetware.org/wiki/Poly_A_RNA_in_situ_protocol) using a Cy-3-oligo-dT₄₀ probe. To ensure this method effectively and specifically detected poly(A)⁺ RNA, HeLa cells were subject to poly(A)⁺ RNA FISH with or without prior treatment with RNase. Poly(A)⁺ RNA FISH of untreated HeLa cells revealed nuclear and cytoplasmic staining (Figure 6.9A), consistent with published data (for example, (Kapadia et al., 2006). RNase treatment effectively abolished the

FISH signal (Figure 6.9B) indicating that the pattern detected in Figure 6.9A corresponds to RNA. Treatment of HeLa cells with arsenite relocalised the cytoplasmic FISH signal to stress granules (Figure 6.9D) indicating that this method specifically detects poly(A)⁺ RNA and can effectively detect changes in poly(A)⁺ RNA distribution.

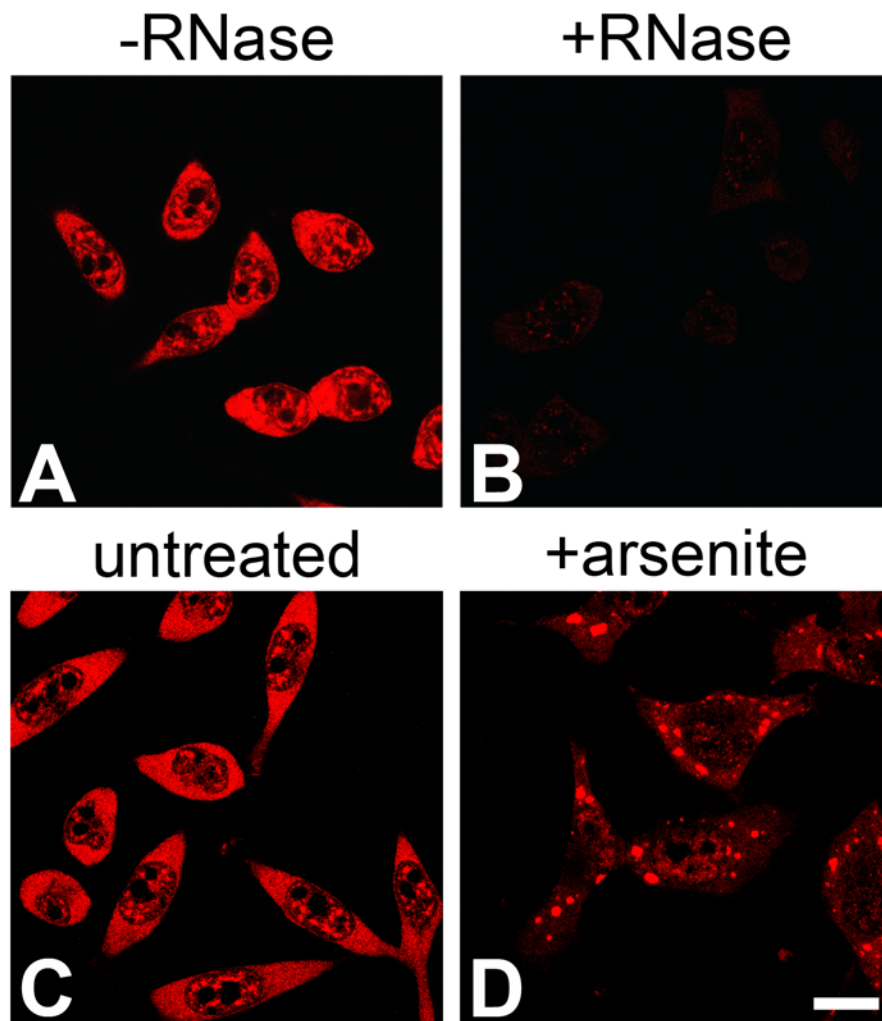


Figure 6.9 Poly(A)⁺ RNA *in situ* controls. HeLa cells were stained by *in situ* using a Cy-3-oligo-dT₄₀ probe. Cells in **A** and **B** were incubated with PBS or 0.1mg/ml RNase A for 30 minutes at 37°C prior to fixation, as indicated. Cells in **C** and **D** were not treated or arsenite stressed (1 hour, 0.5mM) as indicated. Confocal images (x63) are shown; scale bar represents 20µm.

6.2.5.2 Is the distribution of Poly(A)+ RNA affected by UV?

To test whether the distribution of poly(A)+ RNA changes following UV irradiation, HeLa cells were treated with UVC and stained by poly(A)+ RNA FISH followed by immunofluorescence for PABP1 (Figure 6.10 and further low magnification images in Appendix 5A) or PABP4 (Appendix 5B). Surprisingly, poly(A)+ RNA accumulated in the nucleus following UV treatment (Figure 6.10). While a significantly diminished cytoplasmic FISH signal was observed at 6 hours post treatment, by 9 hours post treatment poly(A)+ RNA was very obviously more abundant in the nucleus than the cytoplasm and appeared almost nuclear restricted by 15 hours post treatment (Figure 6.10). Accumulation of poly(A)+ RNA in the nucleus after UV is indicative of a block in mRNA export which has not been previously reported after UV irradiation, however, it is known that transcription and mRNA export are coupled and several mRNA export proteins have been shown to be recruited co-transcriptionally (Reed, 2003). Nuclear accumulation of poly(A)+ RNA appears to precede that of PABP1 and PABP4 relocalisation (Figure 6.10, Appendix 5B), indicating that it may contribute to their relocalisation.

UV induced relocalisation of PABP1 and PABP4 to the nucleus is not unique to HeLa cells and is also observed in mouse 3T3 cells (Figure 5.1). To test whether nuclear accumulation of poly(A)+ RNA is also observed in 3T3 cells after UV these cells were subject to poly(A)+ RNA FISH. In untreated 3T3 cells poly(A)+RNA was detected in the nucleus and throughout the cytoplasm (Figure 6.11, top panel). At 15 hours after UV treatment however, as in HeLa cells poly(A)+ RNA appeared restricted to the nucleus (Figure 6.11, lower panel). This further suggests that nuclear accumulation of poly(A)+ RNA may be a conserved response to UV in mammalian cells.

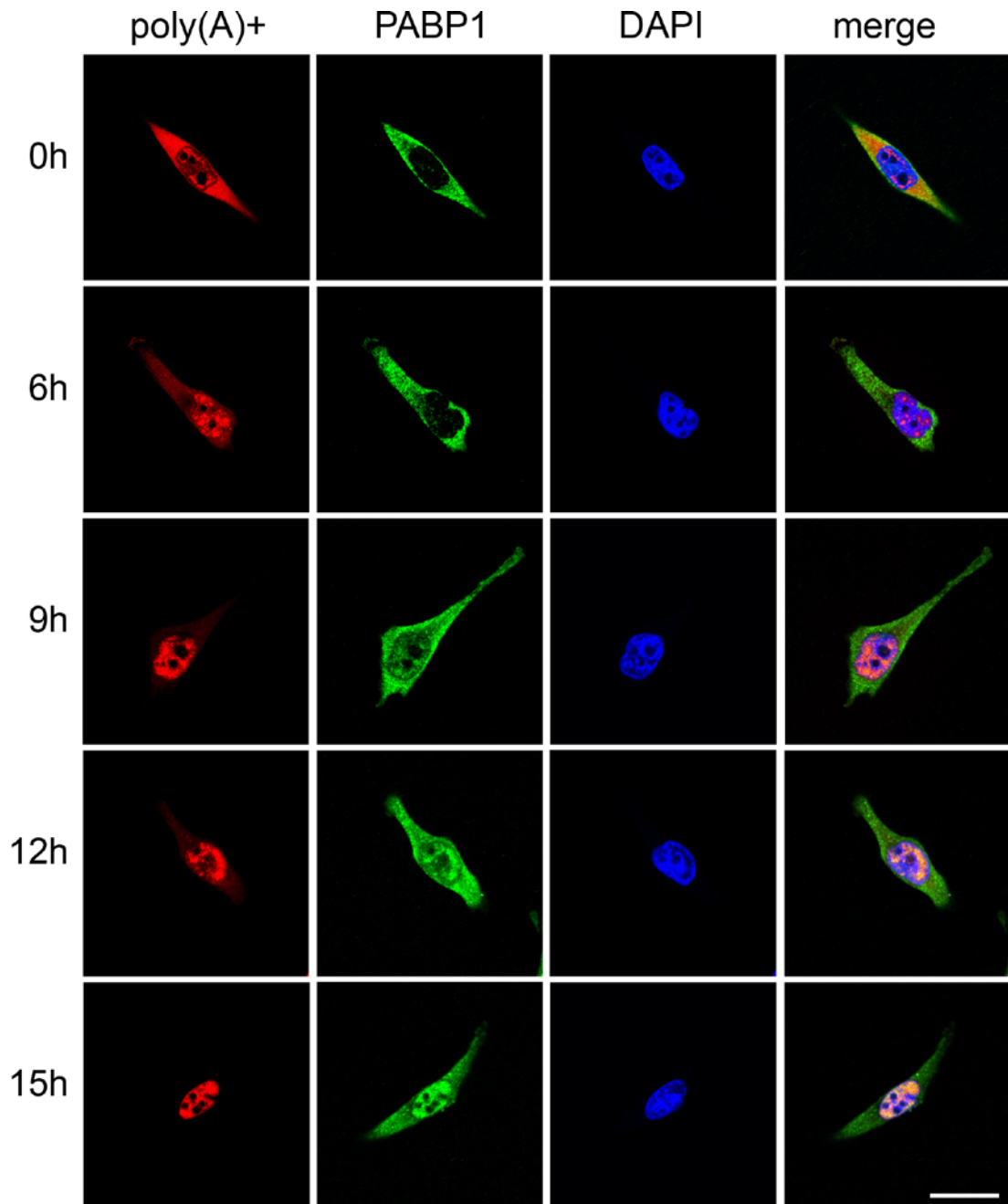


Figure 6.10 Poly(A)+ RNA *in situ* timecourse following UV. HeLa cells were treated with UV ($50\text{J}/\text{M}^2$ UVC) and incubated for the indicated time (hours). Cells were stained for poly(A)+ RNA by *in situ* using a Cy-3-oligo-dT₄₀ probe (red), stained by immunofluorescence for PABP1(green) and counterstained with DAPI (blue). Confocal images (x63) of representative single cells are shown; scale bar represents 20 μm .

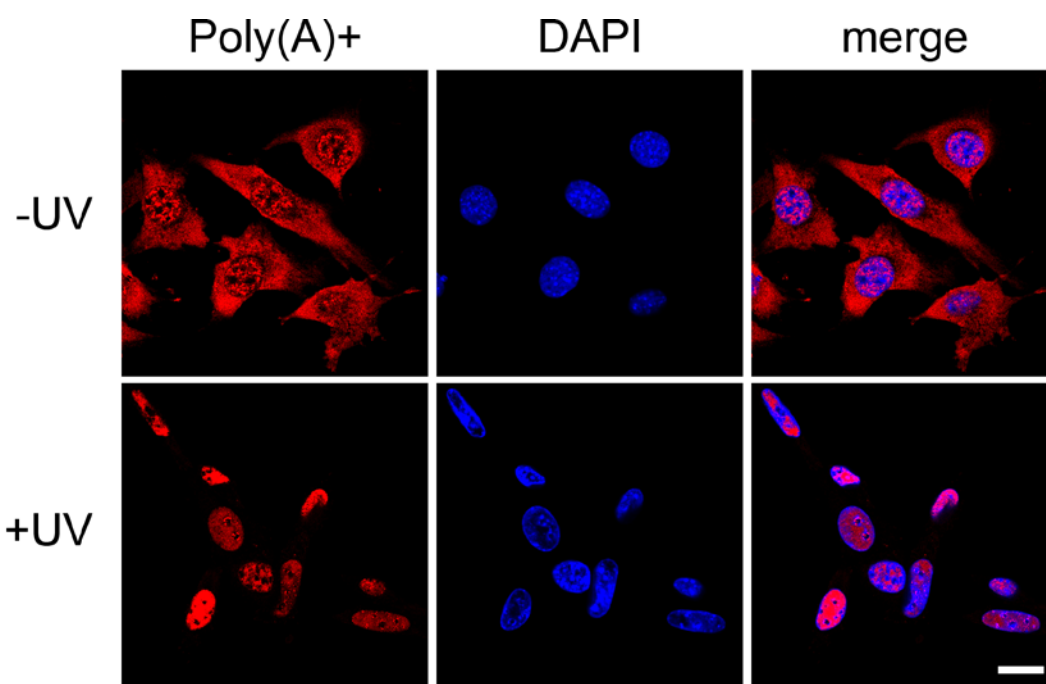


Figure 6.11 Poly(A)+ RNA *in situ* staining of 3T3 cells after UV. Cells were treated with UV ($50\text{J}/\text{M}^2$ UVC +15 hours incubation) and stained for poly(A)+ RNA by *in situ* using a Cy-3-oligo-dT₄₀ probe (red) and counterstained with DAPI (blue). Confocal images (x63) are shown; scale bar represents 20 μm .

6.2.5.3 Is the distribution of Poly(A)+ RNA affected by actinomycin D treatment?

Relocalisation of PABP1 and PABP4 to the nucleus is effected by prolonged actinomycin D treatment, but may be an indirect consequence of transcriptional inhibition (Figures 6.6-6.8). Transcriptional inhibition also inhibits mRNA export (Tokunaga et al., 2006) and a persistent population of poly(A)+ RNA is known to remain in nuclei following prolonged transcriptional inhibition by DRB and α -amanitin (Huang et al., 1994). To examine the distribution of poly(A)+ RNA after actinomycin D treatment, cells were stained by poly(A)+ RNA FISH which was followed by immunofluorescence staining for PABP1.

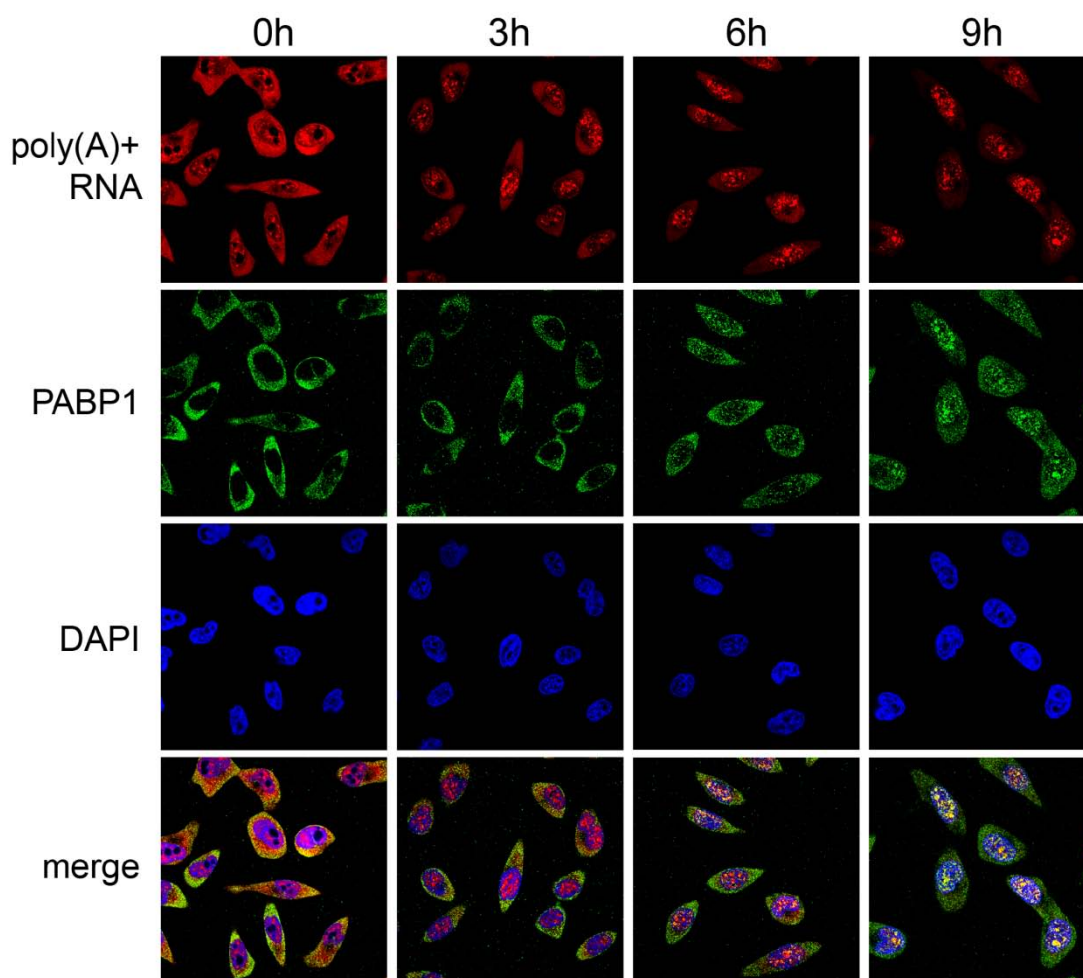


Figure 6.12 Poly(A)+ RNA *in situ* during actinomycin D timecourse. HeLa cells were treated with actinomycin D (5 μ g/ml) for the indicated time (hours). Cells were stained for poly(A)+ RNA by *in situ* using a Cy-3-oligo-dT₄₀ probe (red), stained by immunofluorescence for PABP1(green) and counterstained with DAPI (blue). Confocal images (x63) are shown; scale bar represents 20 μ m.

As expected, a reduction in cytoplasmic poly(A)+ RNA FISH intensity was detected after 3 hours of actinomycin D treatment (Figure 6.12) reflecting the rapid inhibition of transcription. By 9 hours treatment, poly(A)+ RNA was barely detectable in the cytoplasm, however it remained evident in the nucleus (Figure 6.12), consistent with the effect of prolonged treatment with other transcriptional inhibitors (Huang et al., 1994). Localisation of PABP1 and PABP4 to the nucleus following actinomycin D treatment therefore occurs after the reduction in cytoplasmic poly(A)+ RNA but, unlike after UV-irradiation is not accompanied by a significant increase in nuclear poly(A)+RNA.

6.2.5.4 The effect of ICP27 transfection on localisation of PABP1, PABP4 and Poly(A)+ RNA

Recently, HSV-1 infection has been shown to result in nuclear relocalisation of PABP1 (Dobrikova et al., 2010; Salaun et al., Submitted). Infection with wild-type HSV1 does not affect the localisation of poly(A)+ RNA (Johnson and Sandri-Goldin, 2009) suggesting that the mechanism of PABP1 localisation may be different in HSV1 infection to UV irradiation. Transfection with the viral protein ICP27 has been reported to relocalise PABP1 to the nucleus (Dobrikova et al., 2010) however, relocalisation of PABP1 during HSV-1 infection is independent of ICP27 since viruses lacking the protein are able to relocalise PABP1 (Salaun et al., Submitted). ICP27 is a multifunctional nuclear-cytoplasmic shuttling protein which inhibits cellular splicing, binds and facilitates nuclear export of viral RNAs (reviewed in (Sandri-Goldin, 2008; Smith et al., 2005) and is also thought to stimulate translation of specific viral mRNAs (Ellison et al., 2005; Fontaine-Rodriguez and Knipe, 2008; Larralde et al., 2006). ICP27-induced relocalisation of PABP1, while possibly not relevant to infection, does provide a useful tool to examine the requirements of PABP nuclear relocalisation.

Thus, cells were transfected with GFP-tagged ICP27 to test for any effects on the localisation of PABP4 and whether, as for actinomycin D and UV treatment, nuclear relocalisation of PABP1 correlates with any change in the distribution of poly(A)+ RNA. Western blotting for GFP confirmed expression of GFP-ICP27 protein at the predicted size of 90kDa (27kDa GFP + 63kDa ICP27 (Smith et al., 2005)) (Figure 6.13A). Western blotting for RNAPII-ser5p also showed that its levels are unchanged after transfection with ICP27-GFP (Figure 6.13B) indicating that transcription remains active. Immunofluorescence confirmed that ICP27 transfection leads to translocation of PABP1 to the nucleus, which was observed in approximately 75% of GFP-ICP27 expressing cells (Figure 6.13C). PABP4 was also revealed to relocalise to the nucleus in approximately 60% of cells transfected with GFP-ICP27. Poly(A)+ RNA FISH revealed that levels of poly(A)+ RNA appeared decreased in the cytoplasm and increased in the nucleus of ~90% GFP-ICP27 transfected cells (Figure 6.13C).

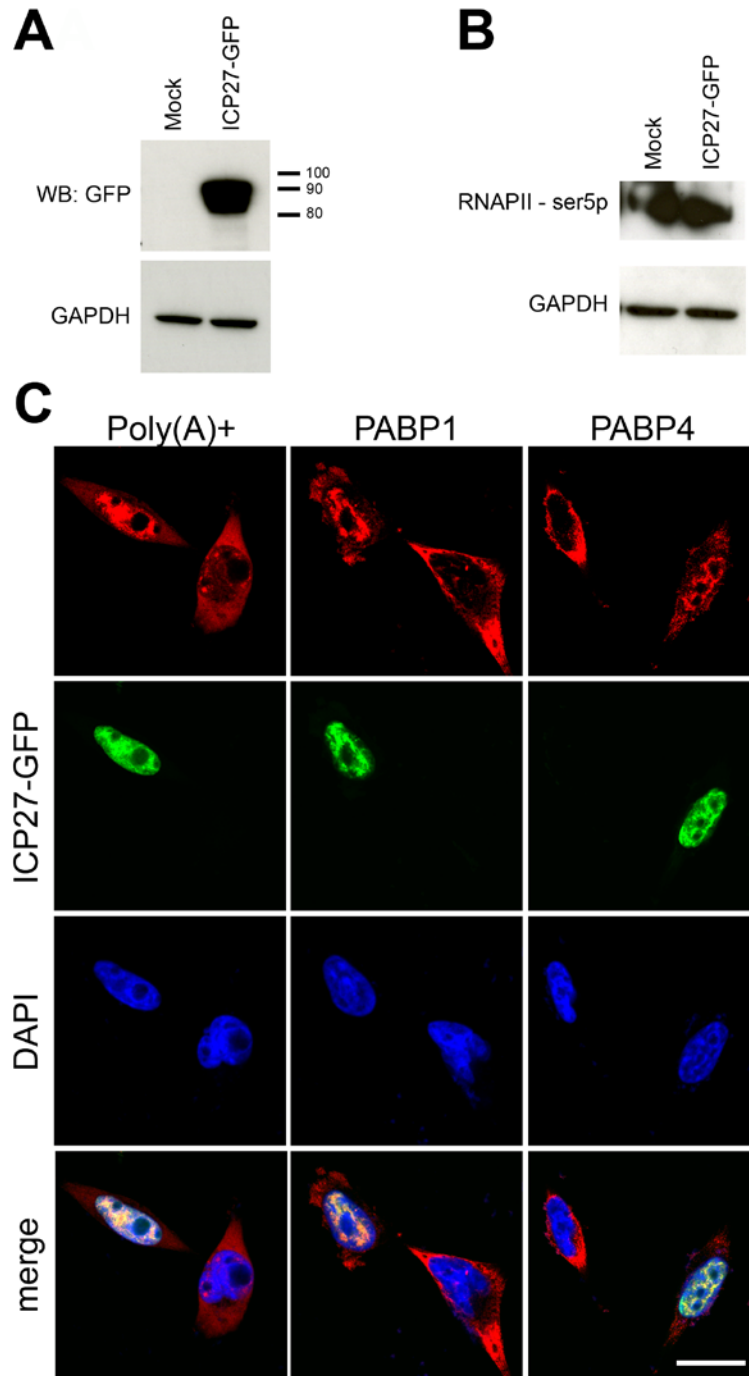


Figure 6.13 ICP27 transfection results in nuclear accumulation of PABP1, PABP4 and poly(A)+ RNA. HeLa cells were transfected with ICP27-GFP (200ng/ml) or mock transfected and incubated for 18 hours. Extracts were western blotted for GFP and GAPDH (**A**) or phosphorylated RNAPII (ser5) and GAPDH (**B**). Cells were stained for poly(A)+ RNA by *in situ* using a Cy-3-oligo-dT₄₀ probe or stained by immunofluorescence for PABP1 or PABP4 (red) as indicated and counterstained with DAPI (blue) (**C**). Confocal images (x63) are shown; scale bar represents 20µm.

This further example of poly(A)⁺ RNA distribution changes coincident with nuclear accumulation of PABP1 and PABP4 supports the notion that mRNA localisation or export may be involved in mechanism of PABP nuclear translocation. The observed block to mRNA export caused by transfected ICP27 has not been previously reported but is consistent with the characterised inhibition of cellular splicing by ICP27 (Hardy and Sandri-Goldin, 1994) which would be expected to inhibit mRNA export (Valencia et al., 2008). During infection ICP27 promotes export of viral mRNAs and binds the export protein TAP and the adaptor protein REF (Chen et al., 2005; Chen et al., 2002; Koffa et al., 2001). Ectopic expression of ICP27 outwith the context of infection may also therefore sequester REF and TAP inhibiting cellular mRNA export.

6.2.5.5 Hypotheses for the mechanism of UV-induced nuclear relocalisation of PABP1 and PABP4

UV irradiation and ICP27 transfection result in nuclear localisation of PABP1 and PABP4 accompanied by an accumulation of poly(A)⁺ RNA in the nucleus consistent with a block in mRNA nuclear export. Actinomycin D treatment also relocalises PABP1 and PABP4, is known to inhibit mRNA export (Tokunaga et al., 2006) and results in a reduced abundance of cytoplasmic poly(A)⁺ RNA but no increase in nuclear poly(A)⁺ RNA. These observations could be explained in four ways. Firstly, poly(A)⁺ RNA and PABPs accumulate in the nucleus after UV independently i.e. mRNA localisation/export does not contribute to localisation of PABPs. Secondly, poly(A)⁺ RNA anchors PABP1 and PABP4 in the nucleus after UV, preventing their nuclear export. Nuclear accumulation of PABPs following actinomycin D treatment, when the nuclear poly(A)⁺ RNA levels do not increase, argues against this explanation. Thirdly, mRNA export is dependent on PABP nuclear export, leading to an accumulation of RNA in the nucleus when PABP1 and PABP4 are localised there by another independent mechanism. The accumulation of poly(A)⁺ RNA after UV appears to precede that of PABP1 and PABP4 raising doubt on this mechanism. Finally, PABP1 and PABP4 may require active mRNA export to exit the nucleus. The latter two hypotheses are directly testable.

6.2.6 Is mRNA export dependent on PABP nuclear export?

Whether PABP1 or PABP4 is important for bulk mRNA export has not been tested in mammalian cells. In order to address this question, HeLa cells were treated with PABP1 and/or PABP4 specific siRNAs (Chapter 4.2.2) and subject to poly(A)+ RNA FISH. In cells treated with siRNAs the distribution of poly(A)+ RNA was unaltered compared to mock treated cells (Figure 6.14) indicating that reduced abundance of PABP1 and/or PABP4 does not affect bulk poly(A)+ RNA nuclear export in HeLa cells.

These results are consistent with experiments in *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, Pab-1 mutants, made viable by a suppressor mutation in a ribosomal protein gene, show no deficit in export of bulk poly(A)+ RNA, although a delay in the export of a specific message was observed, also seen in yeast expressing a Pab1 mutant unable to enter the nucleus (Brune et al., 2005). In *S. pombe* PABP mutants, no changes in bulk poly(A)+ RNA distribution were noticed however, over-expression of PABP could partially relieve an mRNA export block in *rae1* mutants (Thakurta et al., 2002). Therefore, although no inhibition of bulk mRNA export was detected following PABP1/4 knockdown in HeLa cells, there may be subtle or mRNA-specific effects that were not detected by poly(A)+ RNA FISH. Hence, while PABP1 and PABP4 may play a minor role in mRNA export, their nuclear accumulation seems unlikely to underlie the block in poly(A)+ RNA mRNA export observed after UV.

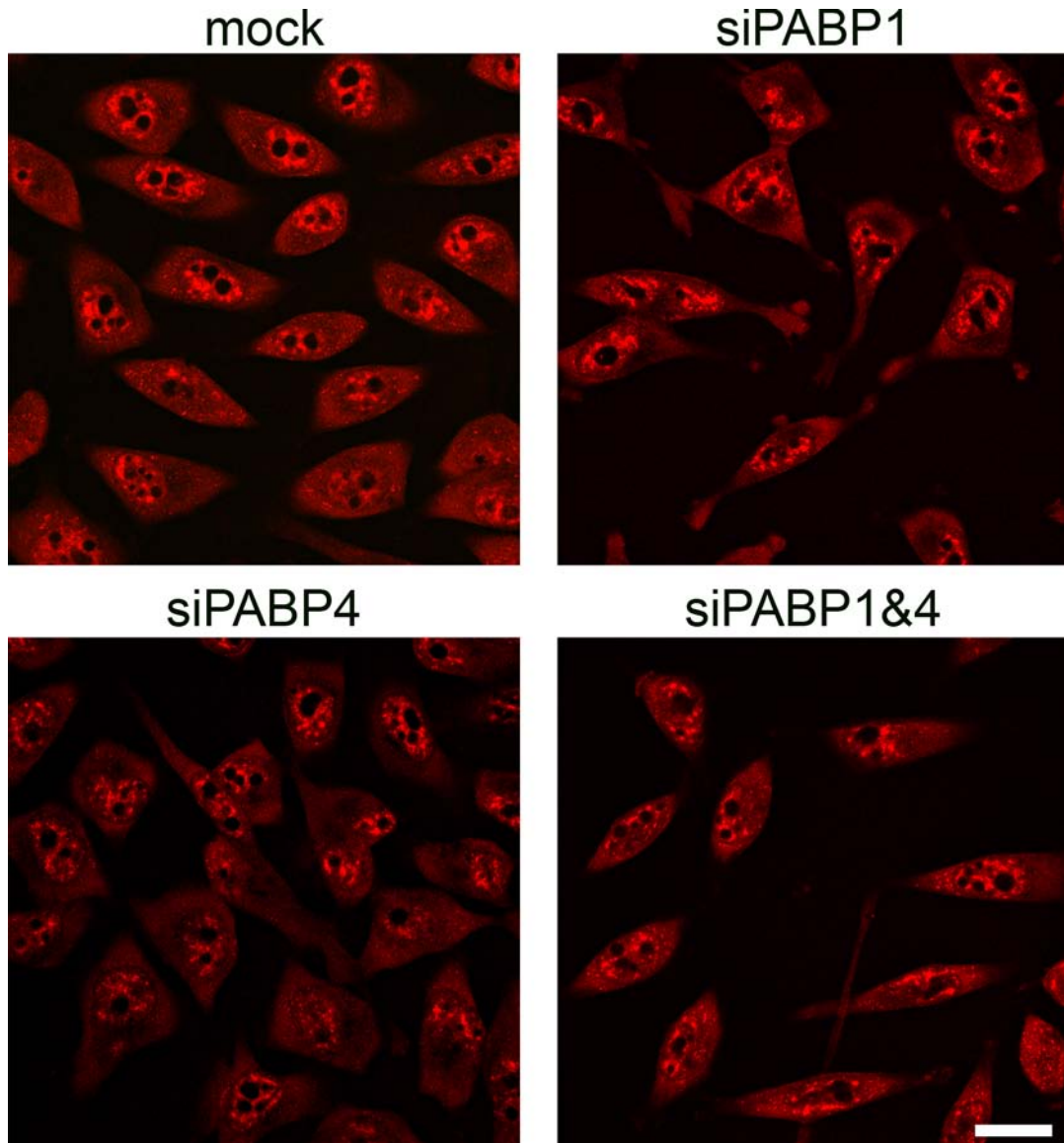


Figure 6.14 Poly(A)+ RNA distribution is not affected by siRNA knockdown of PABP1 and/or PABP4. HeLa cells were transfected with 5nM siPABP1, siPABP4, siPABP1 and siPABP4 or mock transfected as indicated. 48 hours after transfection cells were stained for poly(A)+ RNA by *in situ* using a Cy-3-oligo-dT₄₀ probe. Confocal images (x63) are shown; scale bar represents 20µm.

6.2.7 Is PABP nuclear export dependent on mRNA export?

PABP1 has been shown to bind pre-mRNAs in the nucleus and may exit the nucleus bound to mRNAs. Whether PABP1 or PABP4 nuclear export is dependent on mRNA export or RNA binding has not previously been investigated. To address this question I sought to inhibit bulk mRNA export. Most mRNAs are exported from the nucleus via TAP (see Chapter 1.1.4). TAP forms a heterodimer with p15 to function as an export receptor and also directly binds nucleoporins (Figure 6.15) (Fribourg et al., 2001). Efficient binding of mRNAs requires adapter proteins such as (Hautbergue et al., 2008; Reed and Cheng, 2005). Knockdown of TAP by RNAi has been shown to cause nuclear accumulation of bulk mRNA, detectable by poly(A)⁺ RNA *in situ* (Braun et al., 2002; Hautbergue et al., 2008; Hurt et al., 2009; Johnson et al., 2009; Williams et al., 2005).

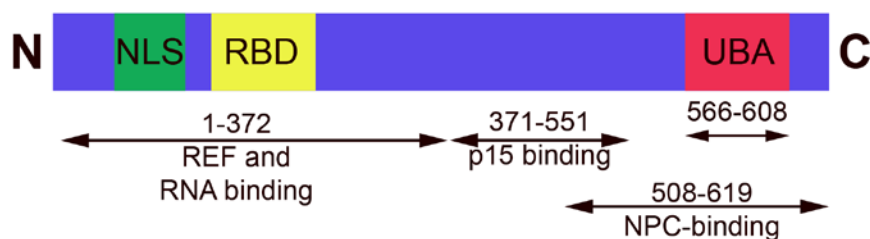


Figure 6.15 Domains and interactions of TAP. Schematic of TAP protein with nuclear localisation sequence (NLS), RNA binding domain (RBD) and ubiquitin-pathway association domain (UBA) labelled. Regions which interact with REF, p15 and the nuclear pore complex (NPC) are indicated. Adapted from (Braun et al., 2001).

6.2.7.1 siRNA knockdown of TAP

In order to inhibit mRNA export, HeLa cells were transfected with four siRNAs targeted against TAP individually or combined and incubated for 48 hours. Western blotting for TAP revealed no significant reduction of TAP protein in siRNA-treated cells compared to GAPDH levels (Figure 6.16A). Extending incubation time post-transfection to 72 hours failed to improve knockdown (Figure 6.16B). As efficient transfection of PABP1 and PABP4 siRNAs was observed under the same conditions, this suggests that these siRNAs are ineffective in the knockdown of TAP.

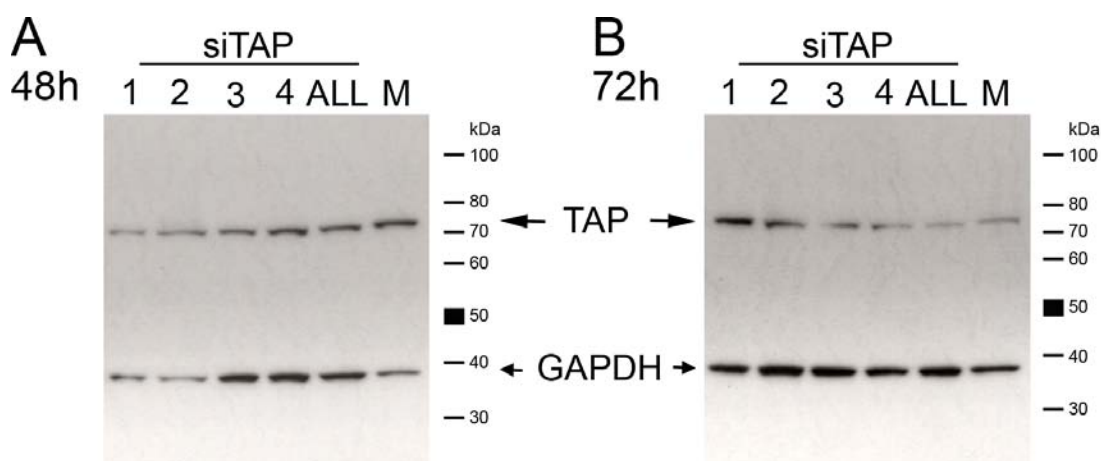


Figure 6.16 Efficiency of TAP siRNA knockdown. HeLa cells were transfected with siRNAs directed against TAP (10nm) or mock transfected (M) as indicated and incubated for 48 hours (A) or 72 hours (B). Extracts (10 μ g) were western blotted for TAP and GAPDH.

6.2.7.2 shRNA knockdown of TAP

As commercially available siRNAs proved ineffective in TAP knockdown (Figure 6.16), I sought to target TAP abundance using reagents with published efficacy. A short hairpin TAP RNAi vector obtained from the lab of Dr SA Wilson (Sheffield, UK) has been demonstrated to knockdown TAP expression and result in a block to mRNA export (Williams et al., 2005). This vector (pSUPERTAP) and the control vector (pSUPER) were transfected into HeLa cells using recommended conditions. Western blotting of transfected extracts showed a small but noticeable decrease in TAP abundance and no change to tubulin levels, blotted as a loading control (Figure 6.17).

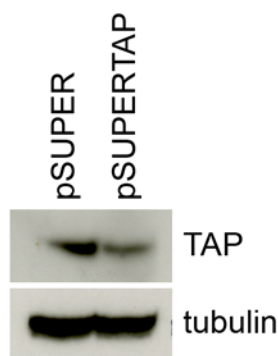


Figure 6.17 Efficiency of TAP shRNA knockdown. HeLa cells were transfected with 1 μ g/ml pSUPER or pSUPERTAP and incubated for 48 hours. Whole cell extracts were western blotted for TAP and α -tubulin.

To test whether a block to mRNA export affects the localisation of PABP1 and PABP4, cells were cotransfected with pSUPER/pSUPERTAP and a GFP-expressing vector (pEGFPC1) to identify transfected cells, then stained by immunofluorescence for PABP1 and PABP4 (Figure 6.18). Approximately ~10% of pSUPERTAP transfected cells were observed to express GFP, indicating that the small decrease in TAP protein abundance detected by western blotting likely reflects a low transfection efficiency rather than poor knockdown efficiency in transfected cells. In ~25% of cells transfected with pSUPERTAP (i.e. GFP-expressing), PABP1 was detectably relocalised to the nucleus compared to ~6% of cells transfected with the control vector (Figure 6.18A and C). Similarly ~22% of cells transfected with pSUPERTAP (i.e. GFP-expressing) showed PABP4 nuclear relocalisation compared to ~4% of cells transfected with the control vector (Figure 6.18B and D). By Fishers two-tailed test the difference between PABP1 and PABP4 localisation (classified as cytoplasmic or detectably relocalised) in cells transfected with the control or TAP RNAi vector was considered to be extremely statistically significant, with a p value in both cases of less than 0.001. This data suggests that nuclear export of PABP1 and PABP4 is at least partially dependent on mRNA export.

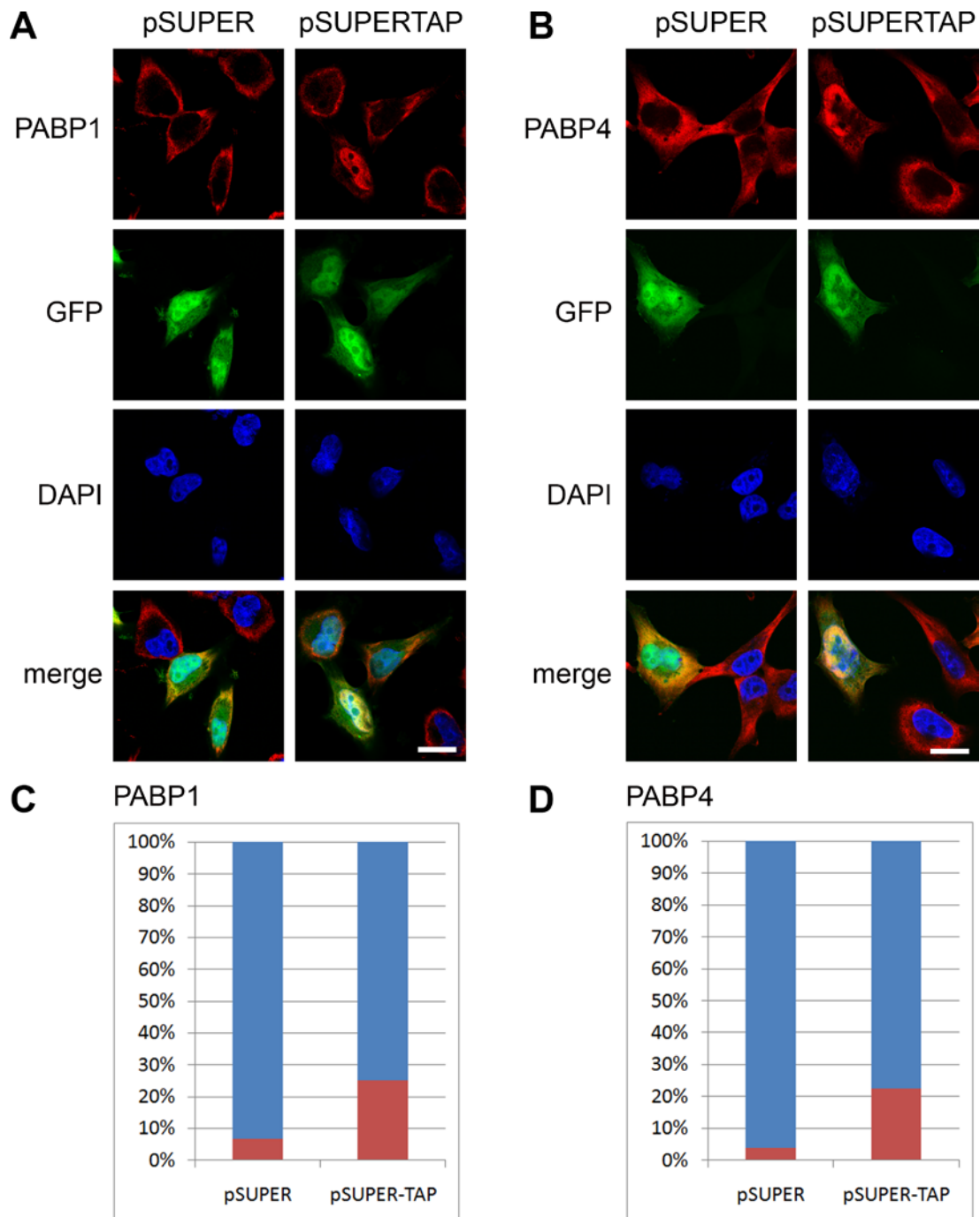


Figure 6.18 Effect of TAP shRNA knockdown on PABP1 and PABP4 localisation. HeLa cells were cotransfected with pEGFP-C1 and pSUPER or pSUPERTAP and incubated for 48 hours. Cells were stained by immunofluorescence for PABP1 (**A**) or PABP4 (**B**) and ~100 GFP-expressing cells were scored for PABP1 (**C**) or PABP4 (**D**) localisation classified as cytoplasmic (blue) or detectably relocalised to nucleus (red). Scale bars represent 20 μ m.

6.3 Discussion

In this chapter I have investigated the mechanism of UV-induced relocalisation of PABPs to the nucleus, systematically addressing the role of the CRM1 pathway, paxillin, transcription and mRNA export.

Inhibition of the CRM1 protein export pathway with leptomycin B did not affect the cytoplasmic localisation of PABP1 or PABP4 (Figures 6.1 and 6.2) indicating that neither protein is reliant on this pathway for its nuclear export. These results are contrary to the results of Woods et al (2002) but consistent with those of Khacho et al (2008). An interaction between PABP1 and the NES-containing protein paxillin has been reported to be important for PABP1 nuclear export however, I could not reproduce this interaction (Figure 6.3B) and so could not assess its importance in the relocalisation of PABPs after UV. Leptomycin B treatment relocalised paxillin to the nucleus (Figures 6.1 and 6.2), though paxillin localisation following UV treatment was unchanged (Figure 6.3A) demonstrating that the localisation of PABP1 is independent of paxillin localisation and vice versa.

The modification status of PABP1 and PABP4 was also investigated by 2D gel electrophoresis and found to be unaltered following UV treatment (Figure 6.4). This indicates that if any changes in post-translational modifications occur on PABP1 or PABP4, they do not affect the pI or the mass/charge ratio of either protein. Detection of PABP1 as a single spot (Figure 6.4) is consistent with published data showing that PABP1 migrates as a single highly basic spot in asynchronously growing HeLa cells (Gorlach et al., 1994). Ma and colleagues however detected additional acidic PABP1 spots in untreated HeLa cells which they suggest represent phosphorylated forms, though the abundance of these additional forms appears to vary between experiments (Ma et al., 2009; Ma et al., 2006). The apparent divergence between the results of myself and Gorlach et al (1994) with Ma et al (2006, 2009) might be explained by differences between HeLa strains in the relative abundance of modified forms of PABP1; work in our lab investigating the modification status of PABP1 in different HeLa strains supports this notion (M. Brook, unpublished data). I found that nuclear localisation of PABP1 after heatshock

reported by Ma et al (2009) was not reproduced in the HeLa cells used here (Appendix 1) and it appears that post translational modification of PABP1 may also be different in the HeLa cells used by our labs.

PABP1 was shown to relocalise to the nucleus following transcriptional inhibition with actinomycin D (Figure 6.6), consistent with published data (Afonina et al., 1998; Khacho et al., 2008b). I further showed that PABP4 also relocalises to the nucleus after actinomycin D treatment (Figure 6.7). Interestingly however, nuclear relocalisation of both proteins was only evident after prolonged treatment. A time-dependent response of PABP localisation to transcriptional inhibitors has not been previously reported, however, other studies have not shown a time course of treatment (Afonina et al., 1998; Khacho et al., 2008b). RNA synthesis is known to be inhibited within minutes of actinomycin D treatment (Sawicki and Godman, 1971) and detection of RNAPII-Ser5p, indicative of active transcription initiation, was almost entirely abolished after 3 hours treatment (Figure 6.8). At 3 hours treatment however PABP1 and PABP4 remained cytoplasmic implying that their nuclear localisation at 6 and 9 hours respectively may require a downstream effect of transcriptional inhibition. Since transcription is inhibited after UV irradiation (Figure 6.5) it is therefore likely that a downstream effect of transcriptional inhibition is also involved in the UV-induced nuclear relocalisation of PABPs.

Analysis of mRNA distribution, revealed that after UV treatment poly(A)+RNA also accumulated in the nucleus of HeLa and 3T3 cells (Figures 6.10 and 6.11). This accumulation appeared to precede PABP1 and PABP4 nuclear accumulation in HeLa cells (Figure 6.10) and is consistent with a block in mRNA export. Cells transfected with the HSV protein ICP27 also accumulated both mRNA and PABPs in the nucleus but showed unaltered levels of RNAPII-Ser5p (Figure 6.13). These observations, and the association of PABP and mRNA nuclear export in budding and fission yeast, lead to the hypothesis that mRNA export may influence PABP nuclear export in mammalian cells.

Nuclear retention of PABPs by anchoring to poly(A)-mRNA in UV-treated and ICP27-transfected cells seems unlikely, since actinomycin D treatment, while associated with inhibition of mRNA export (Tokunaga et al., 2006) and a reduction

in cytoplasmic mRNA, does not result in nuclear accumulation of poly(A)-RNA (Figure 6.12).

Since poly(A)-RNA accumulates in the nucleus of UV-treated cells before PABP1 and PABP4, a role for PABPs in bulk mRNA export seemed unlikely, but had not been formally ruled out. Knockdown of each protein followed by *in situ* demonstrated that bulk mRNA export is not dependent on either PABP1 or PABP4 (Figure 6.14). Since knockdown of PABPN1 leads to nuclear accumulation of poly(A)+ RNA (Apponi et al., 2010), these results underline the divergence of the roles of PABPN1 and cytoplasmic PABPs.

The importance of mRNA export in the export of PABP1 was tested by targeting of the mRNA export protein TAP by RNAi. This led to an accumulation of PABP1 and PABP4 in a significant portion of transfected cells (Figure 6.18). Export of PABP1 and PABP4 therefore appears to be at least partially dependent on active mRNA export.

Recent investigation of the mechanism of KSHV-induced relocalisation of PABP1 appears to support this finding. KSHV infection is associated with an accumulation of poly(A)-RNA in the nucleus detected by poly(A)+ RNA FISH (Lee and Glaunsinger, 2009). This effect is due to the viral protein, SOX (shutoff and exonuclease), transfection of which is sufficient to effect nuclear accumulation of both poly(A)+ RNA and PABP1 (Lee and Glaunsinger, 2009). SOX is usually localised in the nucleus and cytoplasm. When a strong nuclear localisation signal is added to SOX which restricts it to the nucleus there is no change in poly(A) distribution or PABP1 localisation in transfected cells (Covarrubias et al., 2009).

The increased nuclear signal detected by poly(A)+ RNA FISH in KSHV infected and SOX transfected cells is correlated with hyperadenylation of transcripts which is suggested by the authors to induce “host shutoff” i.e. the destruction of cellular messages (Lee and Glaunsinger, 2009). RNAi knockdown of the export protein TAP has also been shown to increase hyperadenylation in addition to inhibiting mRNA export (Hurt et al., 2009). Therefore the increased nuclear poly(A)+ RNA FISH signal observed in UV-treated and ICP27-transfected cells may in fact represent both a block in export and excess polyadenylation since these processes are linked. In SOX transfected cells the hyperadenylation was shown to be

carried out by nuclear poly (A) polymerase (Lee and Glaunsinger, 2009). How the hyperadenylation activity of PAP is activated is unknown. It is tempting to speculate that nuclear PABP1/4 could lead to this hyperadenylation however; whether it precedes PABP1 nuclear localisation remains untested.

Since transcriptional inhibition also results in a block to mRNA export it is difficult to assess whether inhibition of transcription and mRNA export represent separate or overlapping mechanisms affecting PABP localisation after UV-irradiation. There are several approaches we could take to further interrogate the relative contribution of each of these mechanisms. We could attempt to release the block to mRNA export after UV, perhaps by overexpression of the TAP/p15 heterodimer. Coordinate release of nuclear PABP1 and PABP4 to the cytoplasm would indicate that the mRNA export block is the principal basis for their relocalisation. Assuming that the dependence of PABP1/4 nuclear export on mRNA export is mediated by RNA binding, the localisation and response to UV of a mutant PABP unable to bind RNA would also be indicative of the contribution of mRNA export. Conversely, if the regulation of the described PABP1 “transcription-dependent nuclear export motif” (Khacho et al., 2008b) and the homologous region of PABP4 could be shown to be independent of mRNA export, UV-induced nuclear translocation of a reporter protein bearing these motifs would indicate that an alternative effect of transcriptional inhibition, rather than its downstream effect on mRNA export is sufficient to mediate UV-induced relocalisation of PABPs.

In conclusion, I have found that nuclear export of PABP1 and PABP4 is at least partially dependent on mRNA export indicating a novel mechanism for control of PABP localisation. My working model is that after UV treatment, both transcription and mRNA export are inhibited, resulting in nuclear accumulation of PABP1 and PABP4, which may contribute to translational reprogramming. This model requires further interrogation, but may represent a novel mode of regulation of cytoplasmic poly(A)-binding proteins under stress.

Chapter 7: Discussion

PABP4 was previously known to bind poly(A)-RNA and two PABP1 interacting proteins, eRF3 (Cosson et al., 2002a) and TOB (Okochi et al., 2005), and experiments in cell-free extracts hinted that it may support the stability and/or translation of poly(A)- and specific mRNAs (Okochi et al., 2005). Here, I have shown that PABP4 is a predominantly cytoplasmic protein that associates with polysomes (Figures 3.3 and 3.4). Furthermore, PABP4 interacts with eIF4G and PAIP1 (Figure 3.5) - proteins important for PABP1-mediated stimulation of translation. Taken together, these data indicate that PABP4 likely functions similarly to PABP1 to activate translation of mRNAs by promoting end-to-end complex formation.

Knockdown of PABP4 in HeLa cells did not result in a significant reduction of protein synthesis (Figure 4.10). While this may be due to increased abundance or activity of PABP1, it is also possible that PABP4 plays only a minor role in poly(A)-dependent translation in HeLa cells, and may be more important for the regulation of specific mRNAs. However, the ability of PABP4 to stimulate translation remains to be directly tested. A tether assay would allow the ability of PABP4 to stimulate translation to be tested in intact cells, but use of polyadenylated mRNAs in PABP-depleted extracts might also be interesting to test whether a mixed population of PABPs improves translation.

It is also important to investigate whether PABP4 can act as a translational repressor by participating in the regulation of PABP1 translation at the autoregulatory sequence. The increase in PABP1 protein levels upon downregulation of PABP4 (Figure 4.7) suggests that this might be the case and the ability of PABP4 to interact with IMP1 and UNR, form an alternative autoregulatory complex and repress translation should be tested. Interestingly, levels of PABP4 protein did not appear to increase with knockdown of PABP1 suggesting that the translation of PABP4 is not significantly affected by PABP1 levels, even though three out of the four mammalian PABP4 splice forms contain A-rich sequences in their 5'UTR.

The demonstrated interactions between PABP4 and the PAM2-containing proteins PAIP1 (Figure 3.5), eRF3 (Cosson et al., 2002a) and TOB (Okochi et al., 2005), and the high level of conservation of the PABC domain in PABP4 suggests that it likely interacts with other PAM2 containing proteins, such as PAIP2.

Supporting this notion, use of a PAIP2 column to deplete HeLa extract of PABP1 in our lab has been found to also deplete PABP4 (R. Anderson, personal communication), though since PABP4 interacts with PABP1 (Figure 3.5) this could be an indirect effect. A direct interaction between PABP4 and PAIP2 should therefore be tested in the absence of PABP1, for example by a pull-down assay *in vitro*. PAIP2 is involved in maintaining homeostasis of PABP1 activity (Yoshida et al., 2006). An interaction between PABP4 and PAIP2 would therefore imply that PABP4 is similarly regulated, and that PABP4 levels could affect the turnover of PAIP2 protein which would therefore indirectly affect the activity of PABP1.

PABP1 interacts with the Caf1-CCR4 and PAN deadenylases via protein interactions with TOB and PAN3 respectively. PABP4 is known to interact with TOB (Okochi et al., 2005) and may interact with PAN3 since it contains a PAM2 motif and so the ability of PABP4 to stimulate the activity of both the Caf1-CCR4 and PAN deadenylases should be tested. Stimulation of Caf1-CCR4 by PABP4 would explain the dramatic decrease in IL-2 protein synthesis following the addition of TOB with PABP4 to cell-free extracts (Okochi et al., 2005). Whether PABP4 is able to bind GW182 and contribute to microRNA mediated regulation as recently described for PABP1 is also important to test, since miRNAs are emerging as highly important regulators of gene expression. Additionally, the multiple isoforms of PABP4 described here (Figures 3.1 and 3.6) may have different abilities to mediate each of the discussed potential roles for PABP4.

Exploration of the expression patterns of PABP1 and PABP4 in mammalian tissues revealed that these are distinct suggesting functional or regulatory differences between the two proteins may exist. This conclusion is consistent with work on *Xenopus* PABPs in our lab which has found that PABP1, ePABP and PABP4 have non-redundant roles in development. Non-redundant functions would at least go some way to explain the exquisite regulation of PABP1 and PABP4 expression in mammals. PABP1 and PABP4 could differ in several aspects of their function. Firstly, while no incidence of differential protein binding has yet been identified between the two proteins, an exhaustive assessment of the conservation of PABP1's known protein interactome with PABP4 has not been conducted. Therefore, each of these interactions may not be conserved, and where they are, they may not occur

with equivalent affinity. Such differences could have potent effects on PABP4 function, for instance a diminished affinity for PAIP2 would be expected to increase the stability of PABP4's interaction with poly(A) and eIF4G *in vivo* and increase its potential to stimulate translation. Novel protein interactions unique to PABP4 could also lend it new functions not shared with PABP1; the DSP-crosslinking protocol optimized here would be perfectly suited for identifying PABP4 co-immunoprecipitating proteins. Also, since PABP1 is known to bind some RNAs at sequences outside of the poly(A) tail, PABP4 may also bind and affect the translation and metabolism of specific mRNAs. Translational profiling to compare the translation status mRNAs in PABP1- versus PABP4-deficient cells would identify mRNAs that are differentially regulated at the level of translation or mRNA stability. Finally, regulation by post-translational modifications could allow differential control over the functions of PABP1 and PABP4. Differences in PTMs could be identified by mass spectroscopy and their effect on PABP function subsequently interrogated.

While such approaches may well identify differences between PABP1 and PABP4 at the molecular level, the generation of PABP knockout mice would also be informative of the physiological roles of PABPs in mammals. The tissue expression pattern of PABP4 suggests it may be particularly important for muscle or pancreatic function. The type of defects in these tissues might give clues to the type of functions unique to PABP4 and defects in cell-types which express additional PABPs would support a specific and non-redundant role for PABP4 in mammals, as observed in *Xenopus* (Gorgoni et al., Submitted).

Under multiple stress conditions both PABP1 and PABP4 localised to cytoplasmic stress granules (Figures 4.1-4.3). The function of PABP1 at stress granules was previously unknown, however I have now ruled out a scaffolding role since neither PABP1 nor PABP4 were essential for stress granule assembly, consistent with recent findings in budding yeast (Swisher and Parker, 2010). Several FRAP studies have reported that the rate of exchange of PABP1 at stress granules is slower than that exhibited by mRNA (Guil et al., 2006; Kedersha et al., 2000; Kedersha et al., 2005; Mollet et al., 2008; Nadezhdina et al., 2009). This suggests that mRNA and PABP1 may be cycling through SGs independently. To test this

possibility, the exchange of PABP1 and PABP4 at stress granules should be directly compared with that of mRNA in parallel. Whether incorporation of PABPs into stress granules is dependent on mRNA binding could be further addressed by mutation of residues in the RRM of PABP1 and PABP4 likely to be important for RNA binding and tagging with a fluorescent reporter such as GFP. Conveniently, mutations that abolish poly(A)-RNA binding have been characterised for yeast *pab1* (Deardorff and Sachs, 1997). Though protein-protein interactions could also contribute to PABP1/4 recruitment to stress granules, PABP1 is known to interact with several P-body components (GW182 (Eystathioy et al., 2003), PAN3 (Zheng et al., 2008), TOB (Ezzeddine et al., 2007)) but does not localise to P-bodies (Kedersha et al., 1999).

In contrast to early time points after UV irradiation, when PABP1 and PABP4 localise to stress granules (Figures 4.2 and 4.3), at late time points (9 and 12 hours respectively) both were shown to relocalise to the nucleus (Figures 5.3 and 5.4). Given the multiple methods of regulation invested in maintaining active PABP1 levels in the cytoplasm of cells, this relocalisation would be expected to have acute effects on mRNA stability and translation. Protein synthesis following UV irradiation was observed to decrease coincident with PABP1/4 nuclear relocalisation (Figure 5.8), but was also accompanied by a decrease in transcription (Figure 6.5) and a block to mRNA export (Figure 6.10). Thus, while PABP1/4 relocalisation alone might be expected to result in a global inhibition of translation, in the context of reduced cytoplasmic mRNA its contribution to global regulation is less clear. Furthermore the relocalisation was not complete, with PABP4 intensity in the nucleus after UV treatment only ever reaching an intensity equivalent to that in the cytoplasm. Hence relocalisation of PABPs could have more subtle effects than a simple reduction in global translation and could produce a translational reprogramming whereby those mRNAs whose translation is particularly sensitive to PABP levels are disproportionately affected by PABP1/4 relocalisation.

Nuclear relocalisation of PABP1 and PABP4 may reflect a need to remove them from the cytoplasm or a specific requirement for their presence in the nucleus. Knockdown of PABP1 and/or PABP4 did not diminish cell survival after UV (Figure 5.7) indicating that PABPs are not required in the nucleus for a role essential for cell

recovery from UV. However, low levels of PABP1/4 were still present in siRNA treated cells (Figure 4.7) and so an important nuclear function for PABPs cannot be unequivocally ruled out. To address this, the experiment could be repeated in cells in which PABP1 and/or PABP4 are knocked out completely – if indeed these are viable.

Investigation of the mechanism of nuclear relocalisation of PABP1/4 after UV exposure lead to the conclusion that this is likely a consequence of transcriptional inhibition and a block to mRNA export (Figure 7.1). The relative contributions of these overlapping pathways are difficult to dissect, but could be further elucidated as outlined in the discussion of Chapter 6.

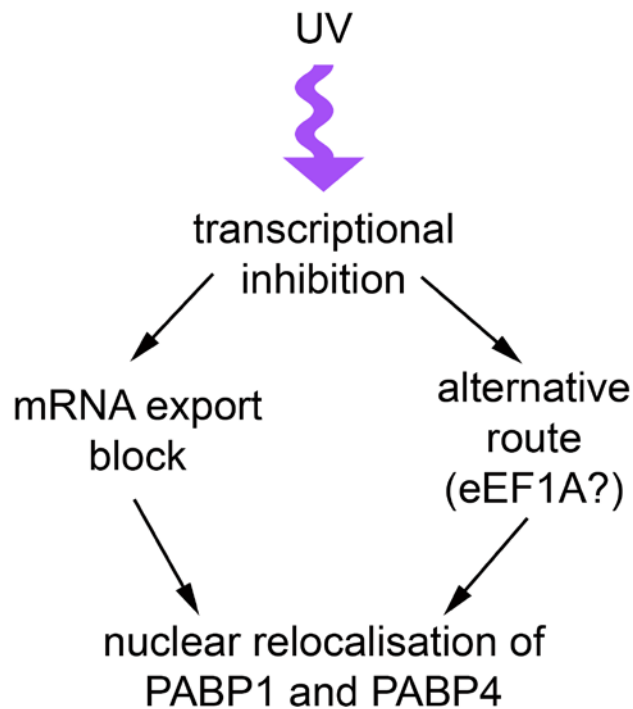


Figure 7.1 Model for the mechanism of PABP1 and PABP4 nuclear relocalisation after UV irradiation

The dependence of PABP1 and PABP4 nuclear export on mRNA can be explained by their ability to bind mRNAs in the nucleus, presumably in competition with PABPN1. Binding to mRNAs would then permit exit through the nuclear pore as part of an mRNP complex exported by the TAP pathway. Transcriptional inhibition may contribute to PABP1/4 relocalisation by blocking mRNA export or

through other means (Figure 7.1). Indeed, a mechanism focusing on the translation elongation factor eEF1A to explain the dependence of transcription on PABP1/4 export has been proposed (Khacho et al., 2008a; Khacho et al., 2008b).

A “transcription-dependent nuclear export motif” (TD-NEM) was characterised in VHL, a tumour suppressor protein that facilitates substrate recognition for an E3 ubiquitin ligase complex (Khacho et al., 2008b). Mutations in VHL that prevent its function or nucleocytoplasmic shuttling lead to Von Hippel–Lindau disease which is associated with tumours in various tissues (Khacho et al., 2008b; Latif et al., 1993). Like PABP1, VHL also relocalises to the nucleus following treatment with transcriptional inhibitors (Lee et al., 1999) and this was demonstrated to be due to a decreased rate of nuclear export (Khacho et al., 2008b). To understand how the export of the TD-NEM might be regulated Khacho and colleagues performed an immunoprecipitation to discover novel binding partners of VHL and identified eEF1A (Khacho et al., 2008a), the second most abundant protein in the cell (Condeelis, 1995). They went on to show that eEF1A associated with the ~20 residue TD-NEM of VHL, that this interaction was diminished upon actinomycin D treatment and that siRNA knockdown of eEF1A resulted in nuclear localisation of GFP-VHL (Khacho et al., 2008a).

To extend these findings to ‘all’ TD-NEM containing proteins, the authors demonstrated that eEF1A co-immunoprecipitated with flag-tagged PABP1, in low stringency conditions and in the absence of RNase, and claimed that knockdown of eEF1A lead to nuclear relocalisation of endogenous PABP1, though this was hardly visible in the figure published (Khacho et al., 2008a). One piece of convincing data was that the TD-NEM of PABP1 (amino acids 296-317) was apparently sufficient to mediate the interaction with eEF1A (Khacho et al., 2008a). While this fragment contains the conserved hexameric RNP2 motif of RRM4, the RRMs are highly structured and so RNA-binding is unlikely to be maintained in this 22 residue peptide. Therefore an interaction of this motif with eEF1A may be direct and not RNA-dependent. However, in order to test the response in localisation of the TD-NEM to UV, I obtained this construct from the Lee group, but found it contained a deletion mutation which removed an aspartic acid residue from the centre of the TD-NEM, casting further doubt on the validity of these interaction experiments.

So how can the apparent nuclear localisation of TD-NEM containing proteins upon eEF1A knockdown be explained? eEF1A is exported from the nucleus with tRNA (Calado et al., 2002) but no role in mRNA export has been reported. One idea might be that active translation mediated by eEF1A is required to keep PABP1 cytoplasmic. Or alternatively constant translation may be required to maintain levels of an unstable protein required for PABP1 nuclear export. However, knockdown of eEF2 resulted in a similar inhibition of protein synthesis to eEF1A knockdown, as measured by metabolic labelling, but did not alter the cytoplasmic localisation of VHL or PABP1 (Khacho et al., 2008a) ruling out these possibilities. However, eEF1A could be involved in the specific regulation of a protein important for this pathway. eEF1A is known to bind the 3' UTR of endothelial nitric oxide synthase (eNOS) mRNA leading to its destabilization (Yan et al., 2008). Interestingly, siRNA knockdown of eEF1A leads to a small increase in eNOS expression (Yan et al., 2008) which would be expected to cause an increase in nitric oxide levels. NO is involved in the signalling to eIF2 α kinases following UV irradiation (Lu et al., 2009) but also leads to oxidative stress which effects transcriptional inhibition (Heine et al., 2008). This is a highly speculative explanation however, until the interaction between eEF1A and PABP1 is reported independently or the role of eEF1A is further explained, I remain sceptical of its validity in the regulation of nuclear export of PABPs.

It is becoming apparent that nuclear relocalisation of PABP1 is a frequent response to viral infection (Smith and Gray, 2010). Since I have found that regulation of PABP4 localisation appears to be similar to that of PABP1, it seems likely that some viruses may also relocalise PABP4. Indeed, our collaborators have found that HSV infection does relocalise PABP4 to the nucleus (S.V. Graham, unpublished). Investigation of other viruses in which PABP relocalisation occurs might help further our understanding of the effects and mechanisms of this relocalisation. In particular, it would be interesting to look at PABP1/4 localisation in cells infected with vesicular stomatitis virus (VSV). VSV inhibits both cellular transcription and mRNA export resulting in an accumulation of polyadenylated mRNAs in the nucleus of infected cells (von Kobbe et al., 2000) and I would predict that PABP1 and 4 would also be nuclear localised.

How do the mechanisms we now believe to influence PABP localisation/nuclear export apply to viral infections known to relocalise PABP? Bunyavirus infection results in a shut-off of host transcription and RNAPII is thought to be dispensable for bunyavirus RNA transcription (Thomas et al., 2004). While the distribution of poly(A)-mRNA in bunyavirus infected cells has not been investigated, bunyavirus mRNAs are not polyadenylated (Blakqori et al., 2009) and so export of poly(A)⁺ mRNAs would be expected to be markedly reduced. KSHV infection results in an accumulation of poly(A)⁺ transcripts in the nucleus (Lee and Glaunsinger, 2009) suggesting that a block to mRNA export may be responsible for PABP1 relocalisation. HSV infection however does not result in a change to poly(A) RNA distribution (Johnson and Sandri-Goldin, 2009), likely because while the transcription and splicing of cellular transcripts is decreased, polyadenylated viral transcripts are synthesized and exported in a TAP-dependent manner (Johnson and Sandri-Goldin, 2009). This implies that the mechanism of PABP1/4 relocalisation during HSV infection is likely different from that after UV and may be regulated by another mechanism, such as interaction with paxillin or viral proteins.

Infection with rotavirus or transfection with the rotavirus protein NSP3 is sufficient to relocalise PABP1 to the nucleus and inhibit cellular translation. (Harb et al., 2008; Montero et al., 2006). NSP3 interacts simultaneously with eIF4G and the cellular protein RoXaN (Vitour et al., 2004). Mutants of NSP3 unable to interact with eIF4G do not relocalise PABP1 and those that cannot interact with RoXaN relocalise PABP1 in fewer cells (Harb et al., 2008) indicating that the interaction with eIF4G is essential for relocalisation and RoXaN promotes it. The most straightforward explanation for these observations is that eviction of PABP1 from translation complexes by NSP3 leads to its nuclear relocalisation. However, in normally growing HeLa cells, PABP1 and PABP4 are almost exclusively cytoplasmic even though significant portions of both proteins do not sediment with ribosomes (Figure 3.4) arguing against this hypothesis. Also, the cellular function of RoXaN is unknown and its effect on the ability of NSP3 to evict PABP1 has not been directly tested, though NSP3 binding to RoXaN does not affect its interaction with eIF4G (Harb et al., 2008). If the only role of NSP3 is to prevent association of

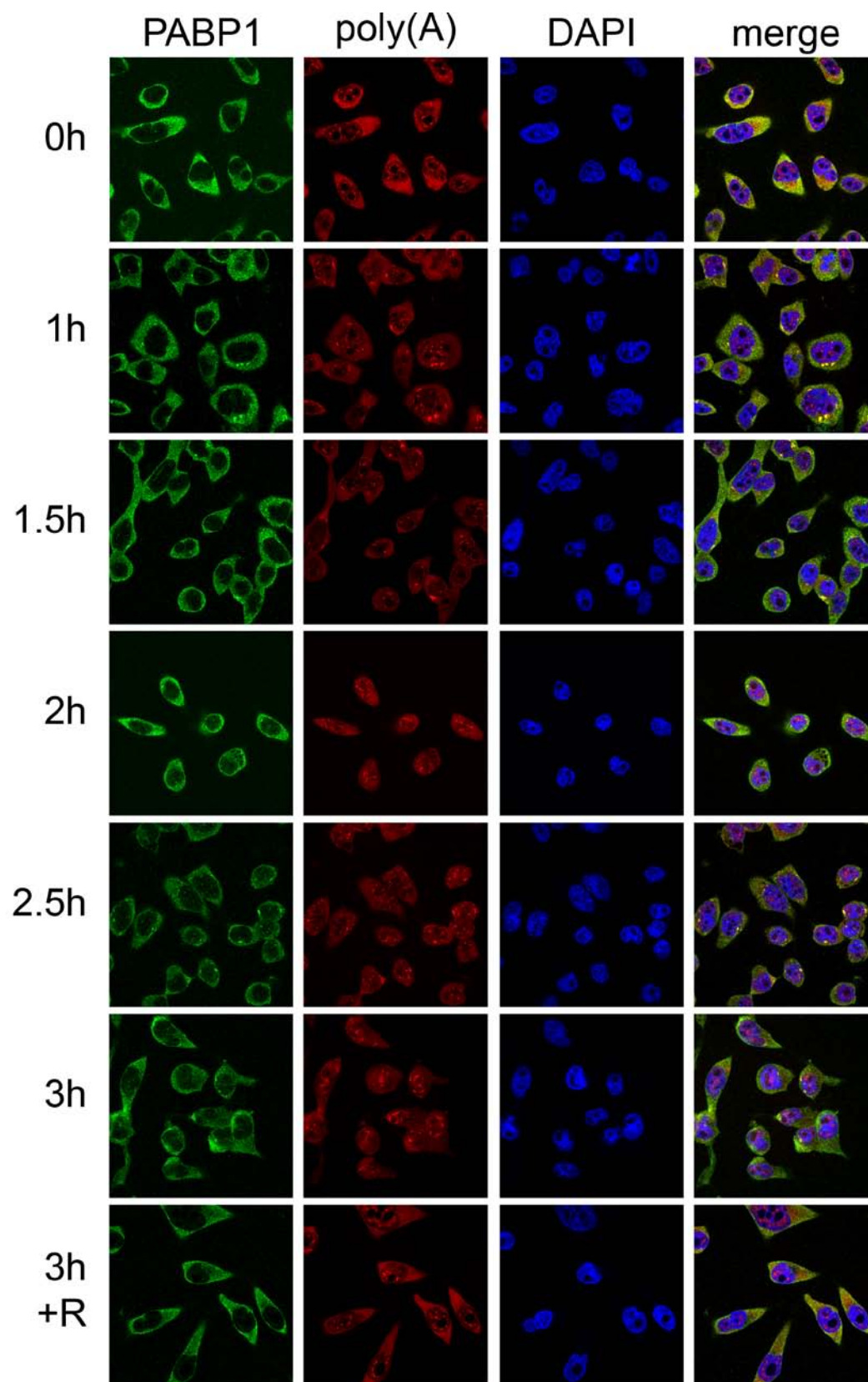
PABP1 with eIF4G we would expect that a mutant PABP1 unable to interact with eIF4G would relocalise to the nucleus, a testable hypothesis.

There remain many questions to solve regarding the regulation and effects of PABP localisation changes. We still know virtually nothing about how PABPs are imported to the nucleus and whether this is subject to regulation. We also do not know of any nuclear function of PABP1 and PABP4 that could justify their energy dependent nuclear import. Could nuclear PABP1 or PABP4 stimulate PAP? Or is binding of cytoplasmic PABPs to messages in the nucleus a strategy to ensure their rapid translation upon entry to the cytoplasm? And how is PABPN1 function affected by the presence of cytoplasmic PABPs in the nucleus - is PABPN1 evicted from the nucleus? Or might PABP1/4 interfere with poly(A)-tail length measurement by PABPN1, which could explain the hyperadenylation of transcripts in TAP knockdown cells and KSHV infected cells.

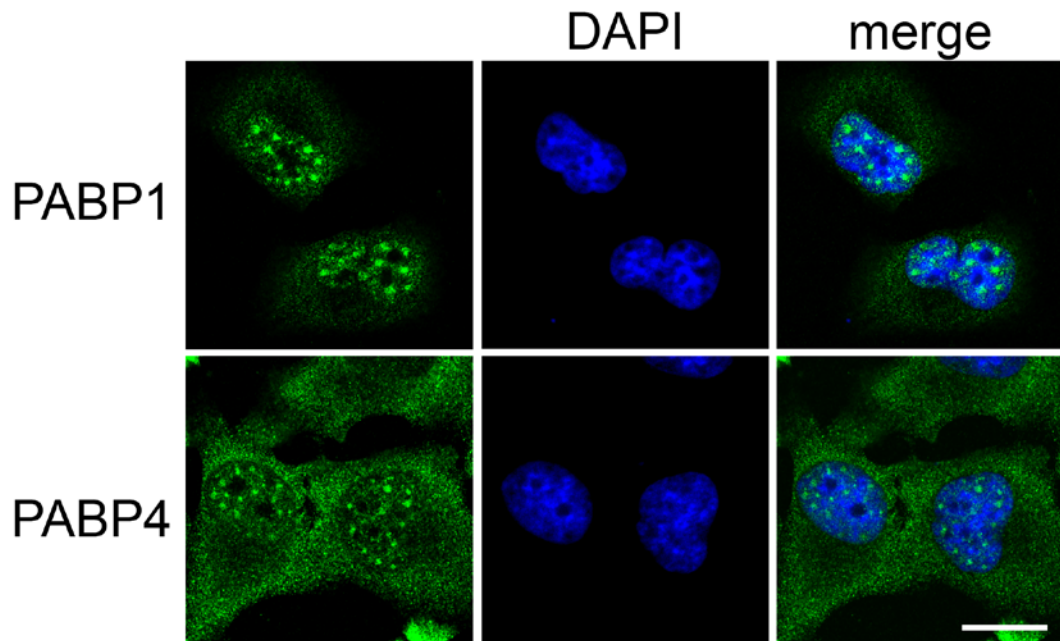
In conclusion, changes in poly(A)-binding protein localisation are emerging as a recurrent consequence of viral infection and certain cell stresses, and the mechanism and effects of this is important to elucidate. We remain far from fully understanding how multiple PABPs function individually, and how their differing functions might complement each other in cells and whole organisms.

Appendices

Appendix 1 PABP1 does not localise to the nucleus after heatshock in HeLa cells. Cells were incubated at 44°C and allowed to recover (R) for 18h where indicated.

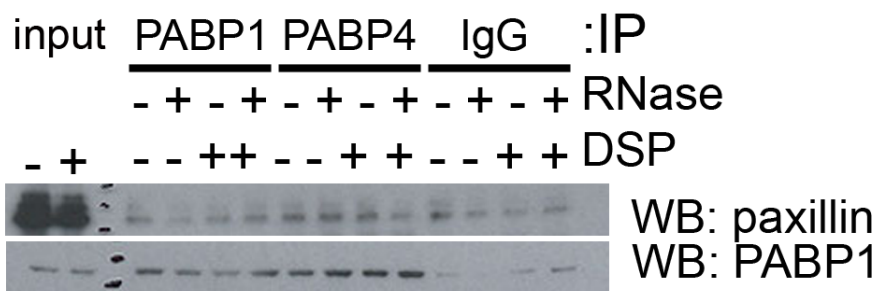


Appendix 2 PABP1 and PABP4 do not colocalise with DAPI-stained DNA after UV treatment. Images from Figure 5.6 merged with DAPI.

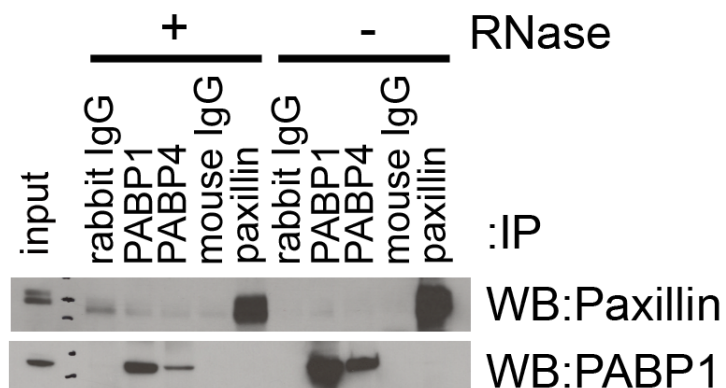


Appendix 3 Paxillin does not co-immunoprecipitate with PABP1 in HeLa cells treated with DSP (**A**) or 3T3 cells (**B**). Immunoprecipitations in A were performed as in Figure 3.5 and immunoprecipitations in B were performed as in Figure 6.3 in the presence of RNase1 where indicated. 10µg is shown as input, corresponding to 2% of material used for each IP.

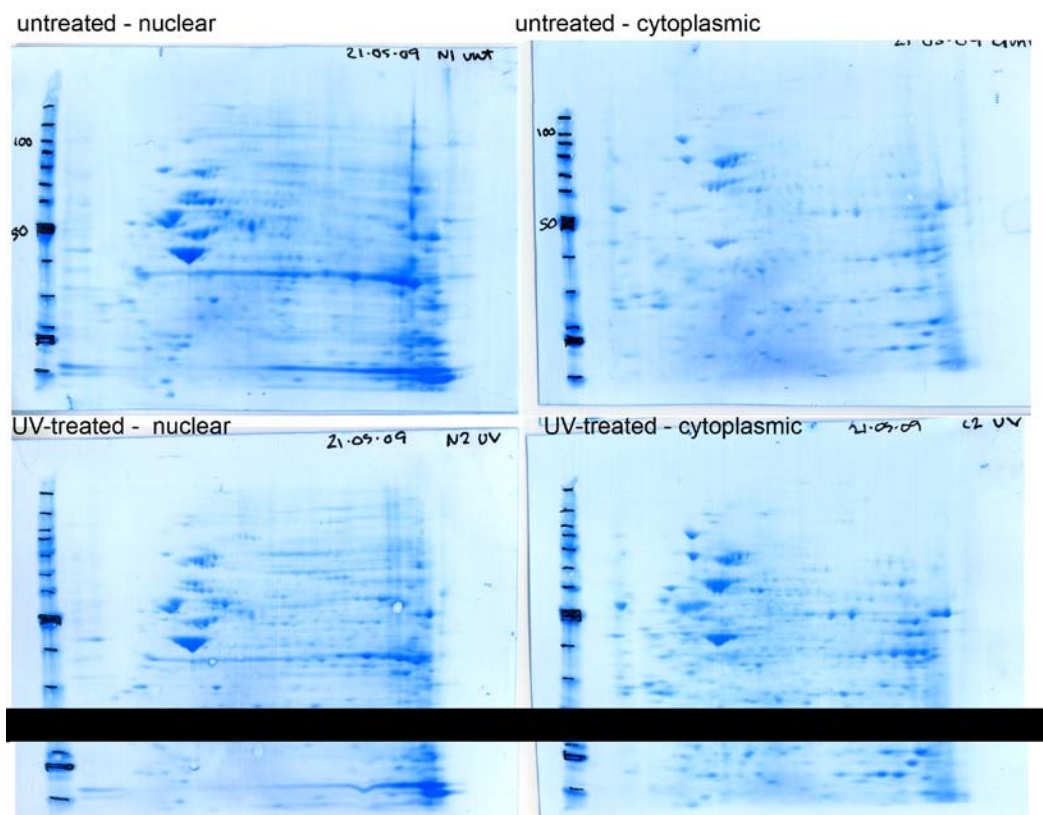
A



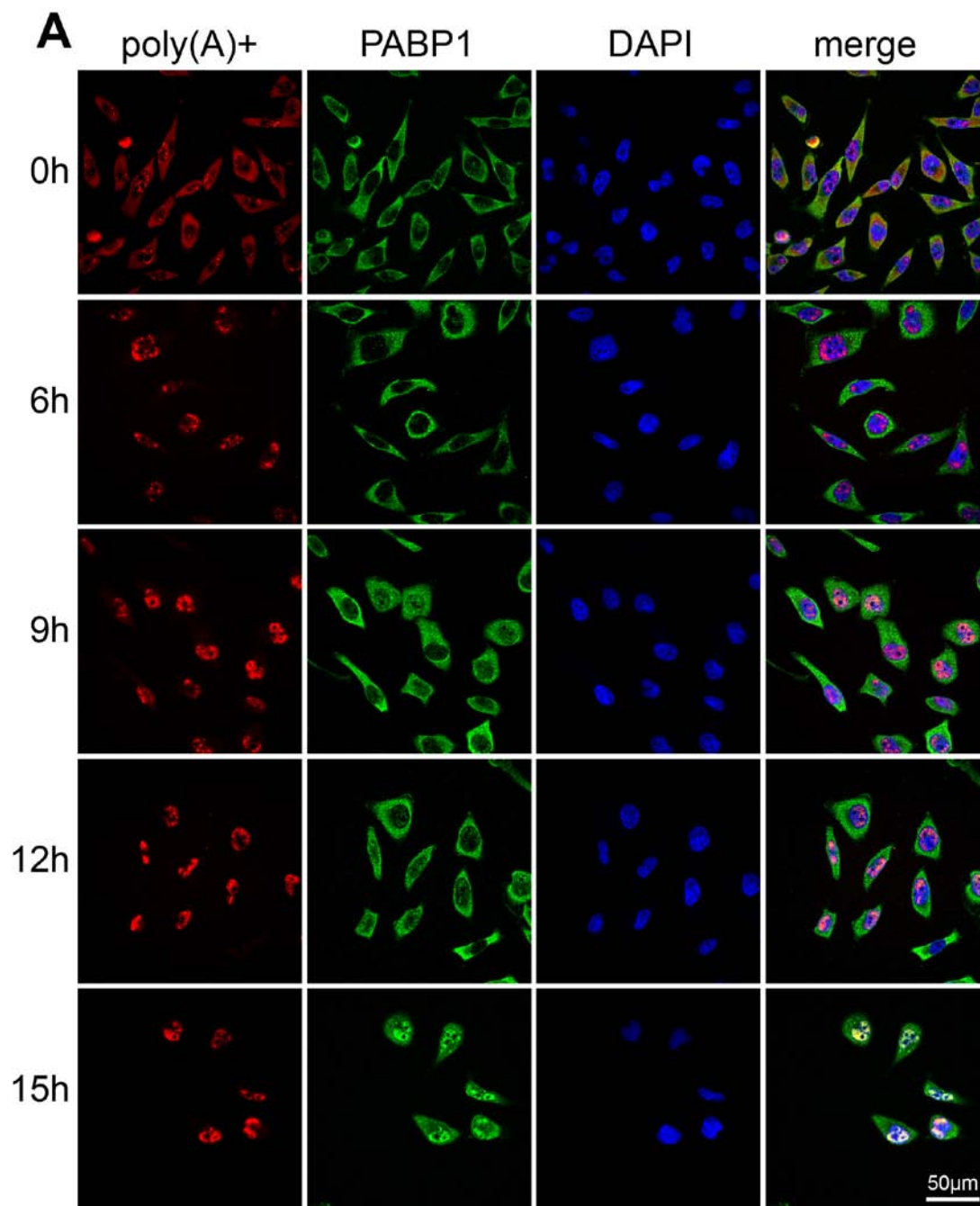
B

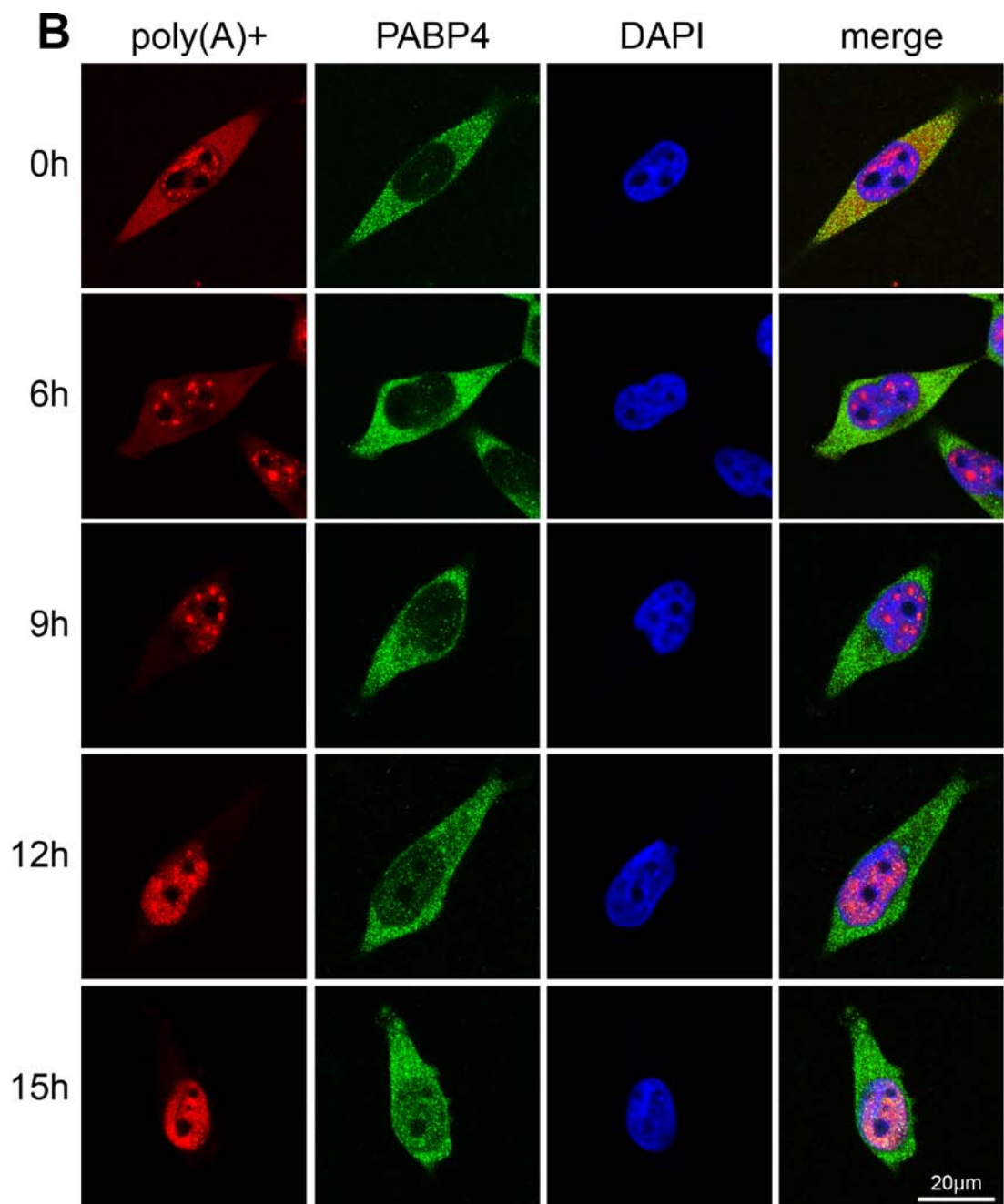


Appendix 4. Total proteins from nuclear and cytoplasmic extracts from UV treated and untreated cells separated by 2D electrophoresis. PVDF membranes were stained with Gelcode Blue prior to western blotting shown in Figure 6.4.



Appendix 5. Additional confocal microscopy images of PABP1 colocalisation with poly(A)+RNA in multiple cells (**A**) and of PABP4 colocalisation with poly(A)+RNA in single cells (**B**) after UV treatment.





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