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Author
Kumar, Gagandeep Renuka

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The Nuclear Functions of Host Cytoplasmic Poly(A) Binding Protein during Viral Infection

By

Gagandeep Renuka Kumar

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Professor Britt Glaunsinger, Chair
Professor Karsten Weis
Professor Matthew Welch

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Abstract

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Poly(A) tail length is emerging as an important marker of mRNA fate, where deviations from the canonical length can signal degradation or nuclear retention of transcripts. Pathways regulating polyadenylation thus have the potential to broadly influence gene expression. Here we demonstrate that accumulation of cytoplasmic poly(A) binding protein (PABPC) in the nucleus, which can occur during viral infection or other forms of cellular stress, causes mRNA hyperadenylation and nuclear accumulation of poly(A) RNA. This inhibits gene expression but does not affect mRNA stability. Unexpectedly, PABPC-induced hyperadenylation can occur independently of mRNA 3' end processing yet requires the canonical mRNA poly(A) polymerase II. We find that nuclear PABPC-induced hyperadenylation is triggered by multiple divergent viral factors, suggesting that altering the subcellular localization of PABPC may be a commonly used mechanism to regulate cellular gene expression in a polyadenylation-linked manner. PABPC is predominantly cytoplasmic at steady state. The molecular events that trigger relocalization of PABPC and the mechanisms by which it translocates into the nucleus to block gene expression are not understood. Here, we reveal an RNA-based mechanism of retaining PABPC in the cytoplasm. Expression of either viral proteins that promote mRNA turnover or of a cytoplasmic deadenylase drives nuclear relocalization of PABPC in a manner dependent on the PABPC RNA recognition motifs (RRMs). Using multiple independent binding sites within its RRMs, PABPC interacts with importin α, a component of the classical nuclear import pathway. Finally, we demonstrate that the direct association of PABPC with importin α is antagonized by the presence of poly(A) RNA but not poly(C) RNA, supporting a model in which RNA binding masks nuclear import signals within the PABPC RRMs, thereby ensuring efficient cytoplasmic retention of this protein in normal cells. These findings further suggest that cells must carefully calibrate the ratio of PABPC to mRNA, as events that offset this balance can dramatically influence gene expression.

Dedicated to my family:

My father
Dr. Krishan Kumar
I miss you.

My mother
Ms. Manjit Kaur Dug

My brother
Mr. Gaurav Kumar

For their endless love.

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Chapter 1

Introduction

Eukaryotic messenger RNA polyadenylation

Eukaryotic messenger RNAs (mRNAs) are protein coding transcripts containing co-transcriptionally added structures at the 5' end and the 3' end, both of which govern mRNA regulation and stability throughout the various stages of the mRNA lifecycle. The 5' end of an mRNA contains a single methylated guanine nucleotide connected in an unusual 5' to 5' triphosphate linkage, known as the 5' cap. However, the 3' end of an mRNA consists of hundreds of adenosine nucleotides, collectively referred to as the 3' poly(A) tail [1]. This 3' poly(A) tail is found on nearly all eukaryotic mRNAs with the exception of cell-cycle regulated histone mRNAs, which instead contain a stem-loop structure at their 3' end [2]. While the body of the mRNA is transcribed from a DNA template, addition of the both the 5' cap and the 3' poly(A) tail are formed through enzymatic processes. Addition of the 5' cap requires action of a phosphatase to remove the phosphate from the 5' end of the nascent RNA, a guanyl transferase that adds a guanine monophosphate in a reverse linkage (5' to 5' instead of 5' to 3'), and a methyl transferase that methylates the guanosine at the 7^{th} carbon position [3]. However, addition of a poly(A) tail is more complex and occurs through cleavage and polyadenylation steps (collectively referred to as 3' end processing), both of which are guided by sequence elements within the RNA, a multitude of protein factors, as well as the poly(A) polymerase [4].

Three core *cis* elements on the mRNA govern the cleavage and polyadenylation process: (a) a hexameric polyadenylation signal (PAS) located 10 to 30 nucleotides upstream of the poly(A) site, (b) a downstream U or GU-rich sequence element (DSE) located up to 30 nucleotides downstream of the poly(A) site, and (c) an auxiliary upstream U-rich sequence element located upstream of the PAS (Fig. 1.1) [5, 6]. The polyadenylation signal, composed of a hexamer motif AAUAAA, was originally discovered through comparison of sequences upstream of the poly(A) site of several mRNAs [7]. This motif is highly conserved and has since been found in nearly all eukaryotic mRNAs [8]. Genome wide sequence based studies have found that nearly 96% of the human and mouse mRNAs contain a PAS, of which 70% contain the canonical AAUAAA motif and about 15% contain the most common variant AUUAAA, whose activity is similar to that of the canonical AAUAAA motif [9-11]. While most other mutations in the PAS inhibit the cleavage and polyadenylation process, select variants exist and generally promote alternative polyadenylation or tissue-specific polyadenylation [5, 11]. The downstream element (DSE) resides within 30 nucleotides of the poly(A) site and affects both the site and efficiency of cleavage of the transcribed mRNA [12, 13]. Unlike the PAS, DSE is a poorly conserved region and can vary across mRNAs, however, the position of the DSE is conserved and deviations result in inefficient cleavage [14, 15]. The DSE contains either one or both of the following: U-rich element comprising of short stretch of U residues or a GU-rich region with a consensus YGUGUUYY (Y=pyrimidines) [16, 17]. Lastly, auxiliary upstream U-rich sequence element [18], located upstream of the PAS, consists either of UUUU or UGUAUAUA sequences and enhances the cleavage and polyadenylation reaction through recruitment of protein factors to the cleavage site [19-23].

While only two enzymatic steps are required for generating the mRNA 3' end, studies have shown that a large number of protein factors are required to facilitate these events. Recent proteomic studies of the human 3' end processing complex found it comprised of nearly 85

proteins, 50 of which were also involved in other mRNA processes, linking mRNA 3' end processing to other stages in mRNA metabolism [24]. The mammalian 3' end processing complex contains sub-complexes that include cleavage and polyadenylation specificity factors (CPSF-30, CPSF-70, CPSF-100, CPSF-160, and hFip1), cleavage stimulation factors (CstF 50, CstF-64 and CstF-77), cleavage factors (Cf I_m and Cf II_m), the poly(A) polymerase (PAP), the nuclear poly(A) binding protein (PABPN), symplekin, and polymerase II C-terminal domain (Pol II CTD) [4-6]. As the polyadenylation signals and sequences become available on a nascently transcribed mRNA, several cleavage and polyadenylation factors are recruited to the mRNA; some interact through the Pol II CTD while others bind directly to the mRNA sequences [6]. For example, Pol II CTD has been shown to interact with CstF 50 and Cf II_m, CPSF-160 has been shown to bind directly to the PAS, CPSF-100 binds to the USE, CstF 64 interacts with the DSE, etc. [4, 6]. These various interactions amongst cleavage and polyadenylation factors facilitate cleavage of the mRNA at the CA dinucleotide at the poly(A) site, a reaction catalyzed by CPSF-73 at the 3' end of the adenosine residue [25, 26]. Following cleavage, the poly(A) polymerase (PAP) is stimulated first by CPSF-160 resulting in distributive polymerization of adenosines at the 3' end of the cleaved fragment [27]. After accumulation of ~10-11 adenosines, nuclear poly(A) binding protein (PABPN) associates with the oligo(A) stretch and stimulation of PAP is facilitated by both CPSF-160 and PABPN synergistically, resulting in processive polymerization of a poly(A) tail of ~250 nucleotides in mammals [28] .

Mammalian mRNA poly(A) tails are consistently found to be in the range of 200 to 300 nucleotides long [29]. In addition to the *in vivo* conditions, this defined length is also maintained in reconstituted *in vitro* polyadenylation assays consisting only of CPSF, PAP, and PABPN [28]. These observations indicate that one or more of these three factors regulates poly(A) tail length control. Indeed, PABPN was recently found to be an active component of the mechanism of poly(A) tail length control as well as the protein that measures the length of a poly(A) tail [30]. The current model suggests that PABPN contributes to poly(A) tail length control through varied interactions with CPSF and PAP throughout the polyadenylation process. Firstly, PABPN ensures that the CPSF binds solely to the AAUAAA PAS and not elsewhere on the mRNA. CPSF association with AAUAAA stimulates PAP to some extent resulting in distributive addition of A residues. Upon addition of ~10-11 A residues, PABPN now associates with the oligo(A), allowing CPSF and PABPN to now synergistically stimulate PAP. Growth of the poly(A) tail results in binding of more PABPN monomers to the tail, facilitating folding back of the RNA, which is required to maintain the contact between CPSF and PAP. Synergistic stimulation of PAP by PABPN and CPSF allow growth of the poly(A) tail in a processive manner, resulting in formation of linear filaments and a 21 nm spherical particle that is thought to serve as a 'molecular ruler'. Once the poly(A) tail exceeds a threshold length of ~250 nucleotides, the spherical particle can no longer be sustained and interaction between CPSF and PAP is disrupted, resulting in reduced stimulation of PAP and termination of polyadenylation [30].

Role of poly(A) tails

When present on an mRNA 3' poly (A) tails serve two major functions: regulating mRNA stability and promoting mRNA translation. The poly(A) tails governs mRNA stability by regulating mRNA decay as the initiation and rate-liming step in mRNA decay is removal of the poly(A) tail by deadenylases [31]. Furthermore, completion of deadenylation stimulates the second step of mRNA decay, which can either be removal of the 5' cap through stimulation of

decapping enzymes, or continued degradation from the 3' end of the mRNA by the exosome complex. Thus presence of the poly(A) tail serves to protect the mRNA ends from aberrant exonucleolytic decay until degradation of the mRNA is desired. A second major role of poly(A) tail includes promoting mRNA translation and protein synthesis. The presence of the poly(A) tail facilitates interaction of the 3' end of the mRNA with the 5' end of the mRNA through various protein factors, therefore resulting in circularization of the translating mRNA [32]. This circularization of the mRNA is hypothesized to further protect the mRNA from degradation by exonucleases, promote protein translation by facilitating efficient ribosomal recruitment to the mRNA, as well as recycling of ribosomes upon termination of a round of protein synthesis.

In contrast, when poly(A) tails are present on non-coding RNAs, they often serve roles in RNA quality control. For example, in yeast, degradation of aberrant ribosomal RNA (rRNA), small nucleolar RNA (snoRNA), or transfer RNA (tRNA) transcripts that fail quality control pathways is mediated through addition of a poly(A) tail. Recently, a polyadenylation complex, called TRAMP, was identified and found to associate with these aberrant transcripts resulting in polyadenylation at the 3' end. This followed recruitment and activation of the exosome complex which rapidly deadenylates the RNA and degrades the transcript in a 3' to 5' direction [18, 33]. Interestingly, the mechanism of polyadenylation-assisted degradation is also found in prokaryotic RNA decay as well as degradation of RNA transcribed in both the mitochondria and the chloroplast [34, 35].

While gene expression is promoted through the presence of a poly(A) tail, it can also be regulating by varying the length of the poly(A) tail. For example, although the mRNAs involved in *Xenopus* oocyte maturation are polyadenylated in the nucleus, they are subsequently deadenylated in the cytoplasm by the deadenylase PARN, resulting in a shortened poly(A) tail that renders the transcript inactive for translation [36]. However, following appropriate signals, the mRNA is re-adenylated by cytoplasmic poly(A) polymerases and translation competence of the mRNA is restored. Similar mechanisms of shortening the poly(A) tail to repress translation and re-polyadenylation to restore translation have also been observed in other process such as meiotic progression, cell senescence, and modulation of neuronal synaptic plasticity [37-39].

In contrast, recent studies have also found mRNAs that possess longer than normal poly(A) tails, a phenotype termed hyperadenylation, under certain conditions. However, hyperadenylation has primarily been documented in yeast, specifically under conditions where factors involved in export of mRNA from the nucleus are disrupted. Yeast mutants defective for several mRNA export factors, including Mex67p, Mtr2p, Rat7p, etc., result in hyperadenylation of newly transcribed mRNAs [40]. Hyperadenylation results in reduced gene expression as these hyperadenylated transcripts are retained in the nucleus at or near the site of transcription, presumably, because they fail quality control checkpoints [40-42]. Hyperadenylation has also been seen in mammalian cells depleted of the mRNA export factor TAP/NXF1, a homolog of Mex67p [43]. However, the mechanism of hyperadenylation of these mRNAs and the mechanism of their nuclear retention remain unknown. Collectively these observations suggest that the existence as well as the length of the poly(A) tail must be carefully monitored as deviations in either direction have important implications for cellular gene expression.

Poly(A) binding proteins (PABP)

In mammalian cells poly(A) tails are decorated with two non-homologous poly(A) binding proteins (PABP) during their lifecycle. During mRNA polyadenylation in the nucleus, the nuclear poly(A) binding protein (PABPN) associates with the nascently synthesized poly(A)

tail, promotes efficient polyadenylation, and accompanies the mRNA into the cytoplasm during export. However, in the cytoplasmic compartment, the majority of the poly(A) tails are bound by the cytoplasmic poly(A) binding protein (PABPC). Since both the nuclear PABPN and cytoplasmic PABPC have been found in the alternate cellular compartments (albeit at reduced levels), it is unclear in which cellular compartment poly(A) tails lose PABPN and acquire PABPC. However, since PABPC has a higher affinity for poly(A) sequences (K_D ~ 2-7nM) as compared to PABPN (K_D ~ 100nM), it is likely that PABPN is rapidly exchanged for PABPC in the cytoplasm, as PABPC is present in the cytoplasmic compartment at a higher concentration [44-46].

Nuclear poly(A) binding protein (PABPN)

PABPN was originally discovered as a component that stimulates PAP during polyadenylation [47]. PABPN has a high affinity for poly(A) and poly(G) sequences, however, a 100x reduced affinity for poly(U), and no detectable interaction with poly(C) [48]. Interaction of PABPN with poly(A) forms oligomeric complexes that are spherical and 21nm in diameter [49]. These structures are thought to control the length of the poly(A) tail [30]. PABPN contains a single central RNA recognition motif (RRM) surrounded by a glutamate-rich N-terminus and an arginine-rich C-terminus (Fig. 1.2). Both the RRM and the C-terminal domain contribute to binding ~10-11 A residues [44]. The C-terminal region also facilitates self-interaction of PABPN. Mutations in PABPN are associated with oculopharyngeal muscular dystrophy (OMPD), a human genetic disease that causes muscle weakness in the eye lids and the pharynx. Individuals with OMPD have an expanded polyalanine tract at the N-terminus resulting in aggregation of PABPN in the nucleus [50].

Cytoplasmic poly(A) binding protein (PABPC)

Cytoplasmic poly(A) binding protein (PABPC) was initially discovered due to its abundant association with eukaryotic poly(A) mRNA [51]. It was shown to 'organize' poly(A) residues in 27-residue repeating structures and this activity was restricted in the cytoplasmic fraction. To date four human isoforms of cytoplasmic PABP have been identified. These include PABPC1, PABPC3, PABPC4 (iPABPC), and PABPC5, of which PABPC1 is the most widely expressed and the most well studied isoform. Four pseudogenes have also been identified in humans [52]. PABPC3 is a testis-specific protein whereas PABPC4 is an inducible form discovered in activated T-cells [53, 54]. PABPC5 is an X-linked isoform encoded by an mRNA lacking introns [55]. Sequence identity among these various human isoforms ranges from 60% to over 90%. PABPC1, PABPC3, and PABPC4 are ~70 kDa proteins that harbor four repeats of a common RNA binding fold at the N-terminus known as the RNA recognition motif (RRM). The four N-terminal RRMs are followed by a proline and glutamine-rich linker that precedes the C-terminal domain, which contains a five-helical region (Fig. 1.2). On the other hand, PABPC5 is a lower molecular weight protein that solely contains the N-terminal RRM domains and lacks the linker and the C-terminal region. While the N-terminal RRMs are conserved across PABPC isoforms and in various species, the C-terminal domain is more variable [53, 56]. PABPC binds strongly to poly(A) sequences via the RRMs, however, it has been shown to associate with poly(U) and poly(G) as well, albeit with reduced affinities. PABPC is unable to bind poly(C) sequences [56]. A single PABPC molecule coats ~25 A residues in a poly(A) tail indicating that a typical tail of ~250 nucleotides will be coated by ~10 PABPC molecules [45, 51]. PABPC RRMs function in pairs to associate with poly(A) RNA. For example, RRMs 1+2 together

associate with ~12 A residues and although RRMs 3+4 alone have a lower affinity for poly(A), it is thought that binding of RRMs 1+2 creates a high local concentration of poly(A) sequences, allowing RRMs 3+4 to further associate with ~12 additional A residues [46, 48]. Crystal structural analysis of RRM 1+2 in complex with A_{11} oligo demonstrates the globular RRM fold to contain four anti-parallel beta sheets backed by two alpha helices [57]. Interaction between the RRM and the A residues are mediated through electrostatic interactions with phosphates, hydrogen bonds with riboses, and stacking interaction between adenine bases and aromatic amino acid side chains.

In addition to binding RNA, PABPC also interacts with several proteins and these interactions are mediated through the RRMs, the linker, and the C-terminal region. PABPC RRMs mediate association of PABPC with scaffolding proteins involved in mRNA translation: eIF4G (eukaryotic initiation factor 4G) and Paip1 (PABPC-interacting protein 1) [58-60]. The linker region of PABPC promotes self-association of PABPC thereby facilitating cooperative binding of multiple PABPC molecules to a poly(A) tail [61]. The C-terminal region of PABPC has been shown to interact with various translation factors including eRF3 (eukaryotic release factor 3) and Paip1 [62, 63]. A negative regulator of translation, Paip2a (PABPC-interacting protein 2), associates with PABPC in a 2:1 stoichiometry by interacting with both the RRM domains and the C-terminal domain [64, 65].

Association of PABPC with several translation factors promotes efficient translation initiation and termination. PABPC facilitates circularization of a translating mRNA through its simultaneous associations with the 3' end and the 5' end of an mRNA (Fig. 1.3) [32]. While bound to the 3' poly(A) tail, PABPC also interacts with scaffolding proteins eIF4G and Paip1. eIF4G and Paip1 are also bound to the 5' mRNA cap binding protein, eIF4E and the helicase eIF4A, respectively, thereby bridging the interaction between the PABPC and the 5' cap. These associations dictate the mRNA closed loop model of translation which is hypothesized to ensure translation of full length transcripts, protect mRNAs from exonucleolytic attacks, and promote recycling of ribosomes [66]. PABPC also promotes translation termination by virtue of its interactions with eRF3, a eukaryotic protein release factor (Fig. 1.3) [62, 63]. Therefore, PABPC contributes to mRNA stability and translation by protectively coating the poly(A) tail as well as through favorable interactions with translation factors.

The majority of interaction partners of PABPC described thus far are involved in mRNA translation. However, associations of PABPC with other cellular factors have also been demonstrated, implying that multiple sub-complexes of this protein exist within a cell. For example, association of PABPC with Paip2a occurs independently of PABPC-translation factor interaction. Paip2a has been shown to deplete polyribosomes and sequester PABPC from translation complexes *in vitro* [64, 65]. PABPC has also been described to interact with paxillin, an adaptor protein associated with focal-adhesion [67, 68]. This interaction is thought to facilitate transport of mRNA to achieve locally regulated protein synthesis at the leading edge of cell migration. Collectively, to date, three sub-complexes of PABPC in the cytoplasm have been described: PABPC complexed with translation factors, PABPC complexed with Paip2a, and PABPC complexed with paxillin. It is, therefore, not unlikely that additional, yet unidentified, subpopulations of PABPC exist within a cell.

PABPC also regulates mRNA decay by directly interfacing with factors involved in deadenylation. PABPC has been shown to interact with TOB, an antiproliferative factor that bridges interaction between the poly(A) tail and poly(A) nuclease complex CCR4-CAF1 [69]. PABPC can also interact with GW182, a component of the miRNA-mediated mRNA decay [70].

PABPC has been shown to inhibit poly(A) specific ribonuclease (PARN) as well as the decapping event since decapping only occurs upon complete removal of the poly(A) tail [31, 71-75]. Interactions of PABPC with eRF3 also block eRF3-UPF1 associations, thereby antagonizing the nonsense mediated mRNA decay (NMD) pathway [76-78].

In addition to regulating global mRNA stability and translation, PABPC is also involved in autoregulating its own expression. The 5'UTR of PABPC mRNA contain short oligo(A) tracts that are bound by the PABPC protein if it is synthesized in excess in the cell. This binding is thought to block 40S ribosomal scanning, resulting in reduced PABPC protein expression [79-81]. These 5' UTR oligo(A) tracts may also regulate PABPC mRNA abundance as they have been implicated in regulating mRNA levels for chloramphenicol acetyl-transferase mRNA [82].

PABPC manipulation during viral infection

Given that PABPC has important regulatory roles in a cell, it is therefore not surprising that divergent viruses manipulate this host factor through a variety of mechanisms. This affords two advantages to a virus infecting a cell: (a) a block to host gene expression that may also aid the virus in evading the cellular immune response and (b) reallocation of resources from the host cell to the virus to avoid competition from the host, resulting in efficient viral replication.

One of the first mechanisms of PABPC manipulation discovered during viral infection was proteolysis of PABPC that resulted in either degradation of PABPC or in some cases utilization of one of the degradation intermediates for viral replication. PABPC proteolysis is carried out by viruses belonging to the genera *Enterovirus*, *Aphthovirus*, *Rhinovirus*, *Hepatovirus*, *Parechovirus*, as well as *Cardiovirus* [83]. The most well studied example is poliovirus which belongs to the *Enterovirus* genus. Poliovirus is the causative agent of poliomyelitis and is contracted through the fecal-oral route. The poliovirus genome consists of a positive single stranded RNA [(+)ssRNA)] that lacks a 5' cap but instead harbors an internal ribosome entry site (IRES) and possesses a 3' poly(A) tail. This (+)ss RNA serves as both a messenger RNA for protein synthesis and a template for genome replication. A single large polypeptide is produced form the viral (+) ssRNA that is further processed by poliovirus encoded proteases 2A and 3C. These proteases were found to also cleave PABPC and an additional translation factor eIF4G [84, 85]. Interestingly, the cleaved products of both PABPC and eIF4G can be bound by the poliovirus (+) ssRNA via the 3' poly(A) tail and the 5' IRES, respectively. Homologous proteins in other *Enteroviruses*, such as Coxsackievirus and Hepatitis A virus, also cleave PABPC and eIF4G similarly [86, 87]. Cleavage of PABPC occurs at multiple sites within the proline and glutamine-rich linker as well as the C-terminal domain, rendering, the N-terminal RRMs intact. It is hypothesized that this cleavage destabilizes the PABPC-poly(A) interaction, therefore, allowing PABPC to dissociate from the host mRNA and instead bind the polyadenylated tail of the viral RNA and facilitate translation and replication [83]. Hepatitis A virus does not bind PABPC as it lacks a poly(A) tail indicating that Hepatitis A virus-mediated cleavage of PABPC may serve solely to dampen host gene expression. Aside from viruses with (+) ssRNA genomes, PABPC is also cleaved by members of the *Retroviridae* family. HIV-1 and HIV-2, the causative agents of acquired immunodeficiency syndrome (AIDS), encode proteases that cleave PABPC and eIF4G [88-90]. In addition to cleaving PABPC at the linker and the C-terminal regions, HIV proteases also cleave PABPC internally in the RRM 3.

A second mechanism employed by viruses is modification of the PABPC-associated translation complex and this is exemplified by the members of the *Reoviridae*, *Flaviviridae*, *Herpesviridae*, *Poxviridae*, and *Togaviridae* families. Rotavirus, a member of the *Reovirus*

genus, is the most common cause of severe diarrhea among infants and children worldwide. Rotavirus contains a double stranded RNA genome that produces mRNAs that are capped but not polyadenylated; they instead contain a structure at the 3' end that associates with the rotaviral non-structural protein NSP3 [91]. Rotavirus NSP3 protein has been shown to associate with and sequester eIF4G, to aid in translation of viral mRNA and concomitantly block host gene expression through eviction of PABPC from the translation complex [92]. A *Flaviviridae* member, Dengue virus, has recently been shown to bind PABPC through the 3' UTR of its (+) ssRNA genome and this interaction modulates dengue virus translation *in vitro* [93]. Herpesviruses and poxviruses generate mRNAs that are similar to host mRNAs in that they contain a 5' cap and a 3' poly(A) tail. As in this case cleavage of PABPC and other translation factors would be detrimental to viral gene expression, these viruses instead have evolved to recruit the translation initiation complex with more efficiency. Members of the *Herpesviridae*, herpes simplex virus (HSV), human cytomegalovirus (HCMV), and Kaposi's sarcoma-associated herpesvirus (KSHV), as well as vaccinia virus (VV) (*Poxviridae*) have evolved to efficiently recruit the eIF4F translation initiation complex through disruption of the a cellular regulatory factor 4E-BP, which otherwise sequesters the 5' cap binding protein eIF4E [94-96]. In the case of HCMV and VV, this also includes upregulation of PABPC (HCMV) [97] and enhanced recruitment of PABPC (HCMV and VV); however, HSV and KSHV accomplish this in the face of reduced cytoplasmic PABPC. Recently, the capsid protein of rubella virus, a member of the *Togaviridae* family, was shown to sequester PABPC and inhibit translation [98].

In the recent years unrelated viruses have been shown to exploit subcellular localization of PABPC, providing an example of a third mechanism of virus-mediated PABPC manipulation. While PABPC has been shown to be a shuttling protein, its steady state localization is largely cytoplasmic [99]. However, nuclear relocalization and nuclear accumulation of PABPC has been documented during infection with several divergent viruses that include rotavirus, bunyamwera virus, as well as select herpesviruses [100-104]. In the case of rotavirus, eviction of PABPC is due to sequestration of eIF4G by NSP3, resulting in nuclear accumulation of PABPC [101]. However, the mechanisms underlying nuclear relocalization of PABPC during bunyamwera virus infection are not well understood. Bunyamwera is a member of the *Bunyaviridae* family that contains a segmented tripartite (-) ssRNA genome (S, M, L fragments). A non-structural protein, NSs2, encoded by the S fragment has been partially implicated in driving nuclear relocalization of PABPC, however, a mutant deleted for NSs2 does not completely rescue nuclear relocalization of PABPC [100]. Herpes simplex virus and Kaposi's sarcoma-associated herpesvirus belong to the *Herpesviridae* family and also promote nuclear relocalization of PABPC. HSV ICP27 has been implicated in this process, however, is not sufficient as exogenous expression of ICP27 results in drastically reduced nuclear accumulation of PABPC as compared to during a viral infection [103, 104]. On the other hand, the KSHV SOX protein causes robust nuclear relocalization of PABPC suggesting it is likely the major determinant of this phenotype [102].

Proteolysis of PABPC and sequestration of PABPC-related factors has been hypothesized to (in some cases shown to) dampen host gene expression and reallocate resources towards viral replication. However, the advantages of nuclear relocalization of PABPC are not immediately clear, especially, for viruses that still utilize canonical mRNA translation machinery for their gene expression or for viruses that harbor 3' poly(A) tails. Furthermore, the cytoplasmic determinants and mechanisms by which PABPC is relocalized into the nucleus remain elusive.

In the current report these unknown avenues have been explored by using the herpesvirus KSHV as a model and the KSHV SOX protein as a tool to manipulate and study PABPC localization.

Kaposi's sarcoma-associated herpesvirus (KSHV)

The *Herpesviridae* are a family of large double stranded DNA viruses that consist of ubiquitous pathogens infecting both vertebrates and invertebrates. More than 100 herpesviruses have been isolated till date and seroprevalence of herpesvirus is more than 90% in the human population. Herpesviruses are divided into three subfamilies based on host range, length of the replication cycle, and establishment of latency. These include the alphaherpesviruses, the betaherpesviruses, and the gammaherpesviruses. The eight human herpes viruses (HHV) are distributed amongst these three subfamilies.

Gammaherpesviruses are lymphotrophic viruses that establish latency in T cells or B cells. Representatives include two important human pathogens: Epstein-Barr virus (EBV, HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8). Both EBV and KSHV infections can result in cancer, especially in immunocompromised patients such as those suffering from acquired immunodeficiency syndrome (AIDS). Importantly, more than 90% of adults in the U.S. have been infected with EBV, the causative agent of infectious mononucleosis. In some cases, persistence of the virus can result in Burkitt's lymphoma or nasopharyngeal cancers, two rare types of cancers in the U.S, but that are more prevalent in other parts of the world [105].

KSHV, the most recently identified human herpesvirus, is the causative agent of Kaposi's sarcoma (KS), the most common neoplasm associated with AIDS patients [106, 107]. KSHV is also associated with two other lymphotrophic disorders: Multicentric Castleman's disease (MCD) and Primary Effusion Lymphoma (PEL) [108]. MCD is an aggressive systemic illness characterized by sustained fever, sweats, weight loss, lymphadenopathy, and enlargement of the spleen. MCD frequently occurs in AIDS patients. PEL is a rare disease of patients with end-stage AIDS, characterized by proliferation of B cells in serosal cavities. However, MCD and PEL occur rarely in contrast to KS.

KS was first described in 1872 by Moritz Kaposi and was thought to be a rare cancer until the AIDS epidemic in the 1980s [107, 109, 110]. KS presents as cutaneous lesions either locally or systemically throughout the skin. There are four forms of KS: classical KS which affects the elderly men in the Mediterranean basin, endemic KS affecting regions in Africa, KS in immunosuppressed individuals, and KS associated with AIDS. KS in the classical or endemic forms usually represents as a single lesion in extremities of the body; however, the sarcoma is more widespread, aggressive, and life-threatening in immunocompromised individuals [111]. With the advent of anti-retroviral therapy KS incidences in the U.S. have decreased dramatically, however, it remains endemic in sub-Saharan Africa. Seroprevalence of KSHV in general populations of U.S., UK, central, Northern, and Western Europe is around 2-7%, with bisexual and gay men at a higher risk. KSHV is frequently found in non-AIDS populations in Mediterranean basin with a prevalence of up to 20%, however, it is widespread in Africa with a seroprevalence of >50%. In Eastern and Southern Africa, KS is responsible for 25 to 50% of soft-tissue sarcomas in children and up to 2 to 10% of all cancers in children [112].

A hallmark of herpesviruses is their biphasic lifecycle as infection with a herpesvirus can be latent or lytic. During a latent infection, the herpesvirus genome is maintained in the nucleus as a closed circular molecule, referred to as the episome, where very few viral genes are expressed. A herpesvirus in the latent state is maintained throughout the lifecycle of the cell

resulting in a persistent infection in the host organism. One the other hand, a lytic infection, similar to many other viruses, is defined by expression of full complement of viral genes resulting in production of progeny virions, which are released and can infect new cells. An important event associated with a lytic KSHV infection is a block in host gene expression.

KSHV-induced host shutoff blocks cellular gene expression

Eukaryotic gene expression is regulated at multiple levels including transcription and translation and these regulatory pathways are often exploited by viruses for their own replicative success. Recent insights into mRNA metabolism indicate that a significant level of gene expression regulation occurs via alternations in mRNA stability. mRNAs are regulated at multiple steps in their life cycle including 5' processing, splicing, 3' end processing, nuclear export, and translation, as well as by quality control surveillance machineries. Changes in mRNA stability also occur during multiple cellular stresses, cellular development, and infection with a variety of unrelated viruses. This virus-mediated dampening of host gene expression is generally referred to as 'host shutoff.'

During a lytic infection with KSHV host shutoff manifests through a rapid and global host transcriptome degradation where nearly 70% of all cellular messages are massively down-regulated with another 20% displaying a modest decrease [113, 114]. This global mRNA destruction is orchestrated by the KSHV shutoff and exonuclease (SOX) protein [115]. As KSHV genetics are challenging, a functional role of host shutoff was instead assessed in a homologous virus, murine herpesvirus 68 (MHV68), which has a tractable genetic system and represents a mouse model of infection for gammaherpesviruses. MHV68 encodes for a SOX homolog termed muSOX. A host shutoff defective virus (MHV68.ΔHS), containing a single point mutation in the muSOX gene, was created and contribution of host shutoff towards viral replication was determined *in vitro* and *in vivo* [116]. Interestingly, host shutoff is dispensable for acute replication in cultured NIH3T3 cells, however, exhibited dramatic consequences for replication in dendritic cells (Abernathy and Glaunsinger, unpublished observations) [116]. Furthermore, infection of mice with MHV68.ΔHS demonstrated that although this virus was similar to wild type during acute replication phase in the mouse lung, it was dramatically attenuated in later stages of infection, suggesting the mutant virus was defective for both viral trafficking (likely due to attenuated replication in certain cell types) and latency establishment (presumably due to reduced delivery of virus to the spleen).

While majority of cellular mRNAs undergo SOX-mediated degradation; a very small number of cellular transcripts, such as IL-6, escape this dramatic fate [117]. Interestingly, viral transcripts are also subject to degradation by this factor. Comparison of MHV68 and MHV68.ΔHS demonstrated that viral mRNAs are also degraded by muSOX in addition to cellular mRNAs (Clyde and Glaunsinger, unpublished observations). It is hypothesized that this muSOX-mediated regulation may contribute to fine tuning the expression levels of viral genes, therefore facilitating choice of latent or lytic infection cycle as well as evading the host immune response.

KSHV-induced host shutoff is linked to relocalization of PABPC

Mechanisms by which KSHV SOX elicits host shutoff are beginning to be understood. Subcellular fractionation studies have shown that the majority of RNA turnover in SOX-expressing cells occurs in the cytoplasm. SOX is thought to behave as an endonuclease that cleaves mRNAs internally, facilitating degradation of the intermediates by the cellular

exonuclease Xrn1 in a 5' to 3' direction [118]. This cytoplasmic turnover is dependent on cellular 3' processing events and/or translational competence, because SOX is unable to promote turnover of reporter mRNAs terminating in a hammerhead ribozyme sequence that do not undergo cellular 3' processing and are not translated [102]. Within the nucleus of SOX-expressing cells, nascent mRNAs have aberrantly long poly(A) tails, termed hyperadenylation, which prevents their nuclear export [102]. These results indicate that KSHV induces gene expression blocks in both cellular compartments. While SOX is present in both the nucleus and the cytoplasm, host shutoff is solely orchestrated by the cytoplasmic fraction of SOX [119]. Furthermore, within the cytoplasm, SOX also drives nuclear relocalization of the cytoplasmic poly(A) binding protein (PABPC) in a host shutoff-dependent manner [102].

Thesis Outline

As previously mentioned, PABPC relocalization to the nucleus is not specific to a lytic KSHV infection because multiple divergent viruses, including Epstein-Barr virus, herpes simplex virus, rotavirus, and bunyamwera virus, also direct PABPC to the nucleus [120, 121]. In addition, PABPC is also directed to the nucleus during non-viral stresses such as transcriptional inhibition, heat shock, hydrogen peroxide induced oxidative stress, and UV irradiation [99, 103, 122, 123]. Furthermore, PABPC has been shown to associate with nuclear pre-mRNA [124]. However, the processes underlying nuclear relocalization and the potential nuclear roles of PABPC have not been elucidated.

In this report I demonstrate mechanistic insights into relocalization of PABPC into the nucleus. I show that subcellular localization of PABPC is directly governed by cytoplasmic mRNA abundance. Divergent viral and non-viral factors that reduce cytoplasmic mRNA levels cause PABPC to leave the cytoplasm and accumulate in the nucleus. Translocation across the nuclear pore is accomplished through interactions with classical nuclear import factors, and this is dependent on the interaction status of PABPC with poly(A) RNA. Importantly, I demonstrate that elevated levels of nuclear PABPC results in nuclear mRNA hyperadenylation and a concomitant mRNA export block, eventually blocking protein expression. PABPC-induced mRNA hyperadenylation and export block is dependent on PABPC poly(A) binding motifs, can occur independently of mRNA 3' end processing, yet requires the canonical poly(A) polymerase. PABPC manipulation during cellular stress is reversible; heat shock induces nuclear accumulation and rescue from heat shock results in PABPC's return to the cytoplasm [125]. Collectively, based on previous observations and the data presented in this report, I propose that nuclear import of PABPC is a stress response pathway that temporarily blocks gene expression, until the cellular stress is alleviated. However, this pathway is exploited by viruses to promote a continued block in host gene expression as PABPC remains nuclear until cell death.

Figures

Figure 1.1

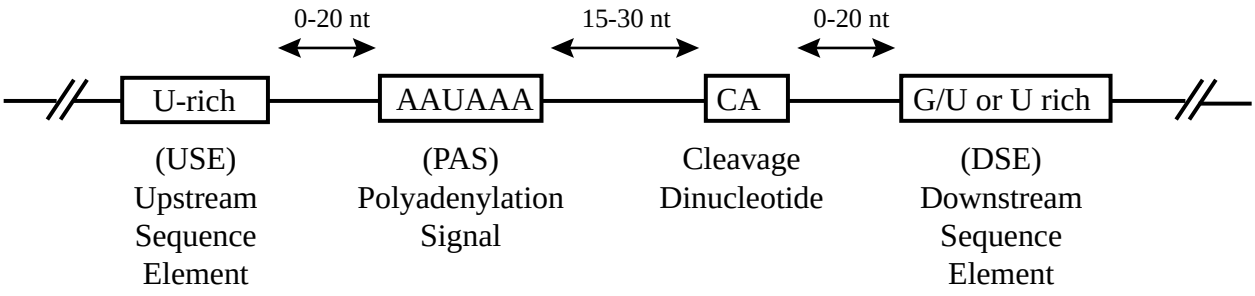

Figure 1.1 Polyadenylation signal of eukaryotic mRNA. Three core *cis* elements on the mRNA govern the cleavage and polyadenylation process: (1) a hexameric polyadenylation signal (PAS) located 15 to 30 nucleotides upstream of the poly(A) site (cleavage dinucleotide), (2) a downstream U or GU-rich sequence element (DSE) located up to 20 nucleotides downstream of the poly(A) site, and (3) an auxiliary upstream U-rich sequence element located upstream of the PAS.

Figure 1.2

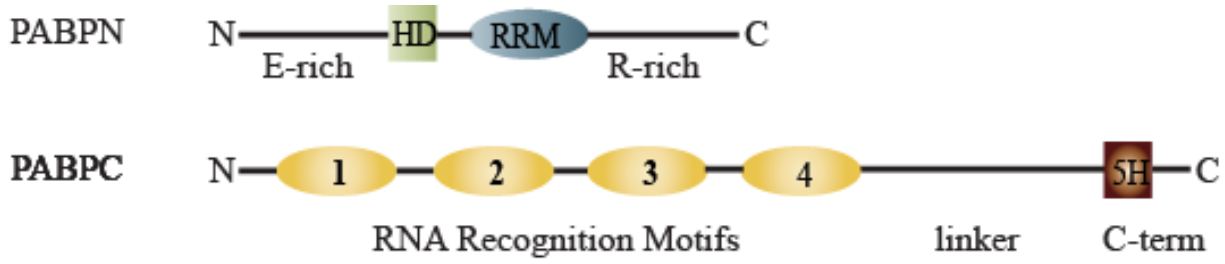

Figure 1.2 Domain architecture of poly(A) binding proteins. The nuclear poly(A) binding protein (PABPN) contains a single central RNA recognition motif (RRM) surrounded by a glutamate-rich N-terminus and an arginine-rich C-terminus. Both the RRM and the C-terminal domain contribute to binding ~10-11 A residues whereas the C-terminus facilitates self-interaction of PABPN. The cytoplasmic poly(A) binding protein (PABPC) contains four N-terminal RNA recognition motifs (RRMs), followed by a proline and glutamine-rich linker that precedes the C-terminal domain, which contains a five-helical region. While poly(A) sequences are bound by the RRMs, protein-protein interactions are mediated by all the domains.

Figure 1.3

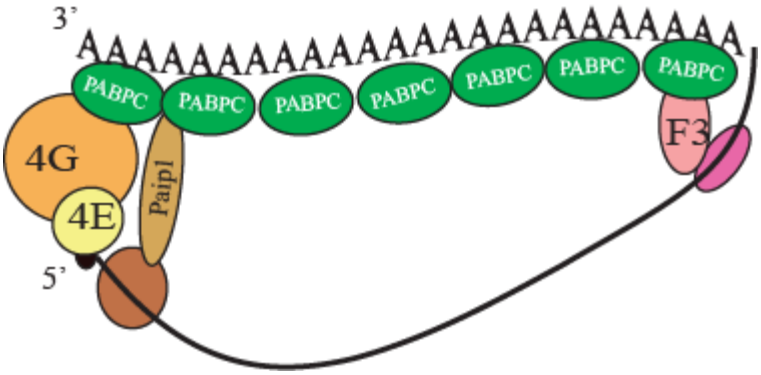

Figure 1.3 Circularization of eukaryotic mRNA facilitated by PABPC. PABPC facilitates circularization of a translating mRNA through its simultaneous associations with the 3' end and the 5' end of an mRNA. While bound to the 3' poly(A) tail, PABPC also interacts with scaffolding proteins eIF4G and Paip1. eIF4G and Paip1 are also bound to the 5' mRNA cap binding protein, eIF4E and the helicase eIF4A, respectively, thereby bridging the interaction between the PABPC and the 5' cap. These associations dictate the mRNA closed loop model of translation which is hypothesized to ensure translation of full length transcripts, protect mRNAs from exonucleolytic attacks, and promote recycling of ribosomes.

Chapter 2

Nuclear import of cytoplasmic poly(A) binding protein restricts gene expression via hyperadenylation and nuclear retention of messenger RNA

Background

Mammalian mRNA poly(A) tails are decorated with two types of non-homologous poly(A) binding proteins with distinct functions. Within the cytoplasm, the cytoplasmic poly(A) binding protein (PABPC) helps modulate the rate of mRNA deadenylation, both through its protective interactions with the poly(A) tail, as well as by interfacing directly with factors involved in deadenylation, including Pan3, TOB, and GW182 [69, 70, 126]. These interactions play key roles in transcript silencing, for example upon micro RNA-mediated repression, because poly(A) tail removal is the rate-limiting step in mRNA degradation and restricts translational competence [31]. PABPC enhances translation efficiency by bridging the mRNA termini via its simultaneous interactions with the poly(A) tail and the cap-binding complex through eIF4G [58]. Formation of this 'closed loop' is hypothesized to promote translation of full-length transcripts, protect mRNAs from exonucleolytic attack, and facilitate recycling of ribosomes [32]. Through additional interactions with the translation release factor eRF3 [62, 63], PABPC is also proposed to enhance the efficiency of termination and inhibit nonsense mediated mRNA decay (NMD) [76, 78, 127, 128], a quality control pathway essential for destruction of messages containing premature termination codons [129, 130].

PABPC is a nuclear-cytoplasmic shuttling protein [99], but its steady state localization is cytoplasmic. Although it has been shown to interact with nuclear pre-mRNA [124], distinct nuclear roles for PABPC remain largely enigmatic. Stimulation of poly(A) polymerase II (PAPII) activity and regulation of mRNA polyadenylation in the nucleus are instead carried out by nuclear poly(A) binding protein (PABPN), which shares little sequence homology with PABPC [28, 30, 48, 131, 132].

While poly(A) tail length clearly has implications for mRNA translation and stability in the cytoplasm, emerging evidence indicates that the extent of polyadenylation in the nucleus also influences RNA fate [133-136]. Messenger RNA poly(A) tail length is generally ~200-250 nucleotides (nt) in mammals and ~70-90 nt in yeast [52]. However, very short poly(A) tails can be found on RNAs that are targets for rapid RNA degradation via nuclear quality control pathways such as the exosome. In yeast, these tails are added by the TRAMP polyadenylation complex, generally upon recognition of RNA processing errors [18, 33, 137]. Conversely, messages with poly(A) tails that extend beyond the canonical length, termed hyperadenylated, accumulate in yeast mutants defective in mRNA export [40-43], although it remains to be established whether hyperadenylation is a cause or a consequence of inefficient nuclear-cytoplasmic trafficking. Errors in RNA processing or ribo-nucleoprotein (RNP) complex remodeling have also been proposed to trigger hyperadenylation [43, 138]. Thus, mRNA

poly(A) tail extension is linked to increased duration of nuclear residence, perhaps as a result of failed quality control checkpoints.

Hyperadenylation is primarily documented in yeast, although recently it has been shown in mammalian cells as well, for example upon expression of the gamma-herpesviral SOX protein [102]. During lytic Kaposi's sarcoma-associated herpesvirus (KSHV) infection, SOX promotes a global restriction of cellular gene expression, through both widespread cytoplasmic mRNA degradation as well as hyperadenylation and retention of cellular messages in the nucleus [102, 139]. Hyperadenylation of nuclear mRNAs is orchestrated exclusively by the cytoplasmic pool of SOX [119], indicating that SOX must stimulate hyperadenylation indirectly, perhaps via another cellular cofactor. Interestingly, an additional SOX activity is the prominent relocalization of PABPC into the nucleus of infected cells [102], although a functional connection between this phenotype and hyperadenylation has not been described. Recently, infection with several other viruses including herpes simplex virus, rotavirus, and bunyavirus, as well as additional non-viral stresses such as heat shock, have also been reported to drive PABPC relocalization [99-101, 104, 125].

In this report, we demonstrate that a functional consequence of accumulation of PABPC in the nucleus is mRNA hyperadenylation and inhibition of poly(A) RNA export, resulting in a restriction of protein expression. Messenger RNAs engineered to bypass cellular 3' end processing can still be hyperadenylated by the canonical poly(A) polymerase when nuclear PABPC levels are elevated, suggesting 3' end processing-independent polymerase activity. Interestingly, several divergent viral proteins that restrict host gene expression promote nuclear relocalization of PABPC and hyperadenylation of transcripts in the nucleus. We therefore propose that manipulation of PABPC localization represents a novel mechanism to globally regulate gene expression both in the nucleus and in the cytoplasm, and has been exploited by diverse viruses perhaps as a means of commandeering cellular resources.

Results

KSHV SOX-induced nuclear retention of endogenous mRNA is dependent upon PABPC.

To better understand mechanisms of hyperadenylation in human cells, we initially focused our studies on the KSHV SOX protein, which induces mRNA hyperadenylation and a coincident mRNA export block. We predicted that if there was a connection between this phenotype and PABPC relocalization, we should be able to block SOX-induced hyperadenylation by depleting PABPC. However, our prior attempts at depleting the major isoform of PABPC (PABPC1) using siRNA oligos failed to yield consistent inhibition of SOX-induced hyperadenylation or nuclear retention of endogenous mRNA [102]. However, there are 3 additional cytoplasmic isoforms of PABPC, two of which (PABPC4, PABPC5) are widely expressed and thus might compensate for PABPC1 function in its absence [53, 55]. Indeed, we detected accumulation of a slightly higher molecular weight protein upon siRNA-mediated PABPC1 knockdown (Fig. 2.1A). Expression of this protein was induced in multiple different cell lines with 2 independent PABPC1 siRNA oligos (Fig. 2.1A). Based on its size, we hypothesized that this protein might be the PABPC4 isoform (also known as inducible PABPC, or iPABPC), which shares 79% aa identity with PABPC1 [53]. The identity of this band as PABPC4 was confirmed by showing its disappearance upon transfection of PABPC4-specific RNAi oligos (Fig. 2.1B). Additionally, α-HA immunofluorescence assays (IFA) on HEK 293T cells expressing an HA-tagged PABPC4 expression plasmid confirmed that, like PABPC1, HA-PABPC4 was relocalized from the cytoplasm to the nucleus upon co-expression with SOX (Fig. 2.1C). Our prior failure to detect mRNA export defects in the presence of SOX upon PABPC1 knockdown could therefore have been masked by the concomitant induction of PABPC4.

Hyperadenylation correlates with a failure to export mRNA from the nucleus, which can be visualized upon oligo(dT) in situ hybridization as an accumulation of endogenous nuclear poly(A) sequences. Thus, to explore whether PABPC played a role in mRNA export defects in SOX-expressing cells, siRNAs against both PABPC1 and PABPC4 or control siRNAs were transfected into HEK 293T cells (Fig. 2.2A). In control siRNA treated cells expressing SOX, oligo(dT) in situ hybridization shows a clear enhancement of endogenous nuclear poly(A) RNA (Fig. 2.2B). However, SOX expression failed to promote nuclear poly(A) RNA accumulation in cells where the levels of PABPC1 and PABPC4 protein were decreased (Fig. 2.2B). These data suggest that PABPC, perhaps upon nuclear import, plays an essential role in SOX-mediated retention of mRNA in the nucleus.

Nuclear accumulation of PABPC1 drives hyperadenylation and inhibits mRNA export.

Two possibilities followed from the above results: 1) that nuclear accumulation of PABPC is the principal driver of mRNA retention in SOX-expressing cells, or, 2) that PABPC is one necessary component of this phenotype but other SOX activities are also required. To distinguish between these, we sought to induce nuclear PABPC accumulation in the absence of SOX and monitor whether hyperadenylation and an mRNA export block ensued. To this end, PABPC1 was fused to an hnRNPC1-derived nuclear retention signal (NRS) [140, 141] and a Flag tag to differentiate it from endogenous PABPC. Importantly, immunoblotting of cell lysates with a PABPC antibody that recognized both endogenous and exogenously expressed protein showed that our transfected PABPC is not expressed above physiologic levels in these cells (Fig. 2.3A). Immunofluorescence assays with α-Flag antibodies confirmed that the NRS tag restricted PABPC1 to the nucleus, and these cells exhibited a dramatic increase in the nuclear

oligo(dT) signal as revealed by in situ hybridization (Fig. 2.3B). It is striking that only a slight elevation in the levels of nuclear PABPC is sufficient to cause nuclear poly(A) RNA accumulation (Figs. 2.3A, 2.3B). Several observations indicate that this increase in nuclear poly(A) RNA is specifically dependent on the accumulation of PABPC in the nucleus. First, it rarely occurred upon expression of Flag-PABPC1 lacking an NRS fusion, except in the occasional cells where Flag-PABPC1 expression was high enough for the protein to enter the nucleus even without the NRS. Second, it did not occur in cells expressing a control Flag-PABPC1 fused to a mutant NRS incapable of nuclear restriction (Flag-PABPC1-NRSmut). Finally, it did not occur upon expression of Flag-PABPC1 fused to a cytoplasmic retention signal derived from APOBEC3G [142] (Flag-PABPC1-CRS) (Fig. 2.3B). Similar transfection efficiencies were observed for each of the PABPC constructs, and thus this is not a contributing factor towards differences in their downstream effects.

To confirm that nuclear PABPC1 indeed directs mRNA hyperadenylation, we used a second assay in which we monitored the length of a GFP reporter message by Northern blotting. SOX-transfected cells were included as a positive control, as we have shown previously they exhibit hyperadenylated GFP mRNA [102]. In the presence of either Flag-PABPC1-NRS or SOX the length of the GFP mRNA was extended in a heterogeneous manner compared with GFP expressed alone (Fig. 2.3C). The mRNA size difference was a result of hyperadenylation, as removal of the mRNA poly(A) tails by hybridization to oligo(dT) followed by RNaseH digestion caused the GFP mRNAs to co-migrate (Fig. 2.3C). Furthermore, fractionation of the nuclear and cytoplasmic RNA populations confirmed that the hyperadenylated GFP mRNAs were largely confined to the nucleus (Fig. 2.3D). Nuclear accumulation of PABPC therefore induces hyperadenylation and nuclear retention of mRNAs.

PABPC1 exerts differential effects on gene expression depending on its localization.

We conducted the above Flag-PABPC1-NRS experiments in cells retaining endogenous PABPC so as not to confound the effect of removal of PABPC from the cytoplasm with the effect of increasing its concentration in the nucleus. Our data suggest that increasing the levels of PABPC in the nucleus causes hyperadenylation and nuclear retention of messages, which presumably would be detrimental to expression of the encoded proteins. Given that this contrasts with established cytoplasmic roles of PABPC in enhancing translation [52], we compared the effect of increasing the concentration of PABPC in the cytoplasm versus the nucleus using the CRS- and NRS- fused PABPC1. Even when attached to nuclear proteins, the CRS has been shown to override an NLS and retain proteins in the cytoplasm [142]. Immunofluorescence assays with α-Flag antibodies confirmed that Flag-PABPC1-CRS remained exclusively cytoplasmic (see Fig. 2.3B). Northern blotting of the GFP reporter was then used to monitor mRNA length and abundance in cells expressing CRS- or NRS-tagged PABPC1. In a dose-dependent manner, cytoplasmic Flag-PABPC1-CRS increased WT-length GFP mRNA levels and failed to promote hyperadenylation (Fig. 2.4A). Conversely, nuclear Flag-PABPC1-NRS expression instead decreased the abundance of WT-length GFP mRNA in a dose-dependent manner and induced accumulation of higher MW GFP mRNA species (Fig. 2.4B).

To determine whether PABPC1-NRS and PABPC1-CRS alter mRNA stability, we measured their effects on the half-life of the GFP message. Cells were treated with actinomycin D (Act D) at 24 h post transfection and RNA was isolated at the indicated times thereafter. Expression of Flag-PABPC1-NRS did not significantly alter the overall stability of the GFP message (Fig. 2.4C). In contrast, expression of Flag-PABPC1-CRS lead to a marked increase in

the GFP mRNA half-life, from ~17 h to >100 h (Fig. 2.4C). These data in cells are in agreement with prior observations showing that cytoplasmic PABPC1 stabilizes mRNAs in cell extracts and when overexpressed in xenopus oocytes [143-145]. In addition, our results suggest that the levels of PABPC in the cytoplasm may be limiting. Alternatively, the CRS fusion could interfere with mRNA deadenylation by Pan2-Pan3, which are recruited via interactions with the C-terminus of PABPC [126].

Although expression of nuclear Flag-PABPC1-NRS and subsequent mRNA hyperadenylation do not lead to changes in the half-life of GFP mRNA, we found that GFP protein levels were dramatically decreased in Flag-PABPC1-NRS-expressing cells (Fig. 2.4D). As endogenous PABPC is present in these cells, the reduced protein levels are likely not a consequence of decreased PABPC in the cytoplasm but rather specific to the ability of nuclear PABPC to induce hyperadenylation and mRNA retention in the nucleus. Conversely, enhanced expression of PABPC in the cytoplasm might boost GFP protein production, at least in part as a consequence of increased mRNA stability. This was indeed the case, as immunoblotting shows enhanced GFP protein levels in cells co-expressing cytoplasmic Flag-PABPC1-CRS (Fig. 2.4D). Collectively, these results indicate that PABPC has profound but opposite effects on gene expression depending on its levels in the nucleus versus the cytoplasm.

Poly(A) binding motifs are required for hyperadenylation.

PABPC1 is a modular protein consisting of four amino-terminal RNA recognition motifs (RRMs), an unstructured linker region, and a conserved carboxyl-terminal helical (5H) region (Fig. 2.5A). RNA binding is primarily carried out by the first 2 RRM domains, although RRM3 and RRM4 can also associate with RNA, albeit with reduced affinity [46, 48, 56, 146, 147]. Protein-protein interactions with factors involved in translation occur via both the RRM regions and the 5H domain [48, 58, 59, 62, 63]. We constructed a variety of deletion mutants to identify the regions of PABPC1-NRS required for promoting hyperadenylation and mRNA retention in the nucleus. Immunoblotting with α-HA and α-Flag antibodies confirmed that the PABPC mutants are expressed to equivalent levels upon transfection into HEK 293T cells (Fig. 2.5B). Using oligo(dT) in situ hybridization and Northern blotting assays, we found that a mutant lacking both RRM1 and RRM2 failed to promote hyperadenylation and mRNA nuclear retention (Fig. 2.5C, 2.5E). In contrast, mutants lacking RRM3 and RRM4, the linker region, or the C-terminal domain retained the ability to hyperadenylate and restrict export of mRNA (Fig. 2.5C, 2.5E).

Having shown PABPC1 RRM1 and RRM2 to be necessary for hyperadenylation, we next asked whether these domains were sufficient for hyperadenylation to occur. Strikingly, expression of RRM1+2 alone fused to an NRS was sufficient to retain poly(A) RNA in the nucleus and drive hyperadenylation (Fig. 2.5D, 2.5E). This region of PABPC1 must be present in the nucleus to block mRNA export, as it failed to cause this phenotype when restricted to the cytoplasm by fusion to a CRS (Fig. 2.5D). Furthermore, expression of RRM3+4 fused to an NRS also failed to promote hyperadenylation and nuclear retention of mRNA (Fig. 2.5D, 2.5E), even though these domains are capable of RNA binding [48, 56]. Therefore, we conclude that RRM1 and RRM2 together are both necessary and sufficient for PABPC1-induced hyperadenylation and nuclear retention of mRNAs.

Dissociation of cellular mRNA 3' end processing from hyperadenylation.

Although hyperadenylation has been shown to occur both in yeast and mammals in association with defects in either mRNA 3' end processing or export, these two processes are closely linked and the underlying mechanisms governing aberrant polyadenylation remain unknown. We therefore assessed the ability of Flag-PABPC1-NRS to promote hyperadenylation of GFP mRNAs that do not undergo cellular 3' end processing, but instead are cleaved by a hammerhead ribozyme [102]. We tested GFP constructs either terminating via ribozyme cleavage just 3' proximal to the stop codon (GFP-HR) or containing a templated 60 nt poly(A) tail upstream of the ribozyme cleavage site (GFP-A_{60}-HR). Unlike GFP that was processed by the cellular 3' end machinery, neither GFP-HR nor GFP-A_{60}-HR appeared efficiently hyperadenylated in the presence of Flag-PABPC1-NRS (Fig. 2.6A). Notably, however, in the presence of Flag-PABPC1-CRS both GFP and GFP-A_{60}-HR mRNA levels increased to a much greater extent than GFP-HR. This is presumably due to mRNA stabilization (see fig. 2.4C). Thus, while cellular 3' end processing enhanced susceptibility of mRNA to PABPC-induced hyperadenylation in the nucleus, the presence of a poly(A) tail is the critical determinant for PABPC-induced mRNA stabilization in the cytoplasm.

We previously showed that treatment of cells with leptomycin B (LMB) stabilized hyperadenylated mRNAs in SOX-expressing cells, greatly facilitating their detection [102]. We therefore tested whether LMB treatment might likewise reveal weak or unstable hyperadenylated GFP-HR or GFP-A_{60}-HR mRNAs in cells expressing Flag-PABPC1-NRS. Surprisingly, we were able to observe nuclear PABPC1-induced accumulation of higher MW GFP-A_{60}-HR species in LMB-treated cells (Fig. 2.6B), although at significantly reduced efficiency relative to normal GFP mRNA. That the higher MW mRNA species represented hyperadenylated products was confirmed by showing their disappearance upon incubation of the RNA with oligo(dT) and digestion with RNaseH (Fig. 2.6C). Hyperadenylation required the presence of the templated A_{60} tail, as it was never detected on the GFP-HR mRNA which lacked any poly(A) tail (Fig. 2.6B). Additionally, we observed that, similar to the case with GFP, nuclear expression of PABPC1 RRM1 and RRM2 alone was sufficient to drive hyperadenylation of GFP-A_{60}-HR in LMB-treated cells, although to reduced levels relative to the full-length protein (Fig. 2.6B). These data show that hyperadenylation can occur in cells, albeit less efficiently, via a mechanism uncoupled to cellular mRNA 3' end processing.

Polyadenylation normally occurs in conjunction with cleavage and polyadenylation specificity factor complex (CPSF)-induced mRNA 3' end cleavage. The canonical poly(A) polymerase, PAPII, is recruited to nascent transcripts via interactions with PABPN and CPSF to processively polyadenylate mRNAs [30]. PAPII also participates in hyperadenylation, as SOX-induced hyperadenylation of CPSF-processed GFP is reduced upon depletion of PAPII [102]. Our current observations, however, suggested that polyadenylation can occur on mRNAs that are not processed by CPSF, as long as they already possess some poly(A) sequence. To determine whether this noncanonical polyadenylation is also carried out by PAPII, we monitored nuclear PABPC1-induced hyperadenylation of GFP-A_{60}-HR upon siRNA-mediated depletion of PAPII (Fig. 2.6D). Indeed, removal of PAPII significantly reduced GFP-A_{60}-HR hyperadenylation (Fig. 2.6D), suggesting that in cells with elevated levels of nuclear PABPC, PAPII can act on mRNAs after or independently of 3' end processing to cause hyperadenylation.

Multiple divergent viral proteins cause nuclear import of PABPC and mRNA accumulation in the nucleus.

Finally, we sought to determine whether PABPC relocalization and subsequent hyperadenylation and nuclear retention of mRNA might be conserved mechanisms used by other viral proteins to restrict host gene expression. In addition to KSHV SOX and its gamma-herpesvirus homologs such as MHV-68 muSOX, the vhs protein of herpes simplex virus (HSV; an alpha-herpesvirus) and the NSP1 protein of SARS coronavirus (SCoV) are well-characterized host shutoff factors that target mRNA [148-153]. We therefore monitored by immunofluorescence assays the endogenous PABPC localization in HEK 293T cells transfected with each of these viral factors. Similar to the gamma-herpesviral SOX and muSOX proteins, HSV-1 vhs and SCoV NSP1 also induced prominent nuclear relocalization of endogenous PABPC (Fig. 2.7A). For vhs this relocalization is linked to its host shutoff activity, as a vhs mutant unable to restrict host gene expression [154] failed to induce PABPC import (Fig. 2.7A), even though it is expressed at levels higher than the WT protein (data not shown). The HSV-1 ICP27 and UL47 proteins have also recently been implicated in PABPC relocalization, suggesting that multiple viral factors may collectively target PABPC during HSV-1 infection [104].

Given their ability to cause PABPC import, we predicted that HSV-1 vhs and SCoV NSP1 should also induce hyperadenylation and nuclear retention of mRNA. Indeed, expression of HSV-1 vhs or SCoV NSP1 resulted in a robust accumulation of endogenous poly(A) RNA in the nucleus and hyperadenylation of the reporter GFP message, similar to SOX and muSOX-expressing cells (Fig. 2.7B, 2.7C). In agreement with the PABPC import data, this mRNA export block was not observed in cells expressing mutant vhs (Fig. 2.7B). Thus, four independent viral proteins known to globally inhibit host gene expression restrict mRNA export via a common mechanism of PABPC relocalization.

A block in mRNA export occurs during viral infection

Thus far we have demonstrated that nuclear accumulation of PABPC (either driven by SOX or through expression of PABPC1-NRS) results in a global mRNA export block. We next sought to determine if this effect was also true during a viral infection. We hypothesized that our current assay to detect endogenous mRNA through oligo(dT) staining would be ineffective during a KSHV infection because KSHV encodes for a highly abundant nuclear poly(A) RNA termed PAN. Therefore we asked this question in a related gammaherpesvirus murine herpesvirus 68 (MHV68). Similar to KSHV, MHV68 also relocalizes PABPC into the nucleus in a host shutoff dependent manner and this effect is due to the activity of the SOX homlog, muSOX protein [116]. Importantly, a host shutoff defective virus did not relocalize PABPC. We infected COS-7 cells with wild type MHV68 and MHV68.ΔHS and visualized endogenous mRNA through in situ hybridization with oligo(dT). We found a global mRNA export block during infection with MHV68, however, it was significantly reduced in cells infected with MHV68.ΔHS (Fig. 2.8). These data indicate that PABPC-induced mRNA export block is also mediated during a viral infection.

Discussion

Although PABPC is a shuttling protein and can be found at low levels in the nuclei of uninfected cells [99], we showed that increasing its nuclear abundance drives hyperadenylation and nuclear retention of mRNAs, thereby inhibiting their expression. Hyperadenylation requires the PABPC poly(A) binding motifs, suggesting it may be triggered upon binding of PABPC to mRNA poly(A) tails either during or after the normal polyadenylation process. In support of this idea is our observation that PABPC can direct PAPII-induced hyperadenylation of ribozyme-terminating transcripts only if the mRNAs contain templated poly(A) sequences. These data additionally indicate that hyperadenylation is not necessarily coincident with 3' end processing, and may be carried out by PAPII after the initial round of polyadenylation has completed.

We envision at least two non-mutually exclusive mechanisms by which nuclear PABPC could trigger hyperadenylation. First, if enhanced levels of PABPC binding to transcripts disrupted or significantly altered the ribonucleoprotein composition of mRNAs, nascent messages might fail nuclear quality control checkpoints required for export. Transcripts that are not efficiently exported may then become susceptible to poly(A) tail extension by PAPII, perhaps because of increased duration of nuclear residence. Reports showing that hyperadenylated mRNAs accumulate in yeast export factor mutants or in mammalian cells depleted of TAP/NXF1 would be consistent with this model [40, 41, 43, 155]. Additionally, the KSHV ORF57 protein, which binds and stabilizes the viral noncoding RNA PAN can also induce PAN hyperadenylation, perhaps as a consequence of increasing its half-life in the nucleus [156]. That said, hyperadenylation and retention of mRNAs in the nucleus are interconnected outcomes, making it difficult to distinguish the initiating event. The interplay between mRNA 3' end processing and export is likely to be complex and involve extensive RNP remodeling and recycling of processing factors [43]. Delineation of which (if any) of these events are disrupted by PABPC will be an important future challenge.

An alternate possibility is that PABPC more directly recruits or affects the regulation of poly(A) polymerase on mRNAs. Recruitment would have to occur through the first two PABPC RRMs, as these are sufficient to stimulate hyperadenylation. A recent report identifying PABPC as a co-purifying component of the mRNA 3' end processing complex may support this model, although whether it actually plays a role in mRNA processing remains to be established [24]. We have yet to observe an interaction between PABPC and PAPII (G.R. Kumar and B. Glaunsinger, unpublished observations), indicating that if PABPC participates in PAPII recruitment it is likely to be via other intermediate interactions. Indeed, *in vitro* biochemical experiments with purified proteins have shown that yeast and mammalian PABPC inhibit the polyadenylation reaction [131, 157]. Competition between PABPC and PABPN for binding nascent poly(A) tails could also disrupt the interplay between PAPII and PABPN, which is proposed to govern poly(A) tail length control [30]. However, this is unlikely to be the sole mechanism of hyperadenylation, given the susceptibility of GFP-A_{60}-HR to this phenotype.

Many viruses globally restrict cellular gene expression, in part as a mechanism of resource reallocation. By dampening cellular mRNA translation, competition is lessened for the gene expression machinery critical for efficient viral gene expression and replication. An additional important advantage of virus-induced restriction of cellular gene expression is immune evasion, as many of the genes whose expression is inhibited are important effectors of the immune response [158, 159]. A diverse group of viruses have thus often evolved to target the same key host proteins and pathways that allow them to commandeer cellular gene

expression machinery. PABPC is a clear cellular target of numerous viruses, many of which encode proteases that cleave it as a means of restricting host translation [83]. However, recent findings that herpesviruses, rotaviruses, and bunyaviruses all drive nuclear import of PABPC underscores the likely importance of its nuclear functions, in addition to its roles in translation, in the global regulation of gene expression [100, 101, 104]. In the case of gamma-herpesvirus SOX, herpes simplex virus vhs, and SARS coronavirus NSP1, host gene expression is repressed on at least two fronts. In the cytoplasm, these viral proteins inactivate host mRNAs through cleavage and/or translational repression [148-153]. The gene expression block is then likely magnified via PABPC relocalization and ensuing hyperadenylation, which would prevent repopulation of the cytoplasm with newly transcribed mRNAs.

For most RNA viruses, whose viral mRNAs are transcribed and processed in the cytoplasm, triggering PABPC-induced hyperadenylation of nuclear mRNAs would be an effective means to selectively block nascent cellular gene expression. Additionally, RNA viruses such as rotaviruses and bunyaviruses produce transcripts lacking poly(A) tails and have evolved PABPC-independent mechanisms of translation [100, 160, 161]. For these viruses, removal of PABPC from the cytoplasm should be detrimental to host, but not viral, gene expression. However, other RNA viruses such as coronaviruses encode mRNAs that are polyadenylated and thus may still depend on some residual cytoplasmic PABPC for efficient expression [162]. In the case of herpesviruses, the advantage of PABPC relocalization is not immediately clear. These DNA viruses utilize the host RNA transcription and processing machinery to produce their transcripts, which must then be exported and translated in a manner presumably analogous to cellular mRNAs. One important future direction will therefore be to reveal how nuclear-replicating viruses such as herpesviruses are able to subvert this nuclear PABPC-induced gene expression block to allow efficient production of their own genes. Interestingly, a recent report suggests that during KSHV infection, translation of viral mRNAs in the absence of abundant cytoplasmic PABPC may be at least partially compensated by enhanced assembly of eIF4F through preferential eIF4G recruitment to the 5' cap [163].

It is notable that nuclear relocalization of PABPC is also triggered during certain cellular stresses, including heat shock or transcriptional block [99, 125]. This phenotype is thus perhaps a normal cellular response to some forms of stress, but which can also be usurped during viral infection. From a cellular perspective, PABPC relocalization could be a mechanism to pause gene expression via inhibition of both translation in the cytoplasm and nascent RNA transport from the nucleus. Under these conditions, cytoplasmic mRNAs may be shuttled to stress granules [164], whereas nuclear transcripts are retained and hyperadenylated. Our half-life analyses indicate that in the absence of viral infection these hyperadenylated transcripts do not undergo enhanced degradation, perhaps suggesting that tail trimming might reinstate their export competence [40]. An important distinction for non-viral stresses that cause PABPC import would therefore be that upon removal of the stress, PABPC could re-enter the cytoplasm, perhaps allowing resumption of mRNA export and translation. Indeed, this is the case following release from heat shock [125]. However, during infection, PABPC remains nuclear until completion of the viral replication cycle [100-102], which generally coincides with cell death.

In summary, our data support a novel role for PABPC in the restriction of cellular gene expression, in addition to its established functions in regulating cytoplasmic mRNA stability and enhancing translation. The ability to globally influence gene expression by controlling the localization of this key regulatory protein would be an efficient means for cells to respond to physiologic stresses, but also represents a potential Achilles heel for viral attack. Further

exploration of PABPC functions in the nucleus should therefore shed new light on how viruses co-opt host gene expression pathways, as well as provide mechanistic insight into the expanding roles of polyadenylation in determining RNA fate.

Materials and Methods

Plasmids

The PABPC1 cDNA (accession number Y00345) was cloned into BamHI and XbaI sites of pCDEF3 and subsequently 5' tagged with a 1X HA or 1X Flag tag to generate pCDEF3-HA-PABPC1 and pCDEF3-Flag-PABPC1, respectively. An 8 glycine (G8) linker followed by either an hnRNPC1-derived wild type nuclear retention signal (NRS) (amino acids [aa] 88 to 165) or mutant version of NRS (NRSmut) [140] that contained aa 98-146 of original NRS fused to 29 aa of GFP to ensure the same size as WT [119] were fused to the C-terminus of Flag-PABPC1 by standard PCR methods to generate pCDEF3-Flag-PABPC1-NRS and pCDEF3-Flag-PABPC1-NRSmut. Likewise, a G8 liner followed by a cytoplasmic retention signal derived from APOBEC3G (aa 51-128) [142] was fused to the C-terminus of Flag-PABPC1 by standard PCR methods to generate pCDEF3-Flag-PABPC1-CRS. HA-tagged PABPC1 deletion mutants fused to the NRS were produced by overlap-extension PCR and cloned into BamHI and XbaI sites of pCDEF3 to generate pCDEF3-HA-PABPC1-ΔRRM1+2-NRS (lacking nt 1-176), pCDEF3-HA-PABPC1-ΔRRM3+4-NRS (lacking nt 191-370), pCDEF3-HA-PABPC1-ΔRRM1-4-NRS (lacking nt 1-370), pCDEF3-HA-PABPC1-ΔLinker-NRS (lacking nt 371-538), and pCDEF3-HA-PABPC1-ΔC-terminus-NRS (lacking nt 539-636). Plasmids pd2-eGFP-HR and pd2-eGFP-A_{60}-HR, [102], pCDEF3-SOX and pCDEF3-muSOX [119], pCDNA3.1-vhs and pCDNA3.1-vhs mut [154] have been previously described. Plasmids pCAGGS-NSP1 and pCDNA3-PABPC4-HA were kindly provided by Dr. Shinji Makino (The University of Texas Medical Branch) and Dr. Tullia Lindsten (University of Pennsylvania), respectively.

Cells, Transfections, and Infections

HEK 293T cells, HeLa cells, and COS-7 cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS. DNA transfections were performed using Effectene (Qiagen) as per manufacturer's instructions. Cells to be processed for in situ hybridization or immunofluorescence (and the corresponding Western blots) were transfected in a 12-well plate with 1 µg total plasmid DNA (900 ng empty vector + 100 ng of the indicated PABPC plasmid). Cells to be harvested for Northern blotting (and the corresponding Western blots) were transfected in a 12-well plate with 1 µg total plasmid DNA (900 ng of the indicated PABPC plasmid or empty vector + 100 ng GFP reporter), unless indicated otherwise in the figure legend.

siRNA Knockdown

PABPC1 and PAPII knockdowns were achieved using previously described siRNA oligos and methods [102]. Knockdowns of PABPC4 (iPABPC) were carried out using the following PABPC4-specific siRNA duplex oligos: siRNA #1: 5'AGGAGAGAAUUAGUCGAUAUCAGGG, siRNA #2: 5'GGAAUUCAACUCAAGGUUUGAAGAC. Nonspecific control siRNAs were purchased from Ambion. HEK 293T cells were transfected with 200nM siRNA oligos using Lipofectamine 2000 (Invitrogen) for 24 h, followed by transfection with DNA for an additional 24 h prior to harvesting for either protein analysis, in situ hybridization, or Northern blotting.

Immunofluorescence Assays and In Situ Hybridization

Cells were grown on coverslips coated with 100µg/mL poly-L-Lysine and processed for IFA as previously described [165]. Briefly, cells were fixed in 4% formaldehyde, incubated for 10 min in permeablization buffer [1% triton x-100 (vol/vol) and 0.1% sodium citrate (w/vol)], then for 30 min in block buffer [1% triton x-100 (vol/vol), 0.5% tween-20 (vol/vol) and 3% BSA (w/vol)], and incubated with either mouse monoclonal PABPC 10e10 (1:25 dilution) (Santa Cruz Biotechnology), rabbit polyclonal SOX J5803 (1:500 dilution) [166], mouse monoclonal HA 12CA5 (1:500 dilution) (Abcam), or mouse monoclonal Flag (1:500 dilution) (Sigma) primary antibodies for 3-12 h in block buffer, followed by incubation with AlexaFluor 488- or 546-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:1500 dilution) (Molecular Probes). Coverslips were mounted in DAPI-containing Vectashield mounting medium (Vector Labs) to visualize nuclei.

For in situ hybridization (http://www.singerlab.org/protocols), cells were fixed with 4% formaldehyde for 10 min, then washed 2X with PBS and permeabilized by treatment with 70% ethanol for 2 h to overnight. Cells were next treated with the following for 5 min each: 1X PBS, 1M Tris (pH8.0), and 1X PBS. Cells were hybridizated overnight at 37°C in 200uL of hybridization buffer [50% formamide (vol/vol), 10% Dextran Sulfate (vol/vol), 0.02% BSA (w/vol), 200µg E. coli tRNA, and 2X SSC] using 2ng/µL of AlexaFluor 546 labeled oligo$(dT)_{15}$ (Molecular Probes). Cells were then processed for IFA as described above.

Cell Extracts, Fractionation, Immunoblots, and Northern Blots

Cell lysates were prepared in RIPA buffer [50mM Tris-HCl [pH8.0], 150mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS)] containing protease inhibitors (Roche), and quantified by Bradford assay (Bio-rad). Equivalent gs of each sample were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and subjected to immunoblot with either mouse monoclonal GFP (1:2000 dilution), rabbit polyclonal PABPC 4992 (1:2000 dilution) (Cell Signaling Technology), or rabbit polyclonal SOX J5803 (1:5000 dilution) [166] primary antibodies, followed by incubation with horseradish peroxidase-conjugated actin antibodies (for loading control), and goat anti-mouse or goat anti-rabbit secondary antibodies (1:5000 dilution) (Southern Biotechnology Associates).

Total cellular RNA was isolated for Northern blotting using RNA-bee (Tel-Test), resolved on 1.2% agarose-formaldehyde gels, and probed with a ^{32}P-labeled GFP DNA probe made using RediPrime II random prime labeling kit (GE Healthcare). Where indicated, cells were treated with 5ng/mL Leptomycin B (Sigma) for 6-12 h prior to RNA isolation to stabilize hyperadenylation products [102], or with 5µg/mL Actinomycin D (Act D) for the indicated times to monitor mRNA half-life. Cellular fractionation was carried out using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) as per manufacturer's instructions, followed by RNA isolation from fractionated extracts using RNA-bee (Tel-Test).

RNaseH digestions were carried out with 5-7µg of total RNA combined with 500 pmol of oligo$(dT)_{15}$ primer in a 25.8µl reaction volume. After incubation at 65°C for 8 min, 1U of RNaseH (New England Biolabs) and 40U of RNasin (Promega) were added with 1X RNaseH buffer, followed by incubation at 37°C for 30 min. Reactions were terminated by the addition of 1µl of 0.5M EDTA (pH 8.0), and the RNA was ethanol precipitated and subjected to gel electrophoresis. Northern blots were analyzed using a Typhoon 8600 phosphorimager and, for half-life experiments, GFP mRNA signal intensity at each timepoint was quantified using Image J software and normalized to 18S signal.

Figures

Figure 2.1

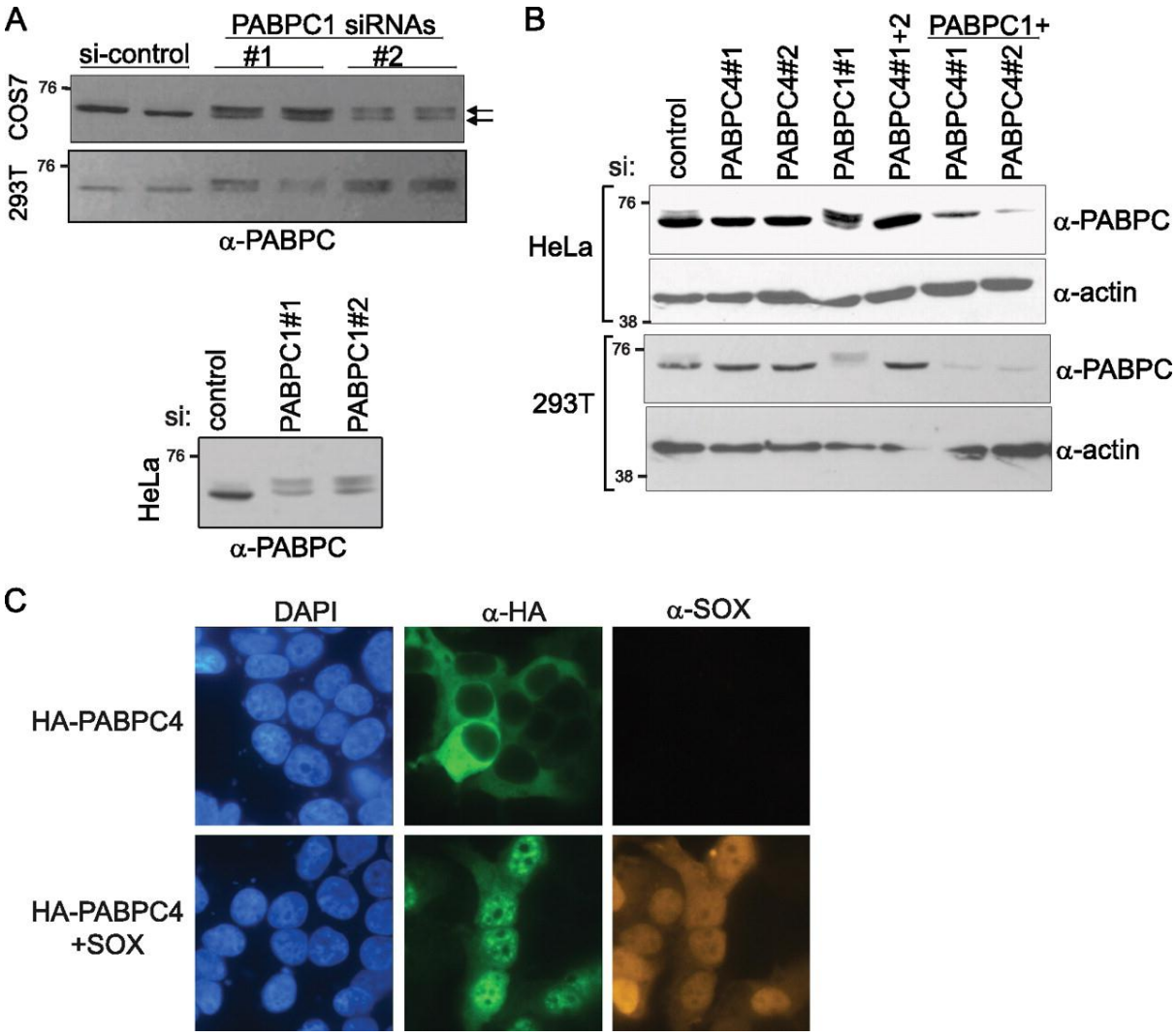

Figure 2.1: **PABPC4 is induced upon PABPC1 depletion and is directed to the nucleus by SOX**. (A) COS7, HEK 293T, and HeLa cells were transfected with either control siRNAs (si) or 2 independent siRNAs specific for PABPC1 (PABPC1#1 and PABPC1#2). siRNA transfections for COS7 and HEK 293T cells were performed in duplicate. At 72 h posttransfection, cell lysates were resolved by SDS-PAGE and immunoblotted with anti-PABPC antibodies. (B) HeLa and HEK 293T cells were transfected with control siRNAs or the indicated siRNAs specific for PABPC1 and/or PABPC4. Two independent PABPC4-specific siRNAs were used (PABPC4#1 and PABPC4#2). Lysates were then harvested and immunoblotted as described in the legend to panel A. (C) HEK 293T cells were transfected with a plasmid expressing HA-tagged PABPC4 alone or together with a plasmid expressing SOX and, 24 h later, subjected to immunofluorescence assays with anti-HA and anti-SOX antibodies. DAPI staining was used to visualize nuclei.

Figure 2.2

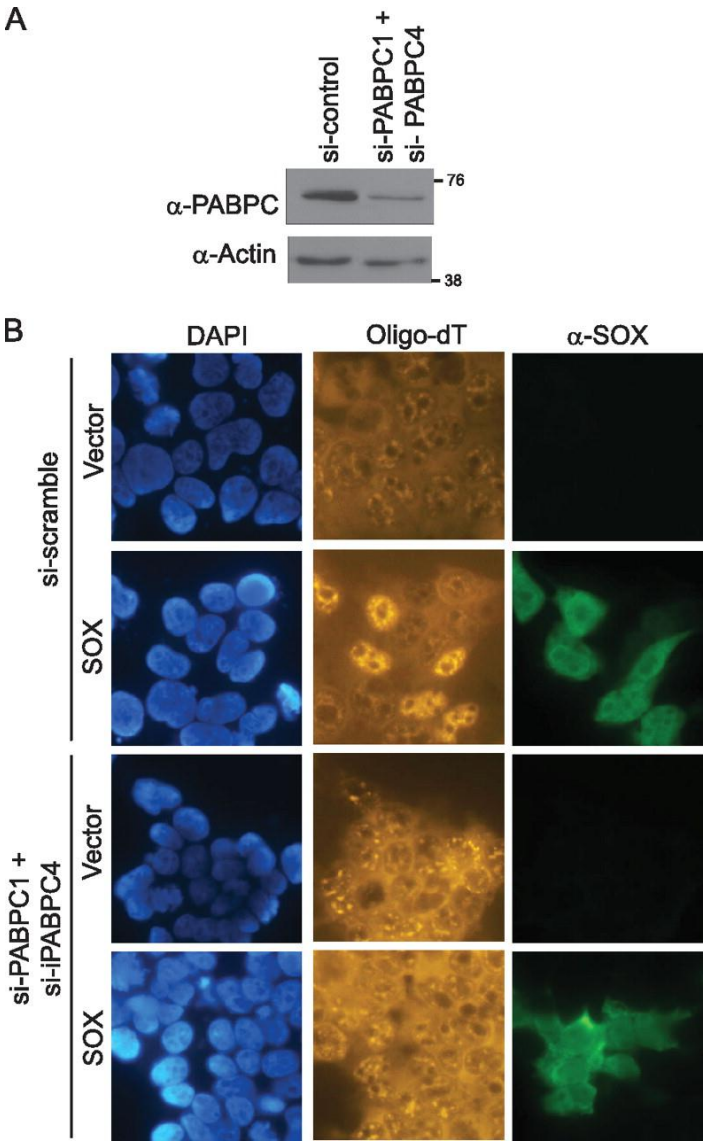

Figure 2.2 PABPC is required for SOX-induced nuclear poly(A) RNA accumulation. (A) HEK 293T cells were transfected with control siRNAs or siRNAs against PABPC1 and PABPC4. Lysates were harvested 24 h posttransfection and immunoblotted with anti-PABPC antibodies to detect the efficiency of PABPC protein depletion. In parallel, antiactin immunoblotting was performed to control for loading. (B) Cells described in the legend to panel A were transfected with either empty vector or a plasmid expressing SOX for 24 h and then processed for in situ hybridization with oligo(dT), followed by staining with anti-SOX antibodies and DAPI to visualize nuclei.

Figure 2.3

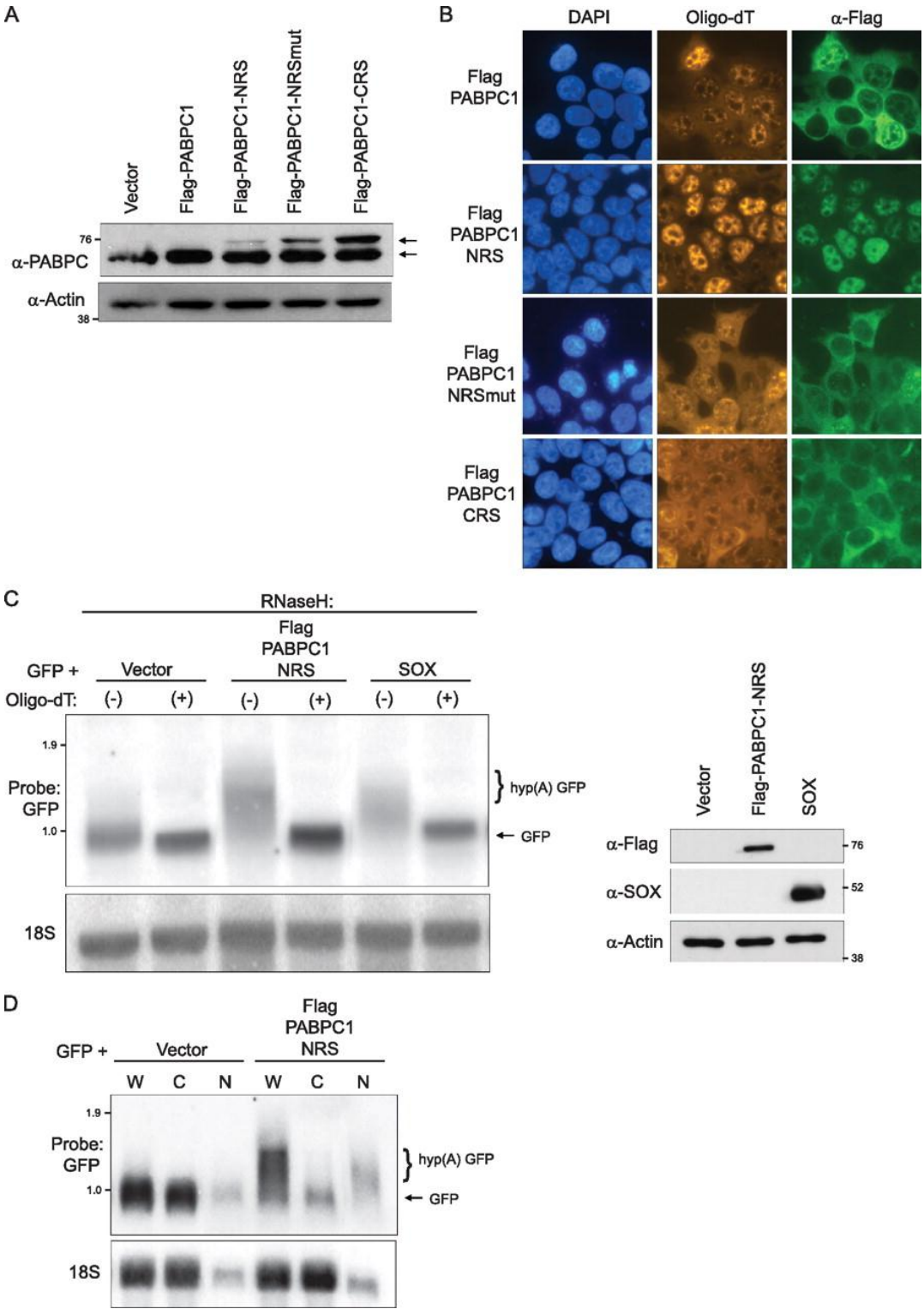
A
Vector
Flag-PABPC1
Flag-PABPC1-NRS
Flag-PABPC1-NRSmut
Flag-PABPC1-CRS
76
α-PABPC
α-Actin
38
B
DAPI
Oligo-dT
α-Flag
Flag PABPC1
Flag PABPC1 NRS
Flag PABPC1 NRSmut
Flag PABPC1 CRS
C
RNaseH:
Flag PABPC1 NRS
GFP +
Vector
SOX
Oligo-dT:
(-)
(+)
1.9
Probe: GFP
1.0
hyp(A) GFP
GFP
18S
Vector
Flag-PABPC1-NRS
SOX
α-Flag
α-SOX
α-Actin
76
52
38
D
Flag PABPC1 NRS
GFP +
Vector
W
C
N
1.9
Probe: GFP
1.0
hyp(A) GFP
GFP
18S

Figure 2.3 Nuclear accumulation of PABPC causes mRNA retention and hyperadenylation. (A) HEK 293T cells were transfected with the indicated PABPC1 expression plasmids for 24 h. Lysates were then harvested, resolved by SDS-PAGE, and immunoblotted with anti-PABPC antibodies to detect both endogenous (bottom arrow) and exogenous (upper arrow) PABPC proteins. Note that Flag-PABPC1 comigrates with endogenous PABPC1 in this blot. In parallel, antiactin immunoblotting was performed to control for loading. (B) HEK 293T cells were transfected as described in the legend to panel A for 24 h and then processed for oligo(dT) in situ hybridization, followed by immunofluorescence assays with anti-Flag antibodies. (C) HEK 293T cells were cotransfected with the indicated plasmids for 24 h. After treatment with leptomycin B (LMB) for 12 h to stabilize hyperadenylated species, total RNA and proteins were isolated. Total RNA was incubated in the presence or absence of oligo(dT) and digested with RNaseH. Products were resolved on a 1.2% agarose-formaldehyde gel and Northern blotted with 32P-labeled GFP and 18S probes (top). Hyperadenylated species are indicated by the labeled bracket [hyp(A) GFP]. Total protein was resolved by SDS-PAGE and immunoblotted using anti-Flag and anti-SOX antibodies (bottom). Actin served as a loading control. (D) Cells were transfected with the indicated plasmid as described above and treated with LMB for 7 h. Total RNA was then isolated from whole cells (W) or cytoplasmic fractions (C) and nuclear fractions (N) fractions and Northern blotted with 32P-labeled GFP and 18S probes. Hyperadenylated species are indicated by the labeled bracket [hyp(A) GFP].

Figure 2.4

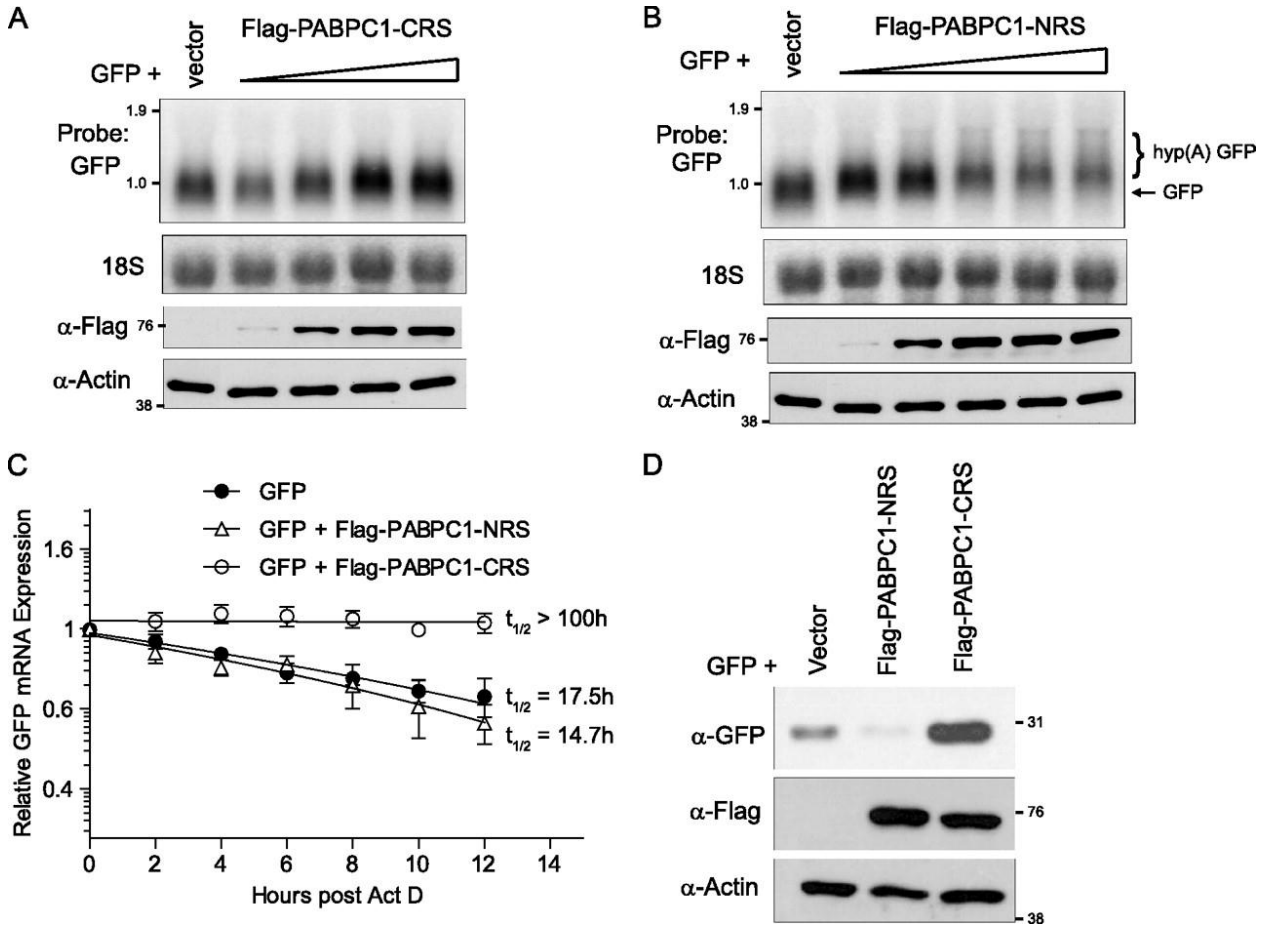

Figure 2.4 Cytoplasmic PABPC and nuclear PABPC have opposing effects on gene expression. (A, B) HEK 293T cells were transfected with GFP and increasing amounts of Flag-PABPC1-CRS or Flag-PABPC1-NRS (100 to 900 ng) for 24 h. Total RNA was then isolated and visualized by Northern blotting with 32P-labeled GFP and 18S probes. Hyperadenylated species are indicated by the labeled bracket [hyp(A) GFP]. To monitor protein levels, protein lysate from the transfected cells was resolved by SDS-PAGE and immunoblotted with anti-Flag or antiactin (loading control) antibodies. (C) HEK 293T cells were transfected with GFP and empty vector, Flag-PABPC1-CRS, or Flag-PABPC1-NRS. At 24 h posttransfection, 5 μg/ml of actinomycin D was added to block transcription, and total RNA was harvested at the indicated time points thereafter. RNA was then visualized by Northern blotting with GFP and 18S probes, and the GFP mRNA half-life was calculated after 18S normalization. Error bars indicate standard errors between samples. Data were derived from five independent experiments. (D) HEK 293T cells were transfected with plasmids expressing GFP and empty vector, Flag-PABPC1-NRS, or Flag-PABPC1-CRS for 24 h. Equivalent amounts of protein lysate were resolved by SDS-PAGE and immunoblotted with antibodies against GFP, Flag, and actin (as a loading control).

Figure 2.5

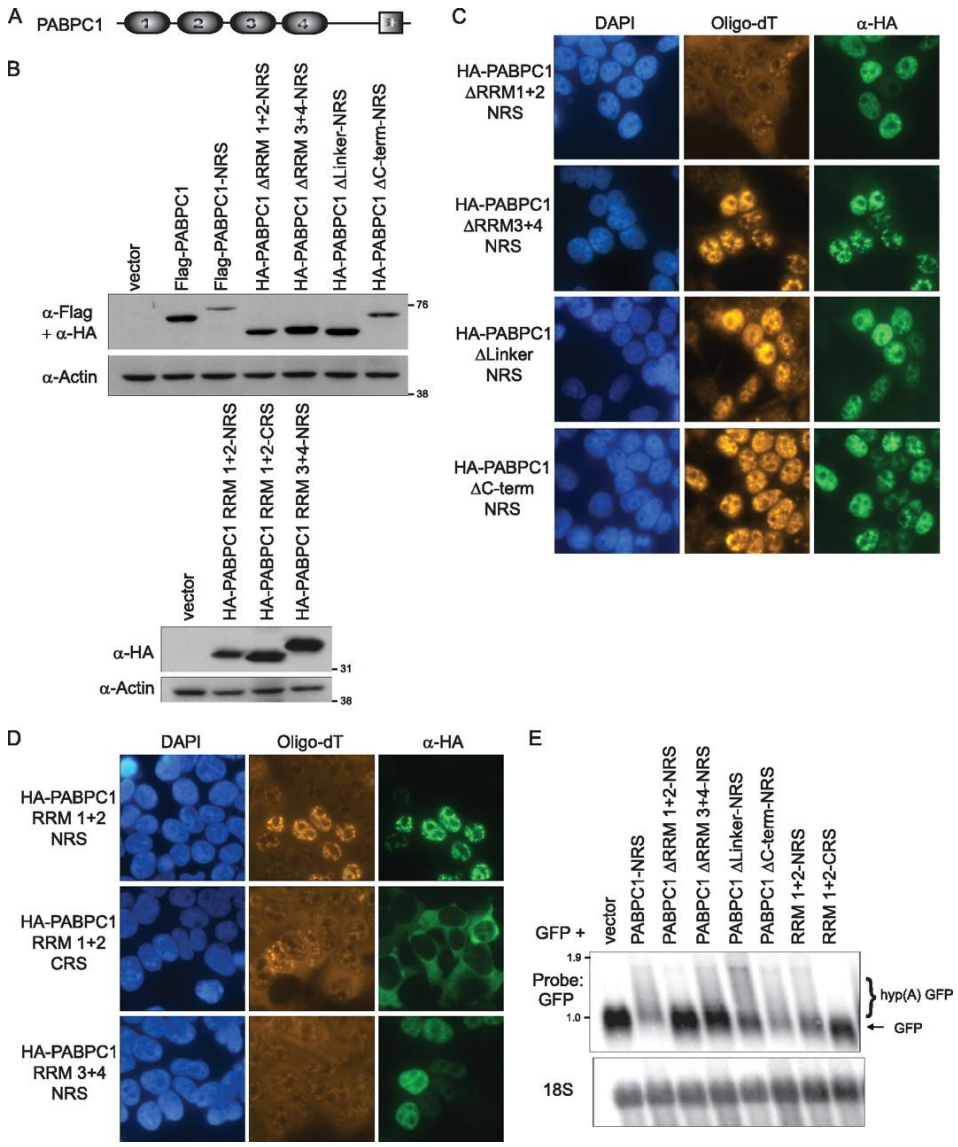

Figure 2.5 PABPC RRM1 and RRM2 are necessary and sufficient to induce hyperadenylation. (A) Diagram of PABPC, showing the 4 RRMs, followed by the linker region and conserved helical carboxyl terminus (square). (B) Expression levels from the indicated PABPC1 WT and mutant constructs were monitored following transfection into 293T cells for 24 h. Protein lysates harvested, resolved by SDS-PAGE, and immunoblotted with a mixture of anti-Flag and anti-HA antibodies or with anti-actin antibody as a loading control. (C and D) HEK 293T cells were transfected with the indicated plasmids for 24 h and then subjected to oligo(dT) in situ hybridization and immunofluorescence assays with anti-HA antibodies. Nuclei were stained with DAPI. (E) HEK 293T cells were transfected with plasmids expressing GFP and either empty vector or the indicated PABPC construct for 24 h. Total RNA was then resolved by agarose-formaldehyde gel electrophoresis and Northern blotted with 32P-labeled GFP and 18S probes. Hyperadenylated species are indicated by the labeled bracket [hyp(A) GFP].

Figure 2.6

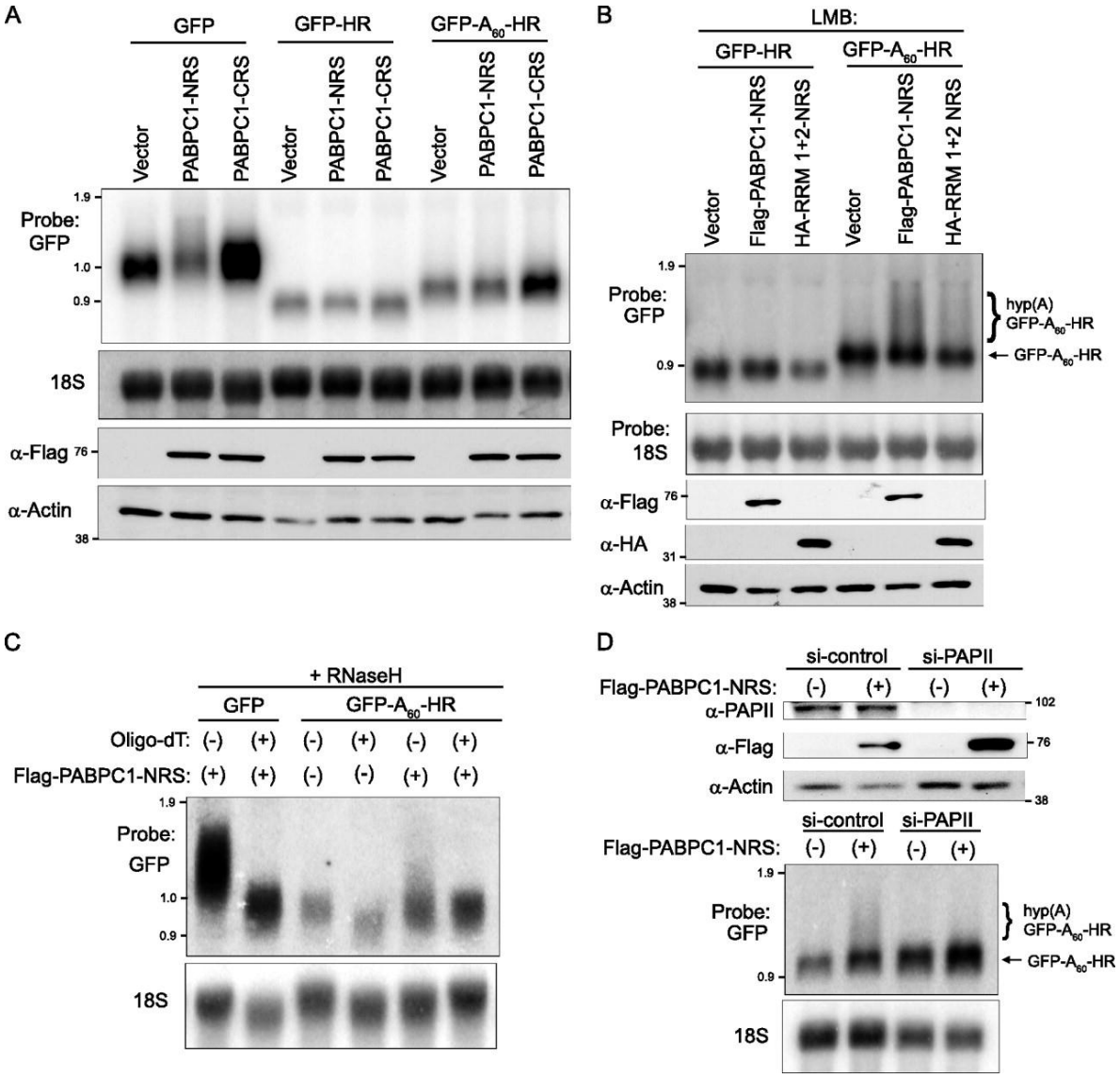

Figure 2.6 Cellular mRNA 3′-end processing enhances but is not required for PABPC-induced hyperadenylation. (A) HEK 293T cells were transfected with the indicated plasmids for 24 h, and then total RNA was isolated and Northern blotted with 32P-labeled GFP and 18S probes. (B) Same protocol as described in the legend to panel A, but cells were treated with 5 ng/ml LMB for 12 h prior to RNA isolation to enhance detection of hyperadenylated species. (C) HEK 293T cells were transfected with the indicated plasmids for 24 h and then treated with 5 ng/ml LMB for 12 h. Total RNA was isolated and incubated in the presence or absence of oligo(dT) and then digested with RNaseH. RNA was visualized by Northern blotting with 32P-labeled GFP and 18S probes. (D) HEK 293T cells were transfected twice over 48 h with either PAPII siRNAs or control siRNAs and then subsequently transfected in duplicate with DNA plasmids expressing GFP-A60-HR alone or together with Flag-PABPC1-NRS for 24 h. Samples were treated with 5 ng/ml LMB for 6 h prior to harvesting either protein (top) or RNA (bottom). Protein lysates were resolved by SDS-PAGE and immunoblotted with antibodies against PAPII,

Flag, or actin (as a loading control). RNA from each sample was Northern blotted with 32P-labeled GFP and 18S probes.

Figure 2.7

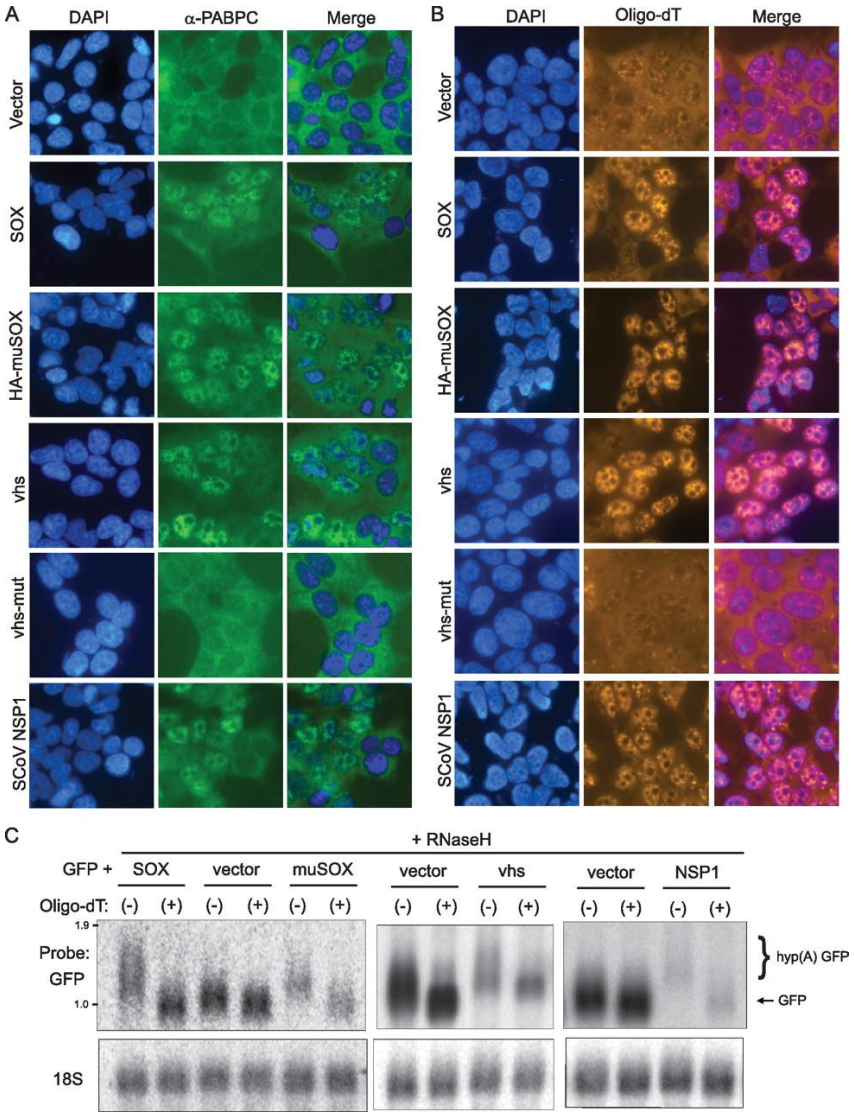

Figure 2.7 Nuclear relocalization of PABPC, nuclear retention of mRNA, and hyperadenylation are phenotypes induced by multiple independent viral factors. (A) HEK 293T cells were transfected with either empty vector or with plasmids expressing SOX, HA-muSOX, vhs, vhs mutant (vhs-mut), or SARS-CoV NSP1 for 24 h and then subjected to immunofluorescence assays (IFA) with anti-PABPC antibodies and stained with DAPI to visualize nuclei. Right panels represent a merge between the IFA and DAPI signals. (B) HEK 293T cells were transfected as described in the legend to panel A, subjected to oligo(dT) in situ hybridization, and stained with DAPI. Right panels represent a merge between the in situ and DAPI signals. (C) HEK 293T cells were cotransfected with a plasmid expressing GFP and either empty vector or plasmids expressing the indicated viral protein for 24 h. Cells were treated with LMB for 15 h, and total RNA was then isolated and digested with RNaseH in the presence or absence of oligo(dT). Products were resolved by agarose-formaldehyde gel electrophoresis and Northern blotted with 32P-labeled GFP and 18S probes.

Figure 2.8

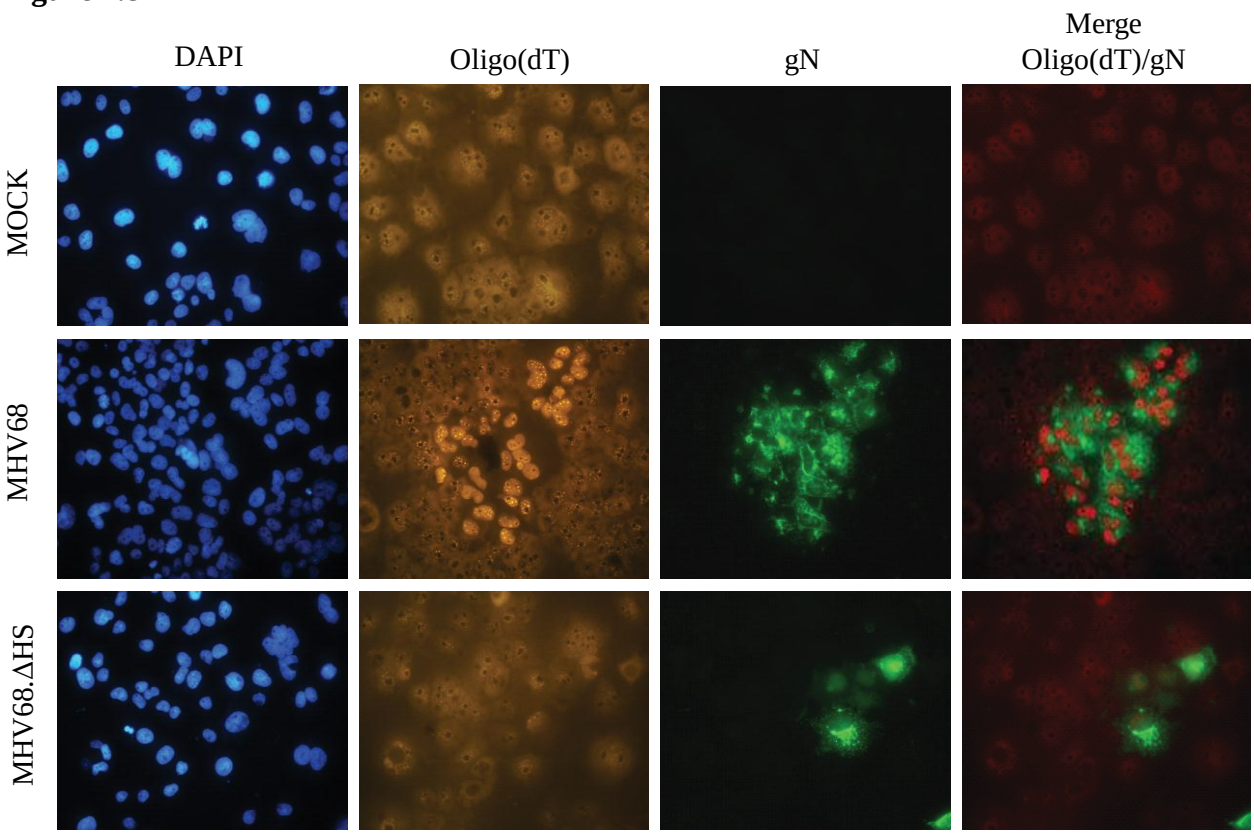

Figure 2.8 PABPC induces an mRNA export block during viral infection. COS-7 cells were infected with MHV68 and MHV68.ΔHS for 24 hours. Cells were processed for oligo(dT) in situ hybridization, followed by immunofluorescence assays with anti-glycoprotien N (gN) antibodies to identify infected cells.

Chapter 3

Importin alpha-mediated nuclear import of cytoplasmic poly(A) binding protein occurs as a direct consequence of cytoplasmic mRNA depletion

Background

Cells regulate multiple processes through subcellular redistribution of proteins, especially upon encountering stressful conditions. Many well characterized examples include a number of transcription factors that are transported to the nucleus to induce expression of specific genes in response to stimuli such as oxidative stress, ultraviolet radiation, heat shock, or infectious agents. For example, the activation of NF-kB, the heat shock response factor, interferon regulatory factors, and p53 leads to their nuclear relocalization and stimulation of gene expression cascades [167-175]. Proteins can also undergo nuclear to cytoplasmic redistribution to affect gene expression posttranscriptionally. This is the case for the RNA binding protein HuR, which translocates from the nucleus to the cytoplasm upon activation of p38 or oxidative stress to stabilize mRNAs containing AU-rich elements [176, 177]. Retaining these regulatory factors in the appropriate locale in the absence of activating signals is critical to preventing inappropriate alterations in gene expression that could affect cellular homeostasis. The mechanisms by which the localization of such factors is restricted in the absence of activating signals are varied, and include interactions with inhibitor proteins, posttranslational modifications such as phosphorylation, and constitutive nuclear export [168, 169, 174-176, 178-180].

Cytoplasmic poly(A) binding protein (PABPC) has recently been shown to exert differential effects on gene expression based on its subcellular localization. At steady state, PABPC is predominantly localized within the cytoplasm where it promotes gene expression by enhancing translation and mRNA stability [48, 52]. Its interactions with the mRNA 3' end via the poly(A) tail and the 5' end through direct and indirect interactions with eIF4G, Paip1, and eIF4E serve to stabilize messages and enhance protein synthesis [32, 58, 59]. Associations of PABPC with the release factor eRF3 promote efficient translation termination, as well as antagonize nonsense-mediated decay (NMD), a quality control pathway that degrades mRNAs bearing premature stop codons [62, 63, 76, 78, 128]. PABPC also enhances message stability by protectively coating the poly(A) tail to prevent exonucleolytic degradation, and influences the rate of mRNA turnover through its interactions with factors involved in deadenylation including Pan3, TOB, and GW182 [69, 70, 181].

Though predominantly present in the cytoplasm, PABPC is a nucleocytoplasmic shuttling protein [99], and it preferentially accumulates in the nucleus upon cellular stresses such as heat shock, oxidative stress, and transcription block, as well as upon depletion of paxillin [68, 99, 103, 125]. In addition, it is driven into the nucleus upon infection with a variety of viruses, including Kaposi's sarcoma-associated herpesvirus (KSHV), murine gammaherpesvirus 68 (MHV68), herpes simplex virus (HSV-1), rotavirus, and bunyamwera virus [100-103, 116]. Following recovery from heat shock, PABPC returns to the cytoplasm, although during viral infection it remains nuclear until cell death [102, 125]. Elevated levels of nuclear PABPC

promote hyperadenylation of nuclear mRNAs and a concomitant RNA export block, thereby restricting gene expression [182]. Hyperadenylation is carried out by the canonical mRNA poly(A) polymerase PAPII, which presumably extends poly(A) tails on PABPC-bound nuclear mRNAs [182]. Thus, PABPC appears to have generally opposing effects on gene expression when concentrated in the cytoplasm versus the nucleus, and changes in its steady state localization are induced in response to various stresses.

Unlike many other gene expression regulators, the specific events that prompt the removal of PABPC from the cytoplasm and the mechanisms by which it translocates into the nucleus to block gene expression are not understood. Interestingly, many viruses that induce PABPC relocalization also suppress protein synthesis by promoting cytoplasmic mRNA turnover [119, 139, 148-153, 166]. Here, we reveal that PABPC localization is directly influenced by cytoplasmic mRNA abundance. Expression of viral proteins that cause widespread mRNA turnover or of a cytoplasmic deadenylase that depletes cytoplasmic poly(A) sequences promotes nuclear accumulation of PABPC. Nuclear import requires the RNA recognition motifs (RRMs) of PABPC, which likely harbor non-canonical nuclear localization signals and which interact directly with importin α, a component of the classical nuclear import pathway. Finally, we show that binding of PABPC1 to the nuclear import machinery is antagonized by the addition of poly(A) RNA but not poly(C) RNA, and enhanced upon ribonuclease (RNase) treatment, arguing that PABPC exhibits mutually exclusive binding to poly(A) or importin α. Collectively, our findings suggest a novel mechanism for controlling the subcellular localization of this critical protein based on the availability of poly(A) mRNA in the cytoplasm, and have important implications for how cells may sense and respond to gross alterations in transcript accumulation.

Results

Viral proteins induce selective nuclear relocalization of PABPC and its dissociation from translation complexes.

One of the more dramatic examples of PABPC relocalization occurs upon viral infection. In the case of KSHV, PABPC relocalization is driven by the viral SOX protein, which promotes widespread mRNA turnover [102, 139]. Because the functional consequences of PABPC nuclear relocalization have been best characterized in KSHV SOX-expressing cells, we initially used this viral protein to investigate the mechanism(s) of PABPC import. We first examined if PABPC-associated proteins were similarly relocalized in SOX-expressing cells, since during heat shock both PABPC and eIF4G are recruited to the nucleus [125]. Immunofluorescence analysis (IFA) revealed that while there was a dramatic nuclear accumulation of endogenous PABPC, the PABPC-associated proteins eIF4G, eIF4E, eRF3, Paip1, and Paip2a remained strictly cytoplasmic (Fig. 3.1). Thus, PABPC appears to be selectively relocalized to the nucleus by SOX.

This observation suggested that either only the population of PABPC not associated with these proteins is targeted, or, that its interactions with one or more proteins are disrupted prior to its relocalization. To distinguish between these alternatives, we monitored interactions of transfected HA-PABPC1 with endogenous eIF4E, eRF3, and Paip2a in SOX-expressing cells. Associations of poly(A) RNA-bound PABPC with eIF4E (through eIF4G) and eRF3 occur during translation [58, 62, 63]. However, interactions of PABPC with Paip2a, a repressor of translation, are RNA-independent and separate from the association of PABPC with translation factors [64, 65, 183]. A reduced amount of eIF4E and eRF3 co-immunoprecipitated with HA-PABPC1 in SOX-expressing cells, however, its level of interaction with Paip2a remained unchanged (Fig. 3.2A). This was also the case in cells expressing two additional viral proteins that induce mRNA turnover and relocalize PABPC, MHV68 muSOX and HSV-1 vhs (Fig. 3.2B, 3.2D). These observations were not an artifact of PABPC overexpression, as endogenous PABPC similarly co-immunoprecipitated dramatically reduced levels of eIF4E but not Paip2a in both KSHV SOX and MHV68 muSOX-expressing cells (Fig. 3.2C). These data suggest that these viral proteins specifically target the population of PABPC in translation complexes.

Expression of a cytoplasmic deadenylase drives nuclear import of PABPC1.

Given that during translation, mRNA-bound PABPC associates with eIF4E and eRF3, and that each of the above viral proteins promotes cytoplasmic mRNA turnover, we hypothesized that the selective relocalization of PABPC occurs in response to mRNA depletion. However, as viral proteins are often multifunctional, PABPC relocalization might also be a consequence of additional cytoplasmic perturbations by SOX, muSOX, or vhs. Therefore, in an effort to specifically assess the extent to which binding to cytoplasmic RNA influences the subcellular distribution of PABPC1, we examined its localization upon depletion of poly(A) mRNA in a virus-independent manner. To this end, we used a cytoplasmic version of human Caf1z, a previously characterized nuclear deadenylase that can function in the cytoplasm upon mutation of its nuclear localization signal (hCaf1z ΔNLS) [184]. hCaf1z ΔNLS has been shown to catalyze rapid deadenylation of reporter mRNAs followed by slow 3'-5' exonucleolytic decay [184]. We first confirmed that all of these proteins were expressed to similar levels and did not influence HA-PABPC1 protein levels (Fig. 3.3A), then showed that overexpression of hCaf1z ΔNLS, but not of wild-type nuclear hCaf1z or a nuclear or cytoplasmic catalytic mutant (hCaf1Z

DEAA, ΔNLS DEAA, respectively) depleted a GFP reporter mRNA in cells (Fig. 3.3B). To determine whether the reduction in mRNA levels in hCaf1z ΔNLS-expressing cells was a consequence of enhanced deadenylation, tetracycline-responsive plasmids expressing GFP and β-globin were co-transfected into HeLa Tet-off cells together with either vector, hCaf1z ΔNLS, or hCaf1z ΔNLS DEAA. Following a pulse of transcription, the decay kinetics of each mRNA was followed over time (Fig 3.3C). Characteristic of enhanced deadenylation, both the GFP and β-globin mRNAs decreased in size and abundance over the 5 h time course specifically in cells expressing hCaf1z ΔNLS (Fig. 3.3C).

We next tested whether enhanced cytoplasmic deadenylation was sufficient to alter the localization of PABPC. Indeed, expression of hCaf1z ΔNLS readily promoted nuclear accumulation of HA-PABPC1 (Fig. 3.3D, 3.3E). This effect was dependent on its ability to promote cytoplasmic deadenylation, as neither the nuclear wild-type protein, nor the nuclear or cytoplasmic catalytic mutants defective for deadenylation was capable of relocalizing HA-PABPC1 (Fig. 3.3D, 3.3E). Thus, the abundance of poly(A) RNA in the cytoplasm directly influences the steady state localization of PABPC1.

PABPC RNA recognition motifs are required for nuclear import

PABPC has four N-terminal RNA recognition motifs (RRMs) that mediate poly(A) binding and interactions with eIF4G, Paip1, and Paip2a [58, 59, 64, 65], followed by a central linker region involved in self-association [61], and a helical C-terminus that interacts with Paip1, Paip2a and eRF3 [60, 63, 64]. In order to determine which regions of PABPC are required for nuclear import, we generated a panel of PABPC deletion mutants that lacked either the first two RRM domains (ΔRRM 1+2), the second two RRM domains (ΔRRM 3+4), all four RRM domains (ΔRRM 1-4), the linker region (ΔLinker), or the C-terminal region (ΔC-term) (Fig. 3.4A, 3.4B). Interestingly, localization of some of the mutants differed from wild-type HA-PABPC1 (Fig. 3.4C). Specifically, in some cells HA-PABPC1 ΔRRM 1+2 was both nuclear and cytoplasmic, and HA-PABPC1 ΔRRM 1-4 was consistently present in both cellular compartments, suggesting that it lacks the determinant(s) that normally prevents nuclear accumulation (Fig. 3.4C). All other mutants exhibited strongly cytoplasmic staining similar to the full-length protein (Fig. 3.4C).

To identify PABPC domains required for nuclear import by the herpesviral proteins and the cytoplasmic deadenylase, we co-expressed them with each HA-PABPC1 mutant and monitored the localization of the mutants by immunofluorescence analysis with anti-HA antibodies. Only the mutant lacking all RRMs (HA-PABPC1 ΔRRM 1-4) was not relocalized in SOX, muSOX, vhs, or hCaf1z ΔNLS-expressing cells (Fig. 3.5A, 3.5B, 3.5C). We have observed that additional mutants lacking RRM 2+3+4, RRM 1+2+3, RRM 1+3, or RRM 2+4 remain subject to at least partial relocalization by SOX (data not shown), indicating that at least one RRM must be present for nuclear import. Collectively, our results suggest that PABPC1 is retained in the cytoplasm via its interactions with RNA through the RRMs, and that widespread depletion of cytoplasmic mRNA liberates this protein to enter the nucleus.

Nuclear import of PABPC is mediated through direct interactions with importin α.

Proteins destined for the nucleus are translocated across the nuclear pore by the classical import machinery. Proteins either bind directly to importin β or to the adaptor importin α, which then engages importin β, and the complex is transported into the nucleus in an energy-dependent manner [185, 186]. We investigated whether PABPC enters the nucleus through the classical

nuclear import pathway by testing its interactions with multiple isoforms of recombinant GST-tagged importin α (α1, α3, α5, and α7) and importin β proteins. GST pull-down assays with protein lysates from HEK293T, HeLa, and COS7 cells revealed that endogenous PABPC interacts predominantly with importin α3, to some extent with other importin α proteins (particularly α7), but not with importin β (Fig. 3.6A).

Although PABPC1 is the most highly expressed isoform, three other cytoplasmic isoforms have been identified with variable expression patterns, namely PABPC3, a testis-specific protein [54], PABPC4, an inducible isoform [53, 182], and PABPC5, an X-chromosome encoded isoform [55]. Similar to PABPC1, PABPC4 and PABPC5 also accumulate in the nucleus upon expression of SOX (data not shown) [182], and therefore may be imported into the nucleus by importin α. Indeed, PABPC4-HA and HA-PABPC5 were also capable of interacting with importin α3 (Fig 3.6B). Interestingly, PABPC5 is composed only of four RRM domains suggesting that the RRMs are sufficient for interactions with importin α as well as nuclear import.

Based on our results thus far, we predicted that the interaction of importin α with PABPC1 would be mediated through its RRMs. GST pull-down assays with recombinant importin α3 confirmed that residues important for binding reside within one or more RRMs, as only the HA-PABPC1 ΔRRM1-4 mutant failed to interact with importin α3 (Fig. 3.6C). This mutant was also unable to bind importin α1, α5, α7 and importin β in GST pull-down assays (Fig. 3.6D). Our observation that mutants HA-PABPC1 ΔRRM 1+2 and HA-PABPC1 ΔRRM 3+4 both interact with importin α3 and accumulate in the nucleus after cytoplasmic mRNA depletion indicate that multiple importin α binding sites exist in the PABPC RRMs (Fig. 3.6C). Consistent with this finding, expression of PABPC RRM 1+2 or RRM 3+4 alone was sufficient for interaction with GST-importin α3 (Fig. 3.6E).

Protein interactions with the importins are generally mediated through a basic nuclear localization signal (NLS) [187]. However, PABPC does not possess a predicted NLS and therefore may instead interact with importin α either indirectly through another NLS-containing cellular factor(s) or directly through one or more non-canonical NLS-like motifs within its RRMs. In order to distinguish between these two possibilities, we tested for an interaction between purified recombinant MBP-tagged PABPC1 and purified GST-tagged importin isoforms. GST pull-down assays demonstrated that MBP-PABPC interacts directly with multiple isoforms of importin α, including α3 and α5, but not with importin β (Fig. 3.7A). The fact that we detected an interaction between PABPC1 and importin α5 *in vitro* but not in cells indicates that the preference for specific isoforms may be influenced by other cellular components. Collectively, these data suggest that PABPC harbors non-canonical NLS-like motifs within the RRMs that mediate direct interactions with importin α.

The interaction of PABPC1 with importin α is antagonized by poly(A) RNA

Our observations thus far indicated that the ability to bind poly(A) RNA plays a significant role in retaining PABPC in the cytoplasm. However, our prior experiments did not allow us to distinguish if this is specifically due to masking of the NLS-like motifs in PABPC by its association with RNA, or as a consequence of its RNA-dependent protein interactions. To resolve these two possibilities, we assessed how addition or depletion of RNA influenced the direct interaction between purified MBP-PABPC1 and importin α3. Addition of 1 μg (representing a 1:1 molar ratio) of poly(A) RNA was, in fact, sufficient to completely block the interaction between GST-importin α3 and MBP-PABPC1 in GST pull-down assays (Fig. 3.7B).

This disruption is not a nonspecific consequence of RNA charge, as addition of poly(C) RNA, which cannot be bound by PABPC [46, 48, 56, 188], does not block the association of PABPC with importin α3 (Fig. 3.7C). Although *E. coli* do not contain abundant polyadenylated RNA, given that PABPC can bind other RNA sequences as well [48, 56, 189], we hypothesized that any co-purifying bacterial RNA might reduce its interactions with importin α. We therefore treated the purified proteins with $RNaseI_f$, which cleaves RNA after each base, prior to performing the GST pull-downs. In agreement with our prediction, the RNase treatment significantly enhanced binding of PABPC to importin α3 (Fig. 3.7B). These data argue that removal of RNA unmasks sequences in PABPC that interact with the nuclear import machinery, and that PABPC forms mutually exclusive interactions with poly(A) RNA and importin α3 within the cytoplasm.

Discussion

PABPC has a very high affinity for poly(A) sequences (2-7 nm K_D), and our observations indicate that this interaction plays a significant role in retaining PABPC in the cytoplasm [45, 46, 48]. In particular, depletion of cytoplasmic mRNA, by divergent viral proteins or a cytoplasmic deadenylase, results in relocalization of PABPC to the nucleus in a manner dependent on its RRMs. Furthermore, poly(A) RNA, but not poly(C) RNA, disrupts interactions of purified PABPC with the nuclear import machinery, whereas RNase treatment enhances them. We therefore propose a model in which the localization of PABPC can be controlled by the levels of poly(A) RNA in the cytoplasm (Fig. 3.8). Normally, the concentration of cytoplasmic mRNA is likely sufficient to retain the majority of PABPC in this locale. However, if this balance is offset, for example by extensive mRNA degradation, an excess of non RNA-bound PABPC accumulates, exposing sequences within the RRMs that associate with importin α, leading to nuclear translocation. It is important to note that a small fraction of PABPC is normally found in the nucleus associated with pre-mRNAs, where it is hypothesized to facilitate mRNA export [124]. However, high levels of nuclear PABPC promote hyperadenylation and inhibit mRNA export [182], highlighting the importance of restricting excess import of PABPC into the nucleus of unstressed cells.

Interestingly, PABPC levels are normally controlled in part through an autoregulatory mechanism. The 5' UTR of the PABPC mRNA contains a 50-70 nt tract with stretches of 7-9 A residues, which are bound by PABPC, presumably once the protein concentration in the cytoplasm reaches a particular threshold [79, 190]. This binding has been shown to block translation, thereby reducing PABPC accumulation [79-81]. One measure of this threshold may be the amount of non-poly(A) tail-bound PABPC, an excess of which could then target the relatively short oligo(A) residues in the PABPC 5' UTR.

PABPC has four RRMs that collectively mediate poly(A) binding, although the first two RRMs are sufficient for wild-type levels of interaction with poly(A) RNA [56, 99]. In contrast, RRMs 3 and 4 have 10-fold reduced affinity for poly(A), but can bind other RNA sequences [56]. Mutational analysis of PABPC supports the conclusion that the poly(A) binding capacity of PABPC is an important determinant of cytoplasmic localization. The mutant lacking RRMs 1+2 accumulates preferentially in the nucleus relative to other mutants, likely because it has reduced affinity for poly(A) RNA in the cytoplasm. In agreement with our model, a deletion in either the linker or the C-terminal domains of PABPC does not affect PABPC localization, as these domains do not interact with poly(A) sequences [99]. Additionally, all mutants that retain at least one RRM domain are subject to nuclear relocalization upon cytoplasmic mRNA depletion, bolstering the idea that cytoplasmic poly(A) RNA is indeed a retention factor for PABPC. In the absence of all four RRMs, PABPC localization is no longer responsive to changes in mRNA levels, although it is constitutively present throughout both the nucleus and the cytoplasm. This may be a consequence of passive diffusion across the nuclear pore, as the ~30 kD size of the ΔRRM 1-4 mutant is below the minimum size required for active transport [191].

Interactions of PABPC with RNA and with importin α appear to be antagonistic, arguing that while bound to RNA, the sequences within PABPC that mediate nuclear import are masked. Given our mutant data, we predict that at least two non-canonical nuclear signals exist within PABPC RRMs. In particular, PABPC RRMs 1+2 or RRMs 3+4 alone are both able to bind importin α. The ability of RRM sequences to interact with the nuclear import machinery is not unprecedented, as RRMs in other proteins have been implicated in facilitating nucleocytoplasmic

transport [192]. For example, both the yeast Lhp1p RRM and the pab1 RRM 4 interact directly with yeast importin Sxm1/Kap108 [193-195]. Furthermore, the RRM domain of the trypanosome *Tc*UBP1 protein behaves as an NLS, mediating nuclear import of this predominantly cytoplasmic protein, particularly under conditions of arsenite-induced stress [192, 196]. Interestingly, *Tc*UBP1 has been shown to interact with *Tc*PABP1, though whether this interaction influences its localization has yet to be determined [197].

Although we have shown that cytoplasmic mRNA abundance is an important determinant of PABPC localization, this is unlikely to be its sole regulator. RNA-dependent protein interactions could also contribute to its nuclear-cytoplasmic distribution. In this regard, it has been calculated that in HeLa cells PABPC abundance exceeds the availability of cytoplasmic mRNA [198]. Presumably, a portion of the excess PABPC is complexed with other cytoplasmic proteins, such as Paip2a and paxillin, although the abundance of these proteins must not be sufficient to retain PABPC in the cytoplasm following widespread mRNA degradation [64, 67]. However, depletion of paxillin, a focal adhesion adaptor protein, results in a modest nuclear accumulation of PABPC in 3T3 cells, suggesting that paxillin also influences PABPC localization [67, 68, 199]. We were unable to detect an interaction between paxillin and PABPC in HEK293T cells, or changes in levels of paxillin in SOX-expressing cells (data not shown), indicating that roles for paxillin in PABPC localization may be cell type or context specific. PABPC relocalization also occurs during a variety of non-viral cellular stresses, in particular upon heat shock, although it has been reported to occur during transcriptional block and oxidative stress as well [99, 103, 125]. Heat shock results in a global decrease in cap-dependent translation and nuclear relocalization of both PABPC and eIF4G [125, 168, 171]. The observation that eIF4G is not relocalized by the KSHV SOX protein suggests that either the mechanism of PABPC import is distinct upon viral infection versus heat shock, or that heat shock also affects separate pathways that influence eIF4G.

Sensing or responding to widespread alterations in cellular transcript abundance via PABPC relocalization may be one means by which cells react to infection with a variety of pathogens. Viruses in particular often come armed with an arsenal of mechanisms to suppress activation of innate immune responses. However, select viruses that benefit from host gene expression shutdown might commandeer this response to facilitate their own replication. Thus, further delineation of the variety of pathways that control PABPC localization are certain to provide insight into how cells broadly regulate gene expression patterns during homeostasis as well as under conditions of stress.

Materials and Methods

Plasmids

Plasmids pCDEF3-HA-PABPC1 [182], pCDEF3-SOX, pCDEF3-muSOX, pCDNA3.1-vhs, pCDNA3.1-vhsmut [119, 154], and pTRE-d2eGFP have been previously described. PABPC1 was cloned into BamH1 and XbaI sites of plasmid pMAL-C2X to generate pMAL-C2X-PABPC1 in order to express and purify MBP-PABPC1 fusion protein from bacterial cells. Plasmid pCDNA3-PABPC4-HA was kindly provided by Dr. Tullia Lindsten (University of Pennsylvania) [53]. Plasmid pDNR-LIB-PABPC5 was purchased from Open Biosystems (Clone ID: 6452933, accession number BC063113) from which PABPC5 was PCR amplified, 5'-tagged with 1X hemagglutinin (HA), and subcloned into the BamH1 and XbaI sites of pCDEF3 to generate pCDEF3-HA-PABPC5. HA-PABPC1 deletion mutants were generated by overlap extension PCR and cloned into the BamH1 and XbaI sites of pCDEF3 to generate pCDEF3-HA-PABPC1 ΔRRM 1+2 (lacking nt 1-528), pCDEF3-HA-PABPC1 ΔRRM 3+4 (lacking nt 571-1110), pCDEF3-HA-PABPC1 ΔRRM 1-4 (lacking nt 1-1110), pCDEF3-HA-PABPC1 ΔLinker (lacking nt 1111-1629), pCDEF3-HA-PABPC1 ΔC-term (lacking nt 1630-1911), pCDEF3-HA-PABPC1 RRM 1+2 (lacking nt 571-1911), pCDEF3-HA-PABPC1 RRM 3+4 (lacking nt 1-525, 1167-1911), and pCDEF3-HA-PABPC1 RRM 1+2+3+4 (lacking nt 1111-1911). Plasmids pGEX-importin α1, pGEX-importin α3, pGEX-importin α5, pGEX-importin α7, and pGEX-importin β were kindly provided by Dr. Riku Fagerlund (National Public Health Institute, Helsinki, Finland). Plasmids pCDNA3-FLAG-hCaf1z, pCDNA3-FLAG-hCaf1z ΔNLS, pCDNA3-FLAG-hCaf1z DEAA, pCDNA3-myc-hCaf1z ΔNLS/DEAA, and pPCβwt-Δ12 were kindly provided by Dr. Jens Lykke-Andersen (University of California, San Diego) [184].

Cells and Transfections

HEK293T, HeLa, HeLa Tet-off, and COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). DNA transfections were performed in 12-well plates using 1 µg total plasmid DNA (100 ng of indicated plasmid plus 900 ng of empty vector) using Effectene (Qiagen) or Lipofectamine (Invitrogen) as per manufacturer's instructions. For kinetic assays, HeLa Tet-off cells were transfected with 100 ng of pPCβwt-Δ12, 100 ng of pTRE-d2eGFP, and empty vector or the indicated hCaf1z plasmid to 1 µg total DNA in the presence of 50 ng/ml tetracycline (Clontech) for 24 hours. Transcription was pulsed by removal of tetracycline for 5 h and then blocked by addition of 1 µg/ml tetracycline. Cells were harvested for total RNA at the indicated time points thereafter and analyzed by Northern blot as described below.

Immunofluorescence assays (IFA)

HEK293T cells were seeded on poly(L)-lysine (100 µg/ml) coated coverslips, transfected for 24 h, and processed for IFA as previously described [165]. Briefly, cells were fixed in 4% formaldehyde for 20 min, incubated in permeabilization buffer (1% [vol/vol] Triton X-100 and 0.1% [wt/vol] sodium citrate in phosphate buffered saline (PBS)) for 10 min, and then in blocking buffer (1% [vol/vol] Triton X-100, 0.5% [vol/vol] Tween 20, and 3% bovine serum albumin [BSA] in PBS) for 30 min. Cells were then incubated with either mouse monoclonal anti-PABPC 10e10 (1:25 dilution, Santa Cruz Biotechnology), rabbit polyclonal anti-eIF4E (1:500 dilution, Cell Signaling Technology), goat polyclonal anti-eIF4G (1:100 dilution, Santa Cruz Biotechnology), rabbit polyclonal anti-eRF3 (1:200 dilution, Abcam), rabbit polyclonal

anti-Paip1 (1:500 dilution, kindly provided by Nahum Sonenberg, McGill University), rabbit polyclonal anti-Paip2a (1:500 dilution, kindly provided by Nahum Sonenberg, McGill University), mouse monoclonal anti-HA 12CA5 (1:500 dilution, Abcam), mouse monoclonal anti-Flag M2 (1:500, Sigma), rabbit polyclonal anti-Flag (1:500, Sigma), or rabbit polyclonal anti-myc (1:200 dilution, BioVision) primary antibodies for 3-12 h at 37°C and washed three times with (PBS). Cells were then incubated with Alexa Fluor 488-, 546-, or 594-conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat secondary antibodies (1:1500 dilution, Molecular Probes) for 1 h at 37°C and washed three times with PBS. Coverslips were mounted in DAPI (4',6-diamine-2-phenylindole)-containing Vectashield mounting medium (Vector Labs) to stain cell nuclei. For all statistical analysis, cells expressing HA-PABPC1 in multiple fields of view from two to three independent experiments were counted and percentage of nuclear HA-PABPC1 is reported graphically. Error bars indicate SEM.

Cell extracts, Western blot, and Northern blot

Cell lysates were prepared in RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) containing protease inhibitors (Roche) for Western blotting or in NETN lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing protease inhibitors (Roche) for immunoprecipitations and GST pull down experiments. Protein lysates were quantified by Bradford assay (Bio-Rad).

Equivalent micrograms of each sample were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinylidene difluoride membrane (PVDF), and subjected to Western blotting using either rabbit polyclonal anti-eIF4E (1:1000 dilution, Cell Signaling Technology), rabbit polyclonal anti-eRF3 (1:1000 dilution, Abcam), rabbit polyclonal anti-Paip2a (1:2000 dilution, kindly provided by Nahum Sonenberg, McGill University), mouse monoclonal anti-PABPC 10e10 (1:2000 dilution, Santa Cruz Biotechnology), rabbit polyclonal anti-PABPC (1:2000 dilution, Cell Signaling Technology), mouse monoclonal anti-HA 12CA5 (1:2000 dilution, Abcam), mouse monoclonal anti-Flag M2 (1:2000 dilution, Sigma), rabbit polyclonal anti-myc (1:2000 dilution, BioVision), or mouse monoclonal anti-β-tubulin primary antibodies (1:1000 dilution, Sigma). Secondary antibodies included horseradish peroxidase-conjugated anti-actin antibodies (1:5000 dilution, Santa Cruz Biotechnology), goat anti-mouse, goat anti-rabbit, or donkey anti-goat antibodies (1:5000 dilution, Southern Biotechnology Associates).

For Northern blots, total cellular RNA was isolated using RNA-bee (Tel-Test), resolved on 1.2% agarose-formaldehyde gels, transferred to a nylon membrane, and probed with ^{32}P-labeled GFP or 18S DNA probes prepared with the Rediprime II random primer labeling kit (GE Healthcare) or ^{32}P-labeled β-globin riboprobes prepared with MAXIscript SP6 kit (Ambion) as per manufacturer's instructions. GFP and 18S bands were quantified using ImageJ software and GFP transcript levels were normalized to 18S levels.

Immunoprecipitations

Immunoprecipitations for endogenous PABPC were performed by binding 1 mg of mouse monoclonal PABPC 10e10 antibody to protein A sepharose 4 fast flow beads (GE healthcare) at 4°C with agitation for 1 h, washing the beads with NETN buffer, and mixing with lysates from transfected HEK293T cells at 4°C with agitation for 3 h. Immunoprecipitations for HA-PABPC1 were conducted by mixing monoclonal anti-HA clone HA-7 beads (Sigma) and

lysates from transfected HEK293T at 4ºC with agitation for 3 h. The beads were then washed 3 times with NETN buffer and bound proteins were eluted with 40 µl 2X Laemmli sample buffer, resolved on 10% SDS-PAGE, and subjected to Western blot.

Production of fusion proteins in *E. coli* and importin binding assays

E. coli strain BL21 was transformed with plasmids encoding MBP alone, MBP-PABPC1, GST alone or GST-fused human importin α1, α3, α5, α7, or β. Overnight cultures grown from single colonies were used to inoculate 100 ml LB media, grown at 37ºC with agitation for 2 h, and induced with 1 mM isopropyl-1-thio-b-galactopyranoside (IPTG) for 4 h. Bacterial cells were pelleted at 10,000 x g, 4°C for 10 min and lysed in L buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 50 mM EDTA, 1% Triton X-100, and 10 mM β–mercaptoethanol) with 3 µg/ml lysozyme (Sigma) and protease inhibitors (Roche) for 15 min with periodic vortexing. Two freeze/thaw cycles were followed by brief sonication and the crude lysates were clarified by centrifugation. MBP and MBP-PABPC1 were purified by mixing crude lysates with amylose resin (New England Biolabs) at 4°C with agitation overnight. The resin was then packed into columns, washed 3 times with RIPA buffer, once with NETN buffer, once with column buffer (20 mM Tris [pH 7.4], 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol with protease inhibitors (Roche)), and MBP-PABPC1 was eluted with column buffer containing maltose (10 mM).

For GST pull-down experiments, GST alone or GST-fused importins were first bound to 25 µl glutathione-Sepharose 4B beads (GE Healthcare) in 300 µl NETN buffer at 4ºC with agitation for 1 h. Beads were washed with NETN buffer and incubated with either protein lysates from HEK293T, HeLa, or COS-7 cells, or purified MBP or MBP-PABPC1 at 4ºC with agitation overnight. Where indicated 1-1000 µg of poly(A) RNA, 1 µg of poly(C) RNA, or 150 U of RNaseI_f (New England Biolabs) was added to the overnight pull-down reaction. After washing the beads 3 times with NETN buffer, bound proteins were eluted with 40 µl of 2X Laemmli sample buffer, resolved by 10% SDS-PAGE, and subjected to Western blot.

Figures

Figure 3.1

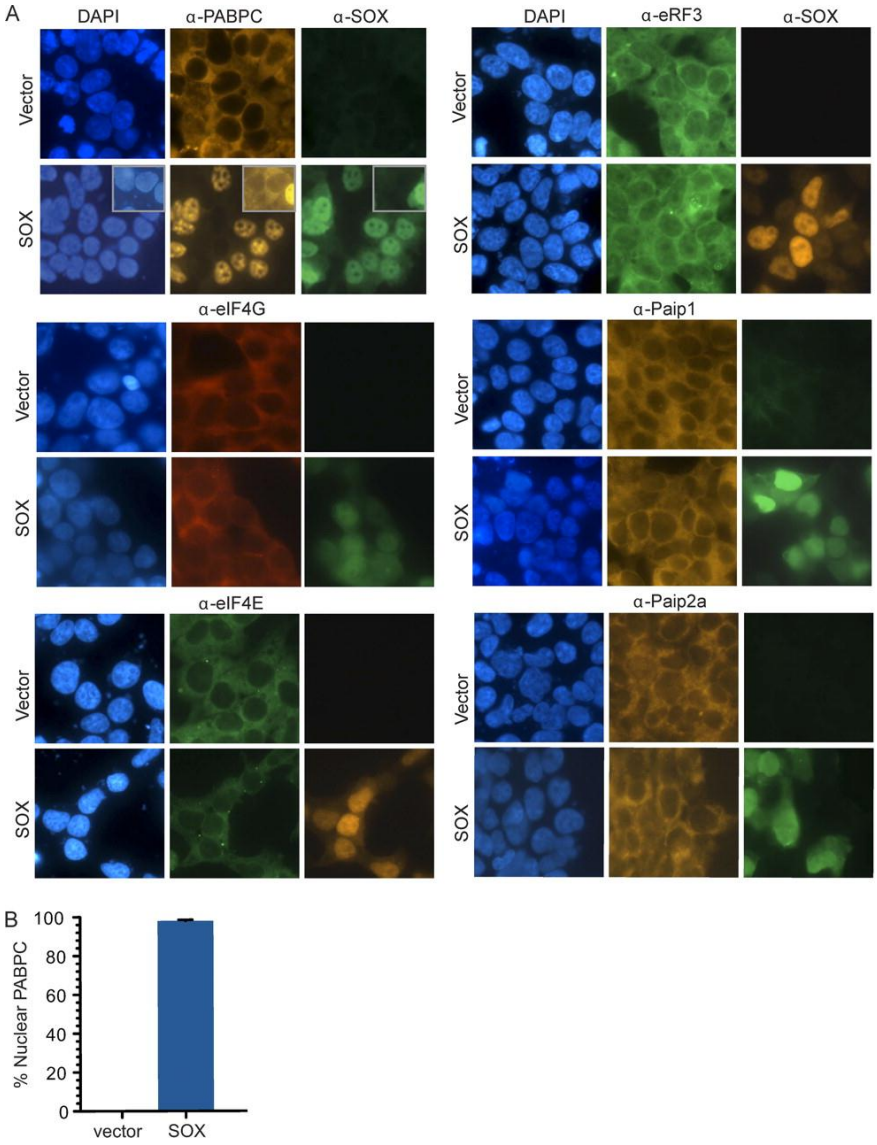

Figure 3.1 PABPC is selectively relocalized to the nucleus by SOX. (A) HEK293T cells were transfected with empty vector or a plasmid expressing KSHV SOX and, 24 h later, subjected to immunofluorescence assays with anti-PABPC, anti-eIF4G, anti-eIF4E, anti-eRF3, anti-Paip1, anti-Paip2a, or anti-SOX antibodies. DAPI staining was used to visualize nuclei. Insets in panels of SOX-expressing cells stained for PABPC represent longer exposures to reveal PABPC in the cytoplasm of cells lacking SOX. (B) Percentages of nuclear PABPC harboring SOX-expressing cells were counted in multiple fields of view from three independent experiments. Error bars indicate SEM.

Figure 3.2

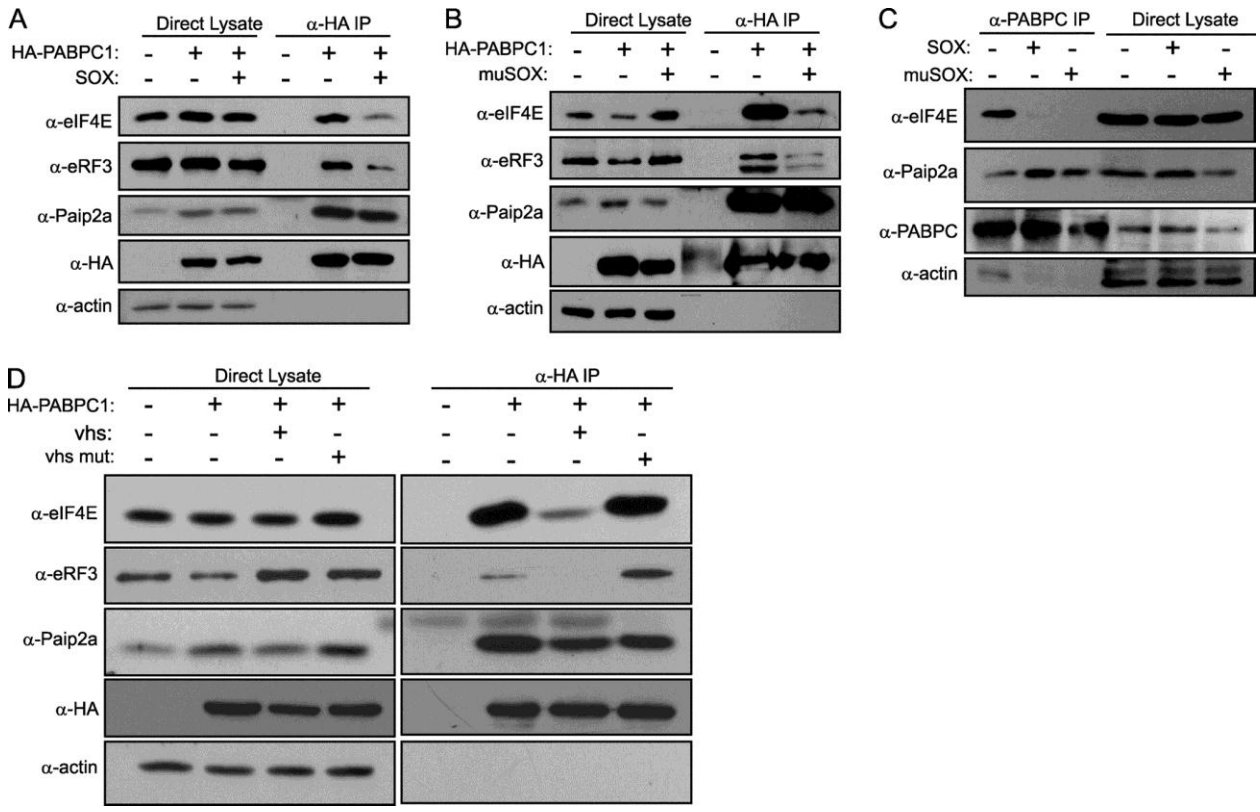

Figure 3.2 Interactions of PABPC with translation factors are disrupted by viral proteins. HEK293T cells were transfected with empty vector or plasmids expressing HA-PABPC1 with or without KSHV SOX (A), MHV-68 muSOX (B), or the wild-type or catalytically inactive mutant (mut) of HSV-1 vhs (D) for 24 h. HA-PABPC1 was immunoprecipitated using anti-HA agarose beads, and bound proteins were resolved by SDS-PAGE and detected by Western blotting with anti-eIF4E, anti-eRF3, anti-Paip2a, anti-HA, and antiactin antibodies. Actin served as a loading and specificity control. (C) HEK293T cells were transfected with empty vector or plasmids expressing SOX or muSOX for 24 h. Endogenous PABPC was immunoprecipitated using anti-PABPC antibodies, and bound proteins were resolved and detected as described above.

Figure 3.3

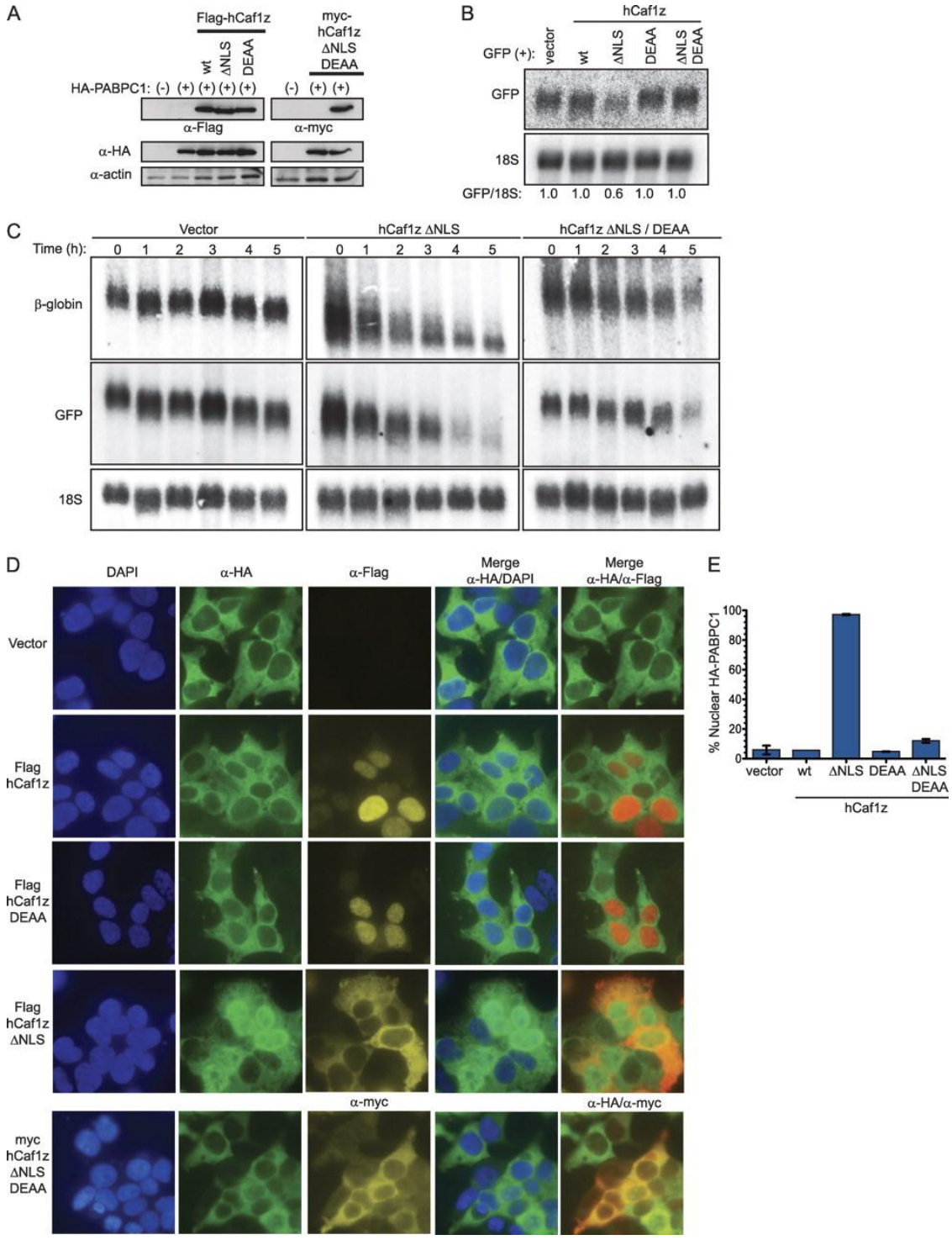

Figure 3.3 Expression of a cytoplasmic deadenylase drives nuclear relocalization of PABPC. (A) HEK293T cells were transfected with the indicated plasmids for 24 h, and protein lysates were Western blotted with anti-Flag, anti-myc, anti-HA, or antiactin (loading control) antibodies. (B) HEK293T cells were transfected as described for panel A, and 24 h later, total

RNA was isolated, resolved by agarose-formaldehyde gel electrophoresis, and Northern blotted with 32P-labeled GFP and 18S probes. GFP and 18S levels were quantified using ImageJ software, and GFP levels were normalized to 18S levels. (C) HEK293T cells were transfected with Tet-responsive GFP and β-globin constructs along with empty vector, hCaf1z ΔNLS, or hCaf1z ΔNLS/DEAA for 24 h in the presence of 50 ng/ml tetracycline. Transcription was pulsed by the removal of tetracycline for 5 h and blocked by the addition of 1 μg/ml tetracycline. RNA was isolated from cells at the indicated time points thereafter, resolved by agarose-formaldehyde gel electrophoresis, and Northern blotted with 32P-labeled GFP, β-globin, and 18S probes. (D) HEK293T cells were transfected with HA-PABPC1 alone or with the indicated Flag-hCaf1z plasmids for 24 h and subjected to immunofluorescence assay with anti-HA and anti-Flag antibodies. DAPI was used to visualize nuclei, and the right panels represent a merge between anti-HA and DAPI signals, anti-HA and anti-Flag signals, or anti-HA and anti-myc signals. (E) HA-PABPC1-expressing cells were counted in multiple fields of view from three independent experiments, and the percentage of transfected cells harboring nuclear HA-PABPC1 was calculated. Error bars indicate SEM.

Figure 3.4

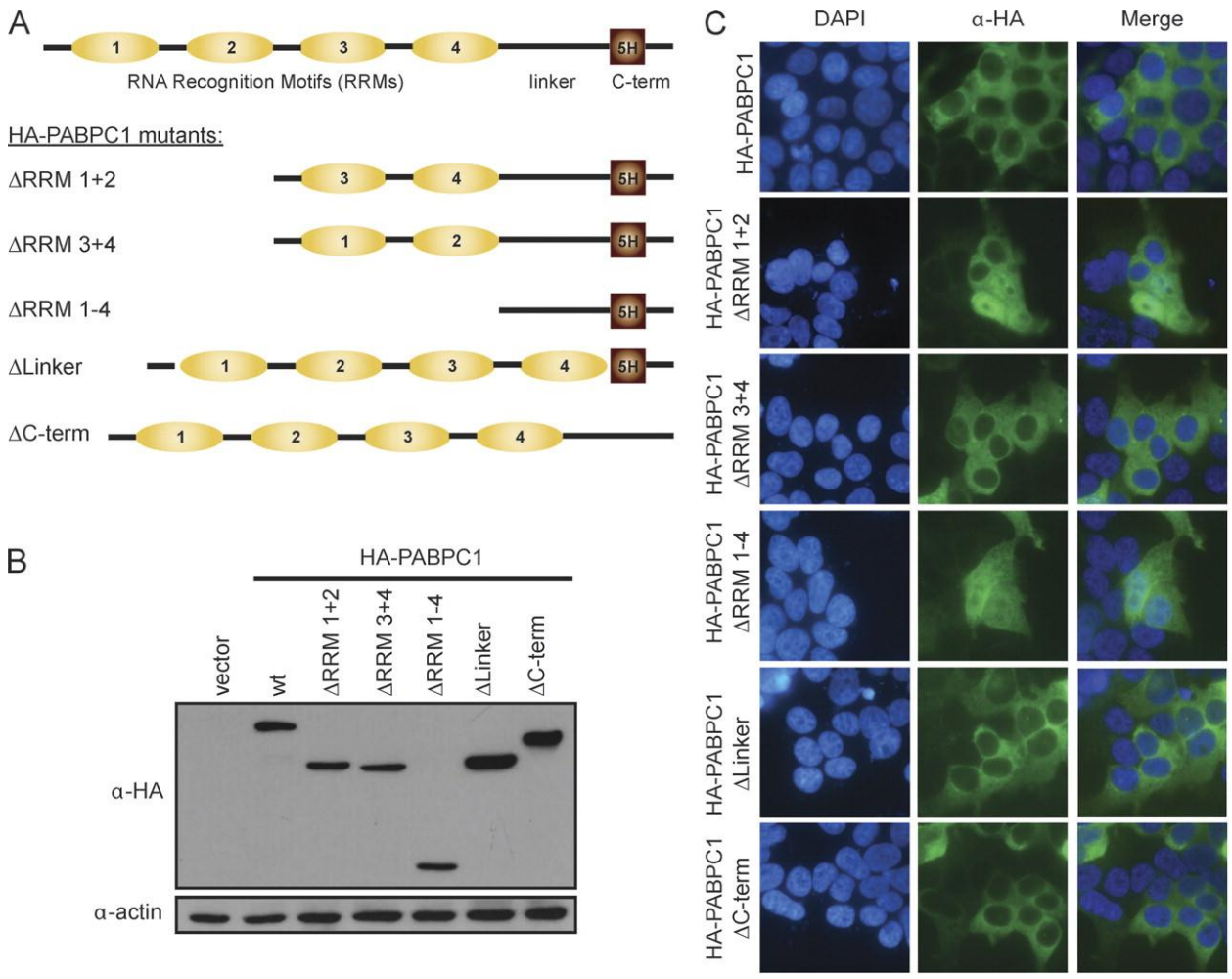

Figure 3.4 Expression and localization of PABPC mutants. (A) Diagram showing wild-type PABPC1, which possesses four N-terminal RRM domains followed by a central linker region and a conserved helical carboxyl terminus (5H), as well as the various HA-PABPC1 mutants. (B) HEK293T cells were transfected with the indicated wild-type and mutant HA-PABPC1 plasmids for 24 h. Equivalent amounts of protein lysate were then Western blotted using anti-HA antibodies. Actin serves as a loading control. (C) HEK293T cells were transfected with the indicated HA-PABPC1 plasmids and subjected to immunofluorescence assays with anti-HA antibodies. Nuclei are stained with DAPI. Right panels represent a merge between anti-HA IFA and DAPI signals.

Figure 3.5

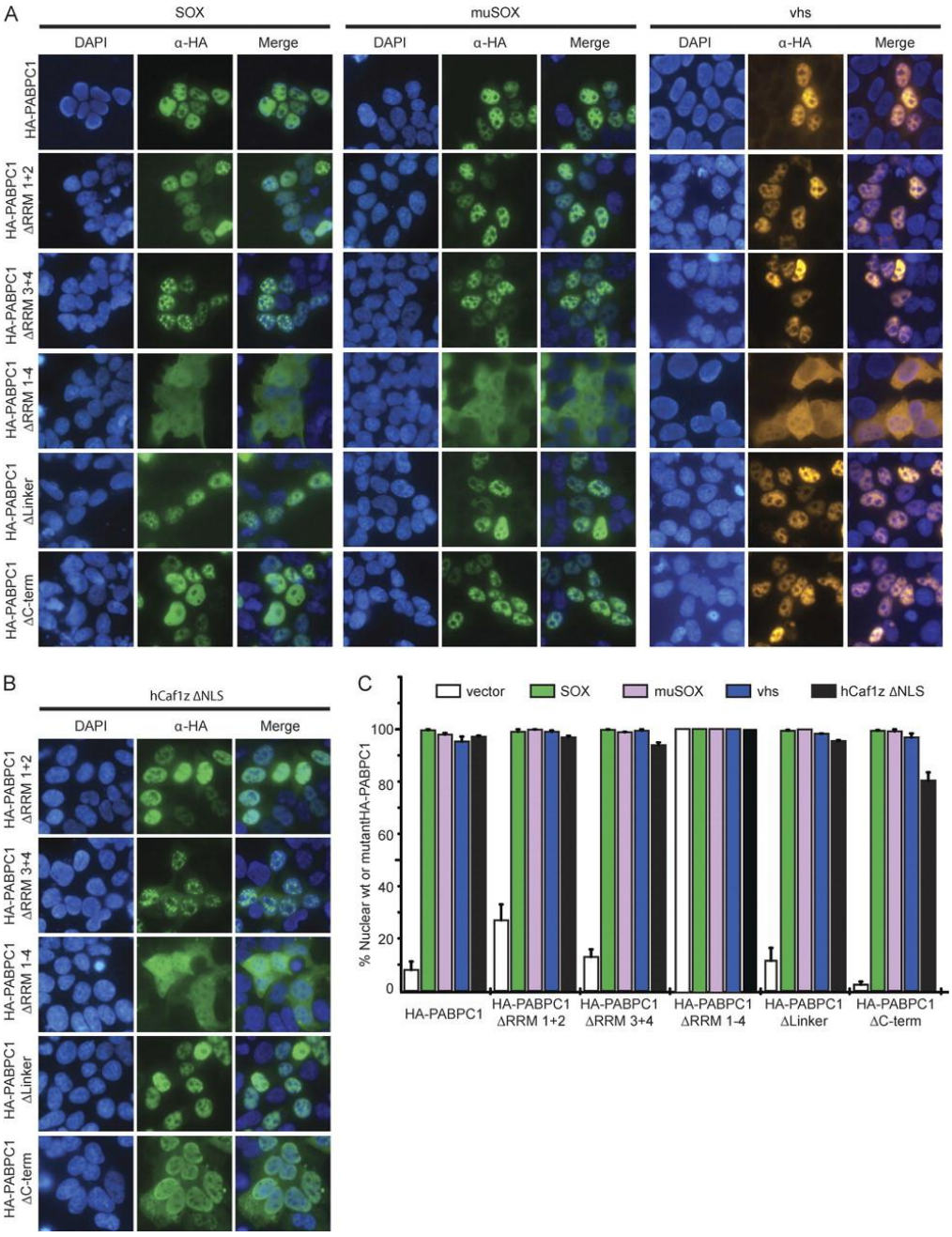

Figure 3.5 PABPC RNA recognition motifs are required for nuclear import. HEK293T cells were transfected with the indicated HA-PABPC1 mutants along with SOX, muSOX, and vhs (A) or Flag-hCaf1z ΔNLS (B) for 24 h and subjected to immunofluorescence assays with anti-HA antibodies. Nuclei are stained with DAPI. Right panels represent a merge between the anti-HA IFA and DAPI signals. (C) HA-PABPC1 wild-type (wt) or mutant-expressing cells were counted in multiple fields of view from two to three independent experiments, and the percentage of transfected cells harboring nuclear wt or mutant HA-PABPC1 was calculated. Error bars indicate SEM.

Figure 3.6

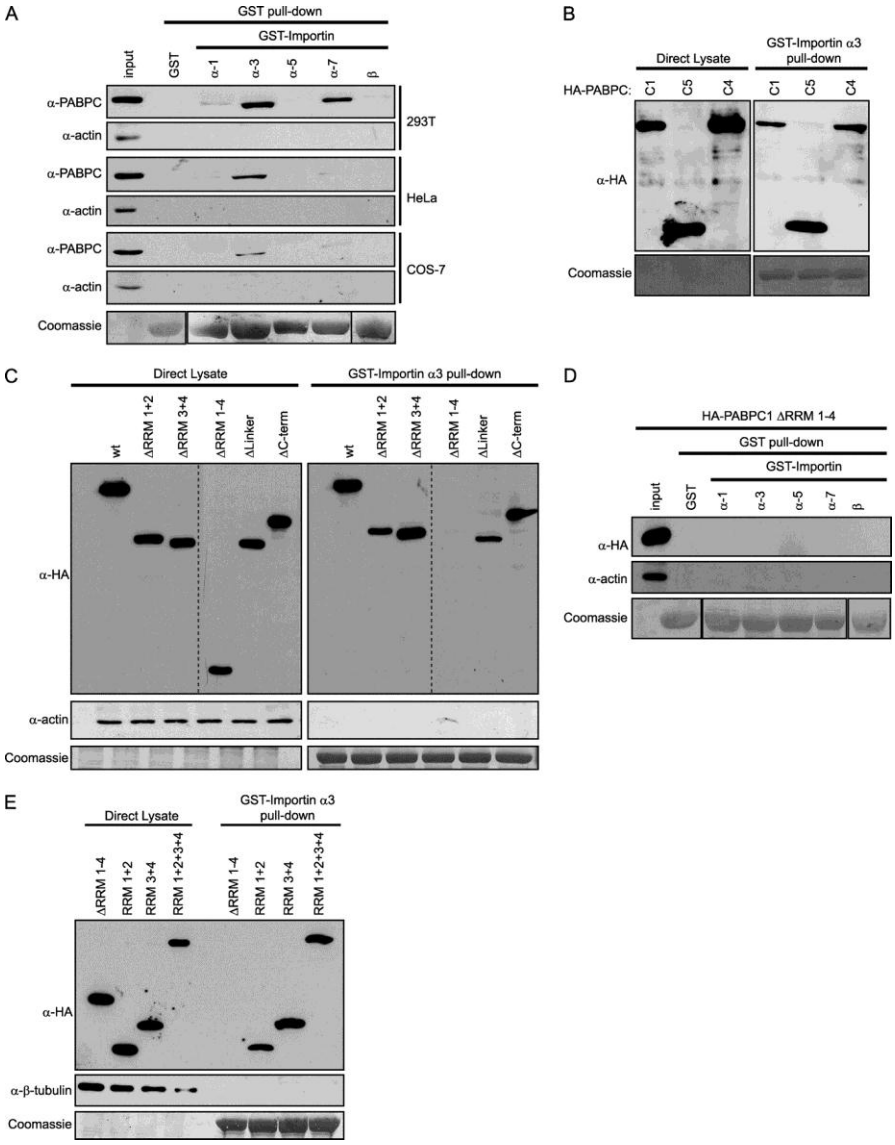

Figure 3.6 PABPC interacts with importin α. (A) Recombinant GST and GST-fused importin α1, α3, α5, α7, and β were purified over glutathione-Sepharose beads and incubated with 300 to 500 μg of lysate from HEK293T, HeLa, or COS-7 cells at 4°C overnight. Bound proteins were resolved by SDS-PAGE and detected by Western blotting with anti-PABPC antibodies. The input was 5% of the lysate used in the pulldown. (B to E) HEK293T cells were transfected either with the indicated HA-PABPC isoform (B) or the indicated HA-PABPC1 mutant (C to E) for 24 h, and the protein lysates were subjected to GST pulldowns with GST-importin α3 (B, C, and E) or additional GST-importin isoforms (D) and Western blotted with anti-HA antibodies. Where indicated, actin or β-tubulin served as a loading and specificity control, and the levels of GST fusion proteins used in each assay are shown by Coomassie staining.

Figure 3.7

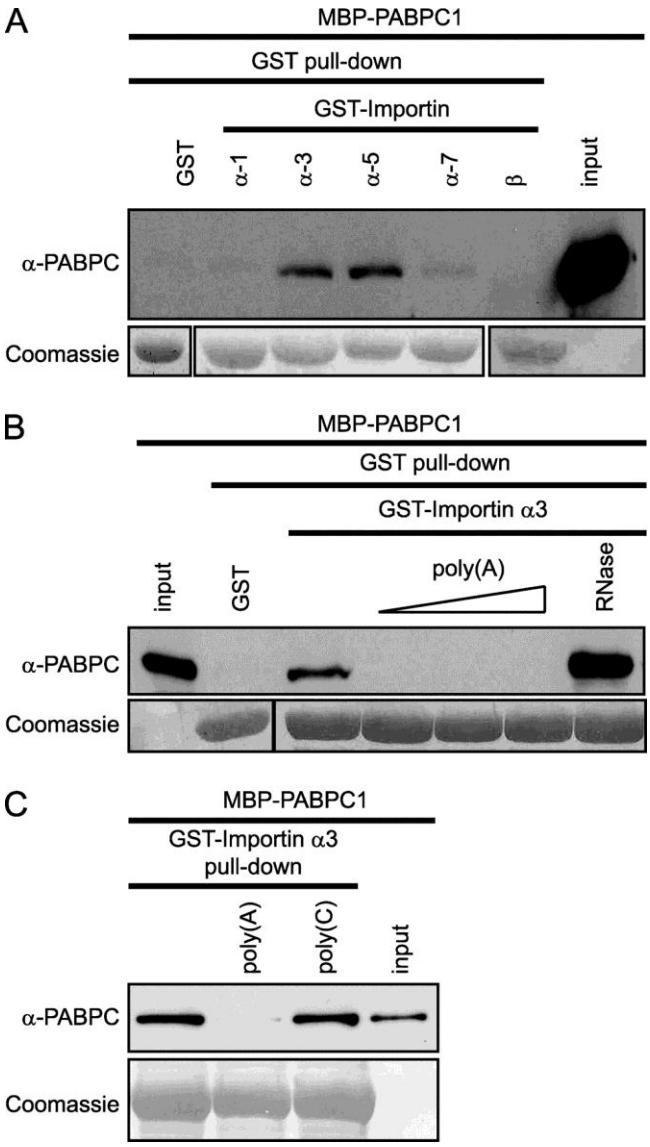

Figure 3.7 Direct interaction between PABPC and importin α is antagonized by poly(A) RNA. (A) Purified MBP-PABPC1 was mixed with purified GST or the indicated GST-importin bound to glutathione-Sepharose beads at 4°C with agitation for 12 h. Bound proteins were resolved by SDS-PAGE and Western blotted with anti-PABPC antibodies. The input was 50% of the purified MBP-PABPC used in the pulldown. (B and C) GST pulldowns were repeated as described for panel A, with the addition of either poly(A) RNA (1 μg [1:1 molar ratio], 10 μg, or 100 μg), 150 U of RNase If, or poly(C) RNA (1 μg [1:1 molar ratio]). The input was 10% (Fig. B) or 1% (Fig. C) of the purified MBP-PABPC1 used in the pulldown. GST and GST-fusion proteins were visualized by Coomassie staining.

Figure 3.8

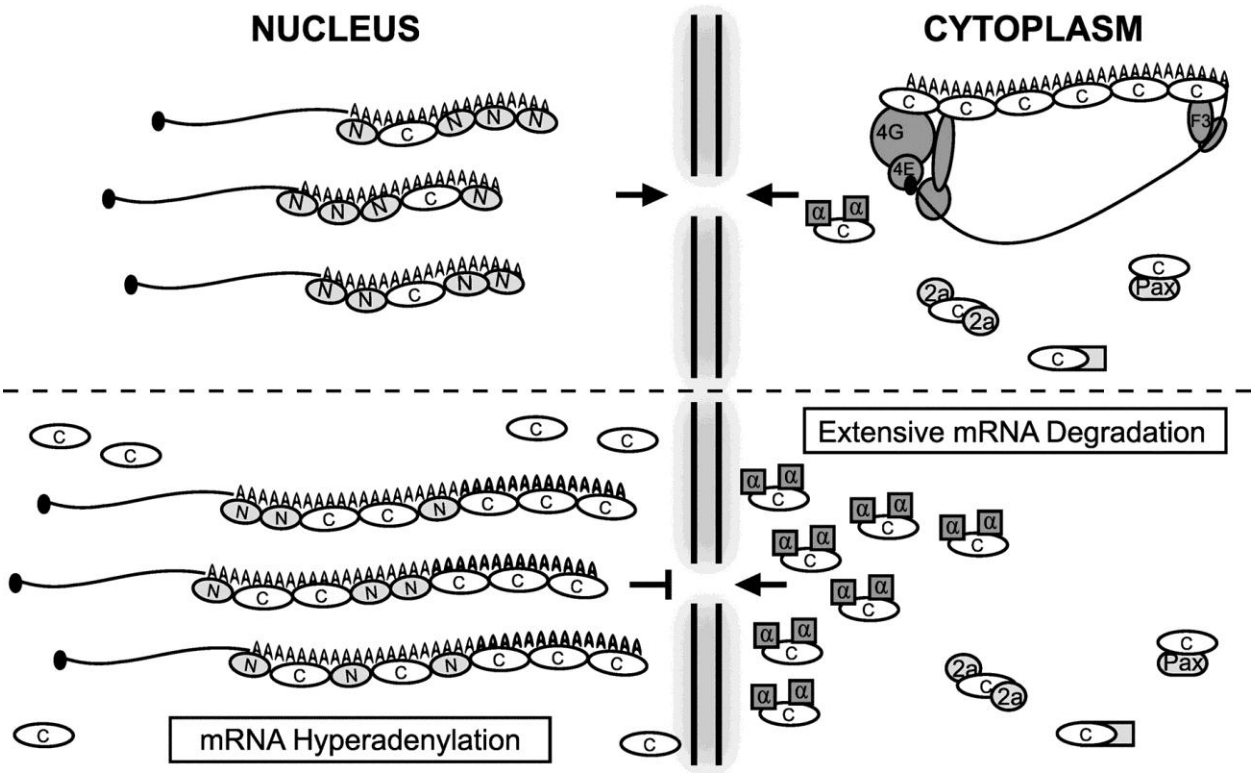

Figure 3.8 Model depicting mRNA turnover-induced nuclear accumulation of PABPC. In normal cells, PABPC is retained in the cytoplasm through its interactions with mRNA or other cellular proteins such as Paip2a and paxillin. At basal levels of mRNA turnover, a small fraction of PABPC shuttles in and out of the nucleus. However, upon widespread mRNA turnover, non-RNA bound PABPC accumulates and can interact with importin α to transit across the nuclear pore. High levels of nuclear PABPC promote hyperadenylation and nuclear retention of mRNA, thereby blocking gene expression. Abbreviations used in the model are as follows: C, PABPC; N, PABPN; α, importin α; 2a, Paip2a; Pax, paxillin; 4G, eIF4G; 4E, eIF4E; F3, eRF3.

Chapter 4

Investigating mechanisms of PABPC-induced hyperadenylation and retention of nuclear mRNA

Background

PABPC was initially discovered as one of the most abundant mRNA binding proteins associated with eukaryotic poly(A) mRNA [51, 188, 200]. Its ability to bind poly(A) RNA was detected in the cytoplasmic fraction, and thus, it was thought to belong to and function in this subcellular compartment. Immunofluorescence assays confirmed these biochemical observations and both yeast and mammalian PABPC were found largely in the cytoplasm of HeLa cells [198]. However, subsequent heterokaryon assays revealed that PABPC is a shuttling protein capable of entering the nucleus, providing the first clues that this cytoplasmic protein may also have nuclear roles [99]. Indeed, PABPC was later found to associate with nuclear pre-messenger RNA (pre-mRNA) implying that it may have roles in pre-mRNA processing and export into the cytoplasm [124]. However, given that majority of PABPC is in the cytoplasmic compartment, this association of PABPC with pre-mRNA would encompass a very small portion of the PABPC molecules in a cell.

Recently, infection of cells with select viruses has demonstrated that, under particular conditions, PABPC localization changes and high levels of PABPC can be found in the nuclear compartment as well [100-104, 116]. Similar nuclear accumulation also occurs during cellular stresses including heat shock, oxidative stress, transcriptional block, and UV irradiation [99, 103, 123, 125]. These observations suggest that manipulating PABPC relocalization is part of the cellular stress response. Indeed, I have shown that nuclear PABPC drives hyperadenylation and nuclear retention of mRNA, resulting in a block in global gene expression (see Chapter 2) [182]. These data indicate that PABPC broadly regulates gene expression under stressful conditions and that viruses exploit this process.

As I have shown in Chapter 2, PABPC-induced hyperadenylation and nuclear retention of the mRNA is dependent on ability of PABPC to bind poly(A) RNA and requires the canonical poly(A) polymerase [182]. However, several questions regarding the underlying mechanisms at play still remain unanswered. For example, it is not yet known which nuclear factors PABPC interfaces with to block gene expression. Furthermore, although bulk mRNA export block is observed when PABPC accumulates in the nucleus, it is yet unclear which specific transcripts undergo PABPC-induced hyperadenylation. Does PABPC hyperadenylate all endogenous mRNA or is this an effect elicited on specific set(s) of transcripts?

As PABPC has previously been reported to associate with pre-mRNA, we hypothesized that PABPC maybe inducing export block and hyperadenylation through interactions with mRNA 3' end processing factors. These interactions may exist normally in cells and could be either enhanced or diminished upon relocalization of PABPC to the nucleus. Alternatively, elevated levels of nuclear PABPC may change the ribonucleoprotein composition of nuclear mRNA. One hypothesis is that PABPC sequesters factors away from proteins involved in poly(A) tail length control, thereby allowing extension of the poly(A) tail beyond the canonical length. In this study, we have tested these possibilities by identifying interactions of PABPC

with 3' end processing factors, as well as by identifying nuclear PABPC-associated proteins using an unbiased proteomics approach.

In our previous report (see Chapter 2) we demonstrated that endogenous mRNAs are subject to PABPC-induced nuclear mRNA retention, however, PABPC-induced hyperadenylation was demonstrated on reporter transcripts. Here, I describe our preliminary attempts to identify specific endogenous transcripts that undergo PABPC-induced hyperadenylation in order to explore whether this phenotype is global or restricted to specific classes of transcripts in a cell. In parallel with nuclearly restricted PABPC (PABPC-NRS), we also employ KSHV SOX and MHV68 muSOX as tools to study these processes.

Results

PABPC interacts with mRNA 3' end processing factors

Control of poly(A) tail length is facilitated by 3' end cleavage and specificity factors (CPSF), nuclear poly(A) binding protein (PABPN), and the poly(A) polymerase (PAP II) [30]. Specifically, the stimulation of PAP II by CPSF results in distributive addition of poly(A) tails. Addition of 10 A residues facilitates recruitment of PABPN, which together with CPSF, stimulates PAP to add 200 – 300 A residues in a processive manner. At this length contact between PABPN, CPSF, and PAP II is difficult to maintain, resulting in reduced stimulation of PAP, which becomes distributive again and eventually polyadenylation terminates. It is possible that high levels of PABPC in the nucleus disrupt this fine-tuned control of poly(A) tail length by either associating with polyadenylation related factors and sequestering them or perhaps by stimulating the PAP itself to facilitate hyperadenylation. The latter is a likely possibility especially because we have previously reported that PAP II is required for PABPC-mediated hyperadenylation [182]. In order to test these hypotheses we assessed whether PABPC is capable of binding to polyadenylation factors.

We transfected 293T cells with HA-PABPC1 in the presence or absence of KSHV SOX for 24 hours, performed anti-HA immunoprecipitations and immunoblotted for various 3' end processing factors. We found that HA-PABPC1 interacts with CPSF 73, CPSF 100, CPSF 160, CtsF 77, and symplekin, but not with CPSF30 (Fig. 4.1A) indicating that PABPC is capable of associating with nuclear proteins involved in 3' end processing of mRNA. Whether these interactions are mediated by RNA or happen to be direct protein-protein interactions remains to be investigated. However, these interactions were not significantly changed in SOX-expressing cells, indicating that PABPC-mediated hyperadenylation and nuclear mRNA retention is unlikely to be a result of differences in the interactions of PABPC with these various factors. In order to ensure that these interactions were not an artifact of PABPC overexpression, we also tested these associations in the context of endogenous PABPC and found that, similar to HA-PABPC1, endogenous PABPC interacts with CPSF 73, CPSF 100, CPSF 160, and CstF 77 but not CPSF 30 (Fig. 4.1B). Again, these interactions were not significantly changed in KSHV SOX- or MHV68 muSOX-expressing cells, suggesting that mRNA hyperadenylation and nuclear retention is not due to altered associations with these 3' end polyadenylation factors.

PABPC1 may directly stimulate PAP II activity, allowing extension of the poly(A) tail beyond the canonical length. In order to test this hypothesis, we determined if PABPC interacted with PAP II. We were unable to detect any interaction between PABPC1 and PAP II (with endogenous or overexpressed proteins) (Fig. 4.1A and 4.1C). However, in normal cells, the majority of the PABPC protein is present in the cytoplasm, suggesting that a lack in interaction between PABPC and PAP II maybe due to low levels of PABPC in the nucleus. In order to elevate levels of nuclear PABPC we transfected 293T cells with either KSHV SOX (to promote nuclear relocalization of PABPC) or a nucleus-restricted PABPC (PABPC1-NRS) and repeated the co-immunoprecipitations. Again, we were unable to detect an interaction between PABPC and PAP II. However, we detected an interaction between PABPC and PAP gamma, another poly(A) polymerase with yet unknown roles (Fig. 4.1B and 4.1C). These data suggest that although PAP II is required for PABPC-induced hyperadenylation [182], stimulation of PAP II by PABPC is not the underlying mechanism that disrupts the poly(A) tail length control.

Interactions of PABPN with two polyadenylation factors are disrupted

Based on the data thus far it appears that PABPC-induced hyperadenylation is not a result of changes in interactions of PABPC with 3' end processing factors. An alternative possibility is that accumulation of PABPC in the nucleus results in more molecules of PABPC binding to the poly(A) tails of nascent mRNAs. This would disrupt the normal association of PABPN with the poly(A) tail as well as with CPSF and PAP II, possibly blocking poly(A) tail length control. This idea is not without precedent, as PABPN has previously been shown to be required for hyperadenylation in KSHV SOX-expressing cells [102]. In order to test this hypothesis we performed co-immunoprecipitations to assess interactions of PABPN with CPSF, PAP, and other 3' end processing factors. As previously reported, we detected interactions between PABPN and several CPSF factors, however, the majority of these interactions were not significantly changed in the presence of SOX (Fig. 4.2).

In contrast to the above observations, association of PABPN with symplekin and nucleophosmin were dramatically reduced in SOX-expressing cells (Fig. 4.2). Symplekin is thought to function as a scaffolding protein that facilitates associations of several cleavage and polyadenylated factors [6]. Nucleophosmin is a nuclear/nucleolar protein that has recently been shown to associate with viral and cellular 3' UTRs following successful polyadenylation and is thought to 'mark' correctly polyadenylated transcripts [201]. A reduced interaction of PABPN and nucleophosmin may suggest that hyperadenylated and nuclearly retained transcripts are sensed by quality control factors. However, whether these changes in symplekin and nucleophosmin binding play a role in PABPC-mediated hyperadenylation and nuclear mRNA retention remains to be investigated.

Unexpectedly, a previously reported interaction between PABPN and PAP II was not detected in this assay [132]. As PAP II has several isoforms, it is possible that the antibody used here does not detect the isoform that interacts with PABPN [202]. If this is the case, reexamination of PABPC and PAP II interactions using an alternative antibody would be informative.

Determining protein interactions of nuclear PABPC using proteomics

Although an interaction between PABPC and 3' end processing factors was observed, the fact that these interactions are unaltered in the presence or absence of SOX suggests that they may not directly contribute to PABPC-mediated hyperadenylation. We therefore considered the possibility that additional, unidentified interactions between PABPC and other proteins in the nucleus may occur, and perhaps these contribute to hyperadenylation. In an effort to obtain a comprehensive list of nuclear interactions of PABPC, we employed a proteomics approach. We transfected 293T cells with Flag-PABPC1, fractionated the cells to separate the cytoplasmic and nuclear proteins, performed anti-Flag immunoprecipitations, and analyzed the eluted proteins through SDS-PAGE followed by a silver stain. Both cytoplasmic Flag-PABPC1 and nuclear Flag-PABPC1 precipitated several proteins (Fig 4.3A). Importantly, nuclear Flag-PABPC1 precipitated different sets and quantities of proteins as indicated by the presence or absence of bands in the nuclear fraction as compared to the cytoplasmic fraction (Fig 4.3A). These samples were analyzed by liquid chromatography mass spectrometry (LC/MS) and several proteins were identified (Table 1). Nuclear PABPC appears to interact with several RNA binding proteins including heterogeneous nuclear ribonucleoproteins A2/B1, zinc finger protein 326, small ribonucleoprotein Sm, among others suggesting this approach may detect valid interactors of nuclear PABPC (Table 4.1). However, in order to gain confidence in LC/MS identified proteins,

additional repeats of the experiment need to be conducted and analyzed to prioritize the proteins for validation.

PABPC is an RNA binding protein and is likely to interact with other RNA binding proteins in an RNA-dependent manner. In order to assess direct protein-protein interactions of nuclear PABPC, we repeated anti-Flag immunoprecipitations of nuclear lysates in the presence of RNase I_f, known to cleave RNA after each base resulting in efficient degradation of poly(A) sequences. Silver stains analysis of eluted proteins revealed that the amount of proteins precipitated with nuclear Flag-PABPC1 is reduced in quantity upon RNase treatment, indicating that several interactions of PABPC are mediated by RNA. Nonetheless, many proteins still co-immunoprecipitate with PABPC and these protein-protein interactions maybe more informative for nuclear functions of PABPC (Fig 4.2B). These samples will be further analyzed by LC/MS to identify direct protein interactions of PABPC.

LC/MS analysis of multiple replicates should identify proteins that reproducibly interact with nuclear PABPC. These interactions will be validated through co-immunoprecipitation experiments in 293T cells as well as in KSHV or MHV68 infected cells. The role of PABPC interactors in PABPC-mediated mRNA retention and hyperadenylation will be evaluated by depleting the PABPC interacting protein using RNA interference. Protein interactors of PABPC that facilitate nuclear functions of PABPC will provide further insights into the underlying mechanisms.

Hyperadenylation of endogenous transcripts

Another approach that may provide clues as to the role of PABPC in the nucleus is to identify specific mRNAs that are subject to PABPC-induced hyperadenylation. For example, a specific subset(s) of mRNAs could be hyperadenylated while others are spared. We have previously demonstrated that nuclear PABPC induces global nuclear mRNA retention (see Chapter 2). These were detected through in situ hybridization using an oligo(dT) probe that binds to all endogenous poly(A) sequences, which largely represent endogenous mRNAs. Importantly, the oligo(dT) signal in SOX-expressing cells was reduced in the cytoplasm and enhanced in the nucleus indicating that majority of nuclear mRNAs are not exported [182].

However, hyperadenylation itself has been studied only on reporter transcripts such as GFP and dsRed. In an effort to understand if endogenous transcripts, in addition to being blocked for export, were also hyperadenylated, we transfected 293T cells with Flag-PABPC1-NRS and analyzed the hyperadenylation state of specific endogenous transcripts. We tested mRNAs encoding heat shock protein 60, heat shock protein 90, L-lactate dehydrogenase, nucleolin, and 60S ribosomal protein L4 mRNAs. We did not observe a mobility shift of any of these transcripts in the presence of nuclear PABPC indicating that these transcripts are not modified by PABPC (Fig. 4.4A). However, it is important to note that since we are analyzing total RNA, the majority of these transcripts represent the cytoplasmic population, which would not be subject to nuclear events. Therefore, it cannot be yet concluded that these mRNAs are not hyperadenylated by nuclear PABPC. Future experiments analyzing only the nuclear fraction of these transcripts will be more informative.

In an effort to expand the repertoire of transcripts that are hyperadenylated by nuclear PABPC, we also analyzed mRNAs of GAPDH and PABPC that were expressed from exogenous plasmids in the presence or absence of nuclear PABPC or KSHV SOX. We found that similar to GFP, GAPDH and PABPC mRNAs were hyperadenylated by both SOX and PABPC-NRS (Fig.

4B). Although these transcripts are not endogenous, these observations demonstrate that the ability of nuclear PABPC to promote hyperadenylation is not limited to GFP or dsRed reporters.

Based on our observations with analyzing endogenous transcripts and GAPDH and PABPC reporters, we hypothesize that nascent transcription is required to detect nuclear PABPC-induced hyperadenylation, because it allows (a) generation of new transcripts to undergo nuclear changes and (b) efficient detection of nuclear transcripts via Northern blot. Consequently, we reasoned that if transcription of specific genes was induced, we could detect hyperadenylation as we would be enhancing the nuclear pool of such transcripts, allowing them to be subject to nuclear-PABPC induced changes. Several cellular stresses induce transcription of specific genes to respond to and manage the cellular stress. One such pathway is the unfolded protein response (UPR) that is triggered by accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) [203, 204]. UPR can be induced by treating cells with dithiothreitol (DTT) or thapsigargin (Tg) resulting in upregulation of UPR-induced genes that include GRP78 and EDEM [205]. GRP78, also known as BiP, is a member of the heat shock 70 protein family that binds to newly synthesized as well as misfolded proteins in the ER and is the master regulator of the unfolded protein response [206]. EDEM (ER degradation enhancing α-mannosidase-like protein) is involved in ER-associated degradation of misfolded glycoproteins [207, 208]. We induced UPR in 293T cells with DTT and Tg in the presence or absence of nuclear PABPC and tested whether GRP78 and EDEM mRNAs were hyperadenylated. While we observed upregulation of both GRP78 and EDEM transcripts when UPR was stimulated by the two drugs, we did not observe any mobility shift of GRP78 or EDEM transcripts indicating they were not modified (Fig. 4C). However, similar to above, the caveat of this experiment is that we are detecting total population of these transcripts, majority of which will be cytoplasmic. Fractionation experiments that allow detection of only nuclear transcripts will provide more interpretable results, especially if these transcripts accumulate in the nuclear fraction, because a second effect of nuclear PABPC is mRNA retention in the nucleus.

Discussion

PABPC interacts with mRNA 3' end processing factors

In this study, we demonstrate that PABPC in the nucleus interacts with several nuclear factors, providing support for the hypothesis that in addition to its roles in the cytoplasm PABPC may also have important roles in the nucleus. PABPC was found to interact with several polyadenylation factors suggesting that it may have roles in mRNA 3' end processing and/or mRNA export (Fig. 1). It will be interesting to determine if any of these factors are required for PABPC-mediated hyperadenylation by reducing their levels via RNA interference. However, as these factors are required for mRNA polyadenylation in a cell, results would be difficult to interpret, as a block in hyperadenylation could merely be due to a block in normal polyadenylation. However, we have previously observed that mRNA hyperadenylation does not necessarily require canonical polyadenylation and can occur post-transcriptionally. This was true for a GFP reporter transcript that contained a templated poly(A) tail of 60 A residues but terminated by a self-cleaving hammerhead ribozyme (HR) instead (Chapter 2) [102, 182]. GFP-A_{60}-HR does not undergo normal polyadenylation, however, is hyperadenylated by nuclear PABPC through the aid of PAP II [182]. Future experiments assessing hyperadenylation of GFP-A_{60}-HR in cells depleted of the various PABPC-interacting polyadenylation factors will be informative to understand if these factors assist PABPC in promoting hyperadenylation. Although PABPC does not associate with PAP II, it is possible that it instead disrupts associations of PABPN with other polyadenylation factors by sequestering them, reducing PABPN-induced stimulation of PAP II. This will be an important future question to address.

Although PABPC was not found to associate with the canonical poly(A) polymerase II (this may be due to poor detection of the correct PAP II isoform), it interacted with another nuclear poly(A) polymerase, PAP gamma. However, although PAP gamma can polyadenylate reporters *in vitro*, its *in vivo* functions are unknown. Based on this observation, it is tempting to speculate that PABPC recruits PAP gamma to promote mRNA hyperadenylation. However, siRNA mediated depletion of PAP gamma did not have any consequences for mRNA hyperadenylation suggesting it does not facilitate PABPC-induced hyperadenylation (see Chapter 2) [182].

Proteomic approaches to identify nuclear interactors of PABPC will provide a global perspective of the nuclear processes that nuclear PABPC may be involved in and may provide further clues to PABPC-mediated hyperadenylation and nuclear mRNA retention.

Interactions of PABPN with two polyadenylation factors are disrupted in SOX-expressing cells

We have previously reported that expression of KSHV SOX disrupts interactions of PABPC with cytoplasmic translation factors (see Chapter 3) [209]. Here we show that interactions of PABPN with two polyadenylation factors, symplekin and nucleophosmin, are also influenced in SOX-expressing cells (Fig 2). Symplekin is a scaffolding protein thought to bridge interactions between various 3' end processing factors and likely facilitates association of PABPN with other polyadenylation factors [6]. Disruption of PABPN and symplekin binding may suggest that the 3' polyadenylation complex composition is altered in the presence of nuclear PABPC, resulting in unregulated poly(A) tail length control.

Nucleophosmin is an abundant nuclear protein that has previously described roles in ribosomal biogenesis. However, recently, it has also been shown to be deposited on correctly

polyadenylated mRNAs [201]. Nucleophosmin was found to be deposited on several endogenous mRNAs, irrespective of the type of polyadenylation signal they possessed implying that this binding may be general. Furthermore, nucleophosmin deposition only occurred following termination of polyadenylation, suggesting that correctly processed transcripts receive this factor. As expected of a protein that associates with polyadenylated mRNAs, it was shown to interact with CPSF proteins indicating that it may be recruited by one of the CPSF factors upon polyadenylation termination. Importantly, depletion of nucleophosmin interfered with poly(A) tail length control resulting in hyperadenylation of mRNAs. Based on these observations it is possible that a disruption of interaction between PABPN and nucleophosmin may play an integral role in PABPC-induced hyperadenylation in SOX-expressing cells. While PABPC was found to interact with nucleophosmin (Fig. 1A), this interaction was not disrupted in SOX-expressing cells indicating that PABPC may instead block interaction between PABPN and nucleophosmin. Future experiments addressing these questions will provide insights into the underlying mechanisms of PABPC-induced hyperadenylation. The current model of nucleophosmin function suggests that its association with CPSF influences PAP activity on the growing poly(A) tail along with PABPN. In addition to the 200 – 300 nucleotide poly(A) tail, presence of nucleophosmin may provide additional strain on association of CPSF and PAP , thereby promoting PAP dissociation and facilitating poly(A) tail length control [201]. Disruption of this interaction may result in continued stimulation of PAP, resulting in hyperadenylated mRNAs. Further analysis of these interactions and their disruption in SOX-expressing cells will provide insights into the exact underlying mechanisms at play.

Hyperadenylation of endogenous transcripts

A complementary approach for determining mechanisms of PABPC-induced hyperadenylation includes identification of specific endogenous transcripts that are subject to PABPC-induced changes. However, the important question of whether endogenous mRNAs are hyperadenylated is still unanswered, mainly because of the challenges associated with detecting hyperadenylated transcripts. First, when steady state levels of transcripts are probed by Northern blot in nuclear PABPC-expressing cells, the majority of the signal corresponds to the cytoplasmic population of the transcript. The strong signal of cytoplasmic mRNA likely masks the weak signal of nuclear mRNA, resulting in inefficient detection of nuclear hyperadenylated mRNAs. Future experiments where mRNA is fractionated into nuclear and cytoplasmic populations may improve the detection of hyperadenylated transcripts. Secondly, basal level of transcription of the probed mRNAs may not be high enough to generate a large nuclear population of mRNA that can be subject to PABPC-induced hyperadenylation. We have attempted to circumvent this issue in two ways: (1) over-expressing endogenous genes using plasmids and (2) inducing transcription of specific genes through cellular stress. With the first approach, we found that overexpressed GAPDH and PABPC mRNAs were indeed hyperadenylated in the presence of nuclear PABPC (Fig. 2B), indicating PABPC-induced hyperadenylation of GFP or dsRed is not due to the nature of the coding regions of the GFP and dsRed reporter. For the second approach, we induced the unfolded protein response with drugs and detected upregulation of UPR regulated genes. However, we did not observe hyperadenylation of these transcripts in the presence of nuclear PABPC. Although there is a transcriptional burst, we may still be largely detecting the cytoplasmic population, thereby masking the nuclear mRNA population. Alternatively, as proteins encoded from these

transcripts are required to combat the cellular stress, these mRNAs may have inherent mechanisms that escape PABPC-mediated hyperadenylation.

Future avenues include testing hyperadenylation of transcripts that are induced during other cellular stresses such as the interferon response or the heat shock response. Additionally, a transcription factor could be overexpressed to induce transcription of very specific gene(s) and assess nuclear PABPC-mediated hyperadenylation of that particular transcript. We have previously reported that addition of Leptomycin B, which blocks CRM-1 mediated protein export, aids in detection of hyperadenylated transcripts [182]. Future experiments include inducing transcription followed by treatment with Leptomycin B for a few hours to facilitate detection of hyperadenylated transcripts.

Materials and Methods

Plasmids

Plasmids pCDEF3-Flag-PABPC1-NRS, pCDEF3-SOX and pCDEF3-muSOX have been previously described [119, 182].

Cells and Transfections.

HEK293T cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected using PolyJet (SignaGen) as per manufacturer's instructions.

Cell extracts, Immunoprecipitations, and Western blots

Cell were lysed in RIPA lysis buffer (50mM Tris-HCl [pH 8.0], 150mM NaCl, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) containing protease inhibitors (Roche) for Western blotting or in NETN lysis buffer (20mM Tris-HCl [pH 8.0], 100mM NaCl, 1mM EDTA, 0.5% Nonidet P-40) containing protease inhibitors (Roche) for immunoprecipitations. For fractionation experiments lysates were separated into nuclear and cytoplasmic fractions using NE-PER as per manufacturer's instructions (Pierce). Protein lysates were quantified by Bradford assay (Bio-Rad).

Immunoprecipitations for HA-PABPC1 and HA-PABPN were conducted by mixing monoclonal anti-HA clone HA-7 beads (Sigma) and lysates from transfected HE293T at 4ºC with agitation for 3 h. Immunoprecipitations for endogenous PABPC were performed by binding 1 mg of mouse monoclonal PABPC 10e10 antibody to protein A sepharose 4 fast flow beads (GE healthcare) at 4°C with agitation for 1 h, washing the beads with NETN buffer, and mixing with lysates from transfected HEK293T cells at 4°C with agitation for 3 h. The beads were then washed 3-5 times with NETN buffer and bound proteins were dissolved with 40µl 2X laemmli sample buffer, resolved on 10% SDS-PAGE, and subjected to Western blot.

Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinylidene difluoride membrane, and subjected to Western blotting using either mouse monoclonal HA12CA5 (1:2000 dilution, Abcam), CPSF30 (1:1000 dilution), CPSF73 (1:1000 dilution, kindly provided by Dr. David Bentley, University of Colorado), CPSF100 (1:1000 dilution), CPSF160 (1:1000 dilution), CtsF77 (1:1000 dilution, kindly provided by Dr. David Bentley, University of Colorado), symplekin (1:1000 dilution, BD Biosciences), nucleophosmin (1:1000 dilution, Abcam), PAP II (1:1000 dilution, Sigma), and PAP gamma (1:1000 dilution) primary antibodies. Secondary antibodies included horseradish peroxidase-conjugated actin antibodies (1:5000 dilution, Santa Cruz Biotechnology), goat anti-mouse, or goat anti-rabbit antibodies (1:5000 dilution, Southern Biotechnology Associates).

Silver stains

SDS-PAGE gels were incubated in 50% methanol twice for 10 mins and 5% methanol twice for 10 mins. After two water rinses, the gel was incubated in 10 µM dithiothreitol (DTT) for 20 mins, 0.1% (w/v) silver nitrate for 20 mins, and rinsed twice with water. The gel was then incubated in developer solution (2.5% (w/v) sodium bicarbonate and 125 µl of 37% formaldehyde) until distinct silver stained bands appeared. The reaction was then stopped with excess citric acid.

Northern blots

Total cellular RNA was isolated using RNA-bee (Tel-Test), resolved on 1.2% agarose-formaldehyde gels, transferred to a nylon membrane, and probed with ^{32}P-labeled GFP, heat shock protein 60, heat shock protein 90, lactate dehydrogenase, nucleolin, ribosomal protein L4, GAPDH, PABPC, EDEM, GRP78 or 18S DNA probes prepared with the Rediprime II random primer labeling kit (GE Healthcare).

Figure 4.1

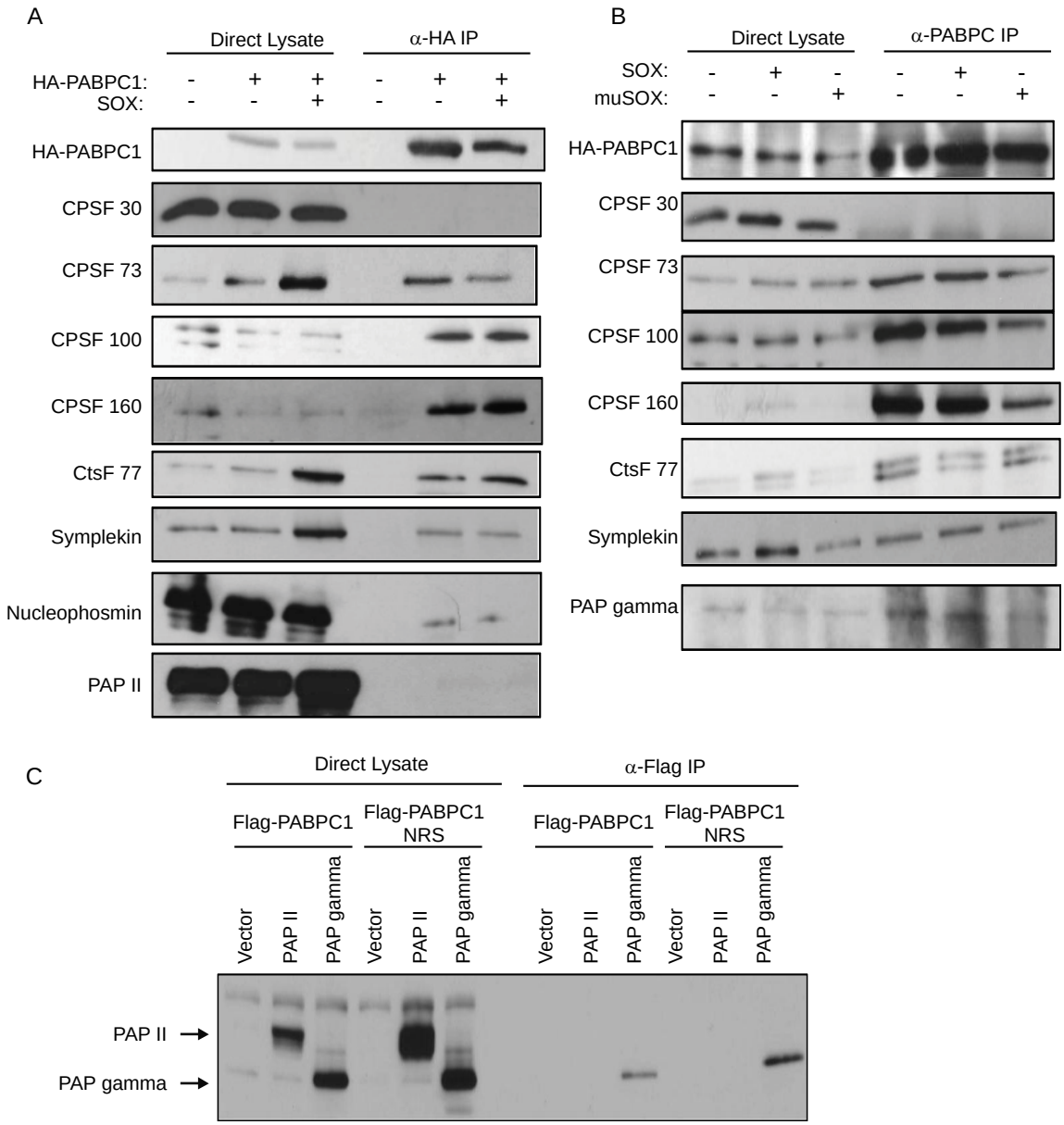

Figure 4.1 Interactions of PABPC with 3' end processing factors. (A) HEK293T cells were transfected with empty vector or plasmids expressing HA-PABPC1 with or without KSHV SOX for 24 h. HA-PABPC1 was immunoprecipitated using anti-HA agarose beads, and bound proteins were resolved by SDS-PAGE and detected by Western blot with antibodies against the indicated proteins. (B) HEK293T cells were transfected with empty vector, KSHV SOX, or MHV68 muSOX for 24 h. Endogenous PABPC was immunoprecipitated using anti-PABPC antibodies, and bound proteins were resolved and detected as above. (C) HEK293T cells were transfected with empty vector or plasmids expressing Flag-PABPC1 or Flag-PABPC1-NRS with or without PAP II and PAP gamma. Flag-PABPC1 was immunoprecipitated using anti-Flag agarose beads, and bound proteins were resolved and detected as above.

Figure 4.2

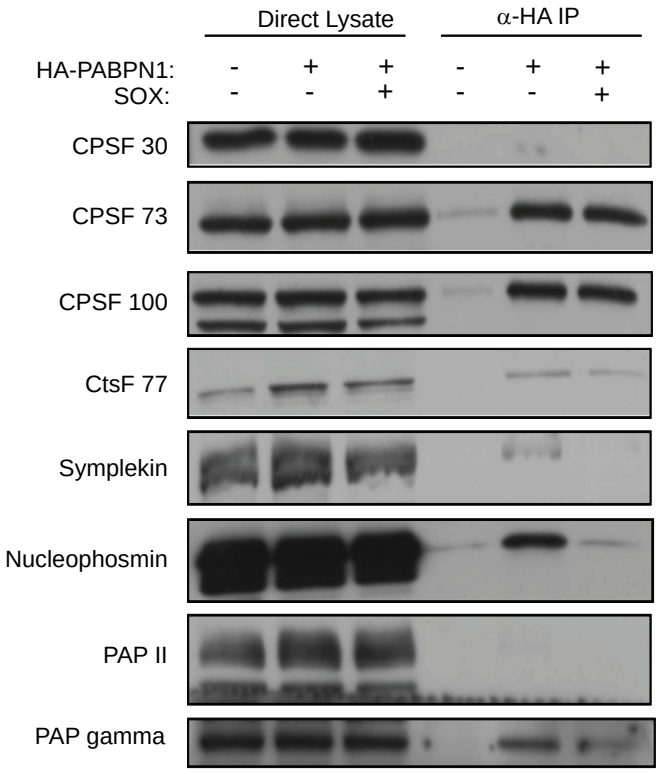

Figure 4.2 Interactions of PABPN with 3' end processing factors. HEK293T cells were transfected with empty vector or plasmids expressing HA-PABPN with or without KSHV SOX for 24 h. HA-PABPN was immunoprecipitated using anti-HA agarose beads, and bound proteins were resolved by SDS-PAGE and detected by Western blot with antibodies against the indicated proteins.

Figure 4.3

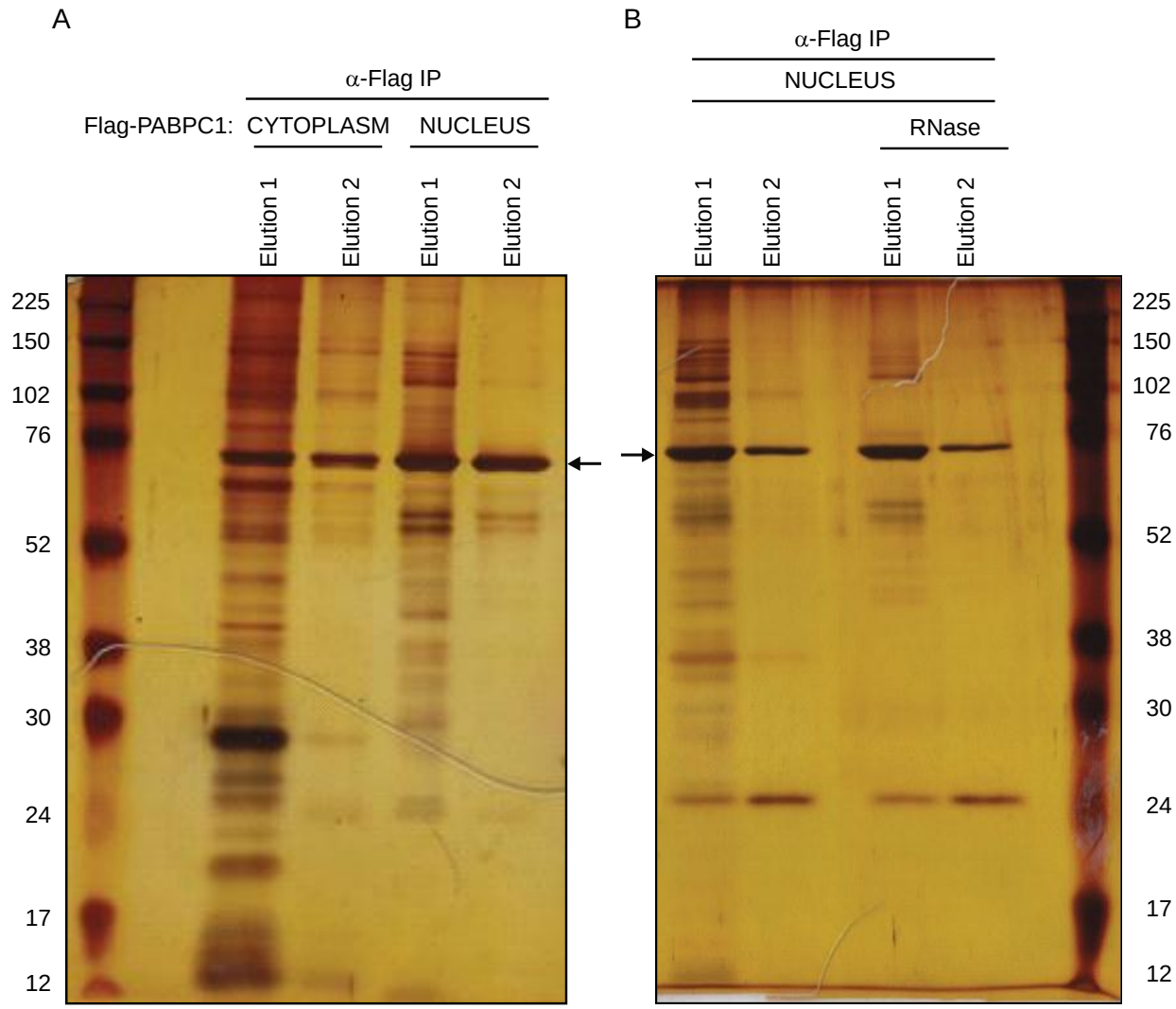

Figure 4.3 Nuclear interactions of PABPC. HEK293T cells were transfected with Flag-PABPC1 for 24 h and fractionated into nuclear (A and B) and cytoplasmic (A) lysates with or without RNaseI_f (B). Flag-PABPC1 was immunoprecipitated using anti-Flag agarose beads and the bound proteins were resolved by SDS-PAGE and detected with silver stain. Arrows mark the Flag-PABPC1 protein.

Figure 4.4

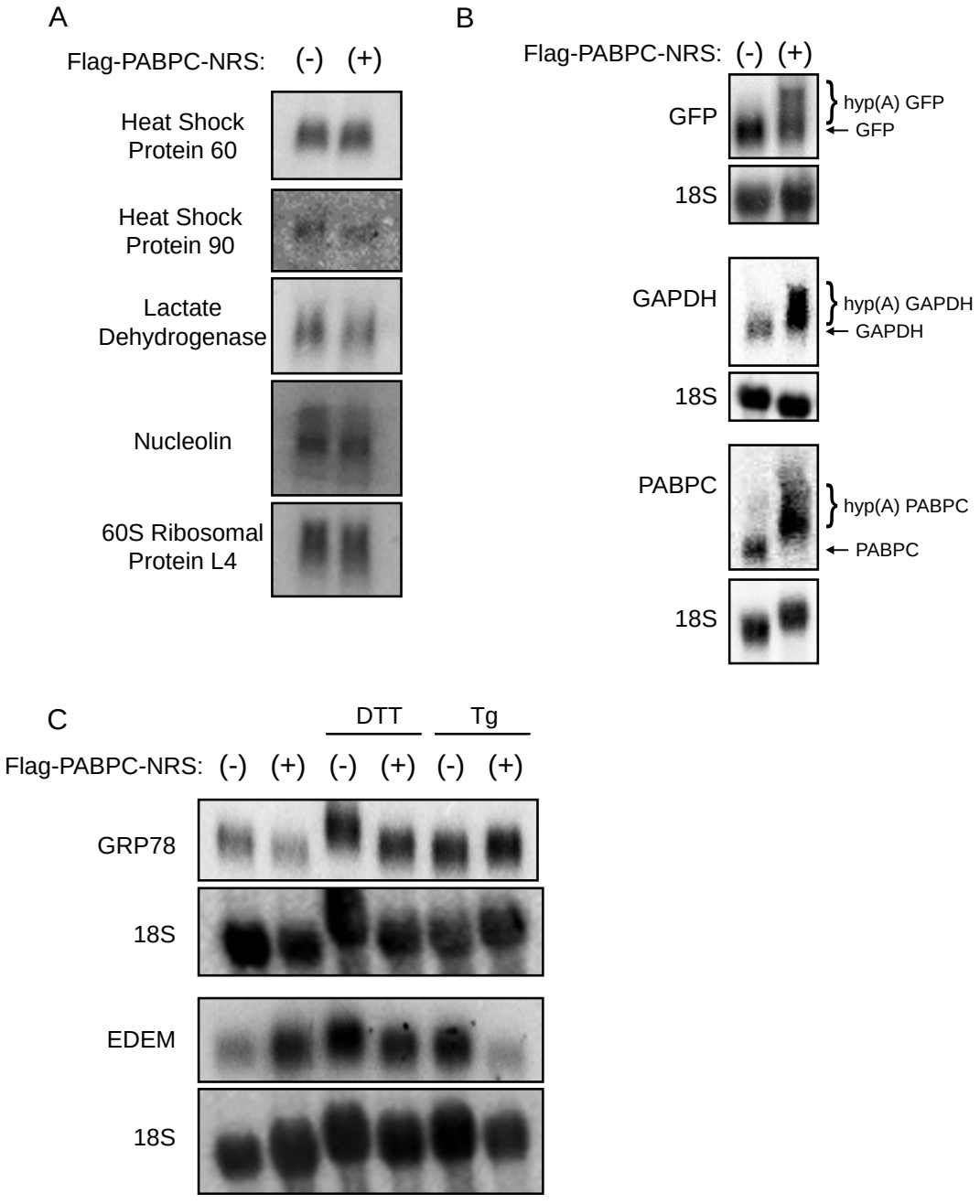

Figure 4.4 PABPC-mediated hyperadenylation of endogenous mRNAs. (A) HEK293T cells were transfected with empty vector or plasmid expressing Flag-PABPC1-NRS for 24 h, total RNA was isolated, resolved by agarose-formaldehyde gel electrophoresis, and Northern blotted with ^{32}P-labeled probes for indicated transcripts. (B) HEK293T cells were transfected vectors expressing GAPDH and PABPC with or without Flag-PABPC1-NRS for 24 h, total RNA was isolated, and analyzed as above. (C) HEK293T cells were transfected with either empty vector or plasmid expressing Flag-PABPC1-NRS for 24 h. Cells were then mock treated, treated with 2 mM DTT or 100 nM Tg for 30 mins. Total RNA was isolated and analyzed as above.

Table 4.1 LC/MS results of protein interactions of nuclear PABPC1.

LC/MS-MS Top Hits:
Zinc finger protein 326 isoform 3
Small nuclear ribonucleoprotein Sm D3
Heterogeneous nuclear ribonucleoprotein A2/B1 isoform B1
Polyadenylate-binding protein 1
Heterogeneous nuclear ribonucleoproteins A2/B1 isoform A2
NHP2-like protein 1 enhancer of rudimentary homolog
Serine/arginine-rich splicing factor 1 isoform 2
U4/U6 small nuclear ribonucleoprotein Prp4
heterogeneous nuclear ribonucleoprotein H
28S ribosomal protein S23, mitochondrial
serine/arginine-rich splicing factor 3
histone H4
40S ribosomal protein S5
general transcription factor II-I isoform 1
heterogeneous nuclear ribonucleoprotein R isoform 4
U4/U6 small nuclear ribonucleoprotein Prp3
heterogeneous nuclear ribonucleoproteins C1/C2 isoform b
serine/arginine-rich splicing factor 9
protein mago nashi homolog
small nuclear ribonucleoprotein E
probable ATP-dependent RNA helicase DDX5
28S ribosomal protein S26, mitochondrial precursor
heterogeneous nuclear ribonucleoprotein R isoform 3
serine/arginine-rich splicing factor 7 isoform 1
heterogeneous nuclear ribonucleoprotein A3
heterogeneous nuclear ribonucleoprotein U
heterogeneous nuclear ribonucleoprotein R isoform 2

Chapter 5

Characterization of nuclear PABPC subdomains during KSHV infection

Background

The kinetics of PABPC import follow the kinetics of Kaposi's sarcoma-associated herpesvirus (KSHV)-induced host shutoff. Host shutoff ensues upon KSHV SOX expression within 12 hours of infection. Similarly, nuclear relocalization of PABPC follows this time course where nuclear import begins around 12 hours and the majority of PABPC is found in the infected nucleus by 24 hours [102]. Nuclear PABPC-induced block in nuclear mRNA export results in a second wave of host shutoff, presumably further ensuring that the virus does not have to compete for cellular resources for its replication. Consequently, while the virus has usurped this cellular stress response pathway for blocking host mRNA export, it must ensure that viral mRNAs are efficiently exported into the cytoplasm. However, as PABPC is a general poly(A) binding protein, it is unclear how viral mRNAs evade PABPC-induced mRNA retention. KSHV ORF57 has been shown to aid export of viral transcripts, suggesting that ORF57 could mediate escape from PABPC-induced mRNA retention [210].

Interestingly, we found that subcellular localization of PABPC is further manipulated during infection with KSHV. During late times in lytic KSHV infection (36-48 hours post infection), nuclear PABPC is found in subdomains in the nuclear periphery. However, they are not formed upon transfection of KSHV SOX indicating that other viral factors are required for their assembly. These types of PABPC structures have not been reported before, therefore, their composition and function is currently unclear. It is possible that during viral infection PABPC is redirected to the subdomains to locally restrict PABPC-induced mRNA retention of cellular transcripts, allowing viral transcripts to escape this fate. In this report, subdomains of nuclear PABPC are characterized in an effort to understand their composition and function during viral infection.

Results

Previous studies had assessed PABPC localization up to 24 hours post KSHV infection and the majority of PABPC was found in the nuclei of virally infected cells. PABPC relocalization has also been reported during certain cellular stresses, including heat shock, oxidative stress, and transcriptional block [99, 103, 125]. Interestingly, upon recovery of cells from heat shock, PABPC has been shown to return to the cytoplasm to resume its roles in cellular mRNA translation [125]. Based on these observations, we wondered if PABPC also returns to the cytoplasm of KSHV-infected cells at late time points to perhaps aid in translation of viral transcripts. We infected telomerase immortalized endothelial cells (TIME) for 36 hours and performed immunofluorescence assay for PABPC and KSHV SOX (to detect lytic cells). Unexpectedly, we observed nuclear PABPC in subdomains around the nuclear periphery (Fig 5.1). Interestingly, PABPC was present in areas of the nucleus where ORF59, a KSHV polymerase processivity factor that would be present at sites of viral transcription, was not enriched (Fig 5.2A, first panel). We next assessed if other nuclear poly(A) binding proteins were also present in such subdomains and stained for PABPN, a protein with roles in mRNA polyadenylation and ZC3H14, a poly(A) binding protein with unknown functions in the cell. We found ZC3H14 to also be present in similar subdomains as PABPC and these were also specifically present in areas that had reduced levels of ORF59 (Fig. 5.2B). PABPN, on the other hand, was not localized in subdomains (Fig. 5.2C). It instead co-localized with ORF59 indicating that PABPN is participating in viral transcription related events whereas PABPC and ZC3H14 are excluded from this locale. A viral DNA binding protein, SSB, also co-localized with ORF59, as expected (Fig. 5.2D).

One of the most abundant transcripts in the cell infected with KSHV is a viral non-coding polyadenylated RNA termed PAN. Recently, PAN was shown to bind PABPC within the nucleus [211]. This observation prompted us to ask if PAN was also present in the PABPC subdomains. We infected TIME cells with KSHV for 36 hours and performed in situ hybridization with PAN probes followed by immunofluorescence assay for ORF59. To our surprise we found that PAN was not present in any subdomains, but it instead co-localized with ORF59 (Fig. 5E). These observations suggest that the interaction between PABPC and PAN maybe temporally and spatially regulated.

While PABPC-induced mRNA export block can aid KSHV in promoting a continued host shutoff effect, the virus must ensure export and translation of viral transcripts for a productive infection. KSHV mRNAs are analogous to cellular mRNAs such that they possess a 5’ cap and a 3’ poly(A) tail. As PABPC associates with poly(A) sequences with high affinity, it is yet unclear as to how viral mRNAs escape PABPC-mediated export block. Based on these subdomains one possibility is that during viral infection there is a localized mRNA export block such that cellular mRNAs are shuttled into PABPC subdomains, preventing PABPC from acting upon viral mRNAs. If this were true endogenous mRNA would also be present in these subdomains. We tested this hypothesis by performing in situ hybridization with oligo(dT) on infected cells followed by immunofluorescence for ZC3H14 to mark subdomains. Remarkably, we observed oligo(dT) staining in subdomains that co-localized with ZC3H14 indicating that a significant portion of mRNA in the cell is present in subdomains (Fig 5.2F).

Discussion

Although nuclear accumulation of PABPC has been observed during several cellular stresses and infection with divergent viruses, its sub-nuclear localization has not been investigated before. Here we show that at late times in KSHV infection PABPC is found in subdomains, often around the nuclear periphery. Characterization of these subdomains showed that they also contain another poly(A) binding protein, ZC3H14 (Fig. 5A). ZC3H14 is a homolog of the yeast Nab2 protein and both have been shown to bind to poly(A) RNA with high affinity using tandem CCCH zinc finger domains, although the cellular function of ZC3H14 is not currently known [212]. However, PABPN was not found in such subdomains, likely because it is required for KSHV transcription. In agreement with that PABPN co-localized with the viral polymerase processivity factor encoded by ORF59 (Fig. 5C).

KSHV encodes for a highly abundant non coding RNA termed PAN (polyadenylated nuclear RNA). While PAN is the most highly expressed transcript in an infected cell, its functions remain elusive. It was recently shown to aid in expression of late genes [211]. In the same study it was also found to bind PABPC, resulting in stabilization of the transcript. This interaction of PABPC and PAN suggested that PAN may also be present in those subdomains, however, this was not observed. PAN was found mostly diffuse in the nucleus and co-localized with ORF59. These data indicate that the previously identified interaction between PAN and PABPC may take place at early time points during KSHV infection, and that they may be disrupted at 36 hours post KSHV infection.

Given that two distinct poly(A) binding proteins are found in nuclear subdomains, it is tempting to hypothesize that these may contain cellular mRNAs that are blocked for export, thereby, reallocating all export resources for viral transcript export. Indeed, bulk mRNA was present in ZC3H14-containing subdomains as demonstrated by oligo(dT) staining. However, it is important to note that oligo(dT) would stain all endogenous mRNAs, all viral mRNAs, as well as KSHV PAN (non-coding RNA) as they all contain poly(A) sequences; the latter is a less likely event as it was found to co-localize with ORF59 instead (Fig 5.2E). An important future challenge includes determining if viral mRNAs are absent from these subdomains, presumably, by virtue of their nuclear export facilitated by ORF57. If true, this would provide a means for viral mRNAs to escape PABPC-induced mRNA retention.

Determining the composition of PABPC subdomains will be important to understand its functions. In addition to identifying specific transcripts that are present in subdomains, future efforts should focus on immunoprecipitating PABPC at late times during KSHV infection and employing proteomics to identify proteins associated with PABPC in that locale. Confocal microscopy analysis will also provide high resolution analysis of PABPC subdomains to achieve further spatial insights into the subdomains, especially, to determine where specifically in the nuclear periphery, in the context of the nuclear pore, these subdomains are located.

Materials and Methods

Cells and Infections.
Telomerase immortalized endothelial cells (TIME) were maintained in EBM-2 medium bullet kits (Clonetics) [213]. TIME cells were infected with KSHV and lytically reactivated for 36 hours with adenoviral vector encoding the KSHV lytic switch protein RTA as previously described [165].

Immunofluorescence Assays and In Situ Hybridization

TIME cells were grown on coverslips, infected for 36 hours, and processed for IFA as previously described [165]. Briefly, cells were fixed in 4% paraformaldehyde (vol/vol), incubated for 10 min in permeabilization buffer [1% triton x-100 (vol/vol) and 0.1% sodium citrate (w/vol)], then for 30 min in block buffer [1% triton x-100 (vol/vol), 0.5% tween-20 (vol/vol) and 3% BSA (w/vol)],and incubated with either mouse monoclonal PABPC 10e10 (1:25 dilution) (Santa Cruz Biotechnology), rabbit polyclonal SOX J5803 (1:500 dilution) [166], mouse anti-ORF59 (1:500 dilution) (Advanced Biotechnologies), rabbit polyclonal ZC3H14 (kindly provided by Dr. Anita Corbett, Emory University), rabbit polyclonal PABPN (kindly provided by Dr. Uwe Kühn, Institut für Biochemie und Biotechnologie), and rabbit polyclonal SSB (kindly provided by Gary Hayward, Johns Hopkins Medicine) primary antibodies for 3-12 hr in block buffer, followed by incubation with AlexaFluor 488- or 546- conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:1500 dilution) (Molecular Probes). Coverslips were mounted in DAPI-containing Vectashield mounting media (Vector Labs).

For in situ hybridization (http://www.singerlab.org/protocols), cells were fixed with 4% paraformaldehyde (vol/vol) for 10 mins, then washed 2X with PBS and permeabilized by treatment with 70% ethanol for 2 hours to overnight. Cells were next treated with the following for 5 mins each: 1X PBS, 1M tris (pH8.0) and 1X PBS. Cells were hybridizated overnight at 37C in 200uL of hybridization buffer [50% formamide (vol/vol), 10% Dextran Sulfate (vol/vol), 0.02% BSA (w/vol), 200ug E. coli tRNA, and 2X SSC (1X SSC is 0.15M NaCl and 0.015M sodium citrate)] using 2ng/uL of AlexaFluor 488 labeled complimentary PAN oligo (CCAATGTTCTTACACGACTTTGAAACTTCTGACAAATGCC). Cells were then stained for IFA as described above.

Figure 5.1

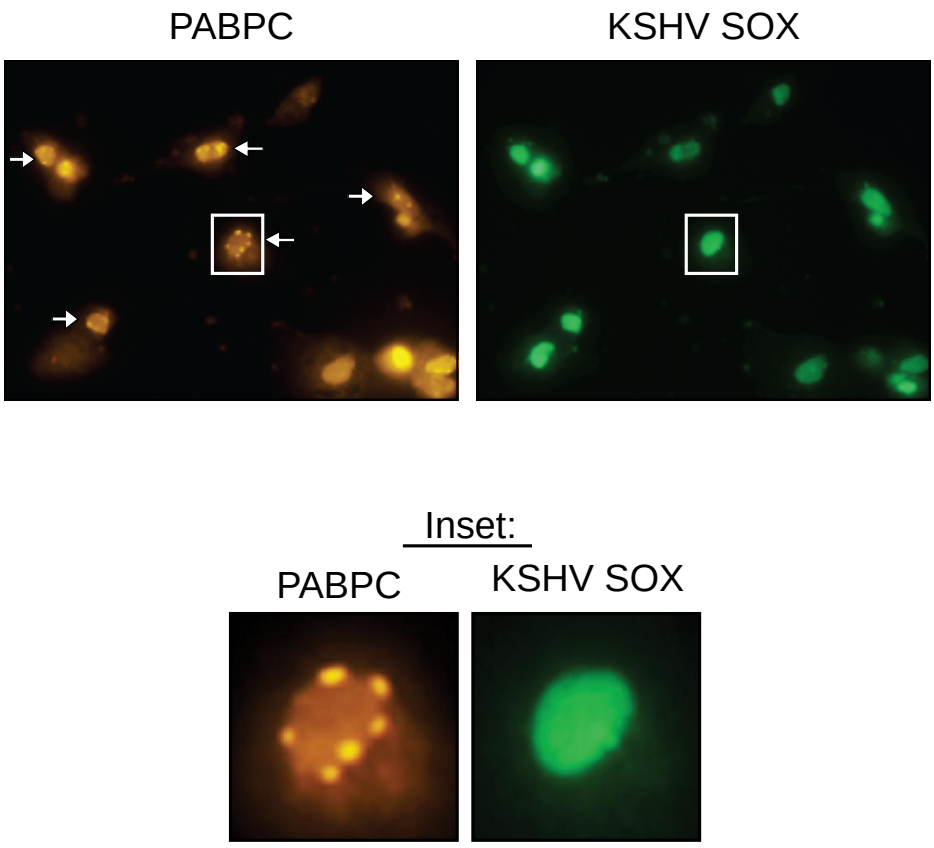

Figure 5.1 PABPC subdomains during late KSHV infection. TIME cells were infected with KSHV and lytically reactivated with using adenoviral expression vector containing the viral lytic transactivator RTA (Ad-RTA) for 36 h. Cells were then processed for immunofluorescence with PABPC and SOX antibodies.

Figure 5.2

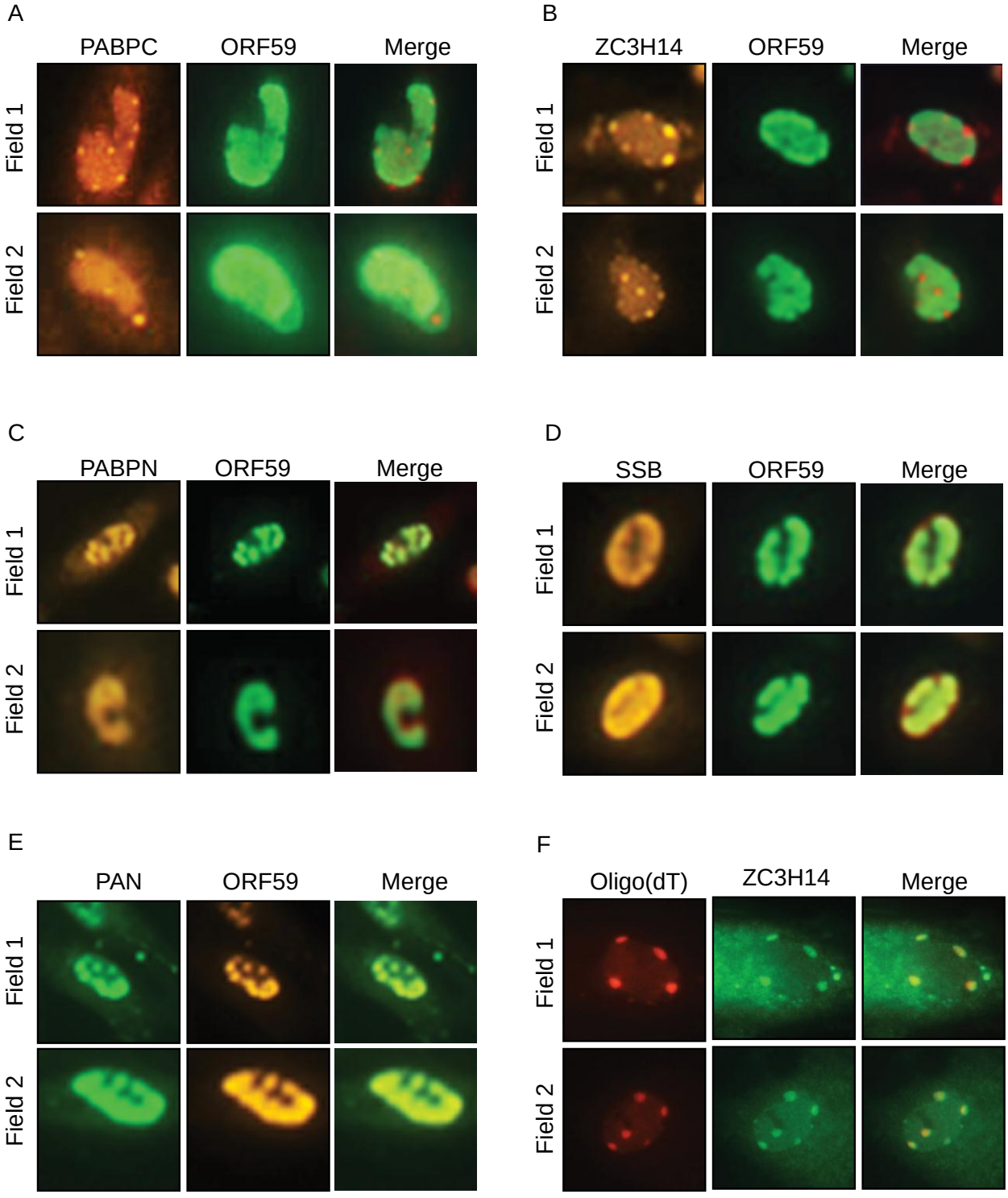

Figure 5.2 Characterization of PABPC subdomains. TIME cells were infected with KSHV and lytically reactivated with using adenoviral expression vector containing the viral lytic

transactivator RTA (Ad-RTA) for 36 h. Cells were then processed for immunofluorescence with PABPC, ZC3H14, ORF59, PABPN, SSB antibodies or in situ hybridization with PAN and Oligo(dT) probes.

Chapter 6

Perspectives

Further Discussion and Future Directions

Gene expression is regulated at multiple levels within a cell. There are abundant examples of transcriptional regulation that control generation as well as levels of mRNA at the desired time. Significant level of regulation also occurs post-transcriptionally throughout the lifecycle of an mRNA, from biogenesis to degradation. Once a protein is translated, post-translational regulation occurs in the form of protein modifications, protein stabilization, and protein interaction partners. Collectively, these networks of regulation play a role in fine-tuning a protein's function and regulate specific cellular processes, both spatially and temporally.

Another prominent level of regulation occurs through differential subcellular localization. An important example includes subcellular organelles in eukaryotic cells such as the nucleus, the mitochondria, the lysosome, etc., that all perform specific functions encased in a specific locale, likely to implement localized regulation and prevent damage to and from other cellular processes. Governing the subcellular localization of a specific mRNA or protein provides another tool to spatially regulate cellular processes, especially, during conditions of stress. This study provides an example of yet another protein whose subcellular localization is manipulated in response to specific stimuli, which then has global effects on mRNA localization, and consequently causes changes in gene expression.

In this report we demonstrated that the subcellular localization of the cytoplasmic poly(A) binding protein (PABPC), a protein with integral roles in mRNA translation, accumulates in the nucleus in response to cellular stress, in particular viral infection, and that nuclear PABPC regulates global gene expression. In the past few decades, the cytoplasmic functions of PABPC have been well characterized; however, its nuclear relocalization and functions are only beginning to be explored. Here we showed that PABPC is relocalized into the nucleus directly in response to cytoplasmic mRNA degradation. Liberation of PABPC from poly(A) sequences results in unmasking of nuclear localization signals within the protein which facilitate PABPC translocation into the nucleus by the cellular nuclear import machinery. Furthermore, we found that nuclear accumulation of PABPC blocked gene expression by preventing mRNA export and inducing mRNA hyperadenylation.

In the past few years the list of viruses that manipulate PABPC localization has been increasing and includes Kaposi's sarcoma-associated herpesvirus, murine herpesvirus 68, herpes simplex virus, rotavirus, and bunyamwera virus. However, what function nuclearly accumulated PABPC could be serving to contribute to viral function was not known prior to this work. Here we have demonstrated that nuclear PABPC causes a block in global mRNA export during viral infection, indicating that viruses manipulate PABPC localization to promote a continued block in cellular gene expression, presumably to allocate cellular resources towards viral replication. Viruses that replicate in the cytoplasm, such as rotavirus, are likely to benefit from the consequences of PABPC accumulation in the nucleus. However, the advantages of PABPC relocalization for viruses that replicate in the nucleus, such as herpesviruses, are unclear.

Herpesviruses utilize the cellular transcription machinery to generate mRNAs that are similar to the host mRNA in that they contain a 5' cap and a 3' poly(A) tail. These observations raise two questions: (a) how are viral mRNAs translated in the face of reduced cytoplasmic PABPC, and (b) how do viral mRNAs escape the PABPC-induced export block? Clues to address the first question come from the observation that KSHV mRNAs more efficiently recruit the translation initiation complex even when cytoplasmic PABPC levels are diminished [163]. On the other hand, mechanisms of how viral mRNAs escape PABPC-mediated affects remain to be investigated.

PABPC is a general mRNA binding protein that has a very high affinity for poly(A) sequences. Since viral mRNAs possess poly(A) tails, the virus must use an active mechanism to escape PABPC-induced export block. This may be accomplished by the action of KSHV MTA (mRNA transport and accumulation protein encoded by ORF57) on viral transcripts, however, it remains to be directly investigated [210]. Another hypothesis is that PABPC-induced mRNA export block is exerted in a specific locale within the nucleus allowing the viral transcripts to escape this fate as they would not come in contact with PABPC. In support of this idea is our finding that subdomains of PABPC accumulate around the nuclear periphery at late times during KSHV infection. These subdomains also harbor another poly(A) binding protein, ZC3H14, and high levels of poly(A) mRNA (see Chapter 5). Intriguingly, these subdomains exclude the cellular PABPN, factor involved in mRNA polyadenylation, ORF59, a viral polymerase processivity factor involved in viral DNA replication, and KSHV PAN, a non-coding RNA with roles in expression of late viral genes. Inclusion of poly(A) RNA and two poly(A) binding proteins but exclusion of components that promote viral replication is suggestive of a localized PABPC-mediated effect, which may allow viral transcripts to escape this fate and be exported. However, currently, the subdomains are poorly understood nuclear bodies and future characterization of their composition should provide important insights into their function.

In addition to viral infection, examples of cellular stresses that promote PABPC relocalization have also emerged in the past few years. These include heat shock, oxidative stress, transcriptional inhibition, as well as ultraviolet irradiation [99, 103, 123, 125]. While PABPC accumulated in the nucleus in response to heat shock, it subsequently returned to the cytoplasm upon recovery from heat shock, indicating that this change in localization of PABPC could be part of a stress response that benefits the cell. These observations, combined with our findings of nuclear PABPC blocking gene expression, suggest that nuclear accumulation of PABPC serves to temporarily block gene expression, until the cellular stress is alleviated. This hypothesis would be supported if mRNA export was blocked by nuclear PABPC in response to cellular stress. Future avenues addressing this hypothesis in the context of various forms of cellular stress that cause PABPC import will be informative towards elucidating functions of nuclear PABPC. These would also provide further insights into how a cell copes with cellular stress, especially if multiple forms of stresses diverge onto similar pathways such as nuclear relocalization of PABPC.

Another phenotype associated with an mRNA export block is extension of the mRNA poly(A) tail beyond the canonical length of 200-300 nucleotides, also known as hyperadenylation. It is currently unclear which event occurs first. Does mRNA retention in the nucleus lead to mRNA hyperadenylation, or, is it that the extension of the poly(A) tail flags transcripts such that they fail quality control checkpoints, blocking their export? This will be a challenging question to explore as thus far mRNA hyperadenylation has always been detected concomitantly with a block in mRNA export. Yeast mutants defective for mRNA export factors

such as Mex67p, Mtr2p, Rat7p, etc., harbor hyperadenylated transcripts [40, 41]. However, the alternative scenario, where an mRNA is blocked for export, but not hyperadenylated, has not been documented, making it difficult to deduce which event occurs first.

Interestingly, hyperadenylated transcripts are also degraded in cells expressing KSHV SOX. However, this is not the case when PABPC is artificially restricted to the nucleus independent of SOX. During viral infection, the goal is likely to remove cellular transcripts to presumably avoid competition for cellular resources; however, it does not seem to be the approach taken under cellular stress. It is possible that during cellular stress, PABPC-induced hyperadenylation confers a temporary arrest in cellular gene expression, and that upon restoration of normal conditions, these hyperadenylated transcripts are trimmed to a normal length, and their export competence is reinstated. This hypothesis is not without precedent as trimming of an mRNA poly(A) tail has been implicated in regulating gene expression [36]. Future avenues focusing on assessing the state of mRNA hyperadenylation upon rescue from cellular stress will provide important insights into this hypothesis.

One important question that should be addressed in the future is to determine which specific mRNAs are blocked for export and hyperadenylated. Are all endogenous mRNAs blocked for export? In situ hybridization experiments conducted with an oligo(dT) probe would represent bulk endogenous mRNA. However, is it possible that select mRNAs escape this fate? This would be especially beneficial in the context of cellular stress as many transcripts would need to be translated to alleviate the stress. Analyzing total nuclear transcripts in the presence or absence of nuclear PABPC through microarray analysis will provide first insights into this question. Furthermore, are all or only a subset of endogenous mRNAs hyperadenylated by nuclear PABPC? Are all mRNAs blocked for export hyperadenylated or are some mRNAs only blocked for export, but not hyperadenylated? These questions should be addressed both by analyzing specific transcripts as well as by studying global hyperadenylation. A recent report demonstrated that fractionation of mRNAs based on their poly(A) tail length can be accomplished [214]. Once a pure population of hyperadenylated transcripts is isolated, these mRNAs can be identified through microarray or deep sequencing analyses. Information obtained from these approaches can provide insights into the composition of the hyperadenylated mRNA population. These analyses together with identification of protein interactions of PABPC in the nucleus will provides clues to the underlying mechanisms of PABPC-induced nuclear mRNA retention and hyperadenylation.

Concluding Remarks

The cytoplasmic poly(A) binding protein (PABPC) is found mostly in the cytoplasmic compartment in normal cells. However, it readily accumulates in the nucleus during infection with several divergent viruses as well as in response to cellular stress. The nuclear translocation and nuclear functions of PABPC remained unknown. In this report we have found that localization of PABPC is directly governed by cytoplasmic poly(A) mRNA abundance and that nuclear translocation is facilitated by the nuclear import machinery. Nuclear accumulation of PABPC resulted in nuclear mRNA hyperadenylation and a concomitant block in mRNA export, thereby preventing protein synthesis. These findings provide insights into a novel function of PABPC that impacts global gene expression. Future investigations into the mechanisms underlying PABPC-mediated effects on nuclear mRNA will be important to understand how PABPC in the nucleus contributes to a stressed and infected cell.

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