# Splicing remodels messenger ribonucleoprotein architecture via eIF4A3-dependent and -independent recruitment of exon junction complex components

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**Pre-mRNA splicing not only removes introns and joins exons to generate spliced mRNA but also results in remodeling of the spliced messenger ribonucleoprotein, influencing various downstream events. This remodeling includes the loading of an exon–exon junction complex (EJC). It is unclear how the spliceosome recruits the EJC onto the mRNA and whether EJC formation or EJC components are required for pre-mRNA splicing. Here we immunodepleted the EJC core component eIF4A3 from HeLa cell nuclear extract and found that eIF4A3 is dispensable for pre-mRNA splicing *in vitro*. However, eIF4A3 is required for the splicing-dependent loading of the Y14/Magoh heterodimer onto mRNA, and this activity of human eIF4A3 is also present in the *Drosophila* ortholog. Surprisingly, the loading of six other EJC components was not affected by eIF4A3 depletion, suggesting that their binding to mRNA involves different or redundant pathways. Finally, we found that the assembly of the EJC onto mRNA occurs at the late stages of the splicing reaction and requires the second-step splicing and mRNA-release factor HRH1/hPrp22. The EJC-dependent and -independent recruitment of RNA-binding proteins onto mRNA suggests a role for the EJC in messenger ribonucleoprotein remodeling involving interactions with other proteins already bound to the pre-mRNA, which has implications for nonsense-mediated mRNA decay and other mRNA transactions.**

messenger ribonucleoprotein remodeling | nonsense-mediated mRNA decay | pre-mRNA splicing

Eukaryotic gene expression requires elaborate posttranscriptional control mechanisms to ensure that the genetic information is precisely transferred from DNA to its final products. The quality control of mRNA synthesis plays a central role in this process (1). During pre-mRNA splicing, introns are removed by the spliceosome, and the exons are ligated to form the mature mRNA. Splicing also affects many other aspects of downstream events, including mRNA export, surveillance, localization, and translation (2). These effects have been linked to the recruitment of specific proteins onto the mRNA during splicing, with the consequent remodeling of the messenger ribonucleoprotein (mRNP) composition and structure.

The exon–exon junction complex (EJC) is loaded onto the newly spliced mRNA ≈20–24 nt upstream of exon–exon junctions in a sequence nonspecific manner (3, 4). Several EJC factors are essential for mRNA localization in oocytes (5), nonsense-mediated mRNA decay (NMD) (5–7), and translational enhancement (8). The crystal structure of an EJC core complex, assembled from individual recombinant proteins and an RNA fragment, provided important details about how the complex is organized and stably binds to its RNA target (9, 10). However, the EJC is normally loaded onto mRNA during the course of pre-mRNA splicing (3), and the molecular mechanisms responsible for splicing-coupled loading of the EJC remain unknown.

Among the EJC components, the ATP-dependent DEAD-box RNA helicase eIF4A3 was proposed to be the key factor in formation of the complex (11), in part because it directly contacts the RNA in the EJC core (7, 9, 10). eIF4A3 is loaded onto mRNA during splicing, but it was not known whether it is essential for pre-mRNA splicing. RNA helicases are involved in many different aspects of RNA metabolism (12). During splicing, the extensive rearrangements and remodeling of the small nuclear RNAs and proteins of the spliceosome occur in an ATP-dependent manner, reflecting the involvement of various RNA helicases required for splicing, including Prp5, Prp18, Prp22, Prp19, and UAP56 (13). eIF4A3, as well as Y14/Magoh, were identified by mass spectrometry as components of purified human spliceosomes (14, 15). Unlike other helicases that function in splicing, eIF4A3 becomes tightly associated with spliced mRNA and plays critical roles in several postsplicing events. It is therefore of interest to determine whether eIF4A3 and other EJC factors play a role in pre-mRNA splicing, as well as to establish the mechanisms by which these factors are loaded onto mRNA. Although pre-mRNA splicing appears to be unaffected by RNAi knockdown of eIF4A3 (6, 8), detailed mechanistic analysis *in vitro* is warranted, because several biochemically well characterized essential splicing factors also appear to be dispensable for splicing *in vivo* after RNAi knockdown (16, 17).

Here we show that eIF4A3 is dispensable for pre-mRNA splicing *in vitro* despite its splicing-dependent association with the spliceosome. We also found that eIF4A3 is required for loading of Y14/Magoh onto mRNA by the spliceosome. Surprisingly, we found that binding of other EJC components onto mRNA does not require eIF4A3, because several factors previously thought to be recruited by the EJC could be loaded onto mRNA independently of EJC assembly. Finally, we show that eIF4A3 assembles onto the EJC at the late stages of splicing and that EJC assembly requires the helicase activity of HRH1/hPrp22.

## Results

**eIF4A3 Is Not Required for Pre-mRNA Splicing *in Vitro*.** To characterize the role of eIF4A3 in splicing, we generated a mouse monoclonal antibody against human eIF4A3 [supporting information (SI) Fig. 5]. We used this antibody to immunodeplete eIF4A3 from HeLa nuclear extract (NE). In high-salt conditions (>750 mM KCl), we depleted >95% of eIF4A3, compared with a control depletion with an antibody against *Escherichia coli* maltose-binding protein (Fig. 1*A*, lanes 1–3). There was no significant change in the amount of several control proteins, including PUF60, SF2/ASF, and U2-B″ (Fig. 1*A*, lanes 1–3). In addition, another EJC core component, Y14, was not codepleted with eIF4A3 under these conditions (Fig. 1*B*), suggesting that the EJC core complex is disrupted in high salt.

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Abbreviations: NMD, nonsense-mediated mRNA decay; EJC, exon–exon junction complex; mRNP, messenger ribonucleoprotein; PTC, premature termination codon; NE, nuclear extract.

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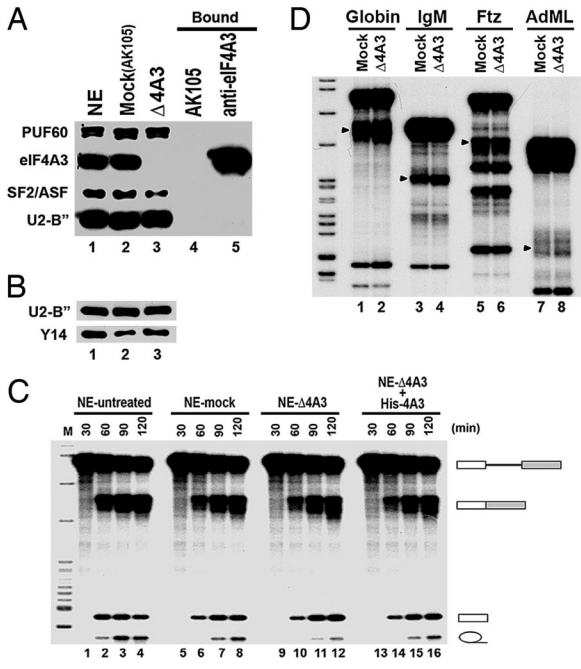


**Fig. 1.** eIF4A3 is dispensable for pre-mRNA splicing *in vitro*. (*A*) Endogenous eIF4A3 is efficiently immunodepleted from HeLa NE. eIF4A3 and the indicated control proteins were detected by Western blotting. AK105 is a control monoclonal antibody against bacterial maltose-binding protein. (*B*) The EJC component Y14 and the small nuclear RNP protein U2-B″ are not codepleted with eIF4A3 under these conditions. (*C*) *In vitro* splicing time course with human β-globin pre-mRNA in untreated NE (lanes 1–4), mock-depleted NE (lanes 5–8), and eIF4A3-depleted NE (Δ4A3 NE) without (lanes 9–12) or with (lanes 13–16) added recombinant His-tagged eIF4A3 (80 ng). The pre-mRNA, mRNA, and intermediates are indicated. (*D*) *In vitro* splicing assays with β-globin, IgM C3-C4, Ftz, and AdML pre-mRNAs in mock-depleted and Δ4A3 NE. The arrowheads show the spliced mRNAs.

We then tested whether the eIF4A3-depleted (Δ4A3) NE is still functional in pre-mRNA splicing. An *in vitro* splicing time course with human β-globin pre-mRNA showed that control-depleted (mock) and Δ4A3 NE had essentially equivalent splicing activity (Fig. 1*C*, compare lanes 5–8 with lanes 9–12). Adding back purified His-tagged recombinant eIF4A3 expressed in *E. coli* did not change the splicing activity (Fig. 1*C*, lanes 13–16). The control-depleted and Δ4A3 NE were equally active with other substrates, e.g., IgM C3-C4, Ftz, and AdML pre-mRNAs (Fig. 1*D*). We conclude that eIF4A3 is dispensable for constitutive pre-mRNA splicing despite its known association with the spliceosome. We also tested a model substrate derived from human β-globin pre-mRNA with a duplicated 5′ splice site (18) and found that eIF4A3 was not required for modulating 5′ splice site selection in response to an increase in (Ser/Arg-rich) protein levels (SI Fig. 6).

**eIF4A3 Is Essential for Loading Y14/Magoh onto Spliced mRNA.** To study the role of eIF4A3 in EJC assembly, we used an *in vitro* assay to measure EJC loading coupled with pre-mRNA splicing. We purified GST-tagged eIF4A3, added the protein to *in vitro* splicing reactions with HeLa NE, and then carried out GST pull-down assays to detect the bound RNAs (19, 20). We used GST-Y14/His-Magoh heterodimer as a control. After splicing, Y14/Magoh and eIF4A3 specifically pulled down spliced mRNA but only trace amounts of pre-mRNA and intron lariat (SI Fig. 7*A*, lanes 9 and 10). A small amount of the 5′ exon was also pulled down, consistent with a report that the 5′ exon provides the binding surface for EJC assembly (21).

Using an RNase H cleavage assay (3), we verified that GST-eIF4A3 and GST-Y14/His-Magoh are loaded onto mRNA upstream of the exon–exon junction during splicing (SI Fig. 7). We next sought to determine the effect of eIF4A3 depletion on EJC assembly. Although eIF4A3 and Y14/Magoh interact (19), how each affects the other's loading onto mRNA was not known. We carried out the above splicing-dependent loading assay using Δ4A3 NE. In the absence of eIF4A3, the Y14/Magoh heterodimer was no longer loaded onto mRNA (Fig. 2*A*, compare lanes 7 and 8). We then added purified recombinant His-tagged eIF4A3 to the Δ4A3 NE and carried out the GST pull-down assay; as the amount of eIF4A3 added increased, the loading of Y14/Magoh onto spliced mRNA was fully restored (Fig. 2*B*, lanes 12–16). This depletion/add-back experiment demonstrates that Y14/Magoh loading onto spliced mRNA requires eIF4A3, suggesting a central role for eIF4A3 in EJC assembly during pre-mRNA splicing.

We then performed the pull-down assays in depleted extract after adding back different mutant forms of human eIF4A3 or its orthologs. Two mutant versions of eIF4A3, K88A and S218L, which lack helicase activity (19), were still active in the Y14/Magoh loading complementation assay, with perhaps a slight decrease in activity compared with the WT (Fig. 2*C*, lanes 12–14), whereas an

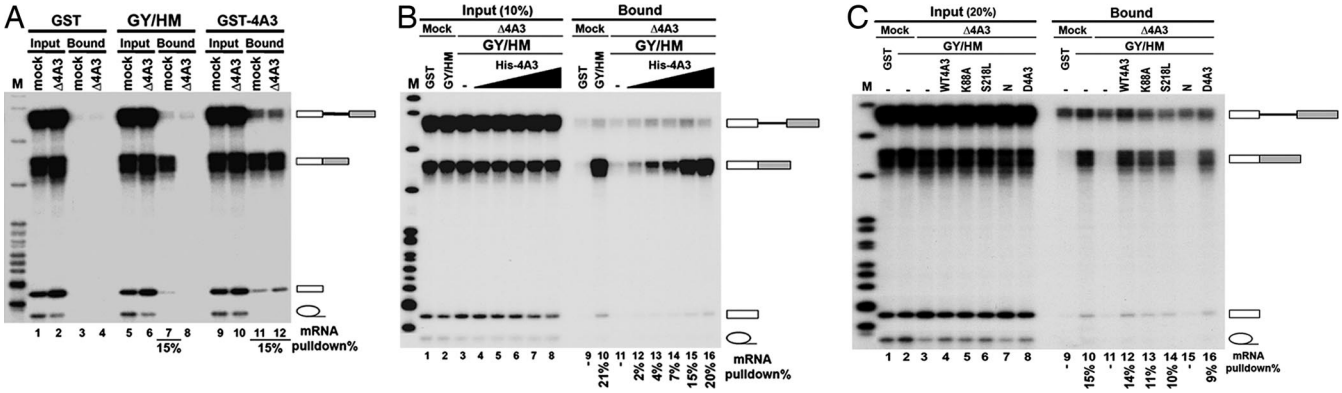


**Fig. 2.** eIF4A3 is essential for loading Y14/Magoh and for EJC core assembly on spliced mRNA. (*A*) Δ4A3 NE cannot load Y14/Magoh onto mRNA during splicing. GST pull-down assays were performed after *in vitro* splicing of β-globin pre-mRNA using mock-depleted or Δ4A3 NE. The input and pull-down samples are shown: lanes 1–4, GST alone; lanes 5–8, GST-Y14/His-Magoh; lanes 9–12, GST-eIF4A3. (*B*) Adding back recombinant eIF4A3 to Δ4A3 NE fully restores loading of Y14/Magoh onto spliced mRNA. GST-Y14/His-Magoh pull-down was carried out from mock-depleted NE (lane 11) and from Δ4A3 NE (lanes 12–16). Purified recombinant His-tagged eIF4A3 (10, 20, 50, 100, and 200 ng) was added to reactions containing 20 μl of Δ4A3 NE (lanes 12–16). A total of 20 μl of untreated NE has ≈400 ng of eIF4A3. (*C*) Comparison of different eIF4A3 mutants or the *Drosophila* ortholog (200 ng each) in restoring the Y14/Magoh-loading activity to Δ4A3 NE. The mRNA pull-down efficiency is indicated below the relevant lanes.

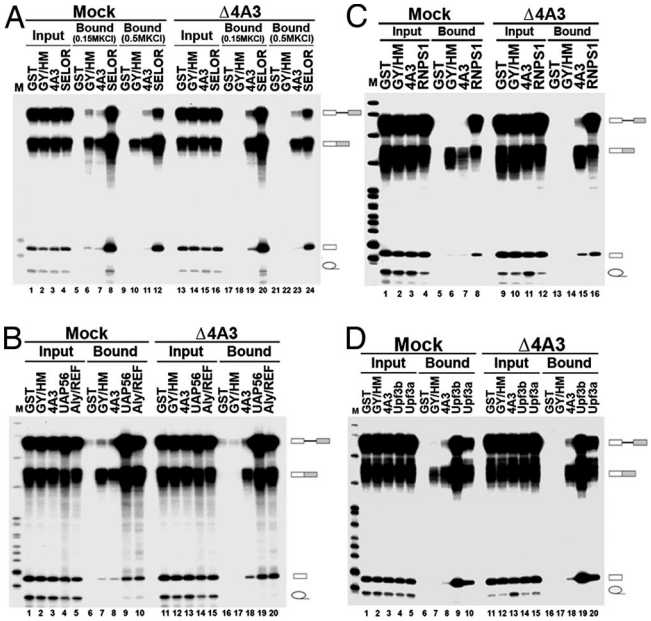


**Fig. 3.** Other EJC-associated factors bind to mRNA independently of eIF4A3. (*A*) eIF4A3 is not required for binding of MLN51 to mRNA but enhances its association with mRNA after splicing. GST pull-down assays were carried out from splicing reactions with β-globin pre-mRNA in the presence of GST–SELOR, GST-Y14/His-Magoh (GY/HM), or GST-eIF4A3 in either mock-depleted NE (lanes 1–12) or ΔeIF4A3 NE (lanes 13–24). After washing with 150 mM KCl buffer (lanes 5–8 and 17–20) or 500 mM KCl buffer (lanes 9–12 and 21–24), the bound RNA was recovered and analyzed by denaturing PAGE and autoradiography. Loading of UAP56 and Aly/REF (*B*), RNPS1 (*C*), and Upf3b and Upf3a (*D*) onto mRNA during splicing is not affected by depletion of eIF4A3. All of the tested proteins were GST-tagged. Fifteen percent to 20% of each reaction was loaded in the input lanes.

N-terminally truncated form of eIF4A3 was totally inactive (Fig. 2*C*, lane 15). This result is consistent with a previous report that the helicase activity of eIF4A3 is not required for EJC assembly and NMD (19). Interestingly, in the absence of human eIF4A3, *Drosophila* eIF4A3 [which is 84% identical and is dispensable for NMD in S2 cells (22)] could functionally replace its human ortholog for loading Y14/Magoh (Fig. 2*C*, lane 16), suggesting that loading of the EJC during pre-mRNA splicing may be an evolutionarily acquired function of the human spliceosome, or, alternatively, that the coupling of the EJC to NMD has been lost in *Drosophila*. We performed a similar EJC assembly assay using *Drosophila* S2 cell NE and observed a strong association of eIF4A3 with pre-mRNA, in contrast to HeLa NE (SI Fig. 8).

**Loading of Other EJC and Associated Components in the Absence of eIF4A3.** Multiple components of the EJC have been identified biochemically (3, 4). To investigate whether these EJC factors can still bind mRNA after eIF4A3 depletion, we again used GST pull-down assays in conjunction with *in vitro* splicing. We first purified GST-tagged Aly/REF, UAP56, RNPS1, SELOR [speckle localizer and RNA binding module, a conserved domain of MLN51 (27)], Upf3b, and Upf3a. We then added these proteins to *in vitro* splicing reactions with either mock-depleted or eIF4A3-depleted NE. In the absence of eIF4A3, all of these proteins were still loaded onto spliced mRNA (Fig. 3), indicating that different mechanisms are responsible for loading of these factors onto mRNA, compared with Y14/Magoh, which requires eIF4A3 for loading. Aly/REF, UAP56, RNPS1, MLN51 (SELOR), and Upf3b/3a not only pulled down the spliced mRNA, but, unlike eIF4A3, they also efficiently pulled down the pre-mRNA, again indicating that the loading of these factors may proceed separately and by a different mechanism from the loading of the EJC core. We also found that, unlike eIF4A3, these five factors could bind mRNA independently of splicing (data not shown).

MLN51 is one of the components of the stable EJC core [although its presence in the EJC has been questioned in a recent report (23)], and the crystal structure of its SELOR domain (amino acids 137–283) was recently solved (9, 10). eIF4A3 directly contacts the bound RNA in the crystal structure, but Phe-188 of SELOR stacks with an RNA base, suggesting that MLN51 enhances the overall RNA-binding affinity of the complex. Moreover, the SELOR domain by itself can bind RNA independently of splicing (24). GST–SELOR associated with mRNA and pre-mRNA in the presence or absence of eIF4A3 (Fig. 3*A*, lanes 8, 12, 20, and 24). Therefore, in addition to its association with eIF4A3, MLN51 is likely to simultaneously and directly bind RNA in the natural mRNP complex. Although SELOR bound both pre-mRNA and mRNA, the latter was considerably enriched in our pull-down assay, indicating that MLN51 may bind to pre-mRNA first, and only then interact with eIF4A3 and Y14/Magoh to form a stable EJC core after splicing. The resulting complex formed during splicing may bind mRNA more tightly, compared with the initial interaction with the pre-mRNA. Consistent with this notion, when we allowed the complex to assemble during splicing, and then increased the salt concentration in the washing step, we pulled down much less pre-mRNA compared with mRNA (Fig. 3*A*, lanes 8 and 12). In the absence of eIF4A3, the RNA-binding affinity of SELOR in high salt (500 mM KCl) was reduced (Fig. 3*A*, compare lanes 20 and 24), again suggesting that the assembled EJC enhances or stabilizes RNA binding by MLN51.

Using RNase H protection, we found that Upf3b, Upf3a, MLN51, Aly/REF, UAP56, and RNPS1 bound both the 5′ and 3′ fragments of the cleaved mRNA in the presence or absence of eIF4A3 (SI Fig. 9 and data not shown), indicating that, unlike binding of the EJC core, binding of MLN51 or Upf3b to mRNA is not restricted to 20–24 nt upstream of the exon–exon junction.

**Stable EJC Assembly onto mRNA Takes Place at the Late Stages of Splicing and Is Coupled with mRNA Release Mediated by HRH1/Prp22.** To address when and how eIF4A3 is recruited and assembled into the EJC, relative to discrete steps in splicing catalysis, we used a mutant substrate that can only undergo the first step of splicing (21). This human β-globin minigene transcript has a shortened exon 2, an improved polypyrimidine track, and an AG to GG mutation at the 3′ splice site to block the second transesterification step (25). When we used a control pre-mRNA with a WT AG dinucleotide (WT-AG), the GST pull-down products for each protein were similar to those we observed above with the standard β-globin pre-mRNA (Fig. 4*A*, lanes 1–12; compare with Fig. 3 and SI Fig. 7*A*). Y14/Magoh and eIF4A3 mainly pulled down spliced mRNA (Fig. 4*A*, lanes 8 and 9), as did Aly/REF, albeit with somewhat lower specificity (Fig. 4*A*, lane 11). RNPS1 preferentially pulled down pre-mRNA and lariat product (Fig. 4*A*, lane 10). Upf3b pulled down all of the RNA species (Fig. 4*A*, lane 12). For the 3′ splice site mutant substrate (mut-GG), because the splicing reaction is blocked after the first catalytic step, if eIF4A3 and other core EJC components have already been assembled into a stable complex on the pre-mRNA or intermediates at this stage, we would expect to pull down these RNA species. However, we observed that eIF4A3 and Y14/Magoh could not pull down the mut-GG pre-mRNA and first-step intermediates, indicating that the core components of the EJC are still loosely associated with the spliceosome and have not yet been loaded onto the RNA. RNPS1, Aly, and Upf3b pulled down the pre-mRNA, indicating that these factors are already stably associated with the pre-mRNA at this stage.

To further test when eIF4A3 is loaded, we purified the spliceosome by adding two MS2-binding sites at the 3′ end of the two splicing substrates. We then used purified MS2-GST fusion protein to pull down the *in vitro*-assembled spliceosomes and probed the

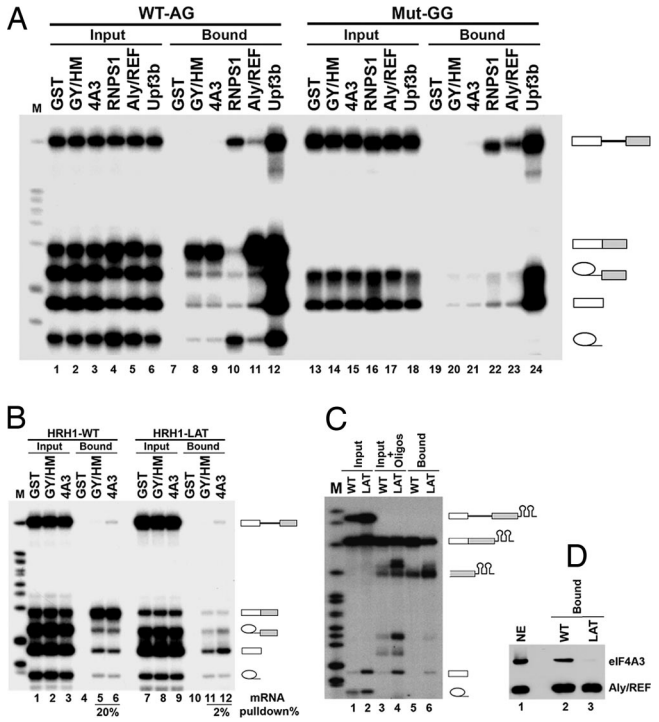


**Fig. 4.** Loading of eIF4A3 and assembly of the EJC core on mRNA occur at the late stages of splicing and require the helicase activity of HRH1/Prp22. (*A*) *In vitro* splicing and GST pull-down using WT (WT-AG; lanes 1–12) and 3′ splice site mutant (Mut-GG; lanes 13–24) pre-mRNAs. The GST fusion proteins added to each reaction are indicated above each lane. The mobilities of the splicing precursor, intermediates, and products are shown on the right. The Mut-GG substrate can undergo only the first step of splicing. (*B*) *In vitro* splicing and GST pull-down in the presence of WT (lanes 1–6) or LAT-mutant HRH1 (lanes 7–12) (200 ng). The mRNA pull-down efficiency is indicated below the gel. (*C*) Purification of *in vitro*-assembled mRNPs after splicing using β-globin pre-mRNA with two MS2-binding sites at the 3′ end of exon 2. Splicing reactions were incubated with GST-MS2 in the presence of WT or LAT HRH1 (lanes 1 and 2). After 3 h, DNA oligonucleotides complementary to the intron were added, and incubation was continued for 20 min (lanes 3 and 4). mRNPs were purified on glutathione-Sepharose, and bound RNAs were extracted and analyzed by denaturing PAGE (lanes 5 and 6). The purified 3′-cleaved products from pre-mRNA are also labeled (lanes 5 and 6). (*D*) Proteins from the purified mRNPs from *B* (lanes 5 and 6) were analyzed by Western blotting using antibodies against eIF4A3 and Aly/REF.

recovered material by Western blotting. We detected much more eIF4A3 and Y14 using the WT-AG substrate than the mut-GG substrate (SI Fig. 10), again indicating that eIF4A3 and Y14 are not yet assembled into a stable complex before the second step of splicing.

The splicing factor HRH1 and its yeast homologue, Prp22, which are ATP-dependent RNA helicases, play dual roles at the late stages of splicing: ligation of the two exons to form mature mRNA in an ATP-independent manner and release of the spliced mRNA from the spliceosome in an ATP-dependent manner (26). To determine whether the EJC assembles onto mRNA after exon ligation but before mRNA release, we took advantage of an HRH1/Prp22 ATPase/helicase dominant-negative mutant, "LAT" (Ser-717 to Leu), which supports exon ligation but blocks the release of spliced mRNA (27, 28). We purified WT and LAT HRH1 proteins expressed in 293E cells, added them to *in vitro* splicing reactions, and confirmed that the LAT mutant indeed inhibits mRNA release from the spliceosome (SI Fig. 11*A*).

We then tested whether the LAT mutant affects EJC assembly when mRNA release is blocked. Using the WT-AG substrate, loading of both Y14/Magoh and eIF4A3 was significantly reduced in the presence of LAT, from 20% pull-down efficiency (Fig. 4*B*, lanes 5 and 6) to 2% (Fig. 4*B*, lanes 11 and 12). The same result was obtained with the standard β-globin pre-mRNA (SI Fig. 11*B*). These data suggest that the EJC is not assembled onto spliced mRNA before the mRNA's release from the spliceosome.

To rule out the possibility that the EJC is actually assembled onto the mRNA but is buried within the spliceosome in such a way that the GST tag is not accessible, we used the above β-globin derivative with two MS2-binding sites at the end of exon 2. We added DNA oligonucleotides complementary to the intron sequence to allow endogenous RNase H to cleave contaminating pre-mRNA (21). In the presence of the LAT HRH1 mutant, the GST-MS2-bound mRNA represented ≈50% of the amount recovered when WT HRH1 was added to the reaction (Fig. 4*C*, lanes 5 and 6). The 3′ half of the cleaved pre-mRNA containing the MS2 sites was also pulled down. We then analyzed the purified mRNP (plus the 3′-cleaved product from pre-mRNA) by Western blotting (Fig. 4*D*). The mRNP generated in the presence of WT HRH1 contained >10-fold more eIF4A3 than the mRNP generated in the presence of the LAT mutant, whereas there was only a slight difference in the amount of Aly/REF (Fig. 4*D*, lanes 2 and 3). We conclude that the mRNA that has not yet been released lacks an assembled EJC core. Thus, the spliceosome may undergo an ATP-dependent conformational change to release the spliced mRNA and to provide the assembly site for deposition of the EJC core at the exon–exon junction.

## Discussion

We have carried out a series of biochemical assays to address several questions about the function of eIF4A3 in pre-mRNA splicing and in splicing-dependent EJC assembly and mRNP remodeling. We provided evidence that eIF4A3 is not required for constitutive pre-mRNA splicing but is essential for assembly of the EJC core, particularly for loading the Y14/Magoh heterodimer onto mRNA in a splicing-dependent manner. We found that other EJC-associated factors, such as MLN51, Aly/REF, UAP56, Upf3b/3a, and RNPS1, can bind mRNA independently of eIF4A3, in contrast to previous suggestions that the EJC core is responsible for recruitment of these factors onto mRNA (4). This eIF4A3-dependent and -independent recruitment of different mRNA-binding proteins onto the mRNA during splicing suggests that the mRNP architecture undergoes a rearrangement through interactions among each EJC and other preloaded mRNA-binding proteins. The resulting conformation of the mRNP might influence downstream events, such as mRNA export, translation, and NMD. We also found that eIF4A3 is assembled onto the EJC late in the splicing reaction, and the final deposition of the EJC onto mRNA requires the helicase activity of the mRNA-release factor HRH1.

**The Role of EJC Core Components in Pre-mRNA Splicing and Their Association with the Spliceosome.** The composition of human spliceosomal complexes has been extensively studied, and ≈200 spliceosomal proteins have been identified (14, 15). Several groups found the EJC core components Y14/Magoh and eIF4A3 in the spliceosome, but whether these factors are involved in pre-mRNA splicing was unclear. We immunodepleted the EJC core component eIF4A3 from HeLa NE and found that eIF4A3-depleted NE is fully functional in splicing, indicating that the EJC core is dispensable for constitutive pre-mRNA splicing. We cannot rule out that residual amounts of eIF4A3 might support splicing of the various pre-mRNAs, but we think that this is very unlikely. First, the extent of eIF4A3 depletion was sufficient to abrogate loading of Y14/Magoh. Second, there was no difference in splicing efficiency between mock- and eIF4A3-depleted NE when we used higher amounts of pre-mRNA (data not shown). Another formal possibility is that other factors may replace eIF4A3 in splicing, e.g., other spliceosomal RNA helicases.

Our observation that eIF4A3 is dispensable for splicing provides further evidence supporting an additional role of the splicing machinery (besides removing introns) in facilitating the loading of various RNA-binding proteins, e.g., the EJC, or altering the reversible modification state of some mRNA-bound proteins during splicing (2). This mRNP remodeling may affect the fate of the mRNA by altering the mRNP structure, and/or through interactions between the loaded proteins and other molecules. It is widely believed that the fate of an mRNA is mainly determined by its associated proteins (29), and an mRNA that has undergone splicing is clearly distinct from an mRNA with identical sequence that is transcribed from an intronless cDNA (30, 31).

**Recruitment of EJC Core Components onto mRNA and EJC Assembly.** We have shown here that the assembly of the EJC proceeds in a stepwise manner: some of the factors are loaded onto pre-mRNA first, and others bind only to the mRNA after splicing, which is consistent with previous work (32). Among the EJC core components, MLN51 appears to bind RNA nonspecifically via its SELOR domain. This binding is not splicing-dependent, because we showed that SELOR binds both pre-mRNA and mRNA. We speculate that MLN51 may bind pre-mRNA on its own, subsequently becoming associated with eIF4A3 on the spliced mRNA. After splicing is completed, eIF4A3 binds specifically to the mRNA upstream of the exon–exon junction, and the loading of eIF4A3 provides a binding surface for loading of Y14/Magoh onto mRNA. Then the prebound MLN51 may join in to form the stable core of the EJC, which in turn can interact with other mRNA-binding proteins that may be already preloaded onto the mRNA, such as Upf3b/3a, to establish a specific mRNP architecture. MLN51 interacts with the EJC component Magoh in a partially RNase-sensitive manner (24). We found that, in the absence of eIF4A3, loading of MLN51 onto mRNA is only slightly reduced, but not abolished, in contrast to loading of Y14/Magoh, which is strongly inhibited. This finding suggests that MLN51 can directly bind RNA independently of the EJC, and its additional interaction with eIF4A3-Y14/Magoh can stabilize the EJC core complex.

eIF4A3 and other EJC factors can be detected in the purified spliceosomal C complex by mass spectrometry (15, 23). Our data indicate that the final assembly of the EJC core takes place after the second step of splicing. We expect that eIF4A3 is recruited by the spliceosome before the second step of splicing but is only loosely associated with the complex. In this scenario, before the second step of splicing, eIF4A3 and other EJC factors may not have been assembled into a stable complex on the mRNA, and thus they would be readily dissociated during our purification. Therefore, we think that stable assembly of the EJC core complex (as opposed to initial recruitment) takes place during or after the second step of splicing. Our data further demonstrate that the helicase activity of the mRNA-release factor HRH1 is required for assembly of the EJC on the mRNA after splicing, indicating that EJC assembly occurs in conjunction with the mRNA's release from the spliceosome. In the spliceosomal C complex, the EJC assembly site near the exon–exon junction region is buried, as the last ≈30 nt of the first exon are protected from RNase H (15). HRH1 releases the spliced mRNA from the spliceosome in an ATP-dependent manner and exposes the binding site for EJC assembly. Hence, HRH1 may facilitate extensive rearrangements near the exon–exon junction, leading to the final release of the spliced mRNA and the deposition of the EJC upstream of the exon–exon junction.

Aly/REF, RNPS1, and Upf3b/3a appear to be loaded onto mRNA by a different mechanism(s) compared with eIF4A3 and Magoh/Y14. They bind not only to the mRNA, but to the pre-mRNA as well, whether directly or indirectly, or perhaps nonspecifically in the context of the prespliceosomal H complex. RNPS1, which is also a general splicing activator, bound preferentially to the pre-mRNA (33). Aly/REF and Upf3b/3a also bound both pre-mRNA and mRNA, but we observed an enrichment for the mRNA in the GST pull-down assays, suggesting that the splicing machinery can facilitate the loading of these factors onto mRNA (or stabilize their preexisting interactions) during splicing. This might reflect splicing-dependent EJC formation, which would stabilize the binding of these proteins onto mRNA through their interactions with the EJC core. It was recently demonstrated that Aly/REF actually binds mRNA upstream of the EJC and forms a stable complex with the CBC (cap-binding complex) and TREX (transcription/export) complex at the mRNA 5′ end that plays a critical role in directing mRNA nuclear export (34).

The EJC-independent binding of Upf3b/3a onto mRNA was unexpected, because the RRM (RNA-recognition motif) of Upf3b interacts with Upf2 instead of binding RNA (35). Using a pull-down assay, we found that full-length Upf3b can bind RNA directly (data not shown), but, in light of its very basic character, we do not know whether Upf3b binds mRNA *in vivo* by direct RNA–protein interaction or through a more elaborate mechanism.

**Remodeling of the mRNP by the EJC and Implications for NMD.** Although most NMD factors are conserved, the mechanisms of NMD appear to be distinct in different species, particularly with respect to how the premature termination codon (PTC) is recognized and distinguished from the normal stop codon (36). NMD requires active translation, and it is thought that cross-talk between the stalled ribosome at a PTC and a downstream feature of the mRNA somehow triggers the NMD response. In yeast and *Drosophila*, the distance between the PTC and the 3′ UTR appears to be critical. In humans, the exon–exon junction boundary and the splicing-dependent assembly of the EJC play important roles (36). In mammalian cells, the prevailing model proposes that the deposition of the EJC during splicing is followed by the recruitment of Upf3b/3a, which in turn interact with Upf2 and Upf1. These proteins form a surveillance complex downstream of the PTC. Cross-talk between this complex and the translation–termination complex when the ribosome reaches the stop codon defines the PTC by an unknown mechanism, triggering the rapid degradation of the target message (37).

Our GST pull-down results are inconsistent with the current EJC-centric model of mRNP assembly (4), according to which it is the assembled EJC core that recruits other factors (Upf3b, Upf3a, RNPS1, and Aly/REF) onto spliced mRNA. We propose instead that these factors can bind to both pre-mRNA and mRNA even before the EJC core is formed. After splicing is completed and the EJC core is assembled, these factors may establish contacts with the EJC through specific protein–protein interactions to form a functional mRNP structure, although at the same time they may retain contacts with other regions of the mRNA.

Our data also suggest that the Upf proteins may actually not be recruited to the mRNA by the EJC during splicing. For instance, Upf3b/3a appear to be preloaded onto the mRNA even before the EJC is assembled. We also found that Upf3b/3a alone have nonspecific RNA-binding activity (data not shown). Both Upf1 and Upf2 bind RNA nonspecifically as well (35, 38). Considering that splicing and EJC loading are required for NMD in mammalian cells, we propose that, after the EJC is loaded onto mRNA, this complex can interact with other mRNA-binding proteins that are already prebound, such as Upf3b/3a, resulting in remodeling of the mRNP conformation. This EJC-directed mRNP remodeling would alter both the local and the overall structure or conformation of the mRNP. The ultimate functional structure may be critical for the downstream fate of the mRNA. Because eukaryotic genes usually contain multiple introns, multiple EJCs can be loaded onto a fully spliced mRNA (34). Each prebound protein on the mRNA may interact with an EJC that is either downstream or upstream, potentially resulting in conformational heterogeneity of the mRNP. When an mRNA harbors a PTC and a translating ribosome reaches it, the local mRNP composition and structure around the PTC may enable the ribosome-termination complex to recognize this stop

codon as an abnormal one. Because of the likely heterogeneity of mRNP structures, some of them may make the PTC easily recognizable; some may cause the recognition of the PTC to be less efficient; and others may mask the PTC in such a way that the mRNA can escape NMD. This heterogeneity may explain why NMD is seldom 100% efficient and why some PTC-containing mRNAs are immune to NMD (39).

For many PTC-containing transcripts, when the distance between the PTC and the last EJC is <50–55 nt, the mRNA can escape NMD, although the reason for this is not known (37). Based on our model, we speculate that this minimal distance constraint may reflect an unfavorable mRNP conformation around such a PTC. Because an elongating ribosome should displace the bound proteins on the mRNA, when it reaches a PTC located very close to the last EJC, the complex interactions between the EJC and the various upstream-bound proteins would already have been disrupted. This may cause a profound change in the local mRNP conformation, which might lead to an unrecognizable PTC.

Concerning the polarity of NMD (40), i.e., the finding that the strength of NMD correlates with the position of the PTC, such that PTCs proximal to the downstream terminal intron tend to elicit robust NMD effects, it is also conceivable that the local mRNP conformation and composition may determine the strength of the NMD effect. A recent report showed that improving the splicing efficiency of an intron downstream of a PTC elicits stronger NMD compared with a weak intron at the same position (41). More efficient and rapid loading of the EJC may account for this NMD-promoting effect by splicing of a particular intron. Based on our model, we think that the spliceosome can remodel the mRNP architecture as splicing of each intron takes place, and thereafter. The timing of each splicing event may therefore influence the local mRNP structure. It is also known that the position of an intron within a gene can affect the fate of the mRNA (30, 42). We can therefore imagine that the intron position dictates where the EJC is deposited, which in turn alters the local or overall mRNP architecture, with consequent effects on translation, localization, export, and turnover of the mRNA.

## Materials and Methods

**Protein Expression and Purification.** All plasmids are described in *SI Materials and Methods*. GST-eIF4A3 was expressed in and purified from *E. coli* strain BL21-DE3(pLysS) using glutathione-Sepharose beads. GST-RNPS1 and GST-Upf3b/3a were expressed in and purified from HEK-293E cells (43). GST-Y14/His-Magoh was purified as described (44). The WT and LAT-mutant HRH1 proteins were overexpressed in 293E cells and purified on Ni-NTA agarose beads. The proteins were dialyzed against buffer D (45).

***In Vitro* Splicing, Immunodepletion, and RNA GST Pull-Down.** *In vitro* splicing was performed as described (45). For RNA GST pull-downs, 4 $\mu$g of GST fusion protein was added to a 50-$\mu$l splicing reaction. After 2 h, 5–10 $\mu$l was kept as the input control; 10 $\mu$l of glutathione beads was added to the remainder, and the volume was increased to 400 $\mu$l with washing buffer [20 mM Hepes, pH 8.0/150 mM KCl/0.05% (vol/vol) Nonidet P-40]. After rocking for 1–2 h at 4°C, the beads were washed four times with cold washing buffer. After addition of 300 $\mu$l of stop solution (45), the RNA was recovered by phenol extraction and ethanol precipitation. Immunodepletion was carried out as described (46).

**Spliceosome Purification and Western Blotting.** One microgram of $^{32}$P-labeled pre-mRNA (WT-MS2 and Mut-GG-MS2) was incubated in a 1-ml splicing reaction with 12 $\mu$g of GST-MS2 protein. Spliceosomal complexes were affinity-purified on glutathione-Sepharose. The beads were washed four times; 1/10th was used for RNA analysis, and the rest was used for Western blotting.

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