A Proline-Tyrosine Nuclear Localization Signal (PY-NLS) Is Required for the Nuclear Import of Fission Yeast PAB2, but Not of Human PABPN1

Pierre-Luc Mallet and François Bachand*

RNA Group, Department of Biochemistry, Université de Sherbrooke, Sherbrooke, QC Canada
*Corresponding author: François Bachand, f.bachand@usherbrooke.ca

Nuclear poly(A)-binding proteins (PABPs) are evolutionarily conserved proteins that play key roles in eukaryotic gene expression. In the fission yeast Schizosaccharomyces pombe, the major nuclear PABP, Pab2, functions in the maturation of small nuclear RNAs as well as in nuclear RNA decay. Despite knowledge about its nuclear functions, nothing is known about how Pab2 is imported into the nucleus. Here, we show that Pab2 contains a proline-tyrosine nuclear localization signal (PY-NLS) that is necessary and sufficient for its nuclear localization and function. Consistent with the role of karyopherin β2 (Kap12)-type receptors in the import of PY-NLS cargoes, we show that the fission yeast ortholog of human Kap12, Kap104, binds to recombinant Pab2 and is required for Pab2 nuclear localization. The absence of arginine methylation in a basic region N-terminal to the PY-core motif of Pab2 did not affect its nuclear localization. However, in the context of a sub-optimal PY-NLS, we found that Pab2 was more efficiently targeted to the nucleus in the absence of arginine methylation, suggesting that this modification can affect the import kinetics of a PY-NLS cargo. Although a sequence resembling a PY-NLS motif can be found in the human Pab2 ortholog, PABPN1, our results indicate that neither a functional PY-NLS nor Kap12 activity are required to promote entry of PABPN1 into the nucleus of human cells. Our findings describe the mechanism by which Pab2 is imported into the nucleus, providing the first example of a PY-NLS import system in fission yeast. In addition, this study suggests the existence of alternative or redundant nuclear import pathways for human PABPN1.

Key words: Kap104, karyopherin, Pab2, PABPN1, PY-NLS, S. pombe

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One of the key features that differentiate a eukaryotic cell from a prokaryotic cell is the presence of a double lipid bilayer that physically isolates the eukaryotic genome from the cytoplasmic content. This membrane-enclosed organelle, the nucleus, allows sophisticated gene regulation, notably by separating transcription and translation processes (1–5). Accordingly, this double lipid bilayer creates a physical barrier that blocks the passive diffusion of molecules larger than 5000 Da. As mRNA translation occurs in the cytoplasm, a large fraction of the eukaryotic proteome thus needs to be imported into the nucleus to accomplish their function (1,2,6). To support nucleo-cytoplasmic trafficking of molecules larger than 5 kDa, the nuclear envelope includes multiple nuclear pore complexes (NPCs). The number of NPCs per nucleus varies widely between organisms, with a typical yeast nuclear envelope containing 70–180 NPCs, whereas these numbers reach 3000–5000 in mammalian cells (7). The NPC is a large multi-protein complex that allows passive diffusion of molecules smaller than 40–60 kDa and requires nucleo-cytoplasmic transporters (karyopherins) to promote active transport of molecules larger than 40–60 kDa (2,8). Karyopherins (Kaps) are members of a protein superfamily that account for the vast majority of the cargo flow through the NPC. Karyopherin-mediated transport across the NPC is driven primarily by the asymmetric distribution of RanGDP in the cytoplasm and RanGTP in the nucleus (2,8,9). During nuclear import, karyopherins in the cytoplasm bind specific cargo proteins via recognition of a nuclear localization signal (NLS), promoting their translocation into the nucleus. Inside the nucleus, RanGTP binds karyopherin-cargo complexes, allowing the release of cargoes into the nucleus (2,8,9). To date, two main types of NLS are known: the classical NLS (cNLS) and the proline-tyrosine NLS (PY-NLS). The cNLS are associated with Kap12, Kap11-mediated nuclear import, which is thought to account for half of the nuclear import trafficking (3). In contrast, the PY-NLS is associated with Kap12-mediated nuclear import. Although few examples of PY-NLS-dependent cargoes have been demonstrated experimentally, in silico predictions suggest that PY-NLS-mediated import accounts for a substantial fraction of the nuclear proteome (4,10). The PY-NLS is composed of three independent epitopes, where epitope I is either a stretch of 4–20 residues enriched in basic (φ) amino acids or a hydrophobic (ε) motif defined as $\Phi_{1-\varepsilon/\phi_{2}} \Phi_{3-\phi_{3}}$, in which $\Phi$ represents any hydrophobic amino acids. Epitope II corresponds to either arginine (R), histidine (H) or lysine (K), which is found 2–5 amino acids N-terminal to epitope III, which is a highly conserved PY-dipeptide. Collectively, the PY-NLS consensus corresponds to [basic/hydrophobic]-X$_1$-[R/H/K]-X$_2$-5-PY (10).

Poly(A)-binding proteins (PABPs) play fundamental roles during multiple steps of eukaryotic gene expression. In most eukaryotic cells, two major PABPs bind the poly(A) tract of mRNAs: PABPC1 in the cytoplasm and...
PABPN1/PABP2 in the nucleus. The ability of PABPN1 to bind poly(A) RNA is mediated by its RNA recognition motif and an arginine-rich domain located at the C-terminus of the protein (11–13). In vitro, PABPN1 promotes poly(A) polymerase processivity and regulates poly(A) tail length (11,13,14), leading to a model whereby PABPN1 is a factor required for general mRNA maturation (11,15). Whereas the genome of Saccharomyces cerevisiae does not encode for an ortholog of human PABPN1 (16), we have previously identified and characterized a PABPN1 ortholog, Pab2, in the fission yeast Schizosaccharomyces pombe (12). Interestingly, human PABPN1 and S. pombe Pab2 are both methylated in their arginine-rich domain (12,13); yet, the functional relevance of this post-translational modification remains elusive. A genome-wide analysis of gene expression changes in cells deleted for pab2 revealed that the expression of most protein-coding genes was unaffected, indicating that Pab2 does not function as a general mRNA maturation factor (17). In contrast, Pab2 was found to function in the maturation of noncoding small nucleolar RNAs (17) and to promote a nuclear pre-mRNA decay pathway that controls the expression of specific intron-containing genes (18). Taking into account the small molecular mass of Pab2 and PABPN1, 18.5 and 32.8 kDa, respectively, they should be able to passively diffuse across the NPC. However, Pab2 and PABPN1 are exclusively nuclear at steady state (12,19–21), suggesting the presence of nuclear targeting signals. Indeed, studies have partly characterized the nucleocytoplasmic transport of mammalian PABPN1 (20), excluding a model in which the nuclear localization of PABPN1 was an indirect consequence of binding to nuclear poly(A) RNA. PABPN1 was shown to bind Kapβ2 in vitro in a RanGTP-dependent manner (20), suggesting the use of a Kapβ2-dependent import pathway. In addition, recent data demonstrate that arginine methylation of PABPN1 reduces its affinity to recombinant Kapβ2 and affects Kapβ2-mediated nuclear import of PABPN1 in vitro import assays using digitonin-permeabilized HeLa cells (22).

Although the aforementioned studies suggest that Kapβ2 mediates PABPN1 nuclear import, the necessity of this karyopherin for PABPN1 nuclear localization has not yet been demonstrated in a cellular system. Furthermore, the exact nature of the PABPN1 NLS remains unclear. To address these issues, we have characterized the mechanism of nucleocytoplasmic transport of the fission yeast PABPN1 ortholog, Pab2. Amino acid sequence analysis revealed the presence of a basic-type PY-NLS located at the C-terminus of Pab2. Importantly, substitutions that perturb the PY domain of this NLS cause Pab2 to accumulate in the cytoplasm, demonstrating the functional importance of this signal for Pab2 nuclear import. Consistent with Pab2 being a PY-NLS cargo, we show direct binding between Pab2 and Kap104, which encodes the fission yeast ortholog of human Kapβ2, and demonstrate that the deletion of kap104 blocks Pab2 nuclear import. In contrast, we find that a putative PY-NLS is not necessary for nuclear import of PABPN1 in human cells. Using a Kapβ2-specific inhibitor, we also demonstrate that PABPN1 is still efficiently imported into the nucleus after inhibition of human Kapβ2. Our findings reveal that whereas a PY-NLS targeting signal is absolutely required for Pab2 nuclear import in fission yeast, its human counterpart, PABPN1, uses alternate or redundant mechanisms of nuclear import.

Results

A PY-NLS targeting signal is necessary and sufficient for nuclear import of S. pombe Pab2

To identify a functional NLS in fission yeast Pab2, we analyzed its amino acid sequence and identified a motif similar to the previously established basic (b) PY-NLS (10).

As can be seen in Figure 1A, the PY-NLS-like sequence of Pab2 is located at the extreme C-terminus of the protein (residues 139–166). Interestingly, amino acid sequence comparison indicated an evolutionarily conserved PY-dipeptide at the extreme C-terminus of Pab2 orthologs (Figure S1). Based on this sequence analysis, we used a green fluorescent protein (GFP)-tagged version of Pab2 to address the functional relevance of this putative bPY-NLS for Pab2 nuclear import. To characterize the putative bPY-NLS of Pab2, we introduced several amino acid substitutions at key residues of the PY-NLS. All of the GFP-Pab2 constructs were expressed from the native pab2 promoter and chromosomally integrated into a pab2Δ strain to prevent overexpression. Western blot analysis confirmed the expression of GFP-Pab2 from all of our constructs (data not shown). We next monitored the localization of Pab2 variants in live S. pombe cells (Figure 1B). As expected, wild-type Pab2 localized to the nucleus (Figure 1B, a,b), whereas the GFP control was diffused across the cell (Figure 1B, o,p). Substitution of proline 165 to alanine [P165A] from the C-terminal PY motif (Figure 1A) caused a clear mislocalization of GFP-Pab2 to the cytoplasm (Figure 1B, c,d); yet, significant GFP-Pab2 signal could still be detected into the nucleus. Notably, substitution of tyrosine 166 to alanine [Y166A] resulted in a more severe defect in nuclear localization than Pab2 [P165A] (Figure 1B, e,f). An assessment of the relative distribution of GFP-Pab2 in the nucleus versus the cytoplasm supports the more important role of Y166 relative to P165 in Pab2 nuclear import (Figure S2A). A version of Pab2 in which the proline and tyrosine residues of the PY motif were both substituted to alanine [P165,166AA] resulted in the complete loss of nuclear accumulation, being nearly excluded from the nucleus (Figure 1B, g,h). In contrast, substitutions of phenylalanine 163 [F163A], arginine 161 [R161A]; epitope II), and residues 156–159 [YRGR156-159AAAA] N-terminal to the PY motif (see Figure 1A) had minimal impact on the nuclear localization of GFP-Pab2 (Figure 1B, i–n, respectively; Figure S2A). These results indicate that the PY dipeptide of the PY-NLS-like sequence of Pab2 is necessary for nuclear import.
A functional PY-NLS sequence is necessary for Pab2 import. A) Amino acids 139–166 of Pab2 that correspond to a putative PY-NLS sequence are underlined. The asterisk corresponds to the C-terminal end of Pab2. B) Visual analysis of pab2Δ cells expressing GFP alone (a,p), wild-type GFP-Pab2 (a,b), and versions of GFP-Pab2 with substitutions in the PY-NLS sequence (c–n). Background fluorescence was subtracted by analyzing pab2Δ cells in which the empty vector was chromosomally integrated (q,r). Direct fluorescence microscopy (GFP) was visualized in live cells (right column) and the corresponding differential interference contrast (DIC) images are shown (left column). Bar 5 μm.

We next addressed whether the putative PY-NLS sequence of Pab2 was sufficient to promote the nuclear localization of a heterologous protein. For this, we used a GST-GFP fusion, which is not efficiently targeted to the nucleus, showing pancellular localization (Figure 2, e,f). Three different sections from the PY-NLS sequence of Pab2 were fused to the C-terminus of GST-GFP: (i) The PY-core alone (aa 161–166), (ii) the PY-core with five N-terminal residues (aa 156–166) and (iii) the entire PY-NLS sequence (aa 139–166). Fusion of the Pab2 PY-core motif to the C-terminus of GST-GFP did not show a noticeable increase in nuclear accumulation as compared to the GST-GFP control (Figure 2, compare panel h to f). The addition of five additional residues, including two basic arginines, enhanced the nuclear accumulation of the GST-GFP protein (Figure 2, i,j); yet, most of the signal remained in the cytoplasm. In contrast, the entire PY-NLS sequence of Pab2 mediated efficient targeting of the GST-GFP fusion into the nucleus (Figure 2, k,l). A measure of the relative distribution of GST-GFP in the nucleus versus the cytoplasm (Figure S2B) provided a quantitative assessment of the results showed in Figure 2. These results indicate that the PY-core sequence lacking the basic arginine-rich region is not sufficient to promote nuclear import.

On the basis of these results, we conclude that the PY-NLS sequence of fission yeast Pab2 is necessary and sufficient for nuclear import.

**PY-NLS-dependent nuclear import of Pab2 is mediated by Kap104**

PY-NLS-containing cargoes are normally recognized by a specific type of karyopherin β receptor: karyopherin β2/transportin (Kapβ2) in humans (9) and Kap104 in S. cerevisiae (4,23). In S. pombe, the ortholog of S. cerevisiae Kap104, SPAC2F3.06c, is encoded by a non-essential gene (24); however, no cargo has yet been identified. To address whether the PY-NLS-mediated nuclear import of Pab2 is dependent on the fission yeast Kap104 ortholog, we deleted the SPAC2F3.06c open reading frame and determined the effect of this deletion on the localization of GFP-Pab2. As can be seen in Figure 3A, the absence of Kap104 caused GFP-Pab2 to mislocalize to the cytoplasm (panels i,j), in contrast to cells that expressed Kap104 (panels g,h). As a control, we also deleted the S. pombe sal3 gene, encoding a putative ortholog of budding yeast Kap121 and human Importin-5 (9,24–26), which are known to share some specific cargoes with Kap104/Kapβ2 (9,27). GFP-Pab2 was properly localized to the nucleus in sal3Δ cells (Figure 3A, k,l), suggesting that the effect of Kap104 on Pab2 nuclear import is specific. We therefore conclude that Pab2 nuclear import is dependent on Kap104.

We next verified whether PY-NLS-mediated nuclear import, as demonstrated using the Pab2 PY-NLS sequence fused to GST-GFP (Figure 2), was dependent on Kap104. We therefore expressed the GST-GFP protein fused
Nuclear import via a cNLS is mediated by human Kapα and Kapβ1 (9), cut15 and Kap95 in fission yeast, respectively, (24), and is therefore not expected to be impaired in the absence of Kap104. Accordingly, the GST-GFP-cNLS protein was localized into the nucleus in kap104-null cells (Figure 3B, o,p), suggesting that Kap104-mediated nuclear import is specific for PY-NLS sequences. Analyses of the relative distribution of GFP-Pab2 and GST-GFP in the nucleus versus the cytoplasm (Figure S2C) provided a quantitative assessment of the results showed in Figure 3.

A direct role for Kap104 in Pab2 nuclear import predicts a physical interaction between these two proteins. To verify whether Kap104 makes direct contact with Pab2, a GST-tagged version of Pab2 and a His-tagged version of Kap104 were expressed in E. coli, and the ability of Pab2 to interact with Kap104 was analyzed in vitro. As can be seen in Figure 3C, GST-Pab2 was specifically enriched using the Kap104-bound resin (lane 7) relative to the nickel beads control (lane 5). As an additional control, GST alone bound poorly to the Kap104-bound resin (lane 6). This result supports a physical interaction between Pab2 and Kap104, consistent with the direct role of Kap104 in Pab2 nuclear import.

**PY-NLS-mediated nuclear import of Pab2 by Kap104 is necessary for protein function**

We have previously described a function for Pab2 in a nuclear pre-mRNA decay pathway that controls the expression of specific intron-containing genes (18). Accordingly, in the absence of Pab2, the spliced mRNA and unspliced pre-mRNA of specific genes, such as the ribosomal protein-coding gene, rpl30-2, are upregulated (Figure 4A, compare lanes 1 and 2). We therefore used this phenotype to test whether the PY-NLS sequence of Pab2 is essential for its cellular function. We first confirmed that our GFP-Pab2 fusion was functional, as it fully restored the altered expression of rpl30-2 observed in a pab2-null strain (Figure 4A, compare lane 4 to 2); in contrast, GFP alone (lane 11) and the empty control vector (lane 3) did not. Alleles of pab2 that expressed [Y166A] and [PY165,166AA] versions of Pab2, which showed the greatest defects in Pab2 nuclear localization (Figure 1), were not able to rescue the accumulation of rpl30-2 mRNA and pre-mRNA in the pab2-null strain (Figure 4A, lane 6 and 7, respectively). Cells expressing GFP-Pab2 [P165A] showed a minimal effect on rpl30-2 expression (Figure 4A, lane 5), which is consistent with the observation that this version of Pab2 retained considerable levels of nuclear accumulation (Figures 1B and S2A). Similarly, versions of Pab2 with PY-NLS substitutions that did not inhibit nuclear import fully rescued the altered expression of rpl30-2 that is detected in pab2-null cells (Figure 4A, lanes 8–10). We conclude that the PY-NLS sequence is important for Pab2 function, suggesting that nuclear import of Pab2 is essential for its function in pre-mRNA decay.

The aforementioned results suggesting that nuclear import is essential for Pab2 function predicts that the
expression of GFP-Pab2 in the absence of Kap104, which directs Pab2 nuclear import (Figure 3), would not rescue the loss of Pab2 function. To test this prediction, we expressed wild-type GFP-Pab2 in the single pab2Δ strain as well as in pab2Δ/kap104Δ and pab2Δ/sal3Δ double mutants. As can be seen in Figure 4B, the expression of GFP-Pab2 in pab2Δ/kap104Δ double mutant cells showed rpl30-2 transcript accumulation (Figure 4B, lane 5) similar to cells that do not express Pab2 (Figure 4B, lane 2). In contrast, expression of GFP-Pab2 in the single pab2Δ strain or in the pab2Δ/sal3Δ double mutant restored the altered expression of rpl30-2 (Figure 4B, lanes 4 and 6,
Figure 4: Pab2 nuclear localization is necessary for protein function. A) Northern blot analysis of total RNA prepared from wild-type (lane 1) and pab2Δ (lanes 2–11) cells expressing GFP alone (lane 11), wild-type GFP-Pab2 (lane 4), and versions of GFP-Pab2 with substitutions in the PY-NLS sequence (lanes 5–10). pab2Δ cells transformed with the empty control vector was also analyzed (lane 3). The blot was hybridized using probes complementary to rpl30-2 and pma1. The pma1 mRNA was used as a loading control. Normalized levels of rpl30-2 mRNA relative to pab2Δ cells expressing the wild-type GFP-Pab2 fusion (lane 4) are indicated beneath each lane. B) Northern blot analysis of total RNA prepared from wild-type (lane 1), pab2Δ (lanes 2–4), pab2Δ kap104Δ (lane 5) and pab2Δ sal3Δ (lane 6) cells that previously transformed with an integration construct expressing GFP-Pab2 (lanes 4–6) and the empty vector (lane 3), or that were not transformed (lanes 1–2). The blot was hybridized using probes complementary to rpl30-2 and pma1. The pma1 mRNA was used as a loading control. Normalized levels of rpl30-2 mRNA relative to pab2Δ cells expressing the wild-type GFP-Pab2 fusion (lane 4) are indicated beneath each lane.

respectively). These results strongly support that Kap104-mediated nuclear import is required for Pab2 function.

Arginine methylation affects the nuclear import of a sub-optimal PY-NLS
The basic region N-terminal to the PY-core motif of Pab2 is arginine methylated by the protein arginine methyltransferase, Rmt1 (12). To address whether arginine methylation affects the kinetics of Pab2 nuclear import, we compared the localization of different versions of GFP-Pab2 (wild-type, [P165A], [Y166A], and [R161A]) between pab2Δ and pab2Δ/rmt1Δ cells. Strikingly, GFP-Pab2 [P165A] showed a noticeable change in subcellular localization in the absence of Rmt1-dependent methylation. Indeed, whereas GFP-Pab2 [P165A] showed significant cytoplasmic signal in the presence of Rmt1 (Figures 5A, i,j and 1B), detection of cytoplasmic GFP-Pab2 [P165A] was almost completely absent in rmt1Δ cells (Figure 5A, k,l). Consistent with our previous results (12), wild-type GFP-Pab2 was localized to the nucleus 

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in the presence and absence of Rmt1 (Figure 5A, e,f and g,h). We quantified the number of cells showing detectable cytoplasmic GFP signal for more than 175 cells per strain (>1000 cells in total for the four strains; Figure S3) and determined the percentage of cells with cytoplasmic GFP-Pab2 (Figure 5B; p < 0.0001 chi-square test). The cell population showing cytoplasmic GFP signal for GFP-Pab2 [P165A] was 18-fold higher than for wild-type GFP-Pab2 (Figure 5B), consistent with the PY-core of Pab2 being important for nuclear import. Significantly, the total number of GFP-Pab2 [P165A]-expressing cells showing cytoplasmic GFP signal was reduced by sevenfold in the absence of Rmt1 (Figure 5B). Because Pab2 can shuttle between the nucleus and cytoplasm (12), the reduction of GFP-Pab2 [P165A] signal in the cytoplasm of rmt1Δ cells could be the consequence of defective nuclear export. If this is the case, inhibition of CRM1-dependent nuclear export with leptomycin B, a potent inhibitor of nuclear export (28), would be expected to decrease the cytoplasmic signal of GFP-Pab2 [P165A]. However, treatment of GFP-Pab2 [P165A]-expressing cells with leptomycin B did not abolish the detection of GFP-Pab2 [P165A] in the cytoplasm (Figure S4), indicating that the redistribution of cytoplasmic GFP-Pab2 [P165A] in the nucleus of rmt1Δ cells is unlikely to be due to defects in CRM1-dependent nuclear export. We thus conclude that arginine methylation by Rmt1 can reduce the kinetics of Pab2 nuclear import in the context of a sub-optimal PY-NLS.

**A functional PY-NLS is not required for the nuclear localization of human PABPN1**

A PY dipeptide is found at the extreme C-terminus of mammalian Pab2 orthologs (Figure S1), suggesting that the PY-NLS/Kap104-dependent mechanism of nuclear import identified for Pab2 could be conserved. However,
we noted that the putative PY-NLS sequence of mammalian PABPN1 orthologs deviates from the PY-NLS consensus, as a spacing of six amino acids is found between epitopes II and III (R298 and PY-core, respectively, in humans; see Figure 6A), which contrasts to the two to five amino acids normally found between epitopes II and III of PY-NLS cargoes (10). This discrepancy explains why PABPN1 was not identified as a PY-NLS cargo using previous *in silico* approaches (10).

To address the importance of the putative PY-NLS sequence of human PABPN1, we introduced amino acid substitutions at key residues in the PY-NLS sequence (Figure 6A) and examined the impact of these substitutions on the nuclear localization of GFP-PABPN1 in HeLa cells (Figure 6B, e,f). In contrast to *S. pombe*, in which substitutions affecting the PY-core motif markedly reduced Pab2 nuclear import (Figure 1), individual substitutions of proline-305 and tyrosine-306 to alanine...
in PABPN1 did not result in detectable cytoplasmic signal relative to wild-type PABPN1 (Figure 6B, compare panels g and i to panel e). Moreover, the combined substitution of proline-305 and tyrosine-306 to alanines [PY305,306AA] had no effect on the ability of GFP-PABPN1 to be imported into the nucleus (Figure 6B, panels k,l). Similarly, the substitution of two arginine residues that include epitope II of the PABPN1 PY-NLS [RR296,298AA] alone, or in combination with substitutions that altered the PY-core epitope [RRPY296,298,305,306AAAA] did not result in the cytoplasmic accumulation of GFP-PABPN1 (Figure 6B, panels m and o, respectively). From these results, we conclude that the putative PY-NLS sequence of human PABPN1 is not required for nuclear import.

Although a PY-NLS-associated karyopherin, Kapβ2, allows the import of PABPN1 into the nucleus of digitonin-permeabilized Hela cells in vitro (22), the requirement of Kapβ2 for PABPN1 nuclear import has never been demonstrated in living cells. Given the persistence of PABPN1 nuclear localization upon the introduction of substitutions that are expected to significantly perturb its putative PY-NLS, we tested whether PY-NLS-dependent karyopherins were required for PABPN1 nuclear import in HeLa cells. We used a previously described Kapβ2-specific nuclear import inhibitor (M9M) that shows a 200-fold increase in binding affinity for Kapβ2 as compared to a natural PY-NLS sequence (29). Expression of a myc-tagged version of the maltose-binding protein (MBP) fused to the M9M peptide competed with the endogenous Kapβ2 substrate hnRNP A1, causing the accumulation of hnRNP A1 in the cytoplasm of Kapβ2-saturated cells (Figure 7, panels e–h, arrowhead); however, hnRNP A1 was exclusively nuclear in cells in which the M9M peptide did not saturate Kapβ2-mediated nuclear import (Figure 7, e–h). In contrast to hnRNP A1, PABPN1 was still predominantly nuclear after saturation of Kapβ2-dependent nuclear import using the MBP-M9M inhibitor (Figure 7, panels m–p). A rescaled (overexposed) image showed low levels of cytoplasmic PABPN1 in Kapβ2-saturated cells (Figure S5).

Altogether, our PY-NLS substitutions and Kapβ2 inhibition experiments strongly suggest that the import of PABPN1 into the nucleus of human cells can be mediated by a mechanism independent from PY-NLS/Kapβ2-mediated import.

Discussion

Nuclear PABPs are evolutionarily conserved proteins that play key roles in eukaryotic gene expression. Yet, the

![Figure 7: Inhibition of Kapβ2-mediated nuclear import does not lead to PABPN1 mislocalization. Hela cells were transiently transfected with an empty control vector (a–d and i–l) or a DNA construct expressing a myc-tagged version of the MBP fused to the M9M peptide (e–h and m–p). The fixed and permeabilized cells were stained for Myc-MBP-M9M (a, e, i and m) using anti-Myc, as well as for endogenous hnRNPA1 (b and f) and endogenous PABPN1 (j and n). DNA stained with DAPI shows the nucleus of each cell (c, g, k and o). Images a–c, e–g, i–k and m–o were merged to form d, h, l and p, respectively. Arrowheads point to cells in which Kapβ2-mediated nuclear import has been saturated by the M9M peptide. Bar 12 μm.](image)
nature of their NLSs and the mechanisms responsible for mediating their transport through the NPC and into the nucleus have remained poorly characterized. In this study, we have identified the functional NLS and the karyopherin responsible for the delivery of the fission yeast nuclear PABPs, Pab2, into the nucleus. Our localization, binding and functional studies collectively show that a basic-type PY-NLS located at the extreme C-terminus of Pab2 is both necessary and sufficient for import into the nucleus via the karyopherin β2 (Kapβ2) receptor, Kap104. This study thus provides the first example of a functional PY-NLS import system in fission yeast. The identification of a PY-NLS-dependent import mechanism for S. pombe Pab2 is consistent with the observation that PY-NLS cargoes in S. cerevisiae and humans tend to be enriched for proteins with RNA-related functions (4,8,10). In silico analyses of the fission yeast proteome should reveal the prevalence of the PY-NLS as a nuclear targeting signal in S. pombe as well as the proportion of PY-NLS cargoes involved in RNA metabolism in this organism. However, prediction of PY-NLS-containing proteins using computational approaches has been complicated by the substantial diversity in PY-NLS sequences (8,23,30). For example, the pattern of basic arginine residues N-terminal to the PY-core motif of the Pab2 PY-NLS does not match the consensus used to predict PY-NLS cargoes in the human proteome (10), and would therefore not be identified as a PY-NLS-containing protein using these criteria. The identification of a PY-NLS import system in fission yeast will therefore provide powerful biochemical and genetic tools to further characterize the properties that govern affinity to Kapβ2 receptors, helping to better define rules for the prediction of PY-NLS cargoes in eukaryotic proteomes.

Our functional dissection of the PY-NLS of Pab2 showed that substitution of tyrosine-166 from the PY-dipeptide had a more profound effect on Pab2 nuclear import (Figure 1) and Pab2 function (Figure 4) than the substitution of proline-165. Similar results were obtained for the Hrp1 protein in S. cerevisiae (4), suggesting a generally more significant contribution of the tyrosine residue within the PY epitope at creating a binding platform for a stable PY-NLS-Kap104 interaction. Although we found a critical role for the PY-dipeptide in Pab2 nuclear import, the PY-core motif (R-X₂₋₅-PY) of Pab2 was not sufficient for nuclear import of an heterologous protein, as the N-terminal basic region was required to efficiently target the expression of unmethylated Pab2 in the cytosol. As two independent studies recently reported that methylation of arginine residues in the vicinity of a PY-NLS decreases its affinity for Kapβ2 (23), we propose that the absence of methylation restored sufficient binding affinity between Pab2 [P165A] and Kap104 to compete with additional PY-NLS cargoes in the cytoplasm, thereby improving the ability of Pab2 [P165A] to be imported into the nucleus. Interestingly, this model differs from the one recently suggested by Fronz et al. (22), which proposed that methylation of PABPN1 in the nucleus would facilitate its dissociation from Kapβ2 and reduce rapid re-export of the PABPN1-Kapβ2 complex in the cytoplasm. The cellular compartment where Pab2 and PABPN1 are arginine methylated remains to be determined, however. Thus, whereas both studies show that arginine methylation can affect the efficiency of PY-NLS-mediated nuclear import of Pab2 and PABPN1, the functional significance of this modification to nuclear import remains elusive, as Pab2 is properly localized to the nucleus in the absence of arginine methylation.

Arginine methylation and PY-NLS-mediated nuclear import

Like mammalian PABPN1, fission yeast Pab2 is methylated at arginine residues within its basic region near the PY-core motif (12). As yet, however, a functional role for Pab2 arginine methylation has remained elusive. Notably, this study provides the first example of a functional role for Pab2 methylation in a cellular system by demonstrating that arginine methylation of Pab2 functions antagonistically to PY-NLS-mediated nuclear import. Indeed, we found that the cytosolic signal resulting from the substitution of the proline residue within the PY-core motif [P165A] of Pab2 was significantly reduced in the absence of Rmt1 (Figure 5), which is the only methyltransferase that catalyzes Pab2 arginine methylation in fission yeast (12). Because the decrease in Pab2 cytosolic signal in rmt1Δ cells is unlikely to be the consequence of nuclear export defects (Figure S4), at least two mechanisms could account for this observation: (i) increase affinity of unmethylated Pab2 to Kap104 or (ii) increase turnover of unmethylated Pab2 in the cytosol. As two independent studies recently reported that methylation of arginine residues in the vicinity of a PY-NLS decreases its affinity for Kapβ2 in vitro (22,31), we favor a model in which Rmt1-dependent arginine methylation within the basic region of Pab2 reduces the binding affinity between its PY-NLS and Kap104. Yet, as we observed no change in the nuclear localization of wild-type Pab2 in the presence or absence of arginine methylation (Figure 5), the role of arginine methylation in Pab2 nuclear import appears to be minor in the case of a robust PY-NLS. However, in the case of a sub-optimal PY-NLS sequence, which is known to impair binding affinity to Kapβ2 (23), we propose that the absence of methylation restored sufficient binding affinity between Pab2 [P165A] and Kap104 to compete with additional PY-NLS cargoes in the cytoplasm, thereby improving the ability of Pab2 [P165A] to be imported into the nucleus. Interestingly, this model differs from the one recently suggested by Fronz et al. (22), which proposed that methylation of PABPN1 in the nucleus would facilitate its dissociation from Kapβ2 and reduce rapid re-export of the PABPN1-Kapβ2 complex in the cytoplasm. The cellular compartment where Pab2 and PABPN1 are arginine methylated remains to be determined, however. Thus, whereas both studies show that arginine methylation can affect the efficiency of PY-NLS-mediated nuclear import of Pab2 and PABPN1, the functional significance of this modification to nuclear import remains elusive, as Pab2 is properly localized to the nucleus in the absence of arginine methylation.

A functional PY-NLS nuclear import pathway is not required for PABPN1 nuclear localization

Although C-terminal PABPN1 truncations were previously shown to cause partial PABPN1 mislocalization (20), the nature of the PABPN1 NLS has remained unclear. Based on the identification of a functional PY-NLS in the C-terminus of S. pombe Pab2, we addressed whether human PABPN1 was also imported into the nucleus via a PY-NLS. However, several lines of evidence presented in this study argue that a PY-NLS import system is not required for PABPN1 nuclear import. First, GFP-tagged versions of PABPN1 in which key residues of its putative PY-NLS were substituted were still efficiently targeted into the nucleus. Second, inhibition of Kapβ2-dependent
nuclear import pathways, which prevents the import of PY-NLS cargoes into the nucleus of human cells [Figure 7 and (29)], did not significantly impair PABPN1 nuclear accumulation. Finally, we noted a spacing of six amino acids between epitopes II and III (R298 and PY-dipeptide, respectively) in the putative PY-NLS sequence of mammalian PABPN1 orthologs (Figure S1), which deviates from the strict two to five amino acids spacing found in PY-NLS cargoes (10). Thus, although direct interactions between PABPN1 and Kapβ2 have been demonstrated in vitro (20,22), our results indicate the Kapβ2 is not the sole nuclear import factor for PABPN1 in human cells, in contrast to S. pombe Pab2. Given that C-terminal truncations partly mislocalize PABPN1 in human cells (20), it is possible that the C-terminus of PABPN1 contains overlapping nuclear targeting signals. Comparison of the C-terminal arginine-rich regions of Pab2 and PABPN1, which also corresponds to epitope I of basic-type PY-NLS, revealed that this basic patch of amino acids is substantially extended in PABPN1 relative to Pab2 (38 versus 21 residues, respectively). Accordingly, this extended basic region in PABPN1 may contain recognition elements for different karyopherins. Such an increase in the complexity of nuclear import pathways from yeast to humans has in fact been reported for the NXF1 mRNA export factor, which can bind five different karyopherins in humans, but a single karyopherin in fission yeast (32). It is therefore likely that multiple redundant nuclear import pathways exist for human PABPN1.

In summary, our findings unveiled the mechanism by which the S. pombe nuclear PAB, Pab2, is imported into the nucleus, providing the first example of a functional PY-NLS import system in fission yeast. Surprisingly, our results indicate that the PY-NLS import pathway is not required for the nuclear localization of PABPN1 in human cells, thus raising the existence of alternative or redundant nuclear import mechanisms for human PABPN1.

Materials and Methods

Strains, growth media, and genetics methods

All the S. pombe strains used in this study are listed in Table S1. Strains were grown to exponential phase at 30°C in Edinburgh minimum medium (EMM) containing appropriate amino acid supplements. PCR-mediated gene disruptions were performed by a two-step approach using 200–300 nucleotides annealing to the target gene, modified from Bahler et al. (33). Gene knockouts were confirmed by colony PCR and RT-PCR. Plasmid integrations at the ade6 locus were performed as previously described (18) using linearized DNA and selecting for adenine prototrophy.

Hela cells were grown in DMEM supplemented with 10% FBS, MEM nonessential amino acids (Wisent), 1 mM sodium pyruvate and penicillin-streptomycin-c-glutamine (Wisent). Transient transfections of DNA constructs were performed by adding 1–4 μg of plasmid constructs (listed in Table S1) with 2–4 μL of lipofectamine 2000 to 2 x 10⁶ cells. After 4 h, the lipofectamine/lipofectamine containing media was removed, replaced with fresh media, and incubated for 16 h before fixation treatment.

Microscopy

The GFP was used to localize Pab2 and PABPN1 in live S. pombe cells and in fixed HeLa cells, respectively. Signals were visualized using a 62HE triple filters (Carl Zeiss Canada) on an Observer.Z1 (Carl Zeiss Canada) equipped with a Cascade II camera (Carl Zeiss Canada). The objectives were a PlanApo 63X/1.4 (Carl Zeiss Canada) and a PlanApo 100X/1.46 (Carl Zeiss Canada). Cellular autofluorescence background signals were removed using the AQUA VISION REL. 4.7 software (Carl Zeiss Canada).

For visual analysis of yeast cells, eight-well slides were coated with 20μL of a 1 mg/mL concanavaline A solution and allowed to dry for 30 min. Exponential phase cultures of S. pombe were dropped into each well and incubated for 10 min followed by two EMM media washes. Slides were mounted with 3 μL of EMM media per well for live cells microscopy.

For visual analysis of human cells, cover slips were pre-coated with poly-ν-cyano-hydrobromide and HeLa cells were allowed to adhere. After 20h post-transfection, HeLa cells were fixed for 15min with a 4% paraformaldehyde/PBS solution and then washed once with PBS. Cells were then permeabilized using a 0.1% triton X-100/PBS solution for 10 min. For the visualization of GFP-PABPN1, cells were washed with PBS and the cover slips were mounted on a slide with slow fade gold antifade reagent with DAPI for microscopy analysis. For the MM9 inhibition assays, cells were equilibrated for 20min in 1% BSA followed by a 90min incubation with primary antibodies dilutions in 1% BSA: 1/100 for anti-Myc (9E10, Santa Cruz), 1/500 for anti-PABPN1 (Epitomics), and anti-hnRNPA1 (a gift from Benoit Chabot, Université de Sherbrooke). Cells were washed three times with PBS/BSA 1% and incubated with secondary antibody dilutions. After three wash with PBS/BSA 1%, the cells were mounted on a slide with slow fade gold antifade reagent with DAPI (Invitrogen) for microscopy analysis.

DNA constructs

The expression of GFP-Pab2 under the control of promoter and terminator sequences of pab2 was performed by cloning PCR products corresponding to the pab2 promoter (400bp), the egfp cDNA, the pab2 cDNA and the pab2 terminator (1000bp) into pBPade6 (34), generating pFB511. Substitutions in the PY-NLS sequence of Pab2 were introduced by site-directed mutagenesis using pFB511 and confirmed by DNA sequencing. The GST-GFP expression construct was generated by PCR amplification of a ~1400-bp DNA fragment corresponding to the GST-GFP coding sequence from pSGST-GFP (35), which was cloned between the promoter and the terminator sequences of pab2, generating pFB548. DNA constructs expressing GST-GFP-Pab2 (161–166), GST-GFP-Pab2 (156–166), GST-GFP-Pab2 (139–166) and GST-GFP-Pab2 (166–166) were generated by PCR amplification of GST-GFP from pFB548 using complementary oligonucleotides that contained the nucleotide sequences coding for these additional amino acids. The GFP-PABPN1 construct was previously described (36). Substitutions in the putative PY-NLS sequence of PABPN1 were introduced by site-directed mutagenesis using the GFP-PABPN1 construct. To generate a polyhistidine-tagged version of S. pombe Kap104, the kap104 cDNA (SPAC2F3.06c) was amplified by RT-PCR and cloned into pET-28a (Novagen), generating pFB674. All constructs were confirmed by DNA sequencing.

Recombinant protein expression and in vitro binding assays

GST and GST-Pab2 were expressed in E. coli as previously described (12). 6×His-tagged Kap104 was expressed by transforming E. coli BL21 DE3 cells (Invitrogen) with pFB674. Kap104 expression was induced by the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside for 20h at 18°C. Following centrifugation, cells were subsequently washed in ice-cold 1% buffer (50μM Tris–HCl pH 7.5, 150μM NaCl, 10% sucrose, 10μg/mL lysozyme, and 0.1% Triton X-100) for 30 min before lysis by sonication. The cleared lysate was supplemented with 10% glycerol and 50μM imidazole before being incubated with N-NTA-agarose matrix (Quiagen).
The resulting 6xHis-Kap104-bound affinity matrix was washed several times in binding buffer (20 mM Hepes pH 7.3, 110 mM KAc, 2 mM DTT, 1 mM EDTA, 2 mM MgAc and 20% glycerol) and used directly for binding assays.

For in vitro binding assays, equal amounts of GST or GST-Pab2 (~1 μg) were added to Kap104-bound Ni-NTA-agarose resin in binding buffer that was adjusted to a final concentration of 350 μM NaCl. Binding assays were performed at 4°C for 1 h with gentle agitation, the unbound fraction was discarded, and the resin was washed four times with binding buffer supplemented with 350 μM NaCl. Proteins were eluted from the resin by the addition of SDS-PAGE sample buffer and the eluted proteins were analyzed by immunoblotting using GST-specific and polyhistidine tag-specific antibodies.

Antibodies
Mouse monoclonal anti-GST B-14 was from Santa Cruz Biotechnology Inc.; mouse monoclonal anti-penta His was from Qiagen; rabbit polyclonal anti-GFP was from Invitrogen. The rabbit polyclonal antibody specific to fission yeast Rps2 was previously described (37). Mouse monoclonal anti-Myc 9E10 was from Santa Cruz Biotechnology Inc. Rabbit monoclonal anti-PABPN1 was from Epitomics. The rabbit polyclonal antibody specific to human hnRNP A1 was a generous gift from Dr Benoit Chabot (Université de Sherbrooke).

Northern blots
To detect rpl30-2 transcripts, 20 μg of total RNA was resolved on a 1.25% agarose-formaldehyde gel and transferred onto nylon membrane by capillary diffusion. Membranes were cross-linked using a UV Stratalinker and probed using a gene-specific oligonucleotide that was 5′-labeled with [γ32P-ATP] using T4 polynucleotide kinase. After extensive washing steps, membranes were exposed to Phosphor Screens and visualized using a Typhoon Trio instrument.

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Supporting Information
Additional Supporting Information may be found in the online version of this article.

Figure S1: Amino acid sequence alignment of the C-terminal region of nuclear poly(A) binding proteins from multiple species. Identical amino acids are shown in black outline and similar amino acids are shown in grey outline. The GenBank accession numbers used for the alignment are as follows: NP_050243.1 for humans; Q28165 for bovine; NP_062275.1 for mouse; AF257236 for Xenopus; AF116341 for Drosophila; CAB16904.1 for S. pombe. Alignments and shading were generated using ClustalW and Boxshade.

Figure S2: Quantitative assessment of subcellular distribution of GFP-Pab2. Histogram showing the number of cells in which a distinguishable nucleus (nuclear > cytoplasmic GFP signal: black box) was observed using GFP fluorescence versus cells for which a nucleus could not be distinguished from the cytoplasmic GFP signal (nuclear ≤ cytoplasmic GFP signal: white box) from (A) cells expressing wild-type and variant versions of GFP-Pab2 as well as the GFP control, (B) cells expressing wild-type GFP-Pab2 and variant versions of GST-GFP fusions and (C) pab2A, pab2A/kap104A and pab2A/sal3Δ cells expressing wild-type GFP-Pab2 or GST-GFP-Pab2 (139–168). At least 50 cells were counted for each strain. A chi-square test confirmed the statistical significance of these results (p-value <0.0001).

Figure S3: Quantitative assessment of subcellular distribution of GFP-Pab2 and GFP-Pab2 [P165E] in the presence or absence of arginine methylation. Histogram showing the number of cells in which a distinguishable cytoplasmic GFP signal (black box) was observed using GFP fluorescence versus cells for which cytoplasmic GFP signal could not be distinguished (white box) from pab2Δ and pab2Δ/mtt1Δ cells expressing wild-type GFP-Pab2 or GFP-Pab2 [P165E]. At least 175 cells were counted for each strain and over 1000 cells were counted in total. A chi-square test confirmed the statistical significance of these results (p-value <0.0001).

Figure S4: Inhibition of CRM1-dependent nuclear export does not abolish the detection of Pab2 [P165A] in the cytoplasm. Visual analysis of pab2Δ cells expressing the empty control vector (A–D), GFP-Pab2 [P165A] (E–H) and GST-GFP-Pap1 NES (I–L) that were previously treated with 100 ng/mL leptomycin B (+LMB) for 6 h or not treated with leptomycin B (~LMB). Direct fluorescence microscopy (GFP) was visualized in live cells and the corresponding differential interference contrast (DIC) images are shown. The GST-GFP-Pap1 NES was used as a positive control for the leptomycin B treatment, as it accumulates in the nucleus after the addition of leptomycin B (Mercier and Labbe, 2009).

Figure S5: Rescaled images showing overexposed saturated signals from PABPN1 indirect immunofluorescence (panels B and F).

Table S1: Strains and plasmids used in this study

References


