

# PYM binds the cytoplasmic exon-junction complex and ribosomes to enhance translation of spliced mRNAs

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**Messenger RNAs produced by splicing are translated more efficiently than those produced from similar intronless precursor mRNAs (pre-mRNAs). The exon-junction complex (EJC) probably mediates this enhancement; however, the specific link between the EJC and the translation machinery has not been identified. The EJC proteins Y14 and magoh remain bound to spliced mRNAs after their export from the nucleus to the cytoplasm and are removed only when these mRNAs are translated. Here we show that PYM, a 29-kDa protein that binds the Y14–magoh complex in the cytoplasm, also binds, via a separate domain, to the small (40S) ribosomal subunit and the 48S preinitiation complex. Furthermore, PYM knockdown reduces the translation efficiency of a reporter protein produced from intron-containing, but not intronless, pre-mRNA. We suggest that PYM functions as a bridge between EJC-bearing spliced mRNAs and the translation machinery to enhance translation of the mRNAs.**

In eukaryotic cells, removal of introns from pre-mRNAs by splicing is essential for gene expression, producing mature mRNAs that properly code for proteins<sup>1,2</sup>. Pre-mRNA splicing not only serves to remove introns but also endows the mRNAs with the capacity to function efficiently in several downstream processes, including 3'-end processing, mRNA export, localization and nonsense-mediated decay (NMD)<sup>3–5</sup>. In addition, pre-mRNA splicing strongly influences translation such that mRNAs produced from intron-containing pre-mRNAs are translated more efficiently than those produced from intronless precursors<sup>6</sup>. Because splicing is a nuclear event and translation occurs in the cytoplasm, the process of splicing must provide spliced mRNAs with one or more factor(s) that remain associated with the RNAs through transport to the cytoplasm and enhance their translation efficiency. The EJC is the most likely source of factors that mediate this effect<sup>7–10</sup>.

The EJC is a splicing-dependent multiprotein complex that associates immediately upstream of the exon-exon junction and contains several proteins, including Y14, magoh, Aly/REF, RNPS1, SRm160, Upf3, MLN51/Barentz and eIF4AIII<sup>11–20</sup>. The EJC thus functions to mark the position of the exon-exon junction in the mature mRNA and thereby influences downstream processes of gene expression. Components of the EJC function in NMD, a quality-control process that scrutinizes mRNAs and selectively degrades those that contain premature termination codons<sup>21–29</sup>. Factors required for NMD have been isolated previously from various organisms and termed Upf1, Upf2 and Upf3. Among these factors, Upf3 has been shown to interact with Y14 and is a component of the EJC<sup>13,24</sup>. These observations indicate that the EJC proteins have the capacity to communicate crucial information on the mRNA from the nucleus to the cytoplasm.

Two components of the EJC in particular, Y14 and magoh, have been implicated in enhancing translation of spliced mRNAs. Magoh and Y14 bind each other and exist as a dimer. These two proteins, along with RNPS1, eIF4AIII and MLN51/Barentz, remain stably associated with newly exported mRNAs in the cytoplasm near the exon-exon junction, whereas most of the other EJC components dissociate during or immediately after mRNA export<sup>11,14,30–32</sup>. Both Y14 and magoh are essential for cell viability, and mutants of Y14 and mago nashi, the *Drosophila melanogaster* homolog of magoh, show mislocalization of *oskar* mRNA in *D. melanogaster* oocytes<sup>33–37</sup>. When Y14, magoh or RNPS1 is tethered to mRNAs, the mRNAs are more efficiently translated<sup>10</sup>. These findings suggest that the cytoplasmic remnants of the EJC (cEJC), comprising at least Y14, magoh, eIF4AIII and RNPS1, have important roles in cytoplasmic events, including translation.

The EJC is required for increased translation of spliced mRNAs; however, it is not known how the EJC components link spliced mRNA to the translation machinery. Here we show that human PYM (also called WIBG), a recently identified binding protein for Y14 and magoh, interacts *in vivo* with the Y14–magoh complex in the cytoplasm<sup>38</sup>. We further show that PYM is also associated with the 40S ribosomal subunit. A domain in PYM that bears great sequence similarity to a domain in the translation initiation factor eIF2A is required for efficient association with the ribosomal 48S preinitiation complex and the 40S ribosomal subunit. Knockdown of PYM expression by RNA interference (RNAi) decreases the translation efficiency of spliced mRNAs but does not affect the translation of mRNAs from intronless precursors. We therefore propose that PYM functions to enhance the translation of spliced mRNAs by directly linking the EJC to the ribosomal 48S preinitiation complex in the cytoplasm.

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**Figure 1** PYM interacts selectively with the Y14–magoh complex. (a) *In vitro* binding of PYM to indicated GST-tagged proteins. Myc-PYM was transcribed *in vitro*, translated in the presence of [<sup>35</sup>S]methionine and incubated with recombinant GST-tagged proteins bound to glutathione beads. Bound PYM was resolved on a 10% SDS-PAGE gel and visualized by fluorography. (b) BiFC of PYM and Y14–magoh. pYN-PYM, pYC-Y14 and Myc-magoh were transiently expressed in HeLa CCL2 cells. No signal was observed for the same YFP fragments alone. Y14 was detected by an immunofluorescent antibody. (c) Immunoprecipitation of Flag epitope-tagged Y14 and magoh and their associated proteins. Y14 and magoh and their associated proteins were immunopurified from stable cell lines expressing Flag-Y14 or Flag-magoh and analyzed by western blotting with antibodies to proteins indicated at left. Input lane contains 2.5% of the extract used for immunoprecipitation.

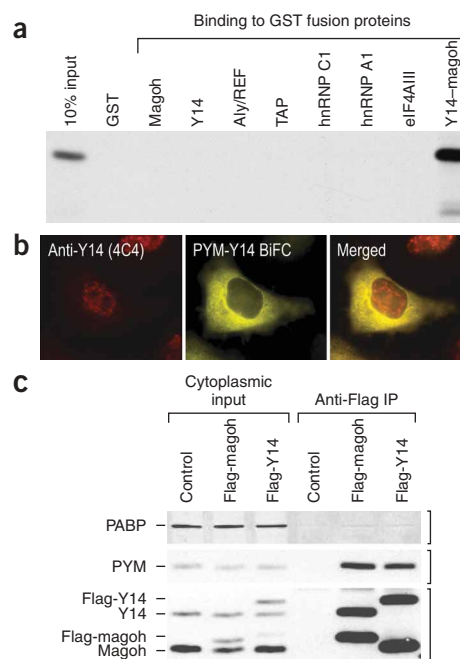
## RESULTS

### PYM interacts with the EJC in the cytoplasm

Y14 and magoh, two core components of the EJC, remain associated with spliced mRNA until it is translated. The Y14–magoh heterodimer has previously been shown to interact with PYM<sup>38</sup>, but the functional significance of this interaction was unknown. We first examined whether PYM also interacts with several other EJC components *in vitro*. PYM was produced by *in vitro* transcription and translation in the presence of [<sup>35</sup>S]methionine and incubated with glutathione S-transferase (GST)-tagged recombinant versions of magoh, Y14, Aly/REF, eIF4AIII, the mRNA-export factor TAP, heteronuclear ribonucleoprotein (hnRNP) C1, hnRNP A1 and the Y14–magoh complex. Bound complexes were then isolated using glutathione affinity beads, and the associated PYM was analyzed by SDS-PAGE. In agreement with previous results, PYM bound specifically and avidly to the Y14–magoh complex but did not show any ability to bind either the other individual components of the EJC, the hnRNP proteins or TAP, suggesting that PYM interacts with the EJC solely through interaction with Y14 and magoh (Fig. 1a).

As the Y14–magoh complex binds mRNAs in the nucleus and remains associated with the mRNAs in the cytoplasm, whereas PYM is predominantly cytoplasmic<sup>11,12,14,38</sup>, we used bimolecular fluorescence complementation (BiFC) to visualize where in cells PYM and the Y14–magoh complex interact. For this, PYM was fused to the N-terminal fragment of yellow fluorescent protein, YFP (pYN-PYM) and Y14 was fused to the C-terminal fragment of YFP (pYC-Y14). The constructs were cotransfected into HeLa CCL2 cells, and the fluorescence signal resulting from a direct interaction of Y14 and PYM *in vivo* was monitored using fluorescence microscopy. For comparison, the cells were also stained for Y14. Although Y14 is predominantly nuclear, PYM and Y14 show strong BiFC signal in the cytoplasm (Fig. 1b), demonstrating that PYM and Y14 are in direct contact in the cytoplasm. The same BiFC pattern was observed for pYC-magoh and pYN-PYM, and no signal was detectable for the YFP fragments alone (data not shown).

We used co-immunoprecipitation experiments to further confirm that PYM interacts with the EJC in the cytoplasm. Flag-tagged Y14 or magoh was expressed in HeLa cells<sup>16</sup>. After induction, we prepared cytoplasmic extracts and isolated the tagged proteins, as well as any associated proteins, by immunoprecipitation with antibodies to Flag. PYM efficiently co-immunoprecipitated with magoh and Y14 from the cytoplasm (Fig. 1c). This association is independent of the presence of RNA, as RNase treatment did not abolish the interaction between PYM, Y14 and magoh (Supplementary Fig. 1 online). The amount of poly(A)-binding protein (PABP) bound to Y14–magoh varies, probably because of variation in the amount of EJC proteins that are still bound to mRNAs in the cytoplasm.



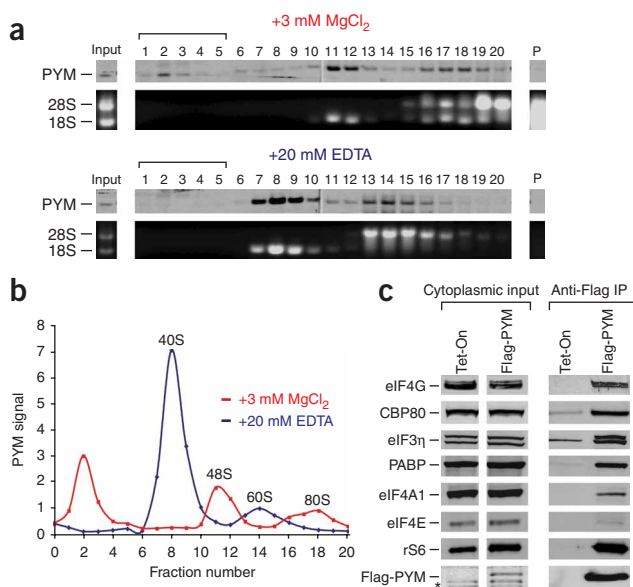
### PYM interacts with ribosomal subunits

The above results show that PYM directly associates with the EJC in the cytoplasm through direct interactions with Y14 and magoh. To further assess PYM function, we identified other proteins that interact with PYM. Flag-PYM, along with its associating proteins, was immunoprecipitated from cytoplasmic extracts of a HeLa Tet-On cell line stably expressing Flag-PYM. The co-immunoprecipitated proteins were resolved by SDS-PAGE and visualized by silver staining (Supplementary Fig. 2 online). The specifically co-immunoprecipitated proteins that were not present in the control immunoprecipitate were analyzed by mass spectrometry and are listed in Table 1. Notably, most are ribosomal proteins and ribosome-associated proteins, suggesting that PYM interacts with components of the translation machinery.

To further test the possibility that PYM is associated with ribosomes or ribosomal subunits, we studied the sedimentation profile of PYM in 5%–20% sucrose gradients. Fractions were collected and analyzed for both protein and rRNA content. Western blotting analysis of the gradient revealed that PYM is found in two distinct peaks, one in fractions 11–12 and the other in fractions 17–18 (Fig. 2a). Total RNAs of each fraction were isolated, separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. Using the 18S and 28S ribosomal RNAs as markers for the small and large ribosomal subunits, respectively, we identified the PYM peak at fractions 11–12 as the 43S or 48S preinitiation complexes and the peak at fractions 17–18 as the 80S ribosomes (Fig. 2a,b). Upon treatment with EDTA,

**Table 1** Proteins identified in a PYM immunoprecipitation

Category	Proteins identified by mass spectrometry
Small ribosomal subunits	S2, S3, S3a, S4, S5, S6, S7, S8, S9, S10, S18, S25
Large ribosomal subunits	L5, L7, L7a, L8, L10a, L11, L12, L13a, L14, L23, L24, L27a
Ribosome-associated	28-kDa protein, BC013949, Vigilin, RNA helicase A, guanine nucleotide-binding protein, eIF3k, PADP1
EJC	Y14, magoh



**Figure 2** PYM associates with the 40S ribosomal subunit and translation factors. **(a)** Sucrose-gradient sedimentation analysis of PYM. Cytoplasmic fractions from HeLa PV cells were separated on a 5%–20% sucrose gradient in either the presence or absence of EDTA. Fractions were collected and analyzed for both PYM protein content (by western blotting, top gels) and rRNA content (bottom gels). **(b)** PYM protein signal in the sucrose-gradient fractions in **a**. **(c)** Flag-PYM immunoprecipitation from cytoplasmic extract. Flag-PYM expressed in a stable cell line was immunoprecipitated along with its associated proteins. Bound proteins were resolved on a 10% SDS-PAGE gel and analyzed by western blotting with antibodies to proteins indicated at left. Asterisk marks a protein associated nonspecifically with the Flag antibody. Input lane contains 2.5% of the extract used for immunoprecipitation.

which dissociates the ribosome into its constituent 40S and 60S subunits<sup>39</sup>, PYM was found to sediment predominantly with the 40S subunit (**Fig. 2a,b**). A much smaller amount of PYM sedimented with the 60S ribosomal subunit.

### PYM associates with ribosomal 48S preinitiation complex

One of the rate-limiting steps in translation is recruitment of the mRNA into the 48S preinitiation complex by the cap-binding complex (eIF4F complex). To determine whether PYM is associated with the 48S preinitiation complex, we analyzed immunoprecipitated Flag-PYM complexes for the presence of several components of the eIF4F complex, including eIF4G and eIF4A1, as well as for the small ribosomal protein S6 and PABP. The results show that PYM interacts with the small ribosomal subunit and with the 48S preinitiation

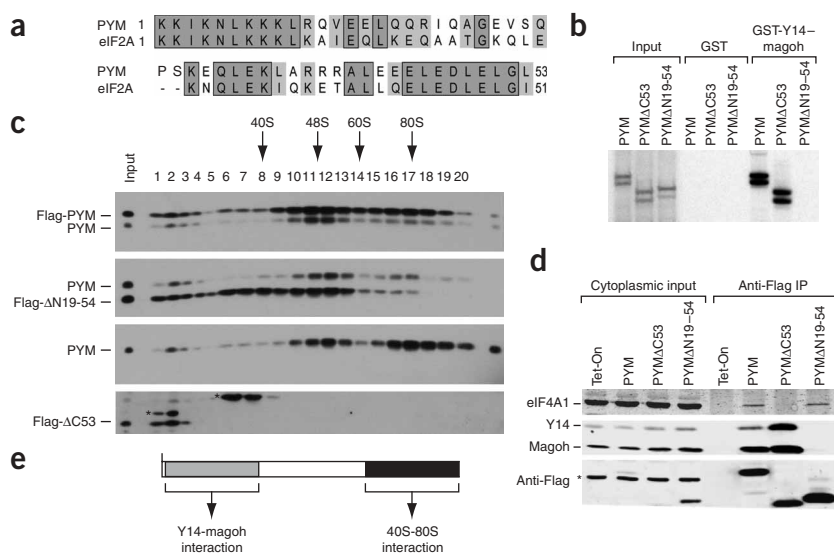
complex and mRNA (**Fig. 2c**). Furthermore, PYM associates with CBP80 but not eIF4E, suggesting that PYM may have a role in the 'pioneer' round of translation, which has previously been linked to EJC-bearing mRNAs (**Fig. 2c**)<sup>9,25,40–43</sup>.

### PYM C terminus associates with the 40S ribosomal subunit

PYM's C-terminal amino acid sequence is highly similar to the last 50 residues of eIF2A (**Fig. 3a**). eIF2A has been proposed to be a minor translation initiation factor on the basis of its ability to interact with tRNA<sup>Met</sup> (ref. 44) and has recently been shown to aid in translation of a viral RNA<sup>45</sup>. Although the exact function of eIF2A is still being elucidated, we deleted the C-terminal region of PYM that has high homology to the C terminus of eIF2A (**Fig. 3a**). We produced an additional mutant, PYMΔN19–54, effectively removing the Y14-magoh interaction domain<sup>38</sup>. We then tested whether these deletion mutants could bind the Y14-magoh complex *in vitro*. Wild-type PYM and the deletion mutants were translated in the presence of [<sup>35</sup>S]methionine *in vitro* and tested for binding to immobilized recombinant GST-Y14-His-magoh complex. The C terminus of PYM is completely dispensable for binding to the Y14-magoh complex; in contrast, as expected, the N-terminal deletion completely abolished the interaction between Y14-magoh and PYM (**Fig. 3b**). Additionally, strong BiFC is evident between PYMΔC53 and Y14,

**Figure 3** The C terminus of PYM is required for association with the 48S preinitiation complex.

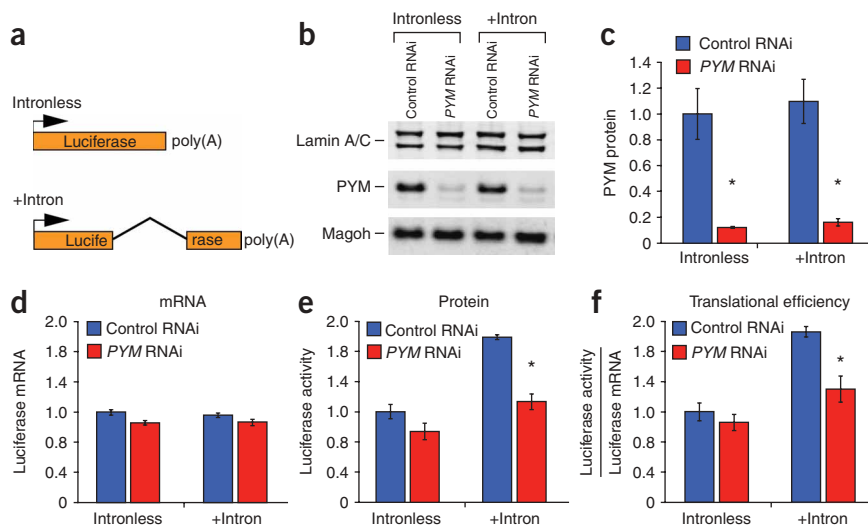
**(a)** Alignment of the C-terminal 53 residues of PYM with the C-terminal 51 residues of eIF2A. **(b)** *In vitro* binding of the Y14-magoh complex with N- and C-terminal deletion mutants of PYM. Myc-PYM, Myc-PYMΔC53 and Myc-PYMΔ19–54 were transcribed *in vitro*, translated in the presence of [<sup>35</sup>S]methionine and incubated with recombinant GST-Y14-His-magoh or GST. Bound protein was resolved on a 10% SDS-PAGE gel and visualized by fluorography. Input lane contains 2.5% of protein used for GST pull-down assay. **(c)** Sucrose-gradient sedimentation analysis of Flag-PYM and its deletion mutants. Cytoplasmic extracts were prepared and analyzed as in **Figure 2a**. Anti-PYM was used to detect endogenous PYM, Flag-PYM and Flag-PYMΔ19–54; anti-Flag was used to detect PYMΔC53. Blot labeled 'PYM' shows endogenous PYM in the PYMΔC53 lysate. **(d)** Immunoprecipitation of Flag-PYM and its deletion mutants from cytoplasmic extract. Flag-tagged PYM constructs were immunoprecipitated from cell extracts, and bound proteins were resolved on a 10% SDS-PAGE gel and analyzed by western blotting with antibodies to indicated proteins. Asterisks in **c** and **d** mark a protein bound nonspecifically to anti-Flag. Input lane contains 2.5% of the extract used for immunoprecipitation. **(e)** Schematic of PYM's two separate protein-interaction domains.



**Figure 4** Knockdown of PYM results in reduced expression from an intron-containing reporter.

(a) Schematic of the reporter constructs. (b) Western blotting of extracts from HeLa CCL2 cells transfected with control (scrambled) or *PYM*-specific siRNA for 40 h, then transfected with intronless or intron-containing luciferase reporter gene. Lamin A/C was used as a loading control. (c) Quantification of *PYM* knockdown by RNAi. \* $P = 0.0015$ . (d) Luciferase mRNA expression from an intron-containing or intronless construct in cells with reduced *PYM* expression or in negative control cells. Reporter constructs were transiently expressed in HeLa CCL2 cells with reduced *PYM* or in control cells. After a short incubation, cells were lysed and analyzed for RNA content by quantitative PCR. (e) Luciferase activity from an intron-containing or intronless construct in cells with reduced *PYM* or in negative control cells. \* $P = 0.00028$ . (f) Relative translational efficiency of cells with reduced *PYM* or negative control cells.

Translational efficiency was defined as luciferase activity/luciferase mRNA, with intronless control samples assigned a relative efficiency of 1. \* $P = 0.002$ . Data in c–f are means from three independent experiments; error bars show s.d.



indicating a direct interaction, but does not occur between PYM $\Delta$ N19–54 and Y14 (Supplementary Fig. 3 online).

The EJC is required for enhanced translation of spliced mRNAs, but exactly how the EJC interacts with the translational apparatus was unknown. To address further the question of whether *PYM* mediates this interaction, we produced doxycycline-inducible stable cell lines expressing Flag epitope-tagged versions of the PYM $\Delta$ N19–54 and PYM $\Delta$ C53 deletion mutants and analyzed their sedimentation patterns on a 5%–20% sucrose gradient, as in Figure 2a. Flag-PYM $\Delta$ N19–54 showed an altered sedimentation pattern compared with endogenous *PYM* and Flag-*PYM*: PYM $\Delta$ N19–54 still peaked in fractions 11 and 12, corresponding to the 48S preinitiation complex, but less was found in the 80S peak with endogenous *PYM* (Fig. 3c). Additionally, a substantial amount of PYM $\Delta$ N19–54 was found in the lighter fractions, suggesting that it may be defective in incorporation into the 40S ribosomal subunit. Notably, deletion of the C-terminal 53 residues of *PYM* (Flag-PYM $\Delta$ C53) caused a complete shift to the top of the gradient, suggesting that this mutant has a severe defect in its ability to associate with the 40S subunit (Fig. 3c). These results were further supported by co-immunoprecipitation experiments with PYM $\Delta$ C53 and PYM $\Delta$ N19–54. As expected, PYM $\Delta$ C53 co-immunoprecipitated the Y14–magoh complex but not the translation initiation factor eIF4A1, whereas the opposite pattern was observed for PYM $\Delta$ N19–54 (Fig. 3d). These data suggest that *PYM* could serve to bridge an EJC-bearing mRNA to the ribosome using the conserved N- and C-terminal *PYM* domains (Fig. 3e).

#### Translational efficiency of spliced mRNAs depends on *PYM*

To examine whether *PYM* has a role in translation, we reduced the expression of *PYM* by RNAi and assessed the effect on translational efficiency of reporter mRNAs resulting from either spliced precursors or intronless, unspliced precursors. The reporter precursors contained a luciferase gene either lacking an intron or having the coding sequence interrupted by a chimeric intron (Fig. 4a). Plasmids encoding these pre-mRNAs were transfected into cells in which *PYM* was knocked down by RNAi as well as into control cells. Using extracts of these cells, *PYM* protein levels were measured by western blotting

analysis, mRNA levels were measured by quantitative PCR, and luciferase protein levels were measured by assays of luciferase enzymatic activity. *PYM* abundance was markedly reduced by RNAi (>80%; Fig. 4b,c). Knockdown of *PYM* did not change the total amount of mRNA, and the amounts of mRNA produced from the intron-containing and intronless precursors were not significantly different ( $0.97 \pm 0.023$  and  $1.0 \pm 0.028$  arbitrary units, respectively;  $P = 0.05$ ; Fig. 4d). However, the efficiency of translation of the intron-containing mRNA, defined here as luciferase activity (Fig. 4e) per luciferase mRNA, was nearly double that of the intronless mRNA ( $1.9 \pm 0.55$  and  $1.0 \pm 0.1$ , respectively; Fig. 4f). Notably, knockdown of *PYM* by RNAi decreased the translation efficiency of the intron-containing mRNA to a level similar to that of the intronless mRNA ( $1.2 \pm 0.14$  and  $1.0 \pm 0.1$ , respectively), removing the enhancement of translation normally observed for spliced mRNAs (Fig. 4f). To test the specificity of *PYM* knockdown, we used four oligonucleotides that target different regions of the *PYM* message. Three of the four oligonucleotides knocked down *PYM* levels by more than 80% and concomitantly reduced translation efficiency (Supplementary Fig. 4 online). Our results, taken together, suggest that *PYM* functions to enhance the translation of EJC-bearing, spliced mRNAs by directly recruiting them to the ribosomal 48S preinitiation complex.

#### DISCUSSION

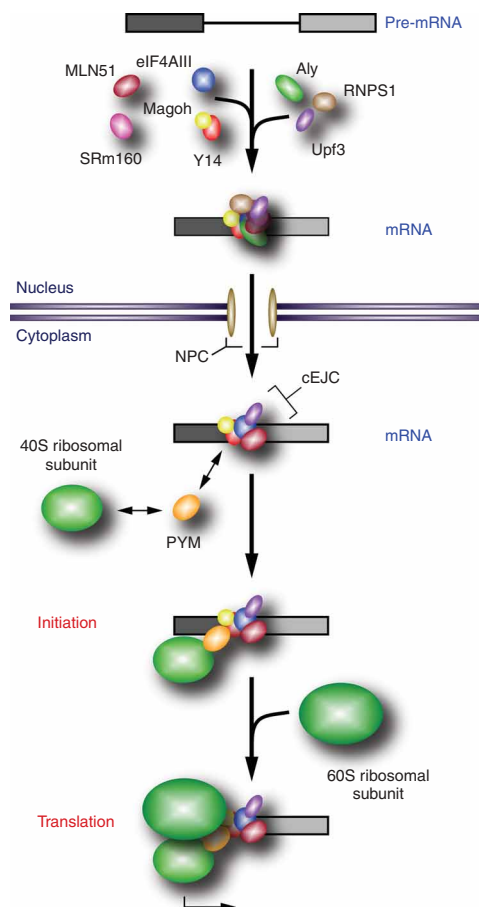
Tethering experiments with overexpressed protein bound to mRNA constructs have suggested that EJC components can increase the translational efficiency of spliced mRNAs<sup>8,10</sup>. However, how the EJC interacts with the translation machinery that mediates this enhancement was not known. Here we show that *PYM* functions as a bridge between the Y14–magoh complex in the cytoplasm and the 48S preinitiation complex and is required for enhancement of the translation of spliced mRNAs. By physically linking two of the core components of the EJC with the translation machinery, *PYM* crucially couples pre-mRNA splicing in the nucleus with translation in the cytoplasm. Figure 5 presents a model summarizing these interactions.

Our model depicts *PYM* interacting with Y14–magoh as part of a core cytoplasmic EJC that contains additional components, including

eIF4A3 and MLN51. However, a core cytoplasmic EJC comprising all four proteins has been detected only in *in vitro* studies using purified recombinant proteins and only in the presence of a non-hydrolyzable ATP analog that stabilizes eIF4A3 (ATP hydrolysis by eIF4A3 disassembles the complex)<sup>32</sup>. It has not yet been demonstrated that these four components form a complex in cells, possibly owing to activation of the ATPase activity of eIF4A3 upon cell lysis and consequent dissociation of the complex. Similarly, using tagged PYM, Y14, magoh, eIF4A3 or MLN51, we were unable to confirm that such a core EJC exists *in vivo* (data not shown). In addition, we have previously shown that Y14 and magoh co-immunoprecipitate only trace amounts of eIF4A3 (ref. 16), and we were also able to immunoprecipitate trace amounts of eIF4A3 with PYM (data not shown). Moreover, we could not detect PYM, Y14 or magoh together with MLN51 in the cytoplasm. Consistent with these data, eIF4A3 has recently been found in purified messenger RNPs, but MLN51 was not present<sup>46</sup>. Furthermore, recent data challenges the concept of an assembled core EJC that recruits other factors<sup>47</sup>. Recruitment of several EJC components seems to be independent of eIF4A3, suggesting that the EJC is a dynamic complex whose components are being loaded and removed constantly during the biogenesis of mRNPs<sup>48</sup>. Thus, a core EJC comprising Y14–magoh, eIF4A3 and MLN51 in the cytoplasm may exist only transiently. An alternative model to the one depicted in **Figure 5** is that PYM interacts with remnants of the EJC including Y14–magoh but not eIF4A3 and MLN51. For example, it is possible that the binding of PYM causes the dissociation of eIF4A3 and MLN51, leaving Y14 and magoh bound to the mRNA. An entirely different mechanism in which PYM associates with Y14 and magoh outside of the EJC, and not bound to mRNA, is unlikely because this cannot explain how the interaction of PYM with Y14–magoh and the ribosome enhances the translation *in cis* of the spliced mRNA reporter but not the translation of the mRNA produced from the intronless gene.

Mass-spectrometric analysis of proteins associated with PYM revealed a large number of ribosomal and ribosome-associated proteins. Notably, PYM was found in translation initiation complexes with eIF4G, eIF4A1, eIF3 $\eta$  and the ribosomal protein S6, but not with the cytoplasmic cap-binding protein eIF4E. Rather, CBP80, the nuclear cap-binding protein, was consistently co-immunoprecipitated with PYM from the cytoplasmic fraction. This suggests that PYM does not have a role in steady-state translation, but rather functions in the initial, pioneer round of translation that has been proposed to take place in the cytoplasm once for each mRNA, as a security checkpoint<sup>41,49</sup>. Aberrant transcripts are then degraded before there is a chance for numerous ribosomes to associate with the mRNA and produce truncated proteins<sup>43</sup>. It will be interesting to determine whether the decrease in translational efficiency seen with reduced PYM levels occurs at the initiation step of translation or at a later step. Although PYM cosediments with the preinitiation complex, it could have a more specific role in the removal of the EJCs, such that the observed block on translation is not inhibition of initiation but rather formation of stalled 80S ribosomes.

In agreement with the recent crystal structure in which the N terminus of PYM is bound to the Y14–magoh complex<sup>38</sup>, deletion of the N-terminal region of PYM completely abolished its association both *in vitro* and *in vivo* with the Y14–magoh complex (**Fig. 3b,d**). Sequence analysis of PYM identified a second domain located in the C terminus with high similarity to the translation initiation factor eIF2A (**Fig. 3a**). Deletion of this C-terminal region of PYM completely abolished PYM's ability to associate with the 40S subunit, whereas deletion of the Y14–magoh–interacting region did not have as



**Figure 5** Model of the assembly pathway and dynamics of the EJC and the role of PYM in the interaction of the EJC with the translation machinery. See Discussion for details. NPC, nuclear pore complex.

strong an effect. Thus, PYM has two independent domains that can associate with both the Y14–magoh complex and the 40S subunit simultaneously (**Fig. 3e**). Although the function of eIF2A eluded researchers for several decades<sup>44</sup>, a number of papers have more recently revealed a role for eIF2A in supplying tRNA<sup>Met</sup> to specific mRNAs<sup>45,50,51</sup>; thus, eIF2A is associated with the 40S ribosomal subunit. It would be interesting to determine whether deletion of the C-terminal 53 residues of eIF2A has a similar effect on association with the ribosome as does the C-terminal deletion in PYM.

## METHODS

**Antibodies.** Mouse monoclonal PYM-specific antibodies were produced by immunizing A/J mice with full-length recombinant GST-tagged PYM. Hybridoma production, screening and ascites fluid preparation were done as described<sup>12</sup>. Anti-Y14 (4C4), anti-magoh (21B12) and anti-PABP (10E10) have been described<sup>12</sup>. The rabbit polyclonal antibody to CBP80 was a gift (see Acknowledgments). Anti-eIF4G, anti-eIF4A1, anti-eIF4E and anti-eIF3 $\eta$  were obtained from Santa Cruz Biotech. Anti-rpS6 was obtained from Abcam and mouse monoclonal anti-Flag (M2) and rabbit polyclonal anti-Flag were obtained from Sigma.

**Plasmid construction.** Full-length PYM complementary DNA was PCR-amplified from a human HeLa Marathon-Ready cDNA library (Clontech) with Pfu polymerase (Stratagene). Deletions were made using an internal primer to remove the N- or C-terminal region (an artificial stop codon was added to the 3' end). To construct plasmids encoding Flag-tagged proteins, the appropriate cDNA sequences were inserted between the BamHI and XhoI sites of

pcDNA3-Flag<sup>52</sup>. To construct plasmids encoding GST-tagged proteins, the cDNAs were cloned into the BamHI and XhoI sites of pGV66 (GST-TEV; see Acknowledgments). To construct bimolecular fluorescence vectors, sequences encoding Y14, magoh, and PYM were cloned into pYN-N1 and pYC-N1 (Invitrogen) at the HindIII and BamHI sites. The intron-containing luciferase reporter gene was generated by inserting a 132-nucleotide  $\beta$ -globin intron at position 1344 of firefly luciferase gene.

#### Production of stable cell lines, cell culture and immunoprecipitation.

Production, characterization and culture of HeLa Tet-On cells expressing Flag-PYM, Flag-PYMAC53 or Flag-PYMA19–54 were done as described<sup>16</sup>. HeLa S3, HeLa PV, HeLa CCL2 and HEK293T cells were cultured as described<sup>11,48</sup>. Cytoplasmic extracts were prepared by the digitonin method as described<sup>11</sup>. Anti-Flag immunoprecipitation assays from HeLa Tet-On stable cell lines was done as described<sup>16</sup>. RNase cocktail (1  $\mu$ l; Ambion) was added to the extract where indicated.

**Recombinant protein production and *in vitro* protein binding.** Proteins were overexpressed in *Escherichia coli* BL21(DE3) codon-plus cells (Stratagene) and purified according to the manufacturer's recommendations (Amersham). *In vitro* protein-binding experiments were done as described<sup>11</sup>.

**Protein microsequencing by mass spectrometry.** Protein bands were excised from a silver-stained polyacrylamide gel (Novex minigel, Invitrogen), and peptides were identified at the New York University Protein Analysis Facility.

**Quantitative western blotting analysis.** Protein samples were resolved by SDS-PAGE and transferred to a 0.2- $\mu$ m nitrocellulose membrane. Membranes were blocked in LI-COR blocking buffer at 4 °C with gentle rocking overnight. Primary antibodies (see figure legends) were diluted in LI-COR blocking buffer containing 0.2% (v/v) Tween-20 and incubated with the membranes for 1 h at room temperature. The membranes were washed three times for 15 min with PBS containing 0.1% (v/v) Tween-20. Secondary antibodies (infrared dye 800, Rockland) were diluted 1:5,000 in LI-COR blocking buffer containing 0.2% (v/v) Tween-20 and incubated with the membranes for 1 h. The membranes were washed four times for 30 min with PBS containing 0.1% (v/v) Tween-20, then rinsed with PBS. Infrared dye-conjugated antibodies were detected with an Odyssey infrared imaging system.

**Sucrose gradients and rRNA preparation.** HeLa S3 cells were grown at 37 °C and 5% CO<sub>2</sub> in complete DMEM. Cycloheximide was added to the medium at a concentration of 100  $\mu$ g ml<sup>-1</sup>, and the cells were further incubated for 2 h to stabilize polysomes. Cytoplasmic extracts were prepared as described above, loaded onto 5%–20% linear sucrose gradients and sedimented by ultracentrifugation in a Beckman SW41.1 rotor at 28,800g for 3 h. After centrifugation, fractions were collected from the top of the gradient with a BioComp fractionator.

One hundred microliters of each sucrose-gradient fraction was transferred to a separate tube, from which total RNA was purified using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Purified RNAs were resuspended in 10  $\mu$ l of RNA loading buffer (Ambion) and analyzed on a 1% (w/v) agarose gel containing formaldehyde and ethidium bromide. RNA bands were visualized by UV irradiation.

**Bimolecular fluorescence complementation assay.** HeLa CCL2 cells were grown on coverslips for 24 h and then transfected, using Effectene (Qiagen) according to the manufacturer's protocol, with a combination of plasmids (0.2  $\mu$ g) containing pYN-N1 and pYC-N1 sequences cloned in frame with the protein of interest (see figure legends). The transfected plasmids were allowed to express protein for 40 h, after which the cells were incubated for 10 min at 30 °C to allow maturation of the YFP molecules. The cells were then stained for Y14 as described<sup>12</sup>. Fluorescing proteins were visualized with a ZEISS Axiom microscope.

**Luciferase assay.** HeLa CCL2 cells were transfected either with a negative control siRNA oligonucleotide (Ambion) or with a PYM-targeting siRNA SMARTpool (Dharmacon) using Oligofectamine (Invitrogen) according to the manufacturer's protocol. The cells were allowed to grow for an additional 40 h, and the intronless or intron-containing luciferase reporters were

transfected using Effectene (Qiagen). The reporters were allowed to express luciferase for 5 h, after which cells were collected and counted with a nucleocounter (New Brunswick Scientific).

Equal numbers of cells from each experiment were lysed with 100  $\mu$ l passive lysis buffer (Promega) according to the manufacturer's protocol. Aliquots of 10  $\mu$ l were used for luminescence measurements and were read in a black 96-well Nunc plate using a Wallac Envision plate reader. The 10- $\mu$ l aliquots were analyzed for PYM protein content by the quantitative western blotting protocol described above. The remaining lysate was used for RNA Trizol (Invitrogen) extraction according to the manufacturer's recommendation. We used 1  $\mu$ g of RNA in a single-step reverse-transcription (RT)-PCR reaction (Qiagen), and the resulting cloned RNAs (cRNAs) were analyzed in a real-time PCR reaction with primers specific for either the luciferase gene or *GAPDH* (loading control) and quantified using a Roche LightCycler.

**Statistics.** Experiments were performed three times, and two-tailed, equal-sample variance Student's *t*-tests were used to determine statistical significance of differences between the data sets. Differences of *P* < 0.01 were considered significant. Measurements of intron-containing RNAs were normalized to those of the intronless control, which were set at 1 for each experiment.

*Note: Supplementary information is available on the Nature Structural & Molecular Biology website.*

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