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## **Multiple factors in the early splicing complex are involved in the nuclear retention of pre-mRNAs in mammalian cells**

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## Abstract

Intron-containing pre-mRNAs are retained in the nucleus until they are spliced. This mechanism is essential for proper gene expression. Although the formation of splicing complexes on pre-mRNAs is thought to be responsible for this nuclear retention activity, the details are poorly understood. In mammalian cells in particular, very little information is available regarding the retention factors. Using a model reporter gene, we show here that U1 snRNP and U2AF but not U2 snRNP are essential for the nuclear retention of pre-mRNAs in mammalian cells, demonstrating that E complex is the major entity responsible the nuclear retention of pre-mRNAs in mammalian cells. By focusing on factors that bind to the 3'-splice site region, we found that the 65-kD subunit of U2AF (U2AF<sup>65</sup>) is important for nuclear retention and that its multiple domains have nuclear retention activity *per se*. We also provide evidence that UAP56, a DExD-box RNA helicase involved in both RNA splicing and export, cooperates with U2AF<sup>65</sup> in exerting nuclear retention activity. Our findings provide new information regarding the pre-mRNA nuclear retention factors in mammalian cells.

## Introduction

The eukaryotic cell prevents the translation of pre-mRNAs in many ways, because aberrant proteins with dominant-negative activities would otherwise be produced. First, pre-mRNAs are retained in the nucleus until they are spliced. This mechanism also ensures efficient pre-mRNA splicing by factors that are confined to the nucleus. Second, mRNA export factors are preferentially recruited to spliced mRNAs (reviewed in, Reed 2003; Kelly & Corbett 2009). Third, pre-mRNAs that still leak into the cytoplasm are degraded by nonsense codon-mediated mRNA decay (reviewed in, Bhuvanagiri *et al.* 2010).

The first of these mechanisms, the pre-mRNA nuclear retention, is the least well understood. This retention is thought to be achieved by the formation of early splicing complexes before the formation of the catalytically active spliceosome (Legrain & Rosbash 1989). The spliceosome is a large complex consisting of over 100 components, both proteins and RNAs (reviewed in, Wahl *et al.* 2009; Valadkhan & Jaladat 2010). The spliceosome is formed through the ordered, stepwise assembly of discrete factors onto the pre-mRNA substrate. The earliest

recognition of pre-mRNAs involves three factors: the U1 small nuclear RNP (snRNP) binds to the 5' splice site (5'-ss) of the pre-mRNA; U2AF (U2 snRNP auxiliary factor), a heterodimer of U2AF<sup>65</sup> and U2AF<sup>35</sup>, binds to the polypyrimidine tract (PPT) and the 3' splice site (3'-ss); and SF1/BBP (splicing factor 1/ branch point binding protein) binds to the branch point sequence (BPS). The resultant complex is called the 'commitment complex' in yeast and the 'early (E) complex' in mammals.

The subsequent step is the replacement of SF1/BBP with the 17S U2 snRNP, which consists of the 12S U2 snRNP and two proteinaceous complexes, SF3a and SF3b. This replacement is achieved by the DExD-box RNA helicase UAP56 (56-kD U2AF<sup>65</sup>-associated protein) in an ATP-dependent manner. UAP56 is also an essential mRNA export factor (Luo *et al.* 2001; Strasser & Hurt 2001). As a result of this replacement, the U2 snRNP becomes tightly associated with the BPS via base-pairing between U2 snRNA and BPS. The resultant complex is called the 'A complex'. The U4/U5/U6 tri-snRNP is then recruited to the A complex to form the B complex, and after some rearrangements of the factors, the catalytically active C complex is finally formed. Importantly, during the

spliceosome assembly processes, there are extensive and dynamic interactions between the components, including protein-protein, protein-RNA and RNA-RNA interactions (Wahl *et al.* 2009; Newman & Nagai 2010; Valadkhan & Jaladat 2010).

The mechanisms underlying the nuclear retention of pre-mRNAs have been studied almost exclusively in budding yeast. A pioneer study by Rosbash and co-workers revealed that early intron recognition is important for nuclear retention (Legrain & Rosbash 1989). Therefore, mutations in the 5'-ss and BPS, as well as mutations in the early splicing factors, lead to the leakage of pre-mRNA into the cytoplasm (Legrain & Rosbash 1989; Rain & Legrain 1997; Luukkonen & Seraphin 1999). Thus, the formation of early splicing complexes appears to be responsible for the nuclear retention of pre-mRNAs. Seraphin and co-workers showed that particular mutations in the yeast SF1/BBP gene induce the leakage of pre-mRNAs into the cytoplasm without affecting the splicing activity *in vitro* (Rutz & Seraphin 2000). The same group subsequently showed that a trimeric protein complex, called the 'RES' (pre-mRNA retention and splicing), was involved in pre-mRNA nuclear retention and splicing in yeast

(Dziembowski *et al.* 2004). Interestingly, Nehrbass and co-workers showed that the depletion of MLP1, a constituent of the fiber-like structures emanating from the nuclear pore complexes (NPCs), impairs the retention of pre-mRNAs without affecting their splicing *in vivo* (Galy *et al.* 2004). This was the first demonstration of a clear separation between nuclear retention and splicing. However, how MLP1 is related to early intron recognition is not well understood.

The mechanism of pre-mRNA nuclear retention must be much more elaborate in vertebrates than in budding yeast because the vast majority of vertebrate genes contain introns, whereas only 5 % of genes in budding yeast contain introns (see for instance, Juneau *et al.* 2007 and references therein). Another difference is that vertebrate splice site sequences are much less conserved than those of budding yeast, and therefore vertebrates often use additional splicing signals that are not seen in yeast (reviewed in, Smith & Valcarcel 2000). We have previously reported that one such type of signal, the purine-rich exonic splicing enhancers, contribute to the nuclear retention of pre-mRNAs in vertebrates (Taniguchi *et al.* 2007). Therefore, it is likely that the mechanism of pre-mRNA nuclear retention is significantly different between yeast and vertebrates.

However, this mechanism has been much less studied and is therefore much less well understood in vertebrates than in yeast, although some earlier work has suggested that early intron recognition is also important in vertebrate (Chang & Sharp 1989). It must be noted that some retroviruses including HIV-1 have systems that induce the nuclear export of intron-containing viral pre-mRNAs, and these phenomena have been quite extensively studied with a view to anti-viral drug development (reviewed in, Cullen 2003). However, these studies have not been very helpful in deciphering the nuclear retention mechanisms of cellular pre-mRNAs.

In conclusion, almost no information regarding the pre-mRNA nuclear retention factors in vertebrates is available. In the only exception, Yoshida and co-workers serendipitously discovered that spliceostatin A (SSA), an anti-tumor drug, targets SF3b and inhibits both the splicing and nuclear retention of pre-mRNAs (Kaida *et al.* 2007). The fact that the yeast SF3b complex physically associates with the above mentioned yeast RES complex (Wang *et al.* 2005) makes mammalian SF3b a likely candidate factor involved in pre-mRNA retention. However, more studies must be performed before the identities of the mammalian pre-mRNA



retention factors are established, for the following reasons. First, because SSA is a splicing inhibitor, the accumulated pre-mRNAs may saturate a hypothetical retention factor(s) other than SF3b, and this may cause the leakage of pre-mRNAs into the cytoplasm. Second, because extensive interaction networks are formed among the splicing factors during the assembly of the early splicing complexes, it is difficult to pinpoint the factor(s) involved in nuclear retention *per se*. Therefore, the nuclear retention activity of these factors must be directly assessed to narrow down the candidate factor(s). Third, because of the elaborate occurrence of introns in mammalian genes, there must be multiple retention factors and mechanisms. These facts prompted us to look for the pre-mRNA retention factors in mammalian cells.

## Results

### Importance of the E complex in the pre-mRNA nuclear retention

To identify the pre-mRNA nuclear retention factors in mammalian cells, we first constructed a reliable and sensitive reporter system for detecting the nuclear retention and cytoplasmic leakage of pre-mRNAs. We inserted the first intron sequence from the p27 gene (Kaida *et al.* 2007) between the cytomegalovirus (CMV) promoter and the green fluorescent protein (GFP) coding region (GFP-intron reporter, Fig.1A). Upon transfection of the reporter plasmid into HeLa cells, the primary transcript from the reporter was spliced at the authentic 3'-ss of the p27 intron and a cryptic 5'-ss, 249 nucleotides downstream from the authentic 5'-ss of the p27 intron (Fig.1E, Ctr lane). Under these conditions, no significant signal from the pre-mRNA was detected in the cells by RNA fluorescent in situ hybridization (RNA FISH) with an intron probe (Fig.1F, Ctr panel). Although this non-canonical splicing was somewhat unexpected, we nevertheless verified that this reporter system was useful. If we treated the cells with SSA, which is known to inhibit splicing and to induce leakage of pre-mRNAs into the cytoplasm (Kaida *et al.* 2007), the splicing of the reporter was strongly inhibited (Fig.1E, SSA lane) and the reporter pre-mRNA leaked into the

cytoplasm as expected (Fig.1F, SSA panel). This demonstrates that this reporter system can be used to detect the nuclear retention and cytoplasmic leakage of pre-mRNAs.

Because early splicing factors are implicated in the nuclear retention of pre-mRNAs in yeast, we first focused on the U1 and U2 snRNPs, which are involved in the formation of the early splicing complexes. It has been reported that if RNA-DNA chimeric antisense oligonucleotides are introduced into cultured cells, the corresponding nuclear RNAs are efficiently degraded by endogenous RNaseH activity (Sasaki *et al.* 2009). When antisense oligonucleotides complementary to the U1 and U2 snRNAs were introduced into HeLa cells by transfection, the endogenous U1 and U2 snRNAs were efficiently and specifically truncated or destroyed, respectively, as demonstrated by northern blotting and quantitative RT-PCR (Fig.1B-D, see Hamm *et al.* 1989; Pan *et al.* 1989). Under these conditions, the splicing of the reporter was partially inhibited (Fig.1E, U1 and U2 lanes). RNA FISH analysis showed that the pre-mRNA was still retained in the nucleus after U2 knock-down (Fig.1F, U2 panel), whereas the pre-mRNA was barely detected after U1 knock-down, for unknown reasons

(Fig.1F, U1 panel). These results indicate that U2 snRNP is not essential for the nuclear retention of pre-mRNAs.

Because we could not obtain a clear result from the U1 knock-down experiment, we next knocked down U1-70K, a specific protein component of U1 snRNP, to inactivate U1 snRNP. When U1-70K was knocked down (Fig.2A, U1-70K lane), the splicing of the reporter was partially inhibited (Fig.2B, U1-70K lane) and the pre-mRNA leaked into the cytoplasm (Fig.2C, U1-70K panel), suggesting that U1 snRNP is important for the nuclear retention of pre-mRNAs. Because we have already shown that U2 snRNP is not essential for nuclear retention, we next knocked down U2AF, which binds to pre-mRNAs before U2 snRNP. U2AF is a heterodimer consisting of U2AF<sup>35</sup> and U2AF<sup>65</sup>. When U2AF<sup>35</sup> was knocked down, the level of U2AF<sup>65</sup> was unaffected (Fig.2A, U2AF<sup>35</sup> lane), and the splicing of the reporter was partially inhibited (Fig.2B, U2AF<sup>35</sup> lane). However, the reporter pre-mRNA was still retained in the nucleus (Fig.2C, U2AF<sup>35</sup> panel). In contrast, when U2AF<sup>65</sup> was knocked down, the level of U2AF<sup>35</sup> was greatly reduced (Fig.2A, U2AF<sup>65</sup> lanes), and the reporter pre-mRNA leaked into the cytoplasm (Fig.2C, U2AF<sup>65</sup> panel), indicating that U2AF, especially U2AF<sup>65</sup>, is

important for the nuclear retention of pre-mRNAs.

The importance of U1-70K and U2AF<sup>65</sup> was further supported by the experiment shown in Fig.2D. In this experiment, HeLa cells were treated with low concentration of SSA (10ng/ml), which induced the leakage of pre-mRNA into the cytoplasm (Fig.2D, vector panel). However, if U2AF<sup>65</sup> or U1-70K was over-expressed, the inhibitory effect of SSA on pre-mRNA splicing was unaffected (data not shown) but the inhibitory effect of SSA on pre-mRNA nuclear retention was reversed (Fig.2D, U2AF<sup>65</sup> or U1-70K panels, respectively). U2AF<sup>35</sup> did not show this activity (Fig.2D, U2AF<sup>35</sup> panel). Taken together, these results show, for the first time, that the E complex is the main entity responsible for the nuclear retention of pre-mRNAs in mammalian cells. Unexpectedly, however, the knock-down of SAP155, a 155-kD component of the retention factor candidate SF3b, did not cause the pre-mRNA to leak into the cytoplasm although splicing was strongly inhibited under these conditions (Fig2A-C). The knock-down of SF1/BBP also did not lead to the leakage of pre-mRNA (data not shown, also see Fig.5C).

## Nuclear retention activity of the candidate factors

Although the assay described above allowed us to identify candidate factors involved in the nuclear retention of pre-mRNAs in mammalian cells, it was useful to more directly assay the nuclear retention activity of the candidates since that would allow us to narrow down the candidates. To this end, we used the MS2 tethering system in which an intronless GFP reporter gene with MS2-binding sequences in its 3'-untranslated region (UTR) was expressed, together with the N-terminal MS2 protein fused to the protein of interest (Fig.3A). If U2AF<sup>35</sup> or a control RNA-binding protein (PHAX) was tethered to the reporter RNA, the RNA was efficiently exported to the cytoplasm (Fig.3B, U2AF<sup>35</sup> or PHAX panels, respectively), indicating that these proteins do not have nuclear retention activity *per se*.

In contrast, if U2AF<sup>65</sup> or U1-70K was tethered, the reporter RNA was retained in the nucleus (Fig.3B, U2AF<sup>65</sup> or U1-70K panels, respectively). These fusion proteins were over-expressed in the cells at similar levels as demonstrated by immunofluorescence (IF) cell staining with an anti-hemagglutinin (HA) antibody (Fig.3B and C, IF panels). Importantly, the nuclear retention activity of these

proteins was counteracted by SSA (Fig.3C), indicating that the retention activity exerted by these tethering events reflected the real retention of pre-mRNAs. Similar results were obtained when the MS2 protein was fused to the C-terminus instead of N terminus (data not shown). These results further supported the idea that U1 snRNP and U2AF are the pre-mRNA nuclear retention factors in mammalian cells.

### **Multiple domains of U2AF<sup>65</sup> are involved in nuclear retention**

For further insight into how nuclear retention is achieved, we focused on U2AF<sup>65</sup> and investigated which domain(s) shows nuclear retention activity *per se* when tethered to the reporter RNA. We made a series of deletion constructs from the U2AF<sup>65</sup> cDNA, many of which are shown in Fig.4A. The U2AF<sup>65</sup> mutant proteins were expressed from these constructs at similar levels (Fig.4B). Nuclear localization signals (NLSs) were included in the tethered mutant proteins to ensure that the proteins were targeted to the nucleus (see Fig.3A). When the nuclear retention activities of the U2AF<sup>65</sup> mutants were examined in the tethering assay, most mutants still retained significant retention activity (Fig.4C and 4D for quantitation). In all cases, the retention activity was reversed by treatment with

SSA, confirming the relevance of the assay to the real retention of pre-mRNAs (data not shown). Among the many mutants examined, only four mutants, RRM1, RRM2, RRM3 and  $\Delta N\Delta link$ , had strongly reduced retention activity (Fig.4C and 4D). These results show that multiple domains of U2AF<sup>65</sup> contribute to its nuclear retention activity.

Several factors are known to interact with U2AF<sup>65</sup>, including SF1, SAP155 (155-kD subunit of SF3b), U2AF<sup>35</sup> and UAP56 (56-Dd U2AF<sup>65</sup>-associated protein). Among these, SF1 and SAP155 interact with the domain near the C-terminus of U2AF<sup>65</sup>, whereas U2AF<sup>35</sup> and UAP56 interact with the domain near the N-terminus. The approximate factor-binding regions are illustrated in Fig.4A (Zhang *et al.* 1992; Fleckner *et al.* 1997; Gozani *et al.* 1998; Rain *et al.* 1998). We investigated the involvement of these factors in the nuclear retention activity of U2AF<sup>65</sup>. We combined the tethering of U2AF<sup>65</sup>, either full length or deletion mutants, with the knock-down of the interacting factors. We initially tried to knock down the expression of all four interacting factors at the same time to examine the effect of this on the nuclear retention activity of full-length U2AF<sup>65</sup>, but efficient knock-down of the four factors at the same time was not possible.



We then tried many different combinations of tethering and knock-down (Supplementary TableS1). Surprisingly, most combinations did not produce a leaking phenotype, suggesting the existence of elaborate layers of fail-safe mechanisms against pre-mRNA leakage. For example, if the C-half and RRM2 mutants are compared (Fig.4A), the former but not the latter has retention activity and the only difference between them is the C-terminal domain, suggesting the importance of the C-terminal domain of in nuclear retention. Because the C-terminal domain largely overlapped with the binding domains of SF1 and SAP155 (Fig.4A), we tethered the C-half mutant and then knocked down SF1 or SAP155 or both (Fig.5A-C). However, none of these inactivated the nuclear retention activity of the C-half mutant (Fig.5C).

Also if we compared the  $\Delta 35\Delta link$  and  $\Delta N\Delta link$  mutants (Fig.4A), the former but not the latter mutant has nuclear retention activity, and the only difference between them is the RS domain, suggesting the importance of the RS domain of U2AF<sup>65</sup> in nuclear retention. (see Discussion).

## **UAP56, a DExD-box RNA helicase, cooperates with U2AF<sup>65</sup> in the nuclear retention activity**

Similarly, when we compared the  $\Delta$ RS $\Delta$ link and  $\Delta$ N $\Delta$ link mutants (Fig.4A), an important clue came. The former but not the latter has the retention activity and the only difference between them is the 35 domain, with which U2AF<sup>35</sup> and UAP56 interact. When we tethered  $\Delta$ RS $\Delta$ link and knocked down U2AF<sup>35</sup>, no effect on nuclear retention was observed (Fig.5D and 5F). Therefore, we next focused on UAP56. In fact, UAP56 has a close relative designated 'URH49' (UAP56-related helicase, 49-kD). UAP56 and URH49 have 90% amino acid identity and 96% similarity and are reported to have both overlapping and distinct roles in mRNA export (Luo *et al.* 2001; Strasser & Hurt 2001; Kapadia *et al.* 2006; Yamazaki *et al.* 2010). However, the role of URH49 in pre-mRNA splicing has not been demonstrated. Knocking down both proteins at the same time led to the extensive death of the cells (data not shown and Kapadia *et al.* 2006). Therefore, we knocked down only one of them at a time.

Knocking down one of the two proteins significantly weakened the nuclear retention of  $\Delta$ RS $\Delta$ linker (Figs.5D, 5F, and 5G for quantitation). In fact, these were

the only two combinations in which cytoplasmic leakage of the reporter RNA was observed (Supplementary TableS1). Under these knock-down conditions, the general gene expression processes such as RNA splicing and export were affected, but only weakly (Fig.5E for pre-mRNA splicing and Supplementary Fig.S1A for export of the GFP reporter mRNA), and cell growth was apparently unaffected (data not shown). Therefore, it is unlikely that this effect was indirect, attributable to the under-expression of a hypothetical retention factor(s). Consistent with this notion is the fact that the retention activity of the C-half mutant was unaffected under these knock-down conditions (Fig.5F).

To confirm that UAP56 is involved in nuclear retention, we assessed the effect of UAP56 overexpression (Fig.6A) on nuclear retention activity. As already shown, the nuclear retention activity of the tethered full-length U2AF<sup>65</sup> was abolished in the presence of SSA (Fig.6B, uppermost panel, also see Fig.3C). However, the over-expression of UAP56 counteracted the inhibitory effect of SSA on pre-mRNA nuclear retention (Fig.6B, the second upper panel) in a dose-dependent manner (Fig.6C, the rightmost section). Under the same conditions, the inhibitory effect of SSA on pre-mRNA splicing was not affected

(data not shown), indicating that the protein level of UAP56 specifically contributes to the nuclear retention activity of U2AF<sup>65</sup>. The over-expression of UAP56 under these conditions did not affect the general gene expression processes such as pre-mRNA splicing (Fig.6D), or the nuclear export of the GFP reporter mRNA (Fig.6B, lower two panels and Supplementary Fig.S1B).

Finally, we assessed the effect of UAP56 over-expression on the cytoplasmic leakage of pre-mRNAs. We have already shown that SSA induced the leakage of the GFP-intron reporter pre-mRNA and that the over-expression of full-length U2AF<sup>65</sup> reversed the SSA effect (Fig.2D). In similar experiments, we found that the over-expression of the U2AF<sup>65</sup>  $\Delta$ RS $\Delta$ link mutant alone did not have this reversal activity (Fig.6E, second panel from the bottom, left half). However, when UAP56 was co-expressed with  $\Delta$ RS $\Delta$ link, the retention activity was strengthened and the SSA effect was reversed (Fig.6E, second panels from the bottom, right half). If  $\Delta$ N $\Delta$ link, which lacks part of the UAP56-binding domain, was used, its co-expression did not have this effect (Fig.6E, bottom panels). Taken together, these results indicate that UAP56 cooperates with U2AF<sup>65</sup> in causing the nuclear retention of pre-mRNAs.

## Discussion

One of the least well understood mechanisms related to pre-mRNA splicing is how intron-containing pre-mRNAs are retained in the nucleus until they are spliced. Pre-mRNA nuclear retention is part of the RNA quality control mechanisms, which ensure the appearance in the cytoplasm of only mature mRNAs. Because almost no information was available about the pre-mRNA nuclear retention factors in vertebrates, we undertook to identify these factors in mammalian cells. Using a model reporter gene, we found that U1 snRNP and U2AF, but not U2 snRNP, are critical for pre-mRNA nuclear retention. We also found that multiple domains of U2AF<sup>65</sup> have nuclear retention activity *per se* and a U2AF-associated DExD-box RNA helicase UAP56 contributes to nuclear retention activity.

Chang and Sharp have previously shown that mutations in either the 5'-ss or 3'-ss weakened the nuclear retention of a model pre-mRNA from the human  $\beta$ -globin gene (Chang & Sharp 1989). Our finding that both U1 snRNP and U2AF are critical for pre-mRNA nuclear retention confirms their conclusion from the

perspective of the factors involved. We also found that the U2 snRNP is not essential for the nuclear retention of pre-mRNAs in mammalian cells. This is consistent with our previous result that showed that the destruction of U2 snRNA did not induce leakage of a model pre-mRNA derived from the *ftz* gene, in *Xenopus* oocytes (Taniguchi *et al.* 2007). There is also no report, as far as we know, that U2 snRNP itself is important for pre-mRNA retention in budding yeast. In conclusion, our results show, for the first time, that the E complex is the major entity responsible for the nuclear retention of pre-mRNAs in mammalian cells.

It should be noted that the GFP-intron reporter pre-mRNA did not leak into the cytoplasm when SF1/BBP was knocked down (data not shown). This result was unexpected because SF1/BBP is implicated in nuclear retention in budding yeast (Rutz & Seraphin 2000). Since the branch point sequence (BPS) is much less conserved in vertebrates than budding yeast, the importance of BPS and its binding factor SF1/BBP may be different between the species. However, we cannot exclude the possibility that SF1/BBP contributes to the pre-mRNA nuclear retention in mammalian cells since human SF1/BBP has a nuclear retention activity in the MS2 tethering assay (data not shown). In contrast to

SF1/BBP, we have good evidence that U2AF is critical for the pre-mRNA nuclear retention in mammalian cells whereas there is no report that U2AF is important for the same process in budding yeast. This may be because there is no clear polypyrimidine tract in the budding yeast introns and therefore U2AF may be less important for the pre-mRNA nuclear retention in budding yeast. This could be another difference between the species.

SF3b is a strong candidate retention factor in mammalian cells (Kaida *et al.* 2007). However, the knock-down of SAP 155, a component of SF3b, did not induce leakage of the GFP-intron reporter pre-mRNA into the cytoplasm (Fig.2). The reason for this difference is not known, but we can at least say that SAP155 is not critical for the pre-mRNA nuclear retention under our experimental conditions. We could not assess the nuclear retention activity of SAP155 with the MS2 tethering assay, because the fusion protein was expressed negligibly in HeLa cells after transfection (data not shown). Therefore, whether SAP155 has nuclear retention activity on its own or only in an interaction with other factors is still an open question. Our study suggests that U2AF<sup>65</sup> may be a good candidate for such a factor.

Our results also indicated that UAP56 cooperates with U2AF in inducing the nuclear retention of pre-mRNAs. This result was also largely unexpected, because UAP56 and URH49 are known to be nuclear export factors rather than nuclear retention factors (Luo *et al.* 2001; Strasser & Hurt 2001; Kapadia *et al.* 2006; Yamazaki *et al.* 2010). UAP56 interacts with the E complex but appears to be missing in the A and B complexes, and it re-appears in the C complex (see Wahl *et al.* 2009 for a review). Thus, whether and how the two distinct roles of UAP56 in pre-mRNA splicing and mRNA export are coordinated is not known.

How does UAP56 contribute to nuclear retention? We failed to detect the nuclear retention activity of UAP56 in the tethering assay (data not shown), so it is reasonable to infer that UAP56 exerts its retention activity by recruiting other factors. During the transition from the E complex to the A complex, U2AF<sup>65</sup> dynamically interacts with SF1/BBP and SAP155, as well as with UAP56. Therefore, SF1/BBP, SAP155 and/or U2 snRNP are candidate factors that support the retention activity exerted by UAP56. However, when we tethered  $\Delta$ RS $\Delta$ link (Fig.4A) and knocked down SF1, SAP155 or U2 snRNA, the nuclear



retention activity of  $\Delta$ RS $\Delta$ link was not affected (Supplementary Fig.S2), excluding the possibility discussed above. Moreover, ATP binding- and ATPase-deficient mutants of UAP56 (K95E and E197Q, respectively, Pause & Sonenberg 1992; Shen *et al.* 2008; Taniguchi & Ohno 2008) still enhanced the nuclear retention activity of U2AF<sup>65</sup> (Supplementary Fig.S3), indicating that the nuclear retention exerted by UAP56 is independent of the recruitment of 17S U2 snRNP to U2AF<sup>65</sup>. Further experiments are required to clarify the nuclear retention activity exerted by UAP56.

How are pre-mRNAs retained in the nucleus? Theoretically, there are two major possibilities. The first is that the nuclear retention factors have an activity that prevents RNA export factors from binding to pre-mRNAs. The second possibility is that the nuclear retention factors actually anchor the pre-mRNAs to some structural entities in the nucleus. The first possibility is reminiscent of the RNA helicase activity that displaces protein factors from RNA templates (reviewed in, Jankowsky 2011). However, such activity has not been demonstrated in the pre-mRNA nuclear retention process. Although we have identified UAP56 as a retention factor, it may be unlikely that UAP56 exerts the retention activity via its

helicase activity, since ATP binding- and ATPase-deficient mutants of UAP56 could still cooperate with U2AF in nuclear retention as discussed earlier.

Yeast MLP1, a constituent of the fiber-like structures emanating from the NPCs, is already implicated in nuclear retention, so it is a strong candidate for the structural entities to which the pre-mRNAs might be anchored (Galy *et al.* 2004). However, it has been recently reported that TPR protein, the vertebrate counterpart of MLP1, may be involved in the same process (Coyle *et al.* 2011). However, the knock-down of TPR induced no cytoplasmic leakage of the GFP-intron reporter pre-mRNA (data not shown).

Another candidate for such a structural entity is the nuclear domain called the 'nuclear speckles' (reviewed in, Zhao *et al.* 2009). An association between pre-mRNAs and the nuclear speckles has been observed (Kaida *et al.* 2007; Ishihama *et al.* 2008), suggesting that the nuclear speckles are the entity to which pre-mRNAs are anchored. The role of the nuclear speckles in RNA retention has previously been suggested (Ishihama *et al.* 2008; Dias *et al.* 2010). In our study, a comparison of the  $\Delta 35\Delta\text{link}$  and  $\Delta N\Delta\text{link}$  (Fig.4A) suggested the

importance of the RS domain of U2AF<sup>65</sup> in nuclear retention. Because the RS domain is the signal that targets proteins to the nuclear speckles (Hedley *et al.* 1995), the importance of the nuclear speckles in nuclear retention is suggested. However, when the retention factor candidates were tethered to the reporter RNA, the RNA stayed in the nucleus but did not preferentially accumulate in the nuclear speckles. Therefore, the nuclear speckles could be only part of the retention mechanisms, and multiple retention mechanisms may be operating. It is likely that extensive interactive networks of retention factors form many layers of fail-safe mechanisms to prevent the leakage of pre-mRNAs into the cytoplasm, especially in mammalian cells. More experiments are required to resolve these highly complex mechanisms.

## Experimental procedures

The sequences of all the PCR-primers, DNA oligonucleotides, chimeric oligonucleotides and siRNAs are listed in the Supplementary Tables S2 and S3.

## DNA constructs

pcDNA3 and pcDNA3.1/myc-His(-) A were purchased from Invitrogen.

The GFP-intron reporter plasmid was constructed as follows. A HindIII-BamHI fragment containing the GFP coding sequence was first cloned into the same sites of pcDNA3 (pcDNA3-GFP). The DNA sequence from the first intron of the p27 gene was then PCR-amplified from the plasmid p27-int-HA (Kaida *et al.* 2007) with the appropriate primers. The amplified DNA was then digested with HindIII and cloned into the HindIII site of pcDNA3-GFP in the sense orientation.

The GFP reporter with MS2-binding sites was constructed by inserting six copies of the MS2-binding sequence (Yoshimoto *et al.* 2009) into the XhoI site of

pcDNA3-GFP.

The plasmid used for the expression of the MS2 fusion proteins (pNMS2-HA-NLSx3) was constructed as follows. The DNA fragment encoding the MS2 protein was PCR-amplified from pGEX-6p-MS2 (Yoshimoto *et al.* 2009). The amplified fragment was cloned into the XbaI-BamHI sites of pcDNA3.1/myc-His(-) A (pNMS2). An HA tag sequence was then inserted into the plasmid by around-the-clock PCR with the appropriate primers (pNMS2-HA). Two oligonucleotides corresponding to the NLS of the SV40 T antigen (PKKKRKV) were annealed and three copies were inserted into the BamHI site of pNMS2-HA (pNMS2-HA-NLSx3).

DNA fragments encoding the proteins of interest were PCR-amplified and cloned into the BamHI-XhoI or BamHI-EcoRI sites of pNMS2-HA-NLSx3.

The plasmids used for over-expression were constructed as follows. DNA fragments corresponding to hU2AF<sup>65</sup>, hU2AF<sup>35</sup> and hU1-70K were PCR-amplified. The hUAP56 fragment was obtained from pGEX-6p-UAP56

(Taniguchi & Ohno 2008) by BamHI-XhoI digestions. These fragments were cloned into the BamHI-XhoI sites of pcDNA3 and a Flag tag sequence was inserted at the HindIII-BamHI sites.

## Antibodies

The monoclonal antibodies directed against DIG, HA, GAPDH, SAP155, SF2, U2AF<sup>65</sup>, U2AF<sup>35</sup> and UAP56 were: clone 1.71.256 (Roche), 3F10 (Roche), 6C5 (Ambion), 16 (MBL), 96 (Zymed), MC3 (SIGMA), N-16 (Santa Cruz Biotechnology) and 2252C4a (Santa Cruz Biotechnology), respectively.

The polyclonal antibodies directed against UAP56 and URH49 (Yamazaki *et al.* 2010) were kind gifts from Dr. Seiji Masuda. The polyclonal antibody against U1-70K was ab51266 (Abcam).

## RNA fluorescence in situ hybridization and immunofluorescence

The DNA fragment for the intron probe was made by PCR amplification of the plasmid p27-int-HA (Kaida *et al.* 2007) with the appropriate primers. The DNA fragment for the GFP probe was made by purifying the GFP fragment from pcDNA3-GFP with HindIII-BamHI digestions. These DNA fragments were cloned into pBluescript SK- and digoxigenin-labeled RNA probes were made by *in vitro* transcription with a DIG RNA Labeling Kit (Roche).

In situ hybridization was performed as described previously (Sone *et al.* 2007), with modifications. HeLa cells, grown on Chamber Slides (Lab-TekR II Chamber Slide™ System; Nunc), were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature (RT), and permeabilized with 0.5% Triton X-100 in PBS for 5 min at RT. The cells were then washed with PBS (three times at RT) and dehydrated by sequential exposure to water, 25%, 50%, 75% and 100% ethanol (30 s each on ice). The cells were dried, pre-hybridized in prehybridization buffer (50% formamide, 1x Denhardt's solution, 2x SSC, 10mM EDTA, 100  $\mu$ g/ml yeast tRNA, 0.01% Tween-20) for 2 h at 55°C, and then hybridized with probes in hybridization

buffer (the prehybridization buffer containing 5% dextran sulfate) for 16 h at 55°C. Cells were then washed with 0.2xSSC (30 min x 2 at 55°C), treated with RNaseH (100U/ml for 30 min at 37°C), and washed sequentially with 0.2xSSC and TBST (5 min each at RT). After the cells were treated with Blocking Reagent (Roche) for 30 min at RT, the hybridization signals were detected by incubating the cells with the primary antibody for 1 h at RT and then with the secondary antibody for 30min at RT. The primary antibodies (anti-DIG, anti-HA, anti-U2AF65, and anti-SF2) are described above. The secondary antibodies were Alexa-Fluor-488-labeled goat anti-mouse IgG1, Alexa-Fluor-568-labeled goat anti-mouse IgG2b, Alexa-Fluor-568-labeled goat anti-rat IgG, and Alexa-Fluor-568-labeled goat anti-rabbit IgG. All were purchased from Molecular Probes. 4',6-Diamidino-2-phenylindole (DAPI) staining was performed during the secondary antibody treatment. The slides were mounted in Fluorescent Mounting Medium (Calbiochem) and observed at RT with an inverted Axio Observer Z1 microscope (Zeiss). The images were captured with an AxioCam MRm (Zeiss) driven by AxioVision 4.7 software (Zeiss).



## RT-PCR

RNA was purified from HeLa cells with Sepasol I Super (Nacalai Tesque), and then treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instruction.

RT-PCR was performed with the AccessQuick™ RT-PCR System (Promega) with the appropriate primers. The amplified DNAs were separated by 6% polyacrylamide gel electrophoresis. The gel was stained with SYBR Gold (Molecular Probes) and scanned with Typhoon (GE Healthcare).

Quantitative RT-PCR was performed with the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) and the StepOnePlus™ Real-Time PCR System (Applied Biosystems).

## Knock-down

Knock-down of the U snRNAs was performed as described previously (Sasaki *et al.* 2009). The corresponding RNA-DNA chimeric oligonucleotides were obtained from IDT. HeLa cells were transfected with the oligonucleotides using Lipofectamine<sup>TM</sup> 2000 (Invitrogen), and typically analyzed at 18 h after transfection.

For the knock-down of protein factors, the corresponding siRNAs were purchased from Invitrogen (Stealth<sup>TM</sup> RNAi). HeLa cells were transfected with the siRNAs using Lipofectamine<sup>TM</sup> RNAiMAX (Invitrogen). In the case of U2AF<sup>65</sup>, a second siRNA transfection was performed 24 h after the first transfection. The cells were then transfected with the plasmids at 24-54 h after the last siRNA transfection, and analyzed 18-24 h after the plasmid transfection.

### Supplementary Material

Supplementary Figs.S1-S3 and Supplementary Tables S1-S3 are available online.

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## Figure legends

### Fig.1 Role of early U snRNPs in pre-mRNA nuclear retention

All the experiments were performed with HeLa cells and the sequences of all the PCR primers and antisense oligonucleotides are shown in Supplementary Tables S2 and S3

(A) Diagram of the GFP-intron reporter. The two short bars in the diagram represent the positions of the primers used in (E).

(B) Northern blotting analysis of endogenous U1 and U2 snRNAs after their knock-down (KD), as described in the Materials and Methods. Size markers are shown on the left.

(C) and (D) Quantitative PCR (qPCR) analysis of the relative amount of U1 and U2 snRNAs after their knock-down (KD). The means and standard deviations of three experiments are shown.

(E) RT-PCR analysis of the splicing of the GFP-intron reporter after U1 and U2 knock-down (KD) or spliceostatin A (SSA) treatment (100ng/ml).

(F) Localization of the GFP-intron pre-mRNAs was examined by RNA fluorescence in situ hybridization (FISH) with the intron probe after U1 and U2



knock-down (KD) or spliceostatin A (SSA) treatment (100ng/ml). On the right, immunofluorescence (IF) cell staining with anti-U2AF<sup>65</sup> antibody (upper three panels) or anti-SF2 antibody (the bottom panel) is shown as a nuclear marker.

## Fig.2 Role of early splicing factors in the nuclear retention of pre-mRNAs

All the experiments were performed with HeLa cells and the sequences of all the siRNAs are shown in Supplementary Table S3.

(A) Immuno-blotting (IB) analysis of early splicing factors after their knock-down by the corresponding siRNAs. GAPDH and CBP80 were used as references.

(B) RT-PCR analysis of the splicing of the GFP-intron reporter after factor knock-down (KD) or spliceostatin A (SSA) treatment (100ng/ml).

(C) Localization of the GFP-intron pre-mRNAs examined by RNA FISH after factor knock-down (KD) or SSA treatment as in Fig.1F. On the right side, immunofluorescence (IF) cell staining with anti-U2AF<sup>65</sup> antibody (upper three panels) or anti-SF2 antibody (lower three panels) is shown as a nuclear marker.

(D) Effect of factor over-expression on the cytoplasmic leakage of the reporter pre-mRNA induced by SSA. The GFP-intron reporter plasmid was transfected to HeLa cells together with a plasmid over-expressing U2AF<sup>35</sup>, U2AF<sup>65</sup>, or U1-70K

or the vector, in the presence of SSA (10ng/ml). RNA FISH was performed as in (C). 4',6-Diamidino-2-phenylindole (DAPI) staining is shown on the right as a nuclear marker.

### Fig.3 Nuclear retention activities of splicing factors

(A) Schematic representation of the MS2 tethering system. A fusion protein consisting of the N-terminal MS2 protein and the C-terminal protein of interest was expressed in HeLa cells together with a CMV-promoter-driven GFP reporter gene without introns but with six copies of the MS2-protein-binding sites in the 3'-UTR. The fusion protein contained tags (HA, myc) and three copies of the nuclear localization signal (NLS) from the SV40 T antigen. The fusion protein should associate with transcripts from the GFP reporter and trap the transcripts in the nucleus if the protein of interest has nuclear retention activity.

(B) The GFP reporter transcripts were localized with RNA FISH using a GFP probe when various factors were tethered to its 3'-UTR. Immunofluorescence (IF) cell staining with anti-HA antibody is shown in the middle, and DAPI staining on the right.

(C) U2AF65 or U1-70K was tethered to the reporter RNA as in (B) except in the

presence of SSA (100ng/ml). RNA FISH and IF staining were performed as in (B).

#### Fig.4 Identification of U2AF<sup>65</sup> domains that exert nuclear retention activity

(A) Schematic representation of the domain structure and deletion mutants of U2AF<sup>65</sup>. The numbers near the bars represent the amino-acid positions in U2AF<sup>65</sup>. The names of the mutants that show strongly reduced retention activity are marked by rectangles. The approximate binding regions of the four factors are illustrated on the top (Zhang *et al.* 1992; Fleckner *et al.* 1997; Gozani *et al.* 1998; Rain *et al.* 1998).

(B) Expression of the deletion mutants was compared by immuno-blotting (IB) using anti-HA (HA) and anti-U2AF<sup>65</sup> (U2AF<sup>65</sup>) antibodies. The position of the band of endogenous U2AF<sup>65</sup> is marked as a reference.

(C) The nuclear retention activity of the deletion mutants assayed as described in Fig.3B.

(D) A quantitative representation of (C). The numbers of cells, in which the GFP reporter RNA localized either mainly to the nucleus (N, blue bars) or in the cytoplasm (C, red bars) or both (C+N, yellow bars), were counted. At least 200

cells were examined per sample. The percentage of each type was calculated.

The means and standard deviations of three experiments are shown.

Fig.5 Effect of UAP56 or URH49 knock-down on the nuclear retention activity of U2AF<sup>65</sup>

(A) Immuno-blotting (IB) with anti-SAP155 antibody with and without the knock-down (KD) of SAP155.

(B) qRT-PCR analysis of SF1 mRNA with and without its KD was performed since anti-SF1 antibodies were not available. The means and standard deviations of six experiments are shown.

(C) The GFP reporter RNA was localized with RNA FISH when the U2AF<sup>65</sup> C-half mutant was tethered and the factors shown on the right were knocked down.

(D) Immuno-blotting (IB) with anti-U2AF<sup>35</sup>, anti-UAP56 and anti-URH49 antibodies with and without their KD.

(E) Splicing of the GFP-intron reporter was examined with and without the knock-down of UAP56 or URH49, as in Fig.1E.

(F) The GFP reporter RNA was localized with RNA FISH when the U2AF<sup>65</sup>

mutants shown on the left were tethered and the factors shown on the right were knocked down.

(G) A quantitative representation of (F) as in Fig.4D, from three experiments.

### Fig.6 Effect of UAP56 over-expression on pre-mRNA nuclear retention

(A) Immuno-blotting (IB) analysis of UAP56 over-expression. Increasing amounts of plasmids expressing Flag-tagged UAP56 were transfected into HeLa cells, and the expression of UAP56 was examined by IB analysis with anti-FLAG and anti-UAP56 antibodies. The expression of endogenous GAPDH is shown as a reference.

(B) The GFP reporter RNA was localized with RNA FISH with and without the tethering of full-length U2AF<sup>65</sup> and with and without UAP56 over-expression, in the presence of SSA (10ng/ml).

(C) A quantitative representation of (B).

(D) Splicing of the GFP-intron reporter and the p27 reporter (Kaida *et al.* 2007) was examined with and without the over-expression of UAP56 or U2AF<sup>65</sup>, as in Fig.1E.

(E) The GFP-intron pre-mRNA was localized with RNA FISH with and without

the over-expression of UAP56 and/or U2AF<sup>65</sup> derivatives, in the presence of  
SSA (10ng/ml).













