Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly

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Recent studies indicate that splicing of pre-messenger RNA and export of mRNA are normally coupled *in vivo*[1–6]. During splicing, the conserved mRNA export factor Aly is recruited to the spliced mRNA–protein complex (mRNP), which targets the mRNA for export. At present, it is not known how Aly is recruited to the spliced mRNP. Here we show that the conserved DEAD-box helicase UAP56, which functions during spliceosome assembly[7–10], interacts directly and highly specifically with Aly. Moreover, UAP56 is present together with Aly in the spliced mRNP. Significantly, excess UAP56 is a potent dominant negative inhibitor of mRNA export. Excess UAP56 also inhibits the recruitment of Aly to the spliced mRNP. Furthermore, a mutation in Aly that blocks its interaction with UAP56 prevents recruitment of Aly to the spliced mRNP. These data suggest that the splicing factor UAP56 functions in coupling the splicing and export machineries by recruiting Aly to the spliced mRNP.

Splicing of pre-mRNA generates a specific mRNP complex that promotes mRNA export by recruiting the mRNA export factor Aly[2]. Previous studies showed that a glutathione *S*-transferase (GST)–Aly fusion protein injected into oocytes of the frog *Xenopus* is specifically incorporated into this spliced mRNP[2]. To screen for factors that mediate the recruitment of Aly to the spliced mRNP, we characterized the proteins that are bound to GST–Aly. Oocyte nuclei containing either GST–Aly or a GST control protein were incubated with glutathione beads and the bound proteins were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1a). Proteins unique to the Aly pull-down were microsequenced revealing the splicing factor UAP56 (ref. 7) as well as several highly abundant nuclear proteins (for example, nucleolin and lamin) (Fig. 1a, lane 2).

UAP56 is a member of the DEAD-box family of splicing factors, which have ATPase and RNA helicase motifs[11]. The counterpart of UAP56 in the yeast *Saccharomyces cerevisiae*, Sub2p, has recently been identified and shown to function during spliceosome assembly[8–10]. Aly also has a counterpart in *S. cerevisiae*, Yra1p, that is essential for mRNA export[12]. The high levels of conservation between the yeast and metazoan splicing and export machineries suggest that the recruitment of Aly/Yra1p to the mRNP involves a conserved factor. Thus, our additional studies focused on the conserved splicing factor, UAP56.

To further investigate the relationship between Aly and UAP56, we asked whether these proteins also interact in mammalian cells. As shown by western analysis (Fig. 1b, top), Aly was specifically

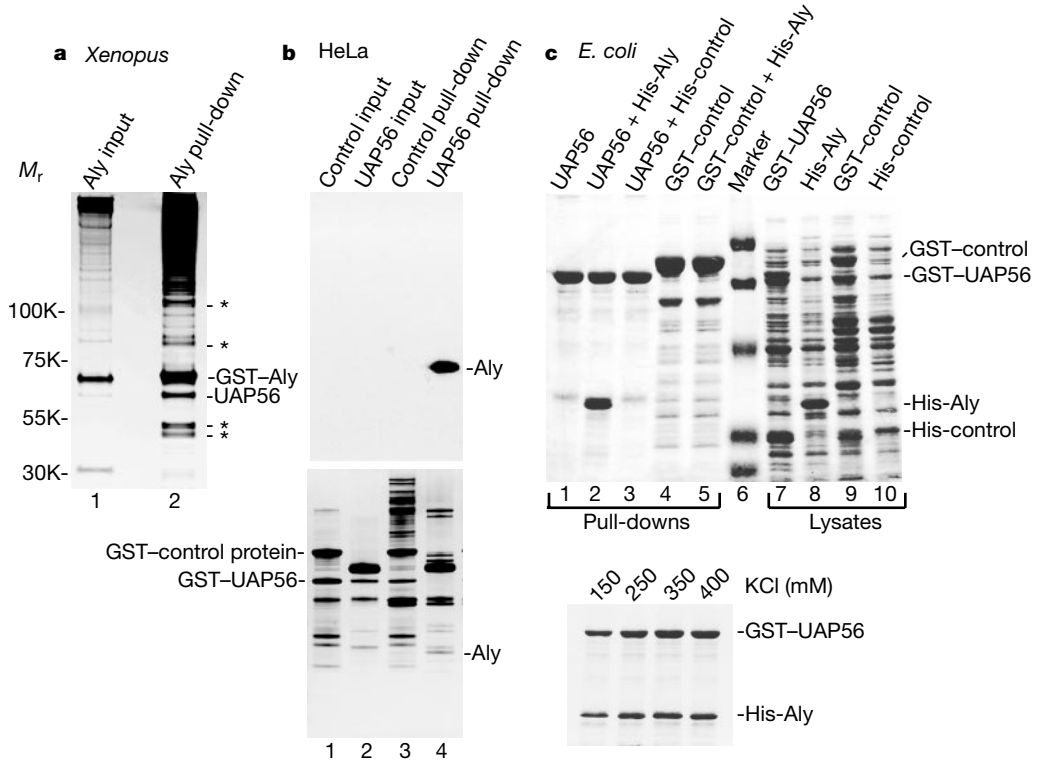

Figure 1 The mRNA export factor Aly interacts with the splicing factor UAP56. Aly interacts with UAP56 in *Xenopus* oocyte nuclei (**a**), HeLa nuclear extract (**b**) and *E. coli* lysate (**c**). **a**, GST–Aly (400 fmol) (lane 1) was injected into oocyte cytoplasm for import into nuclei followed by binding to glutathione beads[2]. Bound proteins were detected on an SDS gel by silver stain. Mass spectrometry identified UAP56 and proteins (from top, indicated by asterisks: nucleolin, lamin L, TAF-Iβ and nucleoplasmin). **b**, GST control protein or GST–UAP56 (1 μg each, lanes 1, 2) was incubated with HeLa nuclear extract (7.5 μl). Proteins bound to glutathione beads (lanes 3 and 4) were analysed by western blot (top) or silver staining (bottom). **c**, *E. coli* lysates containing GST–UAP56, His-Aly or negative control proteins were mixed together in binding buffer containing KCl at 250 mM (top) or the indicated concentrations (bottom). Proteins bound to glutathione beads and lysates were detected by Coomassie stain.

selected from HeLa nuclear extract by GST–UAP56, but not by a negative control GST-fusion protein. A silver-stained gel containing the same samples showed that the pull-down of Aly by GST–UAP56 is highly specific as few bands other than Aly were detected (Fig. 1b, bottom, lane 4; the band designated Aly was confirmed by microsequencing). We conclude that Aly and UAP56 can associate with each other in both *Xenopus* and HeLa nuclei.

To determine whether Aly and UAP56 interact directly, GST–UAP56 and histidine-tagged Aly (His-Aly) were expressed in *Escherichia coli*, the total lysates containing each protein were mixed, and glutathione beads were added. A stoichiometric complex is formed between UAP56 and Aly (Fig. 1c, top, lane 2). The interaction between these two proteins is specific, as GST–UAP56 does not interact with any other protein in the total *E. coli* lysates. Moreover, no interaction was detected between GST–UAP56 and a negative control His-fusion protein (lane 3) nor between His-Aly and a negative control GST-fusion protein (lane 5). The interaction between Aly and UAP56 is also resistant to RNase (data not shown). We conclude that Aly and UAP56 interact directly and with high specificity. Moreover, the complex formed between these two proteins is stable in salt concentrations ranging from 150 to 400 mM, indicating that Aly and UAP56 interact tightly (Fig. 1c, bottom).

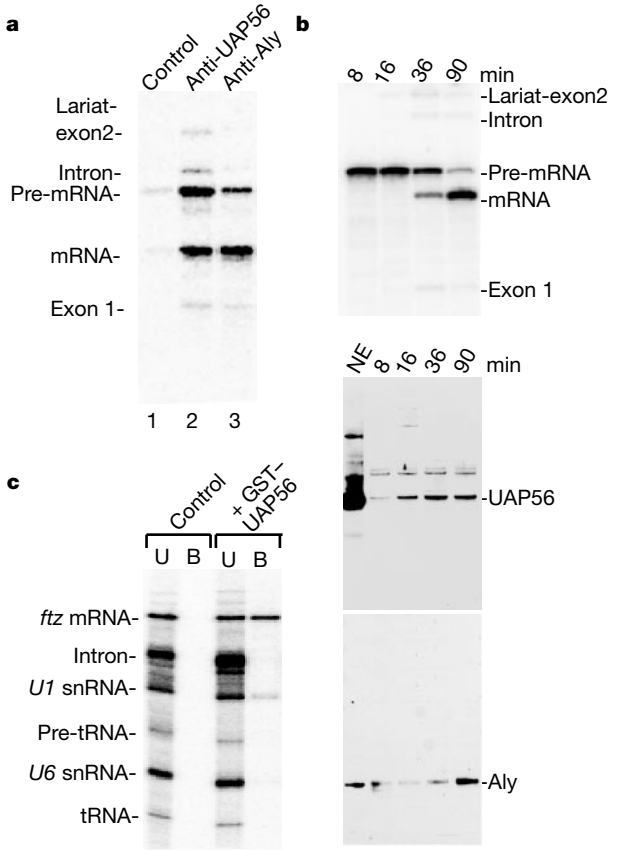

Figure 2 UAP56 is present in the spliced mRNP. **a**, *AdML* pre-mRNA was incubated in HeLa nuclear extract under splicing conditions for 45 min. After gel filtration, the fraction containing spliceosomes and spliced mRNP was incubated with protein A beads coupled to control, UAP56 or Aly antibodies. **b**, Spliceosomal complexes assembled on *AdML* pre-mRNA for the indicated times were purified[2,20] in 60 mM NaCl. RNA (top panel) or western blot of purified complexes were probed with UAP56 (middle panel) or Aly (bottom panel) antibodies. NE, nuclear extract. **c**, Buffer or 55 fmol of GST–UAP56 was injected into oocyte cytoplasm. *ftz* pre-mRNA, pre-tRNA^{ser} and *U1* and *U6* snRNAs were then injected into oocyte nuclei and incubated for 1 h. GST pull-downs were carried out[2] and total unbound and bound RNAs analysed.

UAP56 was originally identified as a protein that interacts with U2AF65, a splicing factor that binds to the 3′ splice site and recruits *U2* snRNP (a small nuclear RNA–protein complex) early in spliceosome assembly[7]. Recent studies indicate that UAP56/Sub2p has multiple tasks in spliceosome assembly, including dissociation of U2AF65 from the spliceosome[8–10]. Because UAP56 also interacts with an mRNA export factor (Aly), we asked whether UAP56, like Aly, is present in the spliced mRNP. Consistent with previous studies, an antibody to UAP56 immunoprecipitates the pre-mRNA (Fig. 2a, lane 2). Significantly, this antibody also immunoprecipitates the spliced mRNA, as does an antibody to Aly (Fig. 2a, lanes 2 and 3). These data suggest that UAP56 is present in the spliced mRNP. To verify that the immunoprecipitation of the spliced mRNP is not due to a crossreacting epitope, western analysis of purified spliceosomal complexes was carried out using the UAP56 and Aly antibodies. UAP56 is detected in the assembling spliceosome and is still present at 90 min, when the spliced mRNA is the major RNA species detected (Fig. 2b). Consistent with previous work[2], Aly is also detected at 90 min (Fig. 2b). Thus, the immunoprecipitation and western data indicate that UAP56 is present with Aly in the spliced mRNP.

To obtain further evidence that UAP56 is associated with the spliced mRNP, we carried out an *in vivo* pull-down assay. To do this, we injected GST–UAP56 into *Xenopus* oocytes and then injected *fushi tarazu* (*ftz*) pre-mRNA and control RNAs. After incubating the oocytes to allow splicing, nuclei were isolated and glutathione beads were added to the lysed nuclei. The spliced mRNA is efficiently bound to GST–UAP56 whereas the intron, *U1* snRNA, pre-tRNA, *U6* snRNA and tRNA are not (Fig. 2c). We conclude that UAP56, like Aly[2], associates with the spliced mRNP both *in vitro* and *in vivo*. Neither UAP56 nor Aly[13] is detected in the spliced mRNP in the cytoplasm (data not shown).

UAP56 and Aly interact directly and both proteins are present in the spliced mRNP; this suggests that UAP56, in addition to functioning in splicing, functions with Aly in mRNA export. To test this possibility, we injected increasing amounts of His-tagged UAP56 into *Xenopus* oocytes and examined the export efficiency of several RNAs. Strikingly, excess UAP56 inhibits the export of *AdML* mRNA in a dose-dependent manner (Fig. 3a). The export of tRNA and *U1* snRNA is not significantly affected by excess UAP56, indicating that the export block is specific to mRNA. The export inhibition seems to be general, as it was also observed with another mRNA (*ftz*, Fig. 3b). Analysis of the kinetics of export showed that excess UAP56 interferes with export throughout the time course (data not shown). We conclude that excess UAP56 functions as a dominant negative inhibitor of mRNA export. Notably, excess UAP56 does not affect export of the constitutive transport element (CTE) (Fig. 3c). This viral RNA element is exported by directly binding to Tip-associated protein (TAP), a late-acting mRNA export factor that interacts with Aly and the nuclear pore complex (reviewed in ref. 14). We conclude that excess UAP56 interferes with a factor or factors upstream of TAP in the mRNA export pathway.

Previous work showed that Aly is a limiting factor for mRNA export in oocytes (refs 2, 4; see also Fig. 4a, lanes 1, 2, 9–12). Thus, excess UAP56 may inhibit mRNA export by binding to and sequestering Aly, thereby preventing its recruitment to the spliced mRNP. Consistent with this possibility, the export block by excess UAP56 can be relieved by excess Aly in a dose-dependent manner (Fig. 4a, lanes 1–8). To determine whether excess UAP56 also prevents the recruitment of Aly to the spliced mRNP, we injected oocytes with GST–Aly without UAP56 or in the presence of increasing amounts of UAP56. Western analysis shows that GST–Aly is present at similar levels in all of the nuclei of injected oocytes (Fig. 4b, top panel). A GST pull-down assay was then used to detect Aly in the spliced mRNP. Excess UAP56 inhibits the recruitment of Aly to the spliced mRNP in a dose-dependent manner (Fig. 4b, bottom panel).

The data presented above provide evidence that UAP56 functions in recruiting Aly to the spliced mRNP. To further test this possibility, we generated a mutation in GST–Aly that inhibits its association with UAP56 (Fig. 5a). As shown by the *in vitro* binding assay (Fig. 5b), wild-type Aly or an Aly RGG-domain mutant (ΔRGG) binds UAP56 efficiently, whereas an Aly carboxy-terminal mutant (ΔC) does not. Both of the mutants are imported into oocyte nuclei (Fig. 5c) and bind TAP (data not shown) as efficiently as wild-type Aly, indicating that the mutants are functional proteins.

To determine whether the Aly mutants can be incorporated into the spliced mRNP, we injected the mutant and wild-type proteins into oocytes and carried out a GST pull-down assay from the oocyte nuclei (Fig. 5d). Significantly, mRNA was efficiently pulled down by wild-type Aly and ΔRGG Aly but not by ΔC Aly. Thus, the Aly mutant that fails to bind UAP56 efficiently also inhibits the recruitment of Aly to the spliced mRNP. These data provide further

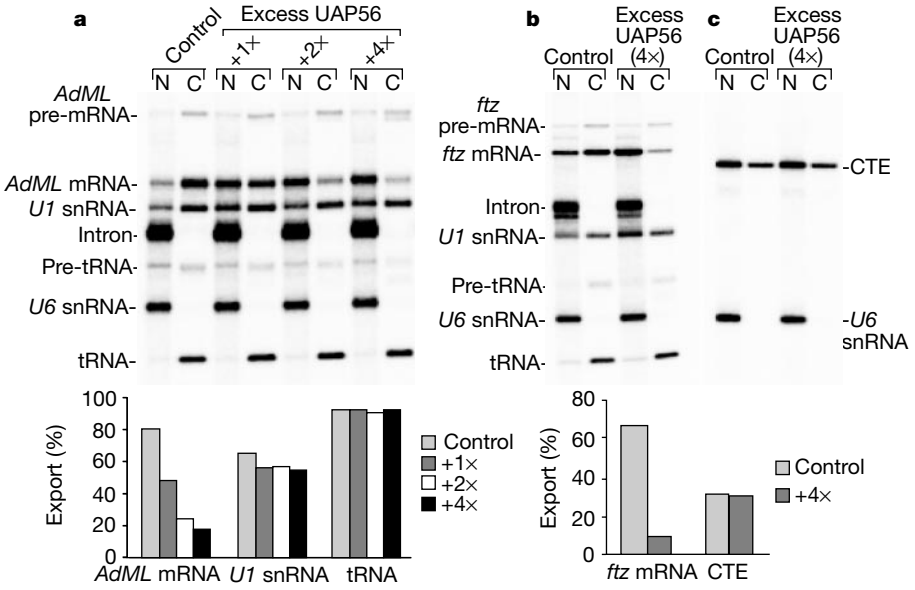

Figure 3 Excess UAP56 inhibits mRNA export. **a**, Buffer or His-UAP56 (1× = 140 fmol) was injected into oocyte cytoplasm. *AdML* pre-mRNA, pre-tRNA^{ser} and *U1* and *U6* snRNAs were then injected into nuclei. Oocytes were incubated for 3 h. **b**, Buffer or 4× His-UAP56 was injected as in **a**. *ftz* pre-mRNA, pre-tRNA^{ser} and *U1* and *U6* snRNAs were then injected into nuclei and incubated for 2 h. **c**, Same as **b** except that CTE RNA and *U6* snRNA were injected into nuclei and incubated for 75 min. RNA from the nucleus (N) or cytoplasm (C) was analysed.

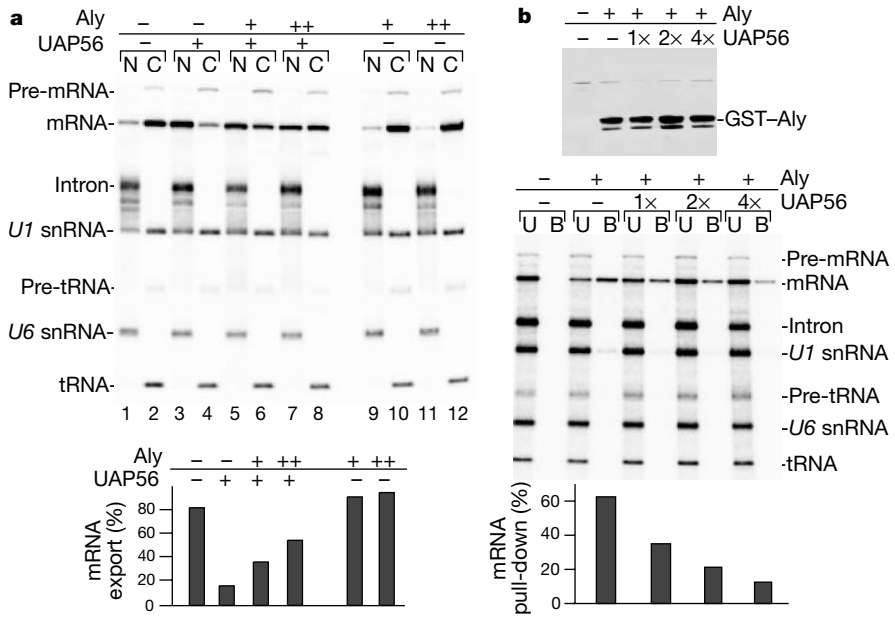

Figure 4 UAP56 recruits Aly to the spliced mRNP. **a**, Excess Aly relieves the UAP56 mRNA export block. Buffer (–), His-UAP56 (280 fmol, +), His-Aly (70 fmol, +) or His-Aly (140 fmol, ++) were injected into oocyte cytoplasm as indicated. *ftz* pre-mRNA, pre-tRNA^{ser} and *U1* and *U6* snRNAs were then injected into nuclei and incubated for 2.5 h. **b**, Excess UAP56 inhibits recruitment of Aly to the spliced mRNP. Buffer or His-UAP56 was injected as in Fig. 3a. After overnight incubation, buffer or 50 fmol of GST–Aly was injected into cytoplasm and incubated for 4 h. Top, GST–Aly in nuclei was analysed by western blot. Bottom, *ftz* pre-mRNA, pre-tRNA^{ser} and *U1* and *U6* snRNAs were injected into nuclei and incubated for 45 min. GST pull-downs from nuclei were carried out.

evidence that UAP56 functions in recruiting Aly to the spliced mRNP.

In this study, we have shown that the mRNA export factor Aly, which is recruited to the mRNA during splicing, specifically interacts with the splicing factor UAP56. This protein is also present with Aly in the spliced mRNP, a complex that targets the mRNA for export. Functional studies show that excess UAP56 is a dominant negative inhibitor of mRNA export and prevents the recruitment of Aly to the spliced mRNP. In addition, a mutation in Aly that inhibits its binding to UAP56 also blocks recruitment of Aly to the spliced mRNP. Together, these data indicate that coupling between the splicing and export machineries involves a direct interaction between UAP56 and Aly. Our previous work indicated that the spliced mRNP is a large spliceosome-sized complex[1] and, thus, is likely to undergo significant remodelling before transit through the pore. The observation that UAP56 is present in the spliced mRNP only in the nucleus raises the possibility that this DEAD-box helicase functions in nuclear remodelling. A number of components of the mRNA export machinery are conserved from yeast to higher eukaryotes, including Aly, TAP/p15, hGle1, hGle2 and hDbp5p (reviewed in ref. 14). Recent studies revealed that the essential yeast splicing factor Sub2p is the homologue of UAP56 (refs 8–10). In an accompanying paper[23], Sub2p is shown to interact physically and genetically with the yeast homologue of Aly, Yra1p, and that Sub2p is required for export of mRNAs derived from both intron-containing and naturally intron-lacking genes. Thus, UAP56/Sub2p may play a general conserved role in recruiting Aly/Yra1p to mRNPs. □

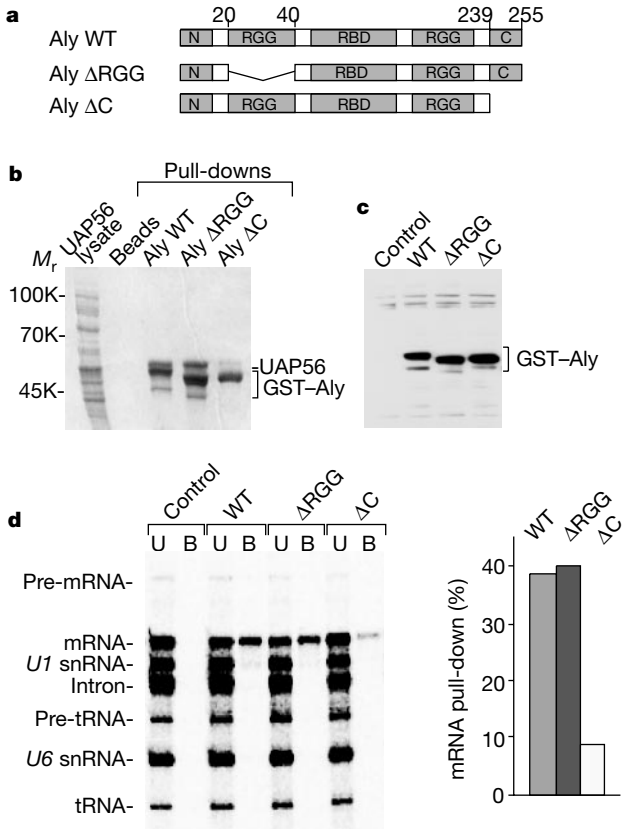

Figure 5 The UAP56–Aly interaction is required for Aly recruitment to the spliced mRNP. **a**, Schematic of Aly showing deletions. WT, wild type. **b**, GST–Aly wild type, ΔRGG or ΔC pre-bound to glutathione beads was rotated with His-UAP56 lysate. Bound proteins were analysed. **c**, Buffer or 50 fmol of GST–Aly wild type, ΔRGG or ΔC was injected into oocyte cytoplasm. GST–Aly in nuclei was analysed by western blot. **d**, Oocytes were injected as in **c**. *AdML* pre-mRNA, pre-tRNA^{ser} and *U1* and *U6* snRNAs were then injected into nuclei and incubated for 1 h. GST pull-downs of nuclei were carried out.

Methods

Plasmids encoding His-Aly (also named BEF)[15], GST–Aly[16], GST–UAP56[7], *AdML* and *ftz* pre-mRNAs, *U1* and *U6* snRNAs and pre-tRNA^{Ser} were described[1,2]. Negative control proteins were GST–Sir2[17] and His-Ran[18]. The plasmid encoding His-UAP56 was constructed by inserting the cDNA of UAP56 into protein expression vector pET9d-HIS6-TB at *Nco*I and *Bam*HI sites. The Aly ΔRGG mutant was obtained by polymerase chain reaction (PCR); the ΔC mutant was constructed by deletion of the *Pst*I fragment that contains the Aly C-terminal coding region from the expression vector[16]. Cytoplasmic injection to import proteins[2], nuclear injection and oocyte isolation were carried out as described[19]. GST pull-down assay of the *in vivo* complexes was as previously described[2]. Spliceosomal complexes were purified using affinity purification with maltose-binding protein[2,20]. Western blots were probed with antibodies against UAP56[7] and Aly[21]. Proteins that interact with GST–UAP56 or GST–Aly were identified by matrix-assisted laser desorption ionization (MALDI) peptide mapping and nano-electrospray tandem mass spectrometry[22].

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