The conserved RNA recognition motif 3 of U2 snRNA auxiliary factor (U2AF⁶⁵) is essential in vivo but dispensable for activity in vitro

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ABSTRACT

The general splicing factor U2AF⁶⁵ recognizes the polypyrimidine tract (Py tract) that precedes 3' splice sites and has three RNA recognition motifs (RRMs). The C-terminal RRM (RRM3), which is highly conserved, has been proposed to contribute to Py-tract binding and establish protein–protein contacts with splicing factors mBBP/SF1 and SAP155. Unexpectedly, we find that the human RRM3 domain is dispensable for U2AF⁶⁵ activity in vitro. However, it has an essential function in *Schizosaccharomyces pombe* distinct from binding to the Py tract or to mBBP/SF1 and SAP155. First, deletion of RRM3 from the human protein has no effect on Py-tract binding. Second, RRM123 and RRM12 select similar sequences from a random pool of RNA. Third, deletion of RRM3 has no effect on the splicing activity of U2AF⁶⁵ in vitro. However, deletion of the RRM3 domain of *S. pombe* U2AF⁵⁹ abolishes U2AF function in vivo. In addition, certain amino acid substitutions on the four-stranded β -sheet surface of RRM3 compromise U2AF function in vivo without affecting binding to mBBP/SF1 or SAP155 in vitro. We propose that RRM3 has an unrecognized function that is possibly relevant for the splicing of only a subset of cellular introns. We discuss the implications of these observations on previous models of U2AF function.

Keywords: RNA-binding proteins; splicing; Py tract; RRM; RNA-binding motif

INTRODUCTION

Pre-mRNA splicing removes introns from nascent transcripts in eukaryotes. It involves a dynamic RNA and protein complex called the spliceosome (Hastings and Krainer 2001; Will and Luhrmann 2001). Signal sequences at the 5' and 3' splice sites of introns help to assemble the spliceosomal components. In higher eukaryotes, the polypyrimidine tract (Py tract) adjacent to the 3' splice site is an essential splicing signal (Moore 2000; Reed 2000). The general splicing factor U2AF specifically recognizes the Py tract and facilitates the recruitment of the U2 snRNP to the pre-mRNA branch site. Human U2AF comprises a large subunit (U2AF⁶⁵) and a small subunit (U2AF³⁵; Zamore et al. 1992;

Zhang et al. 1992). U2AF⁶⁵ has a modular structure, with an N-terminal activation domain and a C-terminal RNA-binding domain (Zamore et al. 1992). The RNA-binding domain is important for binding to the Py tract, and the activation domain is important for the recruitment of the U2 snRNP to the pre-mRNA branch site (Ruskin et al. 1988). The recruitment in part involves stabilization of RNA–RNA base pairing between the pre-mRNA branch site and the U2 snRNA (Valcarcel et al. 1996). The small subunit of U2AF interacts with the 3' splice site AG dinucleotide and plays an important role in the splicing of regulated introns or introns that have weak Py tracts (Merendino et al. 1999; Wu et al. 1999; Zorio and Blumenthal 1999a). In addition, U2AF⁶⁵ interacts with other splicing factors such as UAP56, mBBP/SF1, SAP155, and p54 (Zhang and Wu 1996; Fleckner et al. 1997; Berglund et al. 1998; Gozani et al. 1998). The U2AF⁶⁵ orthologs in the fruit fly, the nematode, and the fission yeast are essential for viability (Kanaar et al. 1993; Potashkin et al. 1993; Zorio and Blumenthal 1999b). The likely budding yeast ortholog, mud2p, although dispensable for viability, has a role in 3' splice site selection (Abovich et al. 1994).

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In addition to the well-characterized role of U2AF⁶⁵ in constitutive splicing, it also plays an important role in regulated splicing (Black 2000; Smith and Valcarcel 2000; Graveley 2001; Singh 2002). For example, competition between U2AF⁶⁵ and the *Drosophila* protein sex-lethal (SXL), which is the master sex-switch in somatic cells, leads to 3' splice site switching for the *transformer* (*tra*) pre-mRNA (Boggs et al. 1987; Sosnowski et al. 1989; Inoue et al. 1990; Valcarcel et al. 1993; Granadino et al. 1997), and intron retention for the *male-specific-lethal2* (*msl2*) pre-mRNA (Zhou et al. 1995; Bashaw and Baker 1997; Kelley et al. 1997; Gebauer et al. 1998; Merendino et al. 1999). Whereas the splicing regulation of *tra* and *msl2* involves blocking an early step during spliceosome assembly, it has been proposed that the exon skipping for *Sxl* pre-mRNA is regulated by blocking the second step of splicing (Sakamoto et al. 1992; Horabin and Schedl 1993; Wang and Bell 1994; Penalva et al. 2001; Lallena et al. 2002). Interactions of SXL with U2AF and U1 snRNP may serve to block the function of these factors (Nagengast et al. 2003) or to further stabilize SXL binding. Similar to the splicing repressor SXL, the polypyrimidine-tract-binding protein (PTB) also represses certain 3' splice sites most likely by competing for the binding of U2AF⁶⁵ (Mulligan et al. 1992; Lin and Patton 1995; Singh et al. 1995; Ashiya and Grabowski 1997; Chan and Black 1997; Gooding et al. 1998; Cote et al. 2001).

Given the modular domain structure of U2AF⁶⁵, fusion of the N-terminal activation domain of U2AF⁶⁵ to the C-terminal RNA-binding domain of SXL converted the splicing repressor SXL into an activator (Valcarcel et al. 1993; Granadino et al. 1997). This chimera, called USx, specifically activates splicing via the non-sex-specific (NSS) Py tract of the *tra* pre-mRNA, which is the cognate SXL-binding site. However, in contrast to the RNA-binding domain of SXL, the RNA-binding domain of U2AF⁶⁵ recognizes a wide variety of Py tracts. It consists of three RNA recognition motifs (RRMs), which is a common RNA-binding domain found in nature (Zamore et al. 1992; Varani and Nagai 1998). Each RRM domain comprises a four-stranded antiparallel β -sheet and two α -helices, in which the central β -strands correspond to the characteristic ribonucleoprotein motifs RNP-1 and RNP-2. It was reported that all three RRMs of U2AF⁶⁵ are important for Py-tract recognition and splicing in vitro (Zamore et al. 1992). In addition, although the sequence of U2AF⁶⁵ is highly conserved from the fission yeast to humans, the RRM3 domain is the only recognizable portion present in the budding yeast protein mud2p (Abovich et al. 1994). Moreover, RRM3-related domains in U2AF⁶⁵ family (PUF60, dPUF68, U2AF⁶⁵, dU2AF⁵⁰, and Mud2p) and in U2AF³⁵ family (dU2AF³⁸, U2AF³⁵, and Urp) of splicing factors define an RRM subfamily, known as the PUMP domain (PUF60, U2AF⁶⁵, Mud2p protein-protein interaction domain; Page-McCaw et al. 1999). These observations suggests that RRM3 plays an important role.

Here, we show that although the RRM3 domain of the

large subunit of U2AF is essential for viability in *Schizosaccharomyces pombe*, it is dispensable for splicing in vitro and likely has an additional unrecognized function.

RESULTS

RRM3 is dispensable for the binding of U2AF⁶⁵ to several natural Py tracts

The RRM3 domain of the general splicing factor U2AF⁶⁵ is highly conserved (Fig. 1A). Intriguingly, in our recent cross-linking studies, RRM3 was not cross-linked to 5-iodouridines incorporated at specific positions within a Py tract (Banerjee et al. 2003). This could be because of limitations of the cross-linking approach such as requirements for specific amino acids in the vicinity and for proper orientation of functional groups that are involved in cross-linking (Willis et al. 1993; Meisenheimer et al. 2000); it is known that certain RNAs can bind with high affinity but not cross-link (Gott et al. 1991; Meisenheimer et al. 1996). Therefore, to determine if RRM3 has any direct role in Py-tract recognition, we tested several natural Py tracts for the binding of U2AF⁶⁵ with (RRM123) or without (RRM12) the RRM3 domain. The Py tracts tested here differ in their length and content of uridines, and thus in their binding affinities for U2AF⁶⁵. For example, U2AF⁶⁵ has about 100-fold and 10-fold lower affinities for the *tra* female-specific (*tra*-FS) and the adenoviral major late (AdML) Py tracts, respectively, compared to the *tra* non-sex-specific (*tra*-NSS) Py tract (Valcarcel et al. 1993; H. Banerjee and R. Singh, unpubl. data). Moreover, the AdML, *tra*-NSS(maxi), and *tra*-FS Py tracts have different lengths of flanking sequences and are present in different sequence contexts. Figure 2 shows that both RRM123 and RRM12 bound to each of these sequences with comparable affinity, which was reproducible within two- to threefold experimental variation typically associated with protein dilutions. The apparent equilibrium dissociation constants (K_d values) for different substrates from three experiments were approximately: AdML (5×10^{-7} M), *tra*-NSS(mini) (1.89×10^{-8} M), *tra*-NSS(maxi) (0.6×10^{-8} M), *tra*-FS (1.5×10^{-6} M), and α -TM(A) and α -TM(P) (1.1×10^{-8} M). In addition, a lack of RRM3 showed no difference in binding affinities for even longer Py tracts [α -TM(A) and α -TM(P)] (Berglund et al. 1998). These results show that RRM3 makes no contribution to the binding affinity of U2AF⁶⁵ for several natural Py tracts that differ widely in their binding affinities.

The RNA-binding properties of RRM123 and RRM12 are indistinguishable

Although deletion of RRM3 had no effect on the binding of four natural Py tracts, it remained possible that RRM3 could interact with specific sequences outside of the Py tract or contribute to Py-tract binding in particular sequence

FIGURE 1. The RRM3 domain is highly conserved. (A) Amino acid sequence alignment of U2AF⁶⁵ RRM3 from various organisms. The RRM3 sequences are from different organisms: Hs, *Homo sapiens*; Sp, *Schizosaccharomyces pombe*; Mm, *Mus musculus*; Sc, *Saccharomyces cerevisiae*; Ce, *Caenorhabditis elegans*; Cb, *Caenorhabditis briggsiae*; Dm, *Drosophila melanogaster*; Np, *Nicotiana plumbaginifolia*; Lc, *Lycopersicon esculentum*; At, *Arabidopsis thaliana*. (B) Alignment of the three RRM3 of the human U2AF⁶⁵ and two RRM3 of the *Drosophila* SXL is shown. (C) Evolutionary relationship among the RRM3 of U2AF⁶⁵ and SXL. Alignment was done using ClustalW.

contexts. The limited RNAs that were tested in Figure 2 might have lacked a binding site for RRM3 to show its effect on binding affinity. Therefore, we performed in vitro selection–amplification, also known as Systematic Evolution of Ligands by EXponential enrichment (SELEX; Tuerk and Gold 1990), from a random pool of RNA to select sequences that bind RRM123 or RRM12. Figure 3 shows sequences of 23 independent clones from each of the selected pools. Both sets of uridine-rich sequences (U123- and U12-series) are comparable in the length and pyrimidine richness, and are similar to natural Py tracts (Mount et al. 1992).

Next, a mixture of RNAs from pools 6 as well as three

individual clones each from pools 6 selected by either RRM123 (U123-pool 6, U123-1, 2, and 3) or RRM12 (U12-pool 6, U12-1, 2, and 3) were directly analyzed for U2AF⁶⁵ binding. Figure 4 shows that each of the sequences tested had comparable binding affinity for both RRM123 and RRM12, which was reproducible within two- to threefold experimental variation. The K_d values for either selected pools or representative RNAs from each pool from three experiments were approximately: U123-pool 6, U12-Pool 6, U12-1, and U123-1 (2×10^{-7} M); U12-2 (0.5×10^{-7} M); U12-3 (1×10^{-6} M); U123-2 and U123-3 (1×10^{-8} M). These results confirm that the RNA-binding properties of RRM123 and RRM12 are indistinguishable, and that RRM3 plays no role in the recognition of natural or artificially selected Py tracts that have different binding affinities for U2AF⁶⁵ and are present in varied sequence contexts.

Given that RRM3 did not contact Py tracts, it was of significant interest to determine if RRM3 interacted with any RNA. Our attempts with in vitro selection–amplification to identify sequences that directly bind RRM3 have so far shown a barely detectable RNA-binding activity, if any, for RRM3 alone (V. Sridharan and R. Singh, unpubl. data).

The deletion of RRM3 does not compromise the splicing activity of U2AF⁶⁵

Because RRM3 does not contribute to Py-tract recognition, we asked if RRM3 is important for the splicing activity of U2AF⁶⁵. Figure 5A shows that reconstitution of a U2AF-depleted nuclear extract, which failed to support splicing in vitro (lane 2), with either the full-length recombinant U2AF⁶⁵ (GST-U2AF⁶⁵ 1-475; lane 3) or the dialyzed guanidine eluate of the proteins bound to the affinity-depletion column (lane 5) fully rescued splicing. However, addition of the U2AF⁶⁵ protein lacking RRM3 (GST-U2AF⁶⁵ 1-346) to the U2AF-depleted nuclear extract also rescued the splicing of the AdML pre-mRNA (lane 4), indicating that RRM3 is dispensable for the splicing of certain introns.

The AdML intron is an example of AG-independent introns, which have strong Py tracts. In contrast, AG-dependent introns such as the IgM intron (Watakabe et al. 1993;

FIGURE 2. (Top) Deletion of RRM3 has no effect on Py-tract binding. Proteins 123 and 12 represent RRM123 and RRM12 of U2AF⁶⁵, respectively. (Bottom) Schematics of RNAs, with respect to the length of Py tracts (filled boxes) and flanking sequences (open boxes). The Py tracts are: *tra*-NSS, *tra* non-sex-specific (mini and maxi); *tra*-FS, *tra* female-specific; AdML, adenoviral major late; α-TM(A), α-Tropomyosin digested with AccI; and α-TM(P), α-Tropomyosin digested with PvuII, as described (Patton et al. 1991; Singh et al. 1995; Banerjee et al. 2003). Given different binding affinities for various Py tracts, only relevant protein concentrations are shown for each Py tract. The protein concentrations for RRM123 were approximately: AdML, 1.23 ng/μL, 3.7 ng/μL, 11.1 ng/μL, 33.3 ng/μL, and 100 ng/μL; *tra*-NSS(mini), 0.14 ng/μL, 0.41 ng/μL, 1.23 ng/μL, 3.7 ng/μL, 11.1 ng/μL; *tra*-NSS(maxi), 0.09 ng/μL, 0.27 ng/μL, 0.82 ng/μL, 2.5 ng/μL, 7.4 ng/μL; α-TM(A), and α-TM(P), 0.17 ng/μL, 0.5 ng/μL, 1.5 ng/μL, 4.5 ng/μL, 13.5 ng/μL; *tra*-FS 2.5 ng/μL, 7.4 ng/μL, 22.2 ng/μL, 66.6 ng/μL, and 200 ng/μL.

Guth et al. 2001) have weak Py tracts, and thus depend on the invariant AG dinucleotide for spliceosome assembly (Reed 2000). It should be emphasized that studies with such introns revealed a requirement for the splicing factor U2AF³⁵ in splicing (Wu et al. 1999). Therefore, we asked if RRM3 was necessary for the splicing of an intron containing a weak Py tract or that of an AG-dependent Py-tract/3' splice site. Figure 5B shows that RRM3 was also dispensable for the splicing of the AG-dependent IgM intron (lanes 3,4 versus 5,6); the presence of U2AF³⁵ had a stimulatory effect on splicing. The RRM3 deletion (U2AF⁶⁵ 1-346) that was used for the splicing experiments also had an RNA-binding activity comparable to that of the full-length U2AF⁶⁵ (data not shown). These results show that RRM1 and RRM2 together provide the necessary Py-tract-binding activity for splicing in vitro. Furthermore, RRM3 contributes neither to Py-tract recognition nor to any other function of U2AF⁶⁵ that is required for the in vitro splicing of the substrates tested in this study.

Although RRM3 is dispensable for splicing in vitro, it remained possible that early events during spliceosome as-

sembly could be more sensitive to a requirement for RRM3. Therefore, we asked if presence of RRM3 would show any detectable effect on spliceosome assembly. To test this argument, spliceosome assembly was carried out using either the full-length AdML or a 3'-half substrate that supported the formation of only the spliceosomal A complex. It is known that certain defects in splicing or spliceosome assembly can be uncovered using minimal splicing signals (Query et al. 1997). Figure 5C shows that the ability of the ΔRRM3 protein to promote complex assembly was reproducibly similar or only slightly reduced compared to that of the full-length protein, particularly for the 3'-half RNA (lanes 8 and 9). Therefore, we conclude that although subtle differences in spliceosome assembly can be detected between the full-length and ΔRRM3 proteins under our experimental conditions, RRM3 is nonetheless dispensable for both spliceosome assembly and splicing of certain introns in vitro.

RRM3 is required in vivo

The large subunit of U2AF is highly conserved from yeast to humans (Fig. 1A). Although sequence conservation between the human and the fission yeast (*S. pombe*) proteins extends over the entire length of protein, the RRM3 domain is the only easily recognizable portion of the budding yeast (*Saccharomyces cerevisiae*) protein mud2p (Fig. 1A). Thus, it was expected from the high degree of sequence conservation that RRM3 would play an important function. Intriguingly, RRM3 is dispensable for the RNA-binding and the splicing activity of U2AF⁶⁵ in vitro (Figs. 2–5). Nevertheless, it remained possible that RRM3 performed an essential function in vivo, which was technically difficult to assess for the human U2AF⁶⁵. Therefore, we deleted the RRM3 domain of U2AF⁵⁹ (*prp2*) (Fig. 6A), which is an essential gene in *S. pombe* (Potashkin et al. 1993; Romfo et al. 1999), and assayed its effect on U2AF⁵⁹ function in vivo. Previously, it was shown that no surviving Ura⁺ spores were obtained from the yeast strain SpCR1 in which one copy of *prp2* (U2AF⁵⁹) had been disrupted with a *ura4* selectable marker, suggesting that U2AF⁵⁹ is essential for vegetative growth in *S. pombe* (Romfo et al. 1999). Using this assay, we showed that two independent deletions of RRM3 (PRP2-ΔRRM3a and PRP2-ΔRRM3b) abolished U2AF⁵⁹ function in vivo, as determined by the inability of the Ura⁺ spores containing

RRM123

[illegible]

BBM12

[illegible]

FIGURE 3. Selection–amplification of sequences by RRM123 and RRM12. Twenty-three sequences each are shown from pools 6 selected by either RRM123 (U123-series) or RRM12 (U12-series), using SELEX from a random pool of RNA.

deletions of RRM3 to grow on EMM2 + A and EMM2 + AL plates (for details, see Table 1). The expression of the PRP2- Δ RRM3a gene was confirmed by both Western analysis of the protein blot (Fig. 7C, lane 4, Δ RRM3; Fig. 7D, lanes 1–4 for loading control) and RT-PCR analysis of cellular transcripts (Fig. 7A, lanes 2,3, –RRM3 band). The authenticity of PCR bands was confirmed by the fact that the +RRM3 band, but not the –RRM3 band, as expected, could be digested and reduced to a smaller size by two appropriate restriction enzymes. These results indicate that RRM3 plays an essential role in vivo.

Amino acid substitutions in RRM3 significantly compromise U2AF⁵⁹ function in vivo

Because RRM3 has two recognizable elements (RNP1 and RNP2) that characterize canonical RRM motifs (Fig. 1A;

Varani and Nagai 1998), we made point mutations for amino acids that are predicted to be on the potential four-stranded antiparallel β -sheet surface of RRM3; this surface has been implicated in RNA binding for other RRM proteins (Varani and Nagai 1998). In the absence of the structure of RRM3, the mutagenesis studies were guided by structure modeling, using the crystal structures of the RRM3s of the U1A and SXL proteins (Varani and Nagai 1998; Handa et al. 1999). Four mutations (H423A, F476A, FY506,7AA, and HF423,476AA) had no detectable effect on viability (Table 1). However, the HFY423,506,7AAA (HFY-m) mutant containing three amino acid substitutions significantly compromised U2AF function. To rule out the possibility that a few colonies that grew for this mutant resulted from gene conversion, we sequenced plasmids recovered from eight independent colonies. All of them contained the mutant sequence (data not shown), indicating that gene conversion was not responsible for the growth of these colonies. We were able to confirm, using a mutant-specific oligonucleotide for RT-PCR, that the mutant transcript was expressed well in vivo (Fig. 7B, lane 5, P3P4 band), and could be translated in vitro (see Fig. 8A). In addition, we analyzed the expression of the HFY-m protein by Western blot analysis. Figure 7C shows that the plasmid-encoded wild-type and HFY-m proteins were expressed well and were stable in vivo (lanes 2,3). Unlike the HFY-m transcript (Fig. 7B, lane 5), the steady level of the HFY-m protein was approximately two- to threefold lower than that of the wild-type protein (RRM123). However, we believe that the slightly reduced level of the plasmid-encoded HFY-m protein was unlikely to be the basis for the mutant phenotype because the levels of HFY-m were significantly higher than those of the chromosomally encoded wild-type protein, which, despite being detectable only upon longer exposures of these blots, evidently supported U2AF⁵⁹ function in vivo (Fig. 7C,D, lanes 1). Furthermore, although the multicopy plasmid contributed to the higher level of expression, this level of the wild-type protein had no detrimental effect on U2AF⁵⁹ function (Table 1). Therefore, we conclude that the potential four-stranded β -sheet surface of RRM3 is important for U2AF function in vivo.

The RRM3 mutation has no effect on binding to mBBP/SF1 or SAP155

The RRM3 domain is known to interact with splicing factors mBBP/SF1 and SAP155 (Berglund et al. 1998; Gozani et al. 1998). Thus, the effect of the HFY-m mutation on viability could be due to its effect on the binding of these proteins or that of an unidentified splicing factor. To distinguish between these possibilities, we analyzed the binding of RRM3 to these proteins. As expected (Berglund et al. 1998; Gozani et al. 1998), the in vitro translated U2AF⁵⁹ protein containing RRM3 bound to the mammalian mBBP/SF1 (Fig. 8A, lane 4) as well as *S. pombe* SAP155 (lane 7)

FIGURE 4. RNA binding for sequences selected by either RRM123 or RRM12. The U123- and U12-series refer to the RNA sequences selected by RRM123 and RRM12, respectively. Pool 6 and three individual clones from the U123- and U12-series from pools 6 were analyzed for binding. Nucleotide sequences of these clones are given in Figure 3. –, no protein; the protein concentrations for RRM123 for each RNA panel were approximately 2.4 ng/ μ L, 9.6 ng/ μ L, 38.4 ng/ μ L, and 153.6 ng/ μ L; the protein concentrations for RRM12 for each RNA panel were approximately 1.6 ng/ μ L, 6.4 ng/ μ L, 26.6 ng/ μ L, and 106.4 ng/ μ L.

proteins, but that lacking RRM3 did not (lanes 6 and 9). The HFY-m mutant also bound to mBBP/SF1 (lane 5) and SAP155 (lane 8). Moreover, there was no detectable difference in binding affinities of the wild-type (RRM123) and mutant proteins for either SAP155 (Fig. 8B, lanes 1–5 versus 6–10, top panel) or mBBP/SF1 (lanes 1–5 versus 6–10, middle panel); the binding was reproducible within twofold of experimental variation. Unavailability of the *S. pombe* BBP cDNA precluded our analysis with the homologous protein. We emphasize that RNase A treatment did not affect binding (Fig. 8A, lanes 4–9, +RNase A panel), suggesting that the interaction is direct rather than through an RNA molecule. We conclude that the HFY-m mutation that compromises U2AF⁵⁹ function in vivo has no detectable effect on its binding to splicing factors mBBP/SF1 and SAP155.

Our combined results show that RRM3 has an important function in vivo that is independent of its interactions with mBBP/SF1, SAP155, or the Py tract.

DISCUSSION

Here we report that the RRM3 domain of the large subunit of the human U2AF does not contribute to Py-tract recognition, and is dispensable for splicing in this assay, at least for the substrates tested. Nonetheless, RRM3 performs an important role in *S. pombe*, and likely also in other organisms, that is unrelated to its binding to mBBP/SF1 or

SAP155. Below we discuss the biological implications of these findings and present a model for the possible role of RRM3 during splicing (Fig. 9).

Two RRM3s are sufficient for Py-tract recognition

We propose that only the RRM1 and RRM2 domains of U2AF⁶⁵ contribute to Py-tract recognition. Accordingly, the RRM3 domain has a function different from Py-tract recognition. Although RRM3, as the name implies, are commonly associated with RNA recognition (Varani and Nagai 1998), several observations suggest that RRM3 in general (Varani et al. 2000) and RRM3 in particular can have other activities. First, RRM3 is more divergent from the other RRM3s of U2AF⁶⁵ and of SXL that bind Py tracts (Fig. 1B,C), and also lacks critical aromatic residues. Second, it is tethered to RRM2 by a long linker, which could make it function independently of the other RRM3s in the protein, that is, binding other targets in *trans* (Shamoo

et al. 1995). Third, it physically associates with other proteins (mBBP/SF1 and SAP155), and genetically interacts with U1 snRNA in the budding yeast, where it is dispensable for viability (Abovich et al. 1994; Berglund et al. 1998). In addition, an RRM3-related domain of PUF60 is dispensable for RNA binding (Page-McCaw et al. 1999). Fourth, it does not cross-link to Py tracts (Banerjee et al. 2003) and does not contribute to binding for short or long Py tracts (Figs. 2–4; Berglund et al. 1998). Fifth, we have not been able to identify RNA aptamers for RRM3 from a random pool of RNA (data not shown). Finally, in proteins that have multiple RRM3s such as U2AF⁶⁵, PABP, HuD, PUF60, SXL, and PTB (Chung et al. 1996; Deo et al. 1999; Handa et al. 1999; Page-McCaw et al. 1999; Conte et al. 2000), two RRM3s are sufficient for binding to cognate RNA sequences, including the Py-tract-binding activity of U2AF⁶⁵ described here. Furthermore, given that RRM3 is the only conserved portion of the *S. cerevisiae* ortholog (mud2p), our results could also help explain why Py tracts are either absent in *S. cerevisiae* introns or can be deleted without affecting splicing of certain introns (Patterson and Guthrie 1991). Thus, RRM3 has a function distinct from Py-tract recognition.

Why is RRM3 dispensable in vitro, but required in vivo?

Paradoxically, RRM3 is essential for viability in *S. pombe* (Table 1) and likely also in *Drosophila* (Kanaar et al. 1993),

FIGURE 5. Deletion of RRM3 has no effect on the splicing of the AdML (A) or IgM (B) pre-mRNA substrates in vitro. (A) NE, nuclear extract; Δ NE, U2AF-depleted nuclear extract (Valcarcel et al. 1997). Recombinant GST-fusion proteins are shown at the top. Δ RRM3 is missing amino acids 347–475. Guanidine is the guanidine-HCl eluate from the affinity column used for U2AF depletion. The splicing product and intermediates are shown. (C) Spliceosome assembly using the full-length AdML (lanes 1–5) or the 3' half of the intron (lanes 6–10). Splicing complexes A, B, and C, and the hnRNP complex H are indicated.

but dispensable in vitro for the splicing or spliceosome assembly activity of the human protein (Fig. 5). Although experiments with the *Drosophila* protein were not designed to specifically test the role of RRM3, the frame-shift mutation in the ~10-kb genomic DNA analyzed by Rio and co-workers, which led to the cloning of the *dU2AF⁵⁰* gene and to the important conclusion that this potential open reading frame encodes an essential function in *Drosophila* (Kanaar et al. 1993), happens to be in the proximity of the boundary between RRM2 and RRM3. Although this mutation was not characterized further and may have affected functions of *Drosophila* U2AF⁵⁰ differently from those associated with RRM3, the results are certainly consistent with its requirement for viability in the fruit fly. In addition, the USx chimera, which lacks the RRM3 domain of U2AF⁶⁵, also supports splicing to the NSS 3' splice site of *tra* both in vitro and in vivo (Valcarcel et al. 1993; Granadino et al. 1997). These observations show that RRM3 function is also dispensable for the splicing of certain introns in vivo. This aspect has largely remained unappreciated, presumably be-

cause it had been assumed that the two RRM3s of SXL merely substituted for the RNA-binding function of the three RRM3s of U2AF⁶⁵. Previous studies concluded that all three RRM3s are required for Py-tract binding and thus splicing (Zamore et al. 1992). We suggest that the most likely reason for this discrepancy is that the U2AF⁶⁵ proteins used here (U2AF⁶⁵ 110–342 and U2AF⁶⁵ 1–346) retain a complete RRM2 (amino acids 258–342; Ito et al. 1999), whereas the protein used in previous studies (U2AF⁶⁵ 64–324) also eliminated a portion of RRM2 that included the β -strand 4 (Zamore et al. 1992), which may have affected the overall folding or binding properties of RRM2. Although RRM3 may behave differently in different organisms, the simplest way to reconcile these observations—that RRM3 is essential in *S. pombe* but dispensable for splicing in HeLa extract—is that RRM3 is required for the splicing of some introns but dispensable for others. It should be pointed out that the RS domain of the small subunit of U2AF has also shown different requirements between in vitro and in vivo assays (for discussion, see Rudner et al. 1998).

Although it remains formally possible that the large subunit of the human and *S. pombe* U2AF proteins may not function or interact with other splicing factors in an identical manner in every respect during splicing or spliceosome assembly, we reiterate that the sequence as well as functions—the site of interaction with pre-mRNA and splicing factors—of U2AF⁶⁵ orthologs in several organisms are remarkably conserved (Fig. 1A), suggesting that the conclusions drawn from cross-species comparisons—dispensability of RRM3 for splicing in vitro and requirement in *S. pombe*—should be biologically meaningful. In fact, the region of SF1 that interacts with RRM3 is also highly conserved between mammals and *S. pombe*; the invariant tryptophan and critical basic residues are present in all SF1 orthologs (Fig. 8C). Therefore, the question arises as to how a highly conserved and essential domain is dispensable for the splicing of certain introns, which is indicated by a broken line in Figure 9. The simplest interpretation is that RRM3 is required for a step(s) during spliceosome assembly that could be rate limiting for some introns, at least under certain conditions. Accordingly, the splicing of certain pre-mRNA substrates (Fig. 5) may not depend on a putative rate-limiting step that involves RRM3. We speculate that the USx chimera lacking RRM3 (Valcarcel et al. 1993; Granadino et al. 1997) most likely supports splicing to the NSS-Py tract/3' splice site because of its higher affinity for the Py tract, possibly by overcoming, directly or indirectly, a rate-limiting step that requires RRM3. The rate-limiting process could be one of many steps during spliceosome assembly, such as recognition of the pre-mRNA branch site by the U2 snRNP or any function prior to the release of U2AF from the spliceosome (Bennett et al. 1992). In addition, presence of negative regulatory elements could also render splicing of certain pre-mRNAs RRM3 dependent.

FIGURE 6. Schematic of the RRM3 domain of *S. pombe* U2AF⁵⁹. (A) The amino acids that were mutagenized in different clones are underlined. For reference, amino acids for predicted β-1 to β-4 strands are also shown. A line represents amino acid identity, absence of a line represents a deletion, and one or two A residues represent alanine substitutions. The mutations are as follows: ΔRRM3a, amino acids 1–401, which correspond to amino acids 1–342 of the human U2AF⁶⁵; ΔRRM3b, amino acids 1–421, in which deletion of adenine 422 generates a translation stop codon at position 425. Nomenclature for other mutants: the numbers refer to amino acid positions, the letter before the number is the wild-type amino acid, and one or more A residues following the number represent the substituted alanine(s). H, histidine; F, phenylalanine; Y, tyrosine; A, alanine. (B) A ribbon diagram of the structure of mammalian RRM3 is shown, redrawn with RasMol v2.6 using atomic coordinates of the U2AF⁶⁵-RRM3/SF1 complex (Protein Data Bank, accession code 1O0P) from the NMR structure (Selenko et al. 2003). Asterisks represent positions of corresponding amino acid substitutions in the *S. pombe* sequence. Four β-strands (β1–β4) and three α-helices (A–C) in RRM3 are labeled. The backbone of the mBBP/SF1 peptide (amino acids 15–25) is shown.

We propose that one or more pre-mRNAs that encode an essential function(s) in vivo require the activity of RRM3. Consistent with this proposal, previous nuclear localization

studies have shown that ΔRRM3 mutants are still localized in the nucleus (Gama-Carvalho et al. 1997). However, we cannot exclude the possibility that RRM3 contributes to

TABLE 1. Deletion of RRM3 abolishes and point mutations in RRM3 significantly compromise U2AF⁵⁹ function in vivo.

Plasmids	Number of colonies on			
	YEA ^a	EMM2 + AL ^b	EMM2 + AU ^c	EMM2 + A ^d
–	90 ± 0 ^e	0	0	0
Empty vector	90	0	90	0
PRP2	90	36 ± 5	90	38 ± 6
ΔRRM3a	90	0	90	0
ΔRRM3b	90	0	90	0
H423A	90	45 ± 8	90	43 ± 9
F476A	90	36 ± 1	90	44 ± 3
FY506,507AA	90	35 ± 12	90	36 ± 14
HF423,476AA	90	49 ± 3	90	43 ± 2
HFY423,506,507AAA (HFY-m)	90	5 ± 1	90	6 ± 3

Total number of haploid spores on defined media, described below, is shown. The diploid *S. pombe* strain SpCR1 (*prp2::ura4/prp2+*, *ade6-M210/ade6-M216*, *ura4d18/ura4d18*, *leu 1-32/leu 1-32*), which is heterozygous for the *prp2* gene disruption, was used for plasmid complementation. The plasmids are: –, no plasmid; empty vector, pIRT3 plasmid alone; *prp2*/pIRT3, plasmid pIRT3 with the wild-type *prp2* gene; and other mutants, also in pIRT3, are described in the legend to Figure 6A. Following transformation of the SpCR1 strain with appropriate plasmids, cells were sporulated, the spores were initially plated on EMM2 + AU media and checked for haploids (red or pink) by plating on a rich medium (YEA). The viable haploids were analyzed for growth by replica plating on defined media (Moreno et al. 1991).

The media were as described previously (Romfo et al. 1999).

^aRich medium (YEA).

^bMinimal medium supplemented with adenine and leucine (100 mg/L each). The SpCR1 cells, in which one copy of *prp2* was disrupted with a *ura4* marker, transformed with the vector alone or plasmids with *prp2* gene deleted of RRM3 (ΔRRM3a and ΔRRM3b) failed to grow because *prp2* encodes an essential function.

^cMinimal medium supplemented with adenine and uracil (100 mg/L each). Cells containing the plasmid (with a functional *leu2* gene) will grow on these plates.

^dMinimal medium supplemented with adenine (Moreno et al. 1991). Cells need both the disrupted allele and the plasmid to grow.

^eIf not shown, the standard deviation was zero.

FIGURE 7. The RRM3 mutants (Δ RRM3 and HFY-m) are transcribed and translated in vivo. (A) The Δ RRM3a transcript is expressed in vivo. Total cellular RNAs from either control cells (SpCR1) or cells containing the plasmid with the Δ RRM3a mutant were analyzed by RT-PCR. (Lane 1) RNA from control cells; (lanes 2 and 3) RNA from cells containing a plasmid with the Δ RRM3a mutant; (lane 3) prior to electrophoresis, the PCR product was digested with MscI and NsiI enzymes, which cut only the wild-type PCR product from the endogenous *prp2* gene, shown as +RRM3, but not that from the plasmid-encoded Δ RRM3a mutant, shown as -RRM3, in which these sites have been destroyed. Asterisks in panels A and B represent primers. (B) The HFY-m transcript is expressed in vivo. Total RNA was isolated from either control cells or cells with plasmids containing either the wild-type or the HFY-m mutant genes. The 3' end of the P4 primer perfectly base pairs with the HFY-m mutant transcript but not the wild-type transcript. In contrast, the P2 and P3 primers hybridize indistinguishably to both the wild-type and mutant transcripts, used here as internal controls. Size markers (in base pairs) are shown. (C) The RRM3 mutant proteins (Δ RRM3a and HFY-m) are stably expressed in vivo. Total cell protein lysates from either control SpCR1 cells (-) or cells containing plasmids with the wild-type (RRM123), HFY-m, or Δ RRM3a genes, indicated above respective lanes, were analyzed by Western analysis using anti-U2AF⁵⁹ polyclonal antibody. Arrows indicate the U2AF⁵⁹ and Δ RRM3 protein bands. (D) For loading controls, a Coomassie-stained gel of the lysates used for Western analysis in panel C is shown. Size markers (in kilodaltons) are shown.

proper subnuclear localization, dynamics, conformation, or turnover, which could potentially explain the *S. pombe* and in vitro splicing differences.

Possible functions of RRM3

The interactions of RRM3 with the Py tract or with known splicing factors (mBBP/SF1 and SAP155) could explain the sequence conservation of RRM3 as well as synthetic lethality of *mud2* (Zamore et al. 1992; Abovich et al. 1994; Berglund et al. 1998; Gozani et al. 1998). However, RRM3 is dispensable for binding to a variety of Py tracts (Figs. 2, 3) and

splicing in vitro (Fig. 5) for at least three splicing substrates. As a corollary, the factors that interact with RRM3 are also dispensable for the splicing of at least a subset of substrates. It is possible that RRM3 plays a kinetic rather than an essential role during in vitro splicing. This proposal is consistent with previous observations that SF1/BBP orthologs in *S. cerevisiae* (Rutz and Seraphin 1999) and humans (Guth and Valcarcel 2000) can be depleted without significant effects on in vitro splicing, and that Mud2p, in which the RRM3 domain is the only recognizable and conserved portion of the protein, is dispensable in *S. cerevisiae* (Abovich et al. 1994). Temperature-sensitive mutants in *S. cerevisiae* showed splicing defects in vivo at restrictive temperatures only for reporter introns with weakened splicing signals, but not for an intron with consensus splice sites (Rutz and Seraphin 2000). Although SF1/BBP and U2AF⁶⁵ containing the RRM3 domain bind synergistically to RNA substrates in a reconstitution system with minimal components (Berglund et al. 1998), SF1/mBBP appears to play an additional role in U2 snRNP recruitment in complete nuclear extracts (Guth and Valcarcel 2000). This is contrary to the expectation that degenerate splicing signals in mammals, in contrast to yeast, should increase their dependence on factors such as SF1/mBBP.

A combination of three point mutations in RRM3 that has no significant effect on the binding of mBBP/SF1 or SAP155 to U2AF⁵⁹ in vitro compromises U2AF⁵⁹ function in *S. pombe* (Fig. 8A; Table 1). It should be noted that the point mutant HFY-m, unlike the RRM3

deletion, significantly compromises rather than completely eliminates the U2AF function. Thus, we propose that the RRM3 domain has an additional, important function that is independent of its binding to these two proteins or to the Py tract (Fig. 9, question mark). Possible function(s) of RRM3 include its interactions with RNA (snRNA or pre-mRNA), protein, or both. Although RRM3 has a noncanonical RRM, the U2AF³⁵ protein, which contains a variant RRM, directly interacts with the 3' splice site when in complex with U2AF⁶⁵ (Merendino et al. 1999; Wu et al. 1999; Zorio and Blumenthal 1999a). Thus, RRM3 could still have a role in RNA recognition. It could serve to stabilize U2AF⁶⁵ binding

FIGURE 8. The HFY-m mutant protein interacts efficiently with splicing factors mBBP/SF1 and SAP155. (A) The RRM3 mutation HFY-m that compromises U2AF⁵⁹ activity in vivo does not disrupt binding to the mammalian mBBP/SF1 or *S. pombe* SAP155 proteins. In vitro translated, ³⁵S-radiolabeled proteins (wild-type RRM123, HFY-m mutant, and Δ RRM3a), indicated by arrows, were incubated with $\sim$ 15–50 ng/ μ L of either GST-mBBP/SF1 (lanes 4–6) or GST-SAP155 (lanes 7–9), captured on GST-agarose beads without (*top* panel) or with (*bottom* panel) RNaseA, washed, and the bound fractions were analyzed on an SDS-polyacrylamide gel. (Lanes 1–3) A fraction (1/10) of the ³⁵S-labeled in vitro translated proteins used for binding. (B) The binding was done as in panel A. The recombinant proteins, GST-mBBP/SF1 and GST-SAP155, were diluted threefold for each successive lane. To calculate the percent bound fraction for each lane, the signal in the GST panel served as background and that in the highest protein concentration as maximum bound (100%). (C) Phylogenetic comparison of the SF1 domain that interacts with the RRM3 domain of U2AF⁶⁵ (Selenko et al. 2003). The SF1 sequences are from the following organisms: Hs, *Homo sapiens*; Sp, *Schizosaccharomyces pombe*; Mm, *Mus musculus*; Sc, *Saccharomyces cerevisiae*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; At, *Arabidopsis thaliana*. The numbers represent starting amino acids for each sequence. Alignment was done using ClustalW.

to Py tracts located within a particular sequence context recognized by RRM3. The RNA-binding and protein-binding activities of RRM3 need not be mutually exclusive. For

example, an RNA could bind to the β -sheet surface of RRM3 and a protein could interact with the backside surface containing two α -helices. In fact, recent NMR analysis suggests that the surface of RRM3 that binds mBBP/SF1 is distinct from the four-stranded β -sheet surface, whose accessibility may also be modulated by helix C (Selenko et al. 2003). On the other hand, a recent X-ray structure provides evidence for extensive protein-protein interactions between the β -sheet surface of Y-14/Tsunagi and Mago Nashi, which could preclude RNA binding (Shi and Xu 2003). Thus, it is also possible that the β -sheet of RRM3 could only provide a protein-binding surface. However, we cannot exclude the possibility that the RNA- and protein-binding regions of RRM3 could be overlapping, but the two binding activities could be temporally separated during spliceosome assembly. In the model (Fig. 9), although RRM3 is tethered by a flexible linker, we cannot exclude the possibility that the linker region, as seen for SXL upon RNA binding (Crowder et al. 1999; Handa et al. 1999), becomes ordered in the spliceosome to favor selection of nearby branch sites (Gozani et al. 1998). It is also possible that the function of RRM3 may be unrelated to splicing.

In conclusion, the RRM3 domain, although essential for viability in *S. pombe*, is dispensable for Py-tract binding and for the splicing of certain introns, an observation that has implications on previous models of U2AF function based on interactions between RRM3 and splicing factors like BBP and SAP155. These studies reinforce the notion that the spliceosome assembly pathway is flexible, that different pre-mRNAs likely have distinct rate-limiting steps, and that there are alternative spliceosome assembly pathways such as the U1-, U2AF-, and now the RRM3-independent pathway (Crispino et al. 1994; Tarn and Steitz 1994; MacMillan et al. 1997; Tange et al. 2001). Future studies should define this previously unrecognized function for RRM3.

FIGURE 9. Model for the function of the RRM3 domain of U2AF⁶⁵. The question mark (?) indicates that the nature of this interacting molecule (RNA or protein) is unknown. The broken line connecting RRM3 reflects that RRM3 is dispensable for the splicing of some introns *in vitro*, and likely also *in vivo*. RRM1 and RRM2 contact the Py tract, shown by a stretch of pyrimidine (Y) residues. The arginine-serine (RS) domain, mBBP/SF1, SAP155 and U2 snRNA interact with the branch site. For simplicity, the temporal order of interactions is not shown. mBBP/SF1 and SAP155 interact with RRM3. UAP56 and U2AF³⁵ interact with the N-terminal region of U2AF⁶⁵.

MATERIALS AND METHODS

Plasmids and mutagenesis

Human U2AF⁶⁵ clones RRM123 (amino acids 110–475) and RRM12 (amino acids 110–342) were obtained by PCR amplification using the following primers and cloned into pGEX-2T digested with BamHI/EcoRI. RRM123: Forward (F) (5'-GCGG GATCCATGCAAGCTGCGGGTCAGA-3') and Reverse (R) (5'-CGGAATTCAAGATCCACGCGGAACCAGGTCGACGAAGTCC CGGCGGTGATAA-3'); RRM12: F (5'-CTGCTGGACGACGAG GAG) and R (5'-CGGAATTCAGTCGACGCTCACCAGCGTG GCATTC-3').

AdML, *tra*-NSS(maxi), and α -TM(A) and α -TM(P) from α -Tropomyosin were described previously (Singh et al. 1995). The *tra*-NSS(mini) RNA was a 33-mer sequence containing the NSS Py tract, and *tra*-FS RNA contained the *tra*-FS Py tract (Banerjee et al. 2003).

For mutations in the *S. pombe prp2* (U2AF⁵⁹) gene, the 3.3-kb SstI DNA fragment of *prp2*/pIRT3 (Potashkin et al. 1993) was cloned into pGEM3 and was used as a template to create point mutations by oligonucleotide-directed mutagenesis using inverse PCR amplification. The double mutants were generated similarly using appropriate single mutants for another round of oligonucleotide-directed mutagenesis. Next, the MscI/NsiI fragments containing various RRM3 domain mutations were substituted for the wild-type portion of the *prp2* gene in the original *prp2*/pIRT3 plasmid (Potashkin et al. 1993). The clones were confirmed by restriction digestion and direct sequencing. The oligonucleotides used for mutagenesis were:

H423A: F (5'-GCTAATTTAATTACTGGGGACGAAATT-3') and R (5'-TAATTGTAAGACTCTAGTTGGAATA-3')

F476A: F (5'-GCTGTACGATCTCCGATATCAGAT-3') and R (5'-AACCTTTCAGTTCCTAATCCC-3')

FY506,7AA: F (5'-GCTGCTGGTGAGGATTGCTATAAAGCT A-3') and R (5'-AGCAATGACAATCGTGCGATCA-3')

HF423,476AA: F (5'-GCTAATTTAATTACTGGGGACGAAAT T-3') and R (5'-TAATTGTAAGACTCTAGTTGGAATA-3'), using the F476A DNA template

HFY423,506,7AAA: F (5'-GCTAATTTAATTACTGGGGAC GAAATT-3') and R (5'-TAATTGTAAGACTCTAGTTG GAATA-3'), using the FY506,7AA DNA template

prp2- Δ RRM3a: The *prp2*/pIRT3 plasmid was digested with MscI and NsiI, which removed RRM3, filled-in with klenow, and religated.

For in vitro transcription/translation experiments with the full-length (RRM123), the HFY423,506,7AAA mutant, and the Δ RRM3 (RRM12) SpU2AF⁵⁹ proteins, appropriate DNA fragments of the *prp2* gene were PCR amplified, digested using BamHI/XbaI, and cloned into pcDNA3. PCR primers were as follows:

RRM123: F (5'-GCGGGATCCACCATGTTGCCAGGCGCTCCT AGA-3') and R (5'-TGCTCTAGATCACCATGCATTAGCTT TATAG-3')

RRM12: F (5'-GCGGGATCCACCATGTTGCCAGGCGCTCCT AGA-3') and R (5'-TGCTCTAGAAAGCTTGATTGAGACCT ACAC-3').

SELEX assay

SELEX was carried out essentially as described (Singh et al. 1995). Briefly, an RNA pool that was randomized at 31 nt (pool size 10^{13} – 10^{14}) was incubated with appropriate recombinant proteins ($\sim 10^{-7}$ M). The protein concentration was 10-fold higher than the equilibrium dissociation constant for the first two rounds, and was reduced threefold every two rounds. The binding reaction was passed through a filter, and the bound RNA was eluted without washing the filter with buffer. Following six rounds, sequences were cloned into pGEM3 and analyzed by direct sequencing. Pool 0 showed no binding, and pools 2 and 4 showed progressive improvement in binding affinity (data not shown).

RNA–protein-binding assay

Gel mobility shift assay was described previously (Singh et al. 1995, 2000). Internally labeled RNAs were incubated with relevant proteins in a 20 μ L reaction containing 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 50 mM KCl, 0.5 U/ μ L RNasin, 0.09 μ g/ μ L acetylated bovine serum albumin, 0.15 μ g/ μ L tRNA, and appropriate dilutions of recombinant proteins in Buffer D (20 mM HEPES at pH 8.0, 0.2 mM EDTA, 20% glycerol, 0.05% NP-40, 1 mM DTT). Protein concentrations were estimated by the staining of an SDS-polyacrylamide gel with Coomassie Brilliant Blue R-250 with bovine serum albumin as a standard.

Splicing assay

The splicing and U2AF-depletion protocols using oligo-dT were described previously (Valcarcel et al. 1997).

GST pull-down assay

³⁵S-labeled proteins were prepared using a TnT reticulocyte lysate system (Promega). Appropriate dilutions of recombinant GST-fusion proteins (mBBP/SF1 or SAP155) were incubated with ³⁵S-labeled proteins in PBST (0.5% Triton X-100) at 4°C for 1 h. Subsequently, glutathione-agarose resin (20 μ L) was added to each binding reaction and allowed to equilibrate at 4°C for 2 h. The binding reaction (200 μ L) was then incubated with or without RNaseA (20 μ g) at 37°C for 1 h, centrifuged, and the resin was washed three times with phosphate buffered saline/1% Triton X-100 (PBST). The bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and the signal was quantified using PhosphorImager and ImageQuant (Molecular Dynamics).

Yeast manipulation

Complementation assays with the wild-type or mutant *prp2* genes were performed by transforming the SpCR1 strain with plasmids carrying appropriate *prp2* alleles. Spores were plated onto EMM2 plates supplemented with uracil and adenine (100 mg/L each). Haploids spores (red or pink) were distinguished from the residual diploids on YEA plates and tested for growth by replica plating on defined media.

Western analysis

Yeast cells (SpCR1 or SpCR1 containing plasmids with Δ RRM3a or HFY-m mutants) were grown to O.D.₅₉₅ = 1. The cells were

collected by brief centrifugation and washed with 20 ml of cold extraction buffer (20 mM Tris at pH 8.0, 150 mM ammonium sulfate, 10% glycerol, 1 mM EDTA, 1 mM DTT) containing protease inhibitor cocktail (Sigma). The cells were resuspended in 600 μ L of extraction buffer and 400 μ L of acid washed glass beads (425–600 microns; Sigma) in screw-capped tubes (SARSTEDT). The cells were subjected to bead beater (BIO 101) three times for 30 sec and centrifuged. The lysate was collected and 5 μ L of the lysate was subjected to 10% SDS-PAGE. The proteins were transferred to PVDF membrane (Millipore) using semi-dry transfer (Hoefer Instruments) in 1 $\times$ Tris-glycine gel running buffer (25 mM Tris, 250 mM glycine at pH 8.3) with 10% (v/v) methanol at 75 mA for 30 min. Membrane was incubated overnight using the primary anti-U2AF⁵⁹ antibody (Ochotorena et al. 2001), washed five times with TBST (50 mM Tris at pH 7.5, 150 mM NaCl, 0.05% Tween-20) incubated with secondary goat antirabbit Ig-G HRP antibody. The signal was detected using the chemiluminiscent detection system of HRP (Pierce).

RT-PCR

The expression of the Δ RRM3a (RRM12) or HFY-m mutants in yeast was analyzed by using RT-PCR with the following primers:

P1: 5'-GCACAATTTGCTTGTGTAGGTC-3'
 P2: 5'-CATTAAGATATTATACTAACCTTTTC-3'
 P3: 5'-GCTAATTTAATTACTGCGGACGAAATT-3'
 P4: 5'-ATAGCAATCCTCACCAGCAGC-3'.

Yeast cells [SpCR1 or SpCR1 containing plasmids with *prp2* (RRM123), HFY-m, or Δ RRM3 mutants] were grown as described in the previous section. Total RNA was isolated from the yeast cells using TRIZOL reagent. The isolated RNA was treated with DNaseI at 37°C for 1 h. For Figure 7A, the RNA was reverse transcribed using the P2 primer at 42°C for 90 min. The cDNA was then PCR amplified using primers P1 and P2 and an annealing temperature of 53°C for 30 cycles. The PCR-amplified DNA from Δ RRM3 was treated with restriction enzymes MscI and NsiI to distinguish between the full-length RRM3 amplified from the endogenous gene and the plasmid-encoded Δ RRM3a. For Figure 7B, the RNA was reverse transcribed using P2 or P4 primer at 42°C for 90 min. The cDNA was then PCR amplified using primers P2 and P3 and an annealing temperature of 59°C for 25 cycles, or P3 and P4 and an annealing temperature of 61°C for 20 cycles.

Plasmid DNA recovery from yeast cells and sequencing

Yeast cells transformed with a plasmid containing the HFY-m mutant were grown as described above. Cells were collected by centrifugation and resuspended in 1.5 mL of 50 mM citrate/phosphate buffer (pH 5.6), 0.2 M sorbitol, and 2 mg/mL Zymolyase-20T. The mixture was incubated at 37°C for 1 h. Cells were pelleted by centrifugation and resuspended in 300 μ L of TE buffer. Thirty-five microliters of 10% SDS were added, mixed, and incubated for 5 min at 65°C. Next, 100 μ L of 5 M potassium acetate were added, mixed, and incubated on ice for 30 min. The reaction mix was then pelleted for 10 min at 4°C. The supernatant was collected in a separate Eppendorf tube. Plasmids were purified from the supernatant using the GeneClean kit (BIO 101). The

isolated plasmids were transformed into *Escherichia coli* and cultured to isolate plasmid minipreps. The rescued plasmids were directly sequenced to confirm the presence or absence of the HFY-m mutation of the RRM3 domain.

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