Molecular genetic analysis of the heterodimeric splicing factor U2AF: the RS domain on either the large or small *Drosophila* subunit is dispensable in vivo

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The pre-mRNA splicing factor U2AF (U2 snRNP auxiliary factor) has an essential role in 3' splice site selection. U2AF binds the intron pyrimidine tract between the branchpoint and the 3' splice site and recruits U2 snRNP to the branch site at an early step in spliceosome assembly. Human U2AF is a heterodimer composed of large (hU2AF⁶⁵) and small (hU2AF³⁵) subunits. Both subunits contain a domain enriched in arginine-serine dipeptide repeats termed an RS domain. The two U2AF RS domains have been assigned essential and independent roles in spliceosome assembly in vitro—the hU2AF⁶⁵ RS domain is required to target U2 snRNP to the branch site and the hU2AF³⁵ RS domain is necessary for protein-protein interactions with constitutive and alternative splicing factors. We have investigated the functional requirements for the RS domains on the *Drosophila* U2AF homolog in vivo. In sharp contrast to its essential role in U2 snRNP recruitment in vitro, the RS domain on the *Drosophila* large subunit homolog (*dU2AF*⁵⁰) was completely dispensable in vivo. Prompted by this unexpected result, we analyzed the RS domain on the *Drosophila* small subunit homolog (*dU2AF*³⁸). Despite its requirement for enhancer-dependent splicing activity in vitro, the *dU2AF*³⁸ RS domain was also inessential in vivo. Finally, we have tested whether the *Drosophila* U2AF heterodimer requires any RS domain. Flies mutant for both the small and large subunits could not be rescued by *dU2AF*⁵⁰ΔRS and *dU2AF*³⁸ΔRS transgenes. Therefore, in contrast to the separate roles assigned to the U2AF RS domains in vitro, our genetic data suggest that they may have redundant functions in vivo.

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The generation of functional mRNAs in eukaryotes requires the accurate removal of noncoding sequences (introns) from pre-mRNAs by a process termed pre-mRNA splicing (Moore et al. 1993; Sharp 1994; Kramer 1996). Pre-mRNA splicing takes place in the spliceosome, a dynamic RNA-protein complex composed of small nuclear ribonucleoprotein particles (snRNPs) and extrinsic (non-snRNP) protein factors. The earliest steps in spliceosome assembly involve recognition of the 5' splice site by U1 snRNP and the branchpoint-3' splice site by U2 snRNP. Targeting of U2 snRNP to the branch site requires the extrinsic splicing factor U2AF (U2 snRNP auxiliary factor) (Ruskin et al. 1988). U2AF binds specifically to the intron pyrimidine tract located between the branchpoint

and the 3' splice site and recruits U2 snRNP to the branch site at an early step in spliceosome assembly (Ruskin et al. 1988; Zamore et al. 1992; Staknis and Reed 1994). Regulation of 3' splice site choice, both positive and negative, can be realized by influencing the pyrimidine tract binding of U2AF (Tian and Maniatis 1993; Valcárcel et al. 1993; Reed 1996).

Human U2AF is a heterodimer composed of a 65-kD large subunit (hU2AF⁶⁵) and a 35-kD small subunit (hU2AF³⁵) (Zamore and Green 1989). Both subunits are highly conserved across species (Zamore and Green 1991); U2AF homologs have been identified in *Drosophila melanogaster* (Kanaar et al. 1993; Rudner et al. 1996), *Schizosaccharomyces pombe* (Potashkin et al. 1993; Wentz-Hunter and Potashkin 1996), and *Caenorhabditis elegans* (Zorio et al. 1997; T. Blumenthal, pers. comm.). The *Drosophila* U2AF large (*dU2AF*⁵⁰) and small (*dU2AF*³⁸) subunit homologs are 50 and 38 kD, respectively (Kanaar et al. 1993; Rudner et al. 1996). The large subunit contains three RNA recognition motifs

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(RRMs) and an amino-terminal arginine-serine-rich (RS) domain (Zamore et al. 1992). The small subunit contains a highly degenerate (pseudo-) RRM (Birney et al. 1993), two putative Zn^{2+} binding motifs (Worthington et al. 1996), and a carboxy-terminal RS domain and glycine-rich region (Zhang et al. 1992).

Biochemical studies of U2AF using extracts depleted of U2AF activity lead to some confusion as to the requirement for the large and small subunits in splicing. Depending on the substrate used and method of U2AF depletion (poly(U)-sepharose or immunoaffinity chromatography), different requirements for the large or large and small subunits were observed (Zamore and Green 1991; Zamore et al. 1992; Kanaar et al. 1993; Valcárcel et al. 1996; Zuo and Maniatis 1996; Gama-Carvalho et al. 1997). Both *Drosophila* U2AF subunits are required for viability suggesting that both subunits are necessary for splicing in vivo (Kanaar et al. 1993; Rudner et al. 1996).

Although both U2AF subunits contain RS domains, these domains have been assigned independent roles in spliceosome assembly. Consistent with a direct role in U2 snRNP recruitment, deletion of the RS domain from hU2AF⁶⁵ (hU2AF⁶⁵ΔRS) had no effect on pyrimidine tract binding yet it completely abolished the ability to restore splicing to U2AF-depleted extracts (Zamore et al. 1992; Valcárcel et al. 1996). Additionally, fusion of a synthetic RS domain containing seven RS dipeptides [(RS)₇] (or any dipeptide repeat that possesses a net positive charge [(RA)₇, (RG)₇, (KS)₇, but not (RD)₇]) to hU2AF⁶⁵ΔRS was sufficient to restore splicing activity (Valcárcel et al. 1996). Based on the sole requirement for a net positive charge, it was proposed that the essential role of the hU2AF⁶⁵ RS domain is to facilitate annealing of the U2 snRNA and the branch site sequence through charge shielding of the RNA phosphodiester backbones (Valcárcel et al. 1996).

Whereas the large subunit RS domain is thought to promote RNA-RNA interactions in U2 snRNP recruitment, the small U2AF subunit RS domain has been implicated in protein-protein interactions with constitutive and alternative splicing factors that serve to stabilize binding of hU2AF⁶⁵ to intron pyrimidine tracts. A role for the small subunit in bridging constitutive and alternative splicing factors and hU2AF⁶⁵ was first suggested by protein-protein interaction studies (Wu and Maniatis 1993; Amrein et al. 1994). These studies revealed that hU2AF³⁵, but not hU2AF⁶⁵, specifically interacts with the SR family of general splicing factors as well as the *Drosophila* alternative splicing factors transformer (TRA) and transformer2 (TRA2).

The SR proteins are a family of conserved splicing factors with similar domain structure and partially overlapping biochemical activities (Fu 1995; Manley and Tacke 1996). SR proteins contain at least one RRM-type RNA-binding domain and a serine-arginine-rich (SR or RS) domain that has been implicated in protein-protein interactions in vitro (Wu and Maniatis 1993; Amrein et al. 1994; Kohtz et al. 1994; Xiao and Manley 1997). In vivo, the *Drosophila* *SRp55/B52* gene and the mammalian *ASF/SF2* gene, including its RS domain, are essential for

viability (Ring and Lis 1994; Wang et al. 1996). SR proteins are required at an early stage in mammalian spliceosome assembly and can promote U1 snRNP and U2AF binding to pre-mRNA in the earliest known mammalian spliceosomal complex (E complex) (Staknis and Reed 1994). In fact, SR proteins can simultaneously interact with both the U1 snRNP 70-kD protein, U1-70K, and with hU2AF³⁵ in the yeast two-hybrid assay (Wu and Maniatis 1993). The RS domain on both U1-70K and hU2AF³⁵ have been implicated in these protein-protein interactions (Wu and Maniatis 1993; Kohtz et al. 1994).

SR proteins also bind exonic enhancer elements located downstream from weak 3' splice sites (Lavigne et al. 1993; Sun et al. 1993; Wang et al. 1995; Tacke et al. 1997). Addition of SR proteins to nuclear extract promotes U2AF binding to pre-mRNA substrates containing these enhancer elements. Consistent with a role for hU2AF³⁵ in bridging SR proteins bound to enhancers and hU2AF⁶⁵ bound to weak pyrimidine tracts, reconstitution of enhancer-dependent splicing in U2AF-depleted extracts requires the addition of recombinant hU2AF³⁵ (Zuo and Maniatis 1996). Addition of hU2AF³⁵ lacking its RS domain (hU2AF³⁵ΔRS) is insufficient for enhancer-dependent splicing, further implicating the RS domain in these critical protein-protein interactions (Zuo and Maniatis 1996).

One of the best characterized examples of enhancer-dependent splicing involves the sex-specific, alternative splicing of *doublesex* (*dsx*) in the sex determination pathway in *Drosophila*. The alternative splicing factors TRA and TRA2 are required for the female-specific, alternative splicing of *dsx* (Baker and Wolfner 1988; Cline and Meyer 1996). TRA and TRA2 activate a weak, female-specific, 3' splice site in the *dsx* pre-mRNA. The resulting mRNA encodes a DSX isoform required for somatic female differentiation. TRA2 has an RRM and both TRA and TRA2 have RS domains. The RS domains on TRA and TRA2 have been implicated in protein-protein interactions with SR proteins and hU2AF³⁵ (Wu and Maniatis 1993; Amrein et al. 1994). Biochemical analysis of the alternative splicing of *dsx* has revealed that TRA and TRA2 bind to exonic enhancer elements downstream of the regulated *dsx* intron and recruit SR proteins to form a splicing enhancer complex (Hedley and Maniatis 1991; Tian and Maniatis 1993). This complex promotes U2AF binding to the weak pyrimidine tract of the female-specific 3' splice site (Zuo and Maniatis 1996). Reconstitution of female-specific *dsx* splicing requires both U2AF subunits as well as TRA and TRA2.

We have undertaken a molecular genetic analysis of the *Drosophila* U2AF homolog in vivo. To define a functional RS domain on the *Drosophila* U2AF large subunit, dU2AF⁵⁰, we analyzed deletions and substitution mutations of the dU2AF⁵⁰ RS domain. Surprisingly, in sharp contrast to the requirement for the hU2AF⁶⁵ RS domain in U2 snRNP recruitment in vitro, we found that the dU2AF⁵⁰ RS domain was completely dispensable in vivo. This unexpected result prompted an analysis of the *Drosophila* small subunit RS domain. Like the dU2AF⁵⁰ RS domain, the dU2AF³⁸ RS domain was completely dis-

pensable in vivo, indicating that neither RS domain is necessary for splicing. Significantly, diplo X flies lacking the dU2AF³⁸ RS domain were 100% viable and phenotypically female. Therefore, in vivo, female-specific, enhancer-dependent splicing of *dsx* was unaffected by the absence of the dU2AF³⁸ RS domain. To determine whether the *Drosophila* U2AF heterodimer requires any RS domain, complementation tests were performed with dU2AF⁵⁰, dU2AF³⁸ double mutant flies. Whereas the combination of two wild-type transgenes could rescue the double mutant flies, the combination of dU2AF⁵⁰ΔRS and dU2AF³⁸ΔRS transgenes could not. Fusion of a synthetic RS domain containing seven RS dipeptides onto dU2AF⁵⁰ΔRS was not sufficient to complement the double mutant in combination with dU2AF³⁸ΔRS. Therefore, at least one RS domain on U2AF is required in vivo and a simple RS dipeptide repeat will not serve as a substitute. In contrast to the separate roles assigned to the U2AF RS domains in vitro, our genetic data suggest that the RS domains have redundant functions in vivo.

Results

The dU2AF⁵⁰ RS domain is dispensable in vivo

We have shown previously that a mutation in the *Drosophila* U2AF large subunit gene is fully penetrant recessive lethal and can be rescued by a genomic transgene that contains dU2AF⁵⁰ (Kanaar et al. 1993). The presence of intervening sequences in and around the amino-terminal RS domain of dU2AF⁵⁰ prohibited a deletion analysis of the dU2AF⁵⁰ RS domain using the rescuing genomic transgene. To facilitate our analysis of the dU2AF⁵⁰ RS domain, we created an in vivo dU2AF⁵⁰ expression vector. The dU2AF⁵⁰ gene in the genomic clone was replaced with an oligonucleotide linker containing unique restriction sites and an improved translation initiation sequence (Cavener and Ray 1991) (see Materials and Methods). A transgene containing the wild-type dU2AF⁵⁰ cDNA inserted into this expression vector rescued a dU2AF⁵⁰ recessive lethal allele as efficiently as the original genomic transgene (Fig. 1A).

To define the functional requirements for the dU2AF⁵⁰ RS domain in vivo, the RS domain from the dU2AF⁵⁰-coding sequence (amino acids 1–34; Fig. 1B) was deleted (dU2AF⁵⁰ΔRS) or replaced with a synthetic RS domain containing seven RS dipeptides [dU2AF⁵⁰(RS)₇] and inserted into the dU2AF⁵⁰ expression vector. Germ-line transformants containing dU2AF⁵⁰ΔRS and dU2AF⁵⁰(RS)₇ transgenes were generated and tested for their ability to complement a recessive lethal dU2AF⁵⁰ allele. Balanced dU2AF⁵⁰ mutant virgin females were crossed to males carrying a dU2AF⁵⁰ transgene. Hemizygous, dU2AF⁵⁰ mutant male progeny carrying the dU2AF⁵⁰ transgene were scored and their percent viability was determined by comparison with their heterozygous mutant sisters. Surprisingly, both dU2AF⁵⁰ΔRS and dU2AF⁵⁰(RS)₇ transgenes efficiently rescued the recessive lethal dU2AF⁵⁰ allele (Fig. 1A).

Figure 1. The dU2AF⁵⁰ RS domain is inessential in vivo. (A) Wild-type (WT) and dU2AF⁵⁰ deletion and substitution derivative transgenes were tested for complementation of a dU2AF⁵⁰ recessive lethal allele. A schematic diagram of the dU2AF⁵⁰ domains is shown. The RS domain (RS), the dU2AF³⁸ interaction domain (int), and the three RNA recognition motifs (RRM) are indicated. The percentages of rescue are from representative transgene lines. (B) Amino acid sequence comparison of the RS domains from dU2AF⁵⁰ and hU2AF⁶⁵. Identities and similarities are shown in black and gray boxes, respectively. Dashes denote gaps. Amino acid positions are shown on the right. The caret below the sequence indicates the dU2AF⁵⁰ RS domain deletion site.

The high degree of sequence similarity between the RS domains on dU2AF⁵⁰ and hU2AF⁶⁵ (Fig. 1B) suggests that the inessential nature of the large subunit RS domain observed in vivo will not be specific to *Drosophila*.

A single RS dipeptide was present in the dU2AF⁵⁰ΔRS rescuing transgene (Fig. 2A). To rule out the possibility that this single RS dipeptide was sufficient for dU2AF⁵⁰ activity in vivo, the serine residue was deleted to create dU2AF⁵⁰ΔRS_{true}. Similar to the findings with dU2AF⁵⁰ΔRS, the dU2AF⁵⁰ΔRS_{true} transgene also efficiently rescued the dU2AF⁵⁰ mutant allele (Fig. 2A). Therefore, dU2AF⁵⁰ does not require any RS dipeptides to support viability.

A positively charged domain is not required for dU2AF⁵⁰ activity in vivo

In the in vitro reactivation experiments that demonstrated an essential requirement for the hU2AF⁶⁵ RS domain, the amino terminus of the hU2AF⁶⁵ protein was deleted up to a conserved proline repeat at amino acid 95 (amino acid 47 in dU2AF⁵⁰, see Fig. 2A) (Valcárcel et al. 1993). In our dU2AF⁵⁰ΔRS_{true} rescuing transgene the RS domain was deleted up to amino acid 37. This deletion left behind three positively charged residues (R37, R38, and K39; Fig. 2A). It was possible that the positively charged residues retained in dU2AF⁵⁰ΔRS_{true} were sufficient for RS domain function. In fact, a hU2AF⁶⁵ RS domain deletion that retained a few positively charged residues weakly reactivated splicing in a poly(U)-depleted extract in vitro (Valcárcel et al. 1993). In an attempt to directly correlate our in vivo complementation data with the published results from the in vitro reconstitution experiments, we deleted the dU2AF⁵⁰ RS domain up to the conserved proline repeat (dU2AF⁵⁰Δ1–46;

Figure 2. (A) Deletion analysis of the dU2AF⁵⁰ RS domain. Wild-type dU2AF⁵⁰ (WT) and deletion and substitution derivative transgenes were tested for complementation of a recessive lethal dU2AF⁵⁰ allele. Amino-terminal amino acids spanning the RS domain and part of the dU2AF³⁸ interaction domain are shown. The amino acids derived from the dU2AF⁵⁰-coding sequence are shown in bold. The amino acids introduced through cloning or fused onto deletion derivatives are in roman type. Dots above the amino acid sequence indicate amino acids required for interaction with dU2AF³⁸. The caret indicates the analogous position on dU2AF⁵⁰ where the hU2AF⁶⁵ RS domain was deleted for the in vitro reconstitution experiments (Valcárcel et al. 1996). The percentages of rescue shown are from representative transgene lines. (B) Protein expression levels of the dU2AF⁵⁰ deletion derivatives. Immunoblot analysis of whole-fly extracts using an anti-dU2AF⁵⁰ antibody. Whole-fly extracts are from *w*¹¹¹⁸ (dU2AF⁵⁰) flies (lanes 1, 11), dU2AF⁵⁰ mutant flies carrying a wild-type (lane 2) or dU2AF⁵⁰ derivative transgene as indicated above the gel (lanes 3, 4, 5, 9, 10) or *w*¹¹¹⁸ flies carrying dU2AF⁵⁰ derivative transgenes (lanes 6–8). Extracts from wild-type (*w*¹¹¹⁸) or dU2AF⁵⁰ mutant flies carrying the same dU2AF⁵⁰ΔRS_{true} rescuing transgene are shown in lanes 4 and 5. The sizes of molecular mass markers are indicated in kD.

see Fig. 2A) to create dU2AF⁵⁰ΔRS_{extreme}. We also fused a synthetic RS domain containing seven RS dipeptides onto this deletion creating dU2AF⁵⁰(RS)_{7extreme}. These dU2AF⁵⁰ derivatives are analogous to the hU2AF⁶⁵ RS deletion and synthetic RS domain fusion proteins used in the in vitro splicing reactivation experiments (Valcárcel et al. 1993). Independent transgenic lines (15–20) of each dU2AF⁵⁰ derivative were generated and tested for complementation of the recessive lethal dU2AF⁵⁰ allele. Consistent with the in vitro reactivation experiments, the dU2AF⁵⁰ΔRS_{extreme} transgene was not able to rescue the dU2AF⁵⁰ mutant allele (Fig. 2A). In contrast to the ability of a synthetic RS domain to restore splicing activity to the analogous deletion in vitro, however, the dU2AF⁵⁰(RS)_{7extreme} transgene failed to rescue the dU2AF⁵⁰ mutant allele (Fig. 2A). The inability of these mutant transgenes to complement the dU2AF⁵⁰ recessive lethal allele was not a consequence of low protein expression levels. Whole-fly extracts from transgenic lines containing either dU2AF⁵⁰ΔRS_{extreme} or dU2AF⁵⁰(RS)_{7extreme} transgenes had mutant protein levels equal to or higher than rescuing dU2AF⁵⁰ transgene lines as assessed by immunoblot analysis using anti-dU2AF⁵⁰ antibodies (Fig. 2B, cf. lanes 7, 8, 9).

The inability of dU2AF⁵⁰ΔRS_{extreme} and dU2AF⁵⁰(RS)_{7extreme} to complement the recessive lethal dU2AF⁵⁰ allele was probably attributable to disruption of the U2AF heterodimer. Recently, we have shown that a triple point mutation (W44A, D45A, and V46A) in dU2AF⁵⁰ abolishes interaction with dU2AF³⁸ completely in an *Escherichia coli* copurification assay and in *Drosophila* embryo extracts (Rudner et al. 1998). Fur-

thermore, we have found that a dU2AF⁵⁰ mutant lacking its RS domain efficiently associates with the small subunit (D.Z. Rudner, K.S. Breger, R. Kanaar, M.D. Adams, and D.C. Rio, in prep.). Consistent with a requirement for U2AF heterodimer formation, we have also shown that the dU2AF⁵⁰ interaction mutant (W44A, D45A, V46A) is unable to complement the dU2AF⁵⁰ recessive lethal allele (Rudner et al. 1998). All three of these critical residues were deleted in dU2AF⁵⁰ΔRS_{extreme} and dU2AF⁵⁰(RS)_{7extreme} (Fig. 2A) as well as in the analogous hU2AF⁶⁵ mutant proteins (Valcárcel et al. 1996). Because the poly(U)-depleted extract does not require the small subunit for reactivation (Zamore et al. 1992), deletion of these conserved residues in hU2AF⁶⁵ would not affect its activity in vitro. The requirement for heterodimer formation in vivo, however, complicates our molecular genetic analysis. We conclude that the inability of the dU2AF⁵⁰ “extreme” derivatives to complement the dU2AF⁵⁰ recessive lethal allele does not address the requirement for the remaining positively charged residues in dU2AF⁵⁰ΔRS_{true}, but it does support the conclusion drawn from our previous study. Consistent with a requirement for both U2AF subunits for splicing in vitro, heterodimer formation is essential in vivo (Rudner et al. 1998).

To address the requirement for the remaining positively charged residues in dU2AF⁵⁰ΔRS_{true}, a final set of dU2AF⁵⁰ mutant transgenes were created. The three positively charged residues remaining at the amino terminus in the dU2AF⁵⁰ΔRS_{true} transgene were deleted to create dU2AF⁵⁰ΔRS_{final} (Fig. 2A). A synthetic RS domain was fused to this final deletion mutant to create

Figure 4. Protein expression of the dU2AF³⁸ deletion derivatives. Immunoblot analysis of whole-fly extracts using an anti-dU2AF³⁸ antibody. Whole-fly extracts are from wild-type, dU2AF³⁸⁺ flies (lane 1); homozygous Δ dU2AF³⁸ (Δ E18) mutant flies carrying a wild-type dU2AF³⁸ rescuing transgene (lane 2); homozygous Δ dU2AF³⁸ carrying a dU2AF³⁸ Δ Gly transgene (lane 3); homozygous Δ dU2AF³⁸ carrying a dU2AF³⁸ Δ RS transgene (lane 4); homozygous Δ dU2AF³⁸ carrying a dU2AF³⁸ Δ RSGLy transgene (lane 5). The size of molecular mass markers are indicated in kD.

taining the dU2AF³⁸ Δ RSGLy transgene were generated and tested for complementation of the dU2AF³⁸ recessive lethal deletion mutation. Surprisingly, the dU2AF³⁸ Δ RSGLy transgene also completely rescued the dU2AF³⁸ null allele (Fig. 3A). In contrast to the requirement for the hU2AF³⁵ RS domain for splicing in vitro, the dU2AF³⁸RS domain and glycine-rich region were completely dispensable in vivo. The ability of the dU2AF³⁸ Δ RSGLy transgene to complement the dU2AF³⁸ null mutant is not a consequence of translational readthrough of the engineered stop codon, as the dU2AF³⁸ deletion mutant was the only protein detected by immunoblot analysis of a whole-fly extract from the rescued flies (Fig. 4, cf. lanes 1, 2, and 5).

Although the human and *Drosophila* small subunit RS domains differ in the placement of the glycine-rich regions, the overall sequence similarity (Fig. 3B) suggests that the lack of requirement for the small subunit RS domain in vivo is not specific to *Drosophila*. In fact, the recent identification of the U2AF small subunit homolog from *S. pombe* (pU2AF²³) reveals substantial amino acid conservation in the amino terminus (77%) but the complete absence of a carboxy-terminal RS domain (Wentz-Hunter and Potashkin 1996). We conclude that the essential function of the U2AF small subunit does not reside in the RS domain and is therefore not necessary for constitutive splicing in vivo.

The dU2AF³⁸ RS domain is not required for enhancer-dependent splicing of doublesex in vivo

If the dU2AF³⁸ RS domain is required for enhancer-dependent, female-specific splicing of the *dsx* pre-mRNA, then female flies lacking the dU2AF³⁸ RS domain (dU2AF³⁸ Δ RS or dU2AF³⁸ Δ RSGLy) should be incapable of efficient female-specific *dsx* splicing. Inefficient female-specific splicing of *dsx* would cause partial or complete sexual transformation of female flies, a phenotype observed in *TRA* or *TRA2* mutant females (Nagoshi et al. 1988; Nagoshi and Baker 1990). Dimorphic body parts on the diplo X, homozygous dU2AF³⁸ mutant flies carrying the dU2AF³⁸ Δ RS or dU2AF³⁸ Δ RSGLy transgenes were analyzed for sexual transformation. The rescued flies were phenotypically female and fully fertile (data not shown). Therefore, in contrast to previous biochemical studies using human splicing extracts and human U2AF³⁵ (Zuo and Maniatis 1996), the dU2AF³⁸ RS domain is not required for enhancer-dependent alternative splicing of *dsx* in vivo. These results are consistent with previous genetic analysis of a semi-lethal (hypomorphic) allele of dU2AF³⁸ (Rudner et al. 1996). In these studies, no genetic interactions were observed between *TRA*, *TRA2*, and the dU2AF³⁸ hypomorphic mutation.

To confirm that the splicing of *dsx* RNA is unaffected in the flies lacking the dU2AF³⁸ RS domain, we analyzed *dsx* transcripts molecularly. Total RNA from *w*¹¹¹⁸ (dU2AF³⁸⁺) and dU2AF³⁸ mutant flies carrying the dU2AF³⁸ Δ RSGLy transgene was isolated. *dsx* splicing was analyzed by reverse-transcription PCR (RT-PCR) using primers specific for male and female *dsx* transcripts (Fig. 5B) (Amrein et al. 1994). No products were observed in the absence of reverse transcription (Fig. 5A, lanes 1,3,5,7). As expected, only male-specific *dsx* mRNA was observed in wild-type and dU2AF³⁸ Δ RSGLy mutant males (Fig. 5A, lanes 2,6). Consistent with our phenotypic analysis of the dU2AF³⁸ Δ RSGLy mutants, only female-specific *dsx* mRNA was observed in both wild-type and dU2AF³⁸ Δ RSGLy mutant females (Fig. 5A, lanes 4,8). Therefore, in the absence of the dU2AF³⁸ RS domain, *dsx* splicing enhancer function is normal.

An RS domain is necessary on the U2AF heterodimer

To determine if any RS domain on the *Drosophila* U2AF heterodimer was required in vivo, the dU2AF³⁸ Δ RS and dU2AF⁵⁰ Δ RS transgenes were tested for the ability to rescue flies mutant for both the small and large subunits. Double mutant flies could be rescued by the combination of a wild-type dU2AF³⁸ and a wild-type dU2AF⁵⁰ transgene, but not by the combination of a dU2AF³⁸ Δ RS and a dU2AF⁵⁰ Δ RS transgene (Fig. 6; see Materials and Methods). We conclude that the presence of at least one RS domain on U2AF is required for viability.

A synthetic RS domain is not sufficient for U2AF activity in vivo

Deletion of the small subunit RS domain provided a genetic background in which to investigate the functional

Figure 5. The dU2AF³⁸ RS domain is not required for enhancer-dependent *dsx* splicing in vivo. (A) RT-PCR analysis of *dsx* splicing. ³²P-labeled RT-PCR products were subjected to electrophoresis through a native polyacrylamide gel and visualized by autoradiography. Total RNA isolated from *w¹¹¹⁸* (dU2AF³⁸⁺) males (lanes 1,2) or females (lanes 3,4) flies or from dU2AF³⁸ mutant males (lanes 5,6) or females (lanes 7,8) rescued by the dU2AF³⁸ΔRSGly transgene was analyzed. The presence or absence of reverse transcriptase (RT) in the reaction is indicated above the lanes. Schematic diagrams of the female-specific (dsx^f) and male-specific (dsx^m) cDNA products are indicated on the left. The markers (M) are ³²P-end-labeled *Msp*I-cleaved pBR322 DNA. (B) Schematic diagram of *dsx* sex-specific alternative splicing. Boxes represent exons, lines represent introns. The male-specific splice and female-specific splice and polyadenylation site (A) are indicated. The primers used for the RT-PCR (Amrein et al. 1994) are shown schematically above the construct.

requirements for the dU2AF⁵⁰ RS domain in vivo. The dU2AF⁵⁰(RS)₇ and the dU2AF³⁸ΔRS transgenes were tested for complementation of the dU2AF³⁸, dU2AF⁵⁰ double mutant. Even though fusion of an identical synthetic RS domain on hU2AF⁶⁵ΔRS will restore splicing activity in vitro (Valcárcel et al. 1996), it did not rescue the double mutant in vivo (Fig. 6). We conclude that, in vivo, a simple RS dipeptide repeat is not equivalent to a U2AF RS domain.

Discussion

The results presented here demonstrate that the dU2AF⁵⁰ RS domain and the dU2AF³⁸ RS domain are completely dispensable in vivo. Although neither U2AF RS domain is required in vivo, we have found that at least one must be present on the U2AF heterodimer for biological activity. Finally, we have shown that a synthetic RS domain containing seven RS dipeptides is not equivalent to a U2AF RS domain.

The U2AF large subunit RS domain is dispensable in vivo

In contrast to the requirement for the hU2AF⁶⁵ RS domain for U2 snRNP recruitment in vitro, our molecular

genetic analysis indicates that this domain is not necessary for splicing in vivo. Resolution of these contradictory results is suggested by the synthetic lethality resulting from deletion of both U2AF RS domains. The requirement for at least one RS domain on U2AF indicates that the two domains might be functionally redundant. If the two RS domains can substitute for each other, a requirement for the dU2AF⁵⁰ RS domain would have been masked by the presence of the dU2AF³⁸ RS domain.

The biochemical analysis of hU2AF⁶⁵ is consistent with functionally redundant U2AF RS domains. The in vitro reconstitution experiments analyzing the requirements for the hU2AF⁶⁵ RS domain were performed in the absence of exogenous hU2AF³⁵ and the RS deletion endpoint would have prohibited stable association with any hU2AF³⁵ retained in the depleted extract (Valcárcel et al. 1996) (see Results). Under these conditions, in the absence of an associated hU2AF³⁵ RS domain, a role for the hU2AF⁶⁵ RS domain could have been revealed. In addition, fusion of the hU2AF³⁵ RS domain onto hU2AF⁶⁵ΔRS was sufficient to restore splicing activity to the poly(U)-depleted extracts (Valcárcel et al. 1996). This result indicates that the hU2AF³⁵ RS domain is capable of U2 snRNP recruitment in vitro.

The results of our genetic assays demonstrate that a synthetic RS domain containing seven RS dipeptides is insufficient for U2AF activity in vivo indicating that a simple RS dipeptide repeat is not equivalent to a U2AF RS domain. Because this identical synthetic RS domain was sufficient for U2 snRNP recruitment in vitro (Valcárcel et al. 1996), it is possible that the synthetic RS domain is not sufficient for interaction with other splicing factors as might be required in vivo (see below). It is also possible there is a species-specific mechanistic difference between the *Drosophila* and human U2AF proteins.

The U2AF small subunit RS domain is dispensable for constitutive and *dsx* enhancer-dependent splicing in vivo

Although the biochemical analysis of hU2AF⁶⁵ is consistent with functionally redundant RS domains, the ex-

transgenes	% rescue of dU2AF ⁵⁰ ⁻; dU2AF ³⁸ ⁻
P[dU2AF ⁵⁰] P[dU2AF ³⁸]	55%
P[dU2AF ⁵⁰ ΔRS] P[dU2AF ³⁸ ΔRS]	0%
P[dU2AF ⁵⁰ (RS) ₇] P[dU2AF ³⁸ ΔRS]	0%

Figure 6. An RS domain on the U2AF heterodimer is required in vivo. Transgenes from wild-type and mutant derivatives of the two dU2AF subunits were tested for complementation of a dU2AF⁵⁰, dU2AF³⁸ double mutant. The combination of dU2AF rescuing transgenes used in each complementation cross are indicated on the left.

periments involving hU2AF³⁵ are not. The protein–protein interaction studies that identified specific interactions between hU2AF³⁵ and the constitutive and alternative splicing factors did not detect interactions between these splicing factors and hU2AF⁶⁵ (Wu and Maniatis 1993). In these experiments, hU2AF⁶⁵ was used as a negative control. The inability of hU2AF⁶⁵ to interact with these splicing factors appears to be inconsistent with the U2AF RS domains having redundant functions.

The domain on hU2AF³⁵ required for interaction with these splicing factors was found to include the hU2AF³⁵ RS domain, but this interaction domain was not thoroughly mapped and might also require another part of hU2AF³⁵ protein (Wu and Maniatis 1993). By analogy, it was shown recently that the RS domain on SRp30a (ASF/SF2) is not sufficient for interaction with the U1 snRNP specific protein U1-70K (Xiao and Manley 1997). If hU2AF³⁵ required both its RS domain and another part of the hU2AF³⁵ protein for interaction with constitutive and alternative splicing factors, then in the context of the U2AF heterodimer, it is possible that the hU2AF⁶⁵ RS domain could satisfy the requirement for the hU2AF³⁵ RS domain. Recently, it was found that the dU2AF⁵⁰ RS domain can substitute for the *TRA2* RS domain in somatic sex determination in vivo (W. Mattox, pers. comm.). This result indicates that the dU2AF⁵⁰ RS domain can function in the protein–protein interactions in which the small subunit has been implicated.

Alternatively, it is possible that the protein–protein interactions between the small subunit and these splicing factors that have been observed in vitro and in the yeast two-hybrid assay are not relevant in vivo. Recently, a transgene containing the *Drosophila* U1 snRNP 70-kD (*dU1-70K*) gene lacking its RS domain was found to rescue a recessive lethal mutation in *dU1-70K* (S. Mount, pers. comm.). This result indicates that the U1-70K RS domain is also inessential in vivo and suggests that the protein–protein interactions observed between U1-70K and SR proteins in vitro are also not necessary for spliceosome assembly in vivo. Recent studies have shown, however, that the mammalian SR protein ASF/SF2 requires its RS domain for viability in vivo (Wang et al. 1996). Interestingly, the *Saccharomyces cerevisiae* U1-70K homolog (Smith and Barrell 1991), like the *S. pombe* U2AF small subunit, lacks an RS domain.

The inability of hU2AF³⁵ΔRS and wild-type hU2AF⁶⁵ to reactivate splicing in the extracts depleted of U2AF by anti-hU2AF³⁵ antibodies (Zuo and Maniatis 1996) is also inconsistent with the RS domains having redundant functions. It is possible that the recombinant hU2AF³⁵ΔRS used in the reconstitution experiments was not active and could not interact with hU2AF⁶⁵. Alternatively, the ability of the hU2AF⁶⁵/hU2AF³⁵ΔRS heterodimer to reactivate splicing might not have been possible to detect in this assay. Because recombinant hU2AF⁶⁵ can associate with endogenous hU2AF³⁵ retained in the immunodepleted extract to reactivate splicing activity, only a small range of hU2AF⁶⁵/hU2AF³⁵ΔRS protein concentrations could be tested (Zuo and Maniatis 1996). Reconstitution of splicing by

hU2AF⁶⁵/hU2AF³⁵ΔRS might require protein concentrations outside this range. Therefore, the requirement for the hU2AF³⁵ RS domain in the reconstitution experiments indicates that the small subunit is important for efficient enhancer-dependent splicing in vitro but cannot address whether it is essential.

Functional redundancy of U2AF RS domains in vivo

Because the U2AF proteins are complexed in a heterodimer and deletion of both RS domains results in synthetic lethality, it is reasonable to hypothesize that the RS domains are redundant and can functionally substitute for each other. An alternative interpretation of the synthetic lethality, however, is that the independent functions assigned to the two RS domains are both redundant with two other activities, one involved in U2 snRNP recruitment and the other in protein–protein interaction. Each redundant activity could individually support viability in the absence of one RS domain but deletion of both RS domains might be too great a burden for these two redundant activities resulting in the observed synthetic lethality. The (h)U2AF⁶⁵ associated protein (UAP56), a human DEAD box protein required for U2 snRNP-branchpoint interaction, could be redundant with the hU2AF⁶⁵ RS domain (Fleckner et al. 1997); and the novel set of bridging interactions between U1 snRNP and hU2AF⁶⁵ suggested by the analysis of the branchpoint bridging protein (BBP) in yeast (Abovich and Rosbash 1997) and the recent identification of a (h)U2AF³⁵ related protein (URP) in mammals (Tronchère et al. 1997), both qualify as potentially redundant with the hU2AF³⁵ RS domain. Although this model is plausible, it does not account for the essential requirement for the individual U2AF RS domains observed in vitro. We favor the first model in consideration of parsimony.

Although we have detected modest splicing defects in dying dU2AF⁵⁰ mutant larvae, it has not been possible to convincingly show that the cause of lethality in *Drosophila* U2AF subunit mutants is a splicing defect (D.Z. Rudner and D.C. Rio, unpubl.). This is likely attributable to the fact that the dying mutant larvae slowly run out of the U2AF protein and/or RNA that was maternally deposited in the mutant embryo. In metazoan nuclei, unspliced nuclear pre-mRNA may simply be degraded. In addition, the splicing of certain introns may be more sensitive to the level of U2AF than others, making detection of a defect in splicing nontrivial. Even with tight temperature-sensitive alleles in certain *S. cerevisiae* splicing factors, it is not always possible to detect a splicing defect in all introns at the nonpermissive temperature. The accumulated biochemical evidence demonstrating an essential requirement for U2AF in constitutive splicing in vitro (Zamore and Green 1991; Zuo and Maniatis 1996; Kanaar et al. 1993) and the requirement for the *S. pombe* U2AF large subunit homolog for splicing in vivo (Potashkin et al. 1993), however, makes it likely that the cause of death in the U2AF mutants in *Drosophila* is a defect in splicing. At this point in time, we cannot rule out the formal possibility that U2AF ac-

tually is dispensable for splicing *in vivo* and its essential function is in some unidentified capacity.

Our early view of the RS domains on U2AF consisted of two domains with highly specialized and independent roles in spliceosome assembly. The hU2AF³⁵ RS domain stabilized hU2AF⁶⁵ on the pyrimidine tract through protein-protein interactions with splicing factors bound to exonic enhancers and the hU2AF⁶⁵ RS domain recruited U2 snRNP to the branch site sequence (Fig. 7A). The molecular genetic analysis of the *Drosophila* U2AF RS domains presented here provides a rather different view of the U2AF RS domains (Fig. 7B). Our analysis suggests that either of the RS domains can perform all the tasks assigned to the individual domains. Both RS domains can recruit U2 snRNP to the branch site and interact with constitutive or alternative splicing factors. This new model for the U2AF RS domains has both mnemonic and predictive value. Genetic experiments using the dU2AF deletion derivatives in combination with biochemical experiments with recombinant heterodimers employing assays that require both subunits will be invaluable in testing the predictions of the redundant function model for the U2AF RS domains.

Materials and methods

dU2AF⁵⁰ *in vivo* expression vector and derivatives

pHSX (Jones and Rubin 1990) was cleaved with *Eco*RI and *Hin*dIII, treated with Klenow DNA polymerase and religated to create pdr1. The rescuing dU2AF⁵⁰ genomic DNA fragment from pHSX-211S12 (Kanaar et al. 1993) was inserted into pdr1 be-

tween the *Bam*HI and *Sal*I sites to create pdr2. pdr2 was cleaved with *Sal*I, treated with Klenow DNA polymerase and religated to create pdr3. Oligonucleotide linkers containing an improved Cavener translational initiation sequence (Schoner et al. 1990), a start codon and unique restriction enzyme sites (top strand: 5'-ATTAATTTTATTAGCAACCAAAATGGCTAGCGGAT-CCGTCGACGAATTCGGTACCA-3'; bottom strand: 5'-GATCTGGTACCGAATTCGTCGACGGATCCGCTAGCCATTTTGGGTGCTAAATAAAATTAAT-3') were annealed and inserted into pdr3 between the *Ssp*I and *Bam*HI sites by partial cleavage to create pdr12. The dU2AF⁵⁰-coding sequence from pdr6 (Rudner et al. 1998) was inserted into pdr12 between the *Bam*HI and *Sal*I sites to create pdr15. The dU2AF⁵⁰ genomic DNA fragment pHSX-211S12 with an *Eco*RI site inserted 3' to the stop codon was cleaved with *Bst*EII and *Not*I and inserted into pdr15 by partial cleavage to create pdr76. pdr76 was cleaved with *Xba*I and *Bam*HI, treated with Klenow DNA polymerase, and religated to create pdr113. pdr113 was cleaved with *Bam*HI, treated with Klenow DNA polymerase, and religated to create pdr141. pdr141 is the wild-type dU2AF⁵⁰ expression plasmid from which all deletion mutants are derived. The dU2AF⁵⁰-coding sequence in pdr6 and all subsequent clones contain a single point mutation at nucleotide 745. This point mutation changes glycine 249 into serine. This mutation is in position 2 of the RNP1 octamer of the second RNA-binding domain. The G249S mutation had no detectable effect on dU2AF⁵⁰ activity *in vivo* (see Fig. 1A).

dU2AF⁵⁰ΔRS was created by insertion of a linker (top strand: 5'-CTAGCGGCGCC-3'; bottom strand: 5'-TCGAGGCGCCG-3') between the *Nhe*I and *Xho*I sites in pdr141 to create pdr134. dU2AF⁵⁰(RS)₇ was created by insertion of a linker (top strand: 5'-CTAGCCGCTCGCGTAGCCGCTCCCGGAGCCGAGC-CGTTCCCGC-3'; bottom strand: 5'-TCGAGCGGGAACG-GCTGCGGCTCCGGGAGCGGTACGCGAGCGG-3') into pdr141 to create pdr137. pdr141 was cleaved with *Nhe*I and *Xho*I, treated with Klenow DNA polymerase, and religated to create dU2AF⁵⁰ΔRS_{true} (pdr157). To create dU2AF⁵⁰ΔRS_{extreme} and dU2AF⁵⁰(RS)_{7extreme} a new *Xho*I site was inserted into pdr6 by site-directed mutagenesis using the oligonucleotide 5'-CGAATCCCGCGGCGGTCTCGAGCAATAAAGCGACG-GCTTGCG-3' to create pdr169. pdr169 was cleaved with *Bam*HI and *Bst*EII and inserted into pdr141 to create pdr177. pdr177 was cleaved with *Nhe*I and *Xho*I, treated with Klenow DNA polymerase, and religated to create dU2AF⁵⁰ΔRS_{extreme} (pdr182a). The same oligonucleotide linker used to create dU2AF⁵⁰(RS)₇ was inserted between the *Nhe*I and *Xho*I sites in pdr177 to create dU2AF⁵⁰(RS)_{7extreme} (pdr179b). To create dU2AF⁵⁰ΔRS_{final} and dU2AF⁵⁰(RS)_{7final} the 5' end of dU2AF⁵⁰ was PCR amplified using the following 5' primers: ΔRS_{final}, 5'-CCGGATCCGCTAGCCCGTCTCGCTTTATTGGGATG-3'; and (RS)_{7final}, 5'-CCGGATCCGCTAGCCCGTCTCGCTAGCCCGCTCCCGGAGCCGAGCCGTTCCCGCTCGCCGCTCGCTTATTGGGATG-3'; and a 3' primer downstream of the *Sph*I site in the dU2AF⁵⁰-coding sequence. The PCR products were cloned into pGem3Zf(+) (Promega) between the *Bam*HI and *Sph*I sites to create pdr219b and pdr220b. The *Nhe*I-*Sph*I DNA fragments from pdr219b and pdr220b were ligated with an *Sph*I-*Bst*EII dU2AF⁵⁰ fragment from pdr6 into pdr141 cleaved with *Nhe*I and *Bst*EII in a three-way ligation to create pdr236 (dU2AF⁵⁰ΔRS_{final}) and pdr226 (dU2AF⁵⁰(RS)_{7final}). All PCR products, oligonucleotide linkers, and DNA fragments containing site-directed changes were confirmed by sequencing (U.S. Biochemical). *Not*I DNA fragments from the wild-type expression plasmid and all deletion or substitution derivatives were subcloned into a unique *Not*I site in the *Drosophila* transformation vector pw8 (Ashburner 1989).

Figure 7. Old and new models for U2AF RS domains. (A) The two U2AF RS domains were originally assigned separate functions in spliceosome assembly. The large subunit (65) RS domain recruits U2 snRNP (U2) to the branch site “A” and the small subunit (35) RS domain interacts with the RS domains on constitutive and alternative splicing factors (SR) to stabilize the large subunit binding to the intron pyrimidine tract (py). (B) Both U2AF RS domains participate in all the tasks assigned to U2AF RS domains in the redundant function model. Both RS domains can target U2 snRNP to the branch site and interact with constitutive and alternative splicing factors.

dU2AF³⁸ expression plasmids

The *dU2AF³⁸* genomic clone was subcloned into pHSX (Jones and Rubin 1990) between the *EcoRI* and *ClaI* sites to create pdr115. *dU2AF³⁸ΔGly* was made by oligonucleotide linker insertion (top and bottom strand: 5'-GGATCCTTAGC-3') into an *XmaI* site in the *dU2AF³⁸*-coding sequence in pdr115 to create pdr160. *dU2AF³⁸ΔRS* was made by oligonucleotide linker insertion (top strand: 5'-CTCTACGGC-3'; bottom strand: 5'-CCGGGCCGTAGAGGTAC-3') between the *KpnI* and *XmaI* sites in the *dU2AF³⁸*-coding sequence in pdr115 to create pdr162. *dU2AF³⁸ΔRSGLy* was created by oligonucleotide linker insertion (top and bottom strand: 5'-CTCTACTAACGGATCCGT-TAGTAGAGGTAC-3') into a *KpnI* site in the *dU2AF³⁸*-coding sequence in pdr115 to create pdr192. All the cloning junctions and oligonucleotide linkers were sequenced. The *dU2AF³⁸* genomic clones from pdr160, pdr162, and pdr192 were subcloned into a unique *NotI* site in the *Drosophila* transformation vector pw8 (Ashburner 1989). Interestingly, we observed a reproducible 10-fold difference in transformation efficiency depending on the orientation of insertion into pw8. This phenomenon was also observed with the *dU2AF⁵⁰* transgenes.

Complementation analysis

Germ-line transformation of the wild-type *dU2AF⁵⁰* transgene and derivatives into *w¹¹¹⁸* embryos was as described (Spradling 1986). Independent transformant lines (10–30) were generated for each derivative. All autosomal insertions were tested for complementation of the *dU2AF⁵⁰* recessive lethal allele, *9-21^{XR15}*. *y, w, 9-21^{XR15} f/Bins* (*y, w, sn, B*) virgin females were mated to *w/Y; P[w⁺; dU2AF⁵⁰]/+* males. *y, w, 9-21^{XR15}, f/Y; P[w⁺; dU2AF⁵⁰]/+* males were compared with their unbalanced *y, w, 9-21^{XR15}, f/w; P[w⁺; dU2AF⁵⁰]/+* sisters. At least 150 progeny were scored in each complementation cross. At least one unbalanced stock (*y, w, 9-21^{XR15}, f/y, w, 9-21^{XR15}, f; P[w⁺; dU2AF⁵⁰]/+*) was established for each of the rescuing *dU2AF⁵⁰* derivatives. For the *dU2AF⁵⁰* derivatives that did not rescue, we tested >15 independently isolated transgene lines to rule out the possibility of genomic position effect. No complementation was observed for any of these lines.

Two of the five *dU2AF⁵⁰* wild-type transgene lines tested complemented *9-21^{XR15}*. The percent rescue ranged from 75% to 123%. Isolate C8 28.1 is shown in Figures 1 and 2. Eight of the 12 *dU2AF⁵⁰ΔRS* transgene lines tested complemented *9-21^{XR15}*. The percent rescue ranged from 52% to 150%. Isolate C18 9a is shown in Figures 1 and 2. Eight of the 11 *dU2AF⁵⁰(RS)₇* transgene lines tested complemented *9-21^{XR15}*. The percent rescue ranged from 35% to 122%. Isolate C21 9b is shown in Figures 1 and 2. Three of the four *dU2AF⁵⁰ΔRS_{true}* transgene lines tested complemented *9-21^{XR15}*. The percent rescue ranged from 56% to 104%. Isolate 5d is shown in Figure 2. Sixteen *dU2AF⁵⁰ΔRS_{extreme}* transgene lines were tested for complementation of *9-21^{XR15}*. No rescue was observed. *dU2AF⁵⁰(RS)_{7extreme}* transgene lines (24) were tested for complementation of *9-21^{XR15}*. No rescue was observed. Eleven of the 20 *dU2AF⁵⁰ΔRS_{final}* transgene lines tested complemented *9-21^{XR15}*. The percent rescue ranged from 11% to 100% (most were 80%–100%). Isolate C43 2a is shown in Figure 2. Seven of the 12 *dU2AF⁵⁰(RS)_{7final}* transgene lines tested complemented *9-21^{XR15}*. The percent rescue ranged from 61% to 157%. Isolate 2a is shown in Figure 2.

The *9-21^{XR15} dU2AF⁵⁰* allele has not been characterized molecularly. It is fully penetrant recessive lethal and no endogenous *dU2AF⁵⁰* protein is detected by immunoblot in *9-21^{XR15}* mutant flies (Fig. 3; our unpublished observations).

dU2AF⁵⁰ΔRS can also rescue a *dU2AF⁵⁰* deletion mutation, *9-21^{XR26}*. *9-21^{XR26}* was not used in these studies because the deletion disrupts an adjacent essential gene complicating the genetic analysis.

Germ-line transformation of *dU2AF³⁸* mutant transgenes into *w¹¹¹⁸* embryos was as described previously (Spradling 1986). Independent transformant lines (5–10) were generated for each *dU2AF³⁸* derivative. All insertion lines on the X and third chromosomes were tested for complementation of the recessive lethal *dU2AF³⁸* null allele, *ΔE18*. *w¹¹¹⁸; ΔE18/Sm6β* (*Cy, RoI*) virgin females were mated to *w¹¹¹⁸/Y; ΔE18/Sm6β* (*Cy, RoI*; *P[w⁺; dU2AF³⁸]/+* males. Rescued, *w¹¹¹⁸; ΔE18/ΔE18*; *P[w⁺; dU2AF³⁸]/+* progeny were scored and percent viability was determined by comparison with *w¹¹¹⁸; ΔE18/Sm6β*; *P[w⁺; dU2AF³⁸]/+* siblings. At least 150 progeny were scored in each complementation cross. Unbalanced stocks (*w¹¹¹⁸; ΔE18/ΔE18*; *P[w⁺; dU2AF³⁸]*) were established with all three *dU2AF³⁸* deletion transgenes.

Two out of three *dU2AF³⁸* wild-type transgene lines tested complemented *ΔE18*. The percent rescue ranged from 82%–114%. Isolate 9a is shown in Figure 1. Both of the *dU2AF³⁸ΔGly* transgene lines tested complemented *ΔE18*. The percent rescue ranged from 30%–90%. Isolate 1a is shown in Figure 1. Six of the seven *dU2AF³⁸ΔRS* transgene lines tested complemented *ΔE18*. The percent rescue ranged from 55%–130%. Isolate 6b is shown in Figure 1. All eight of the *dU2AF³⁸ΔRSGLy* transgene lines tested complemented *ΔE18*. The percent rescue ranged from 70%–140%. Isolate 4a is shown in Figure 1.

To test the requirement for an RS domain on the *dU2AF* heterodimer, the third chromosome, rescuing *dU2AF³⁸* transgenes were *P[w⁺; dU2AF³⁸ 9]* and *P[w⁺; dU2AF³⁸ΔRS 2a]*. Both transgenes fully complement the *dU2AF³⁸* recessive lethal allele, *ΔE18*. The third chromosome *dU2AF⁵⁰* rescuing transgene lines used were *P[w⁺; dU2AF⁵⁰28.1]*; *P[w⁺; dU2AF⁵⁰ΔRS 9a]* and *P[w⁺; dU2AF⁵⁰(RS)₇ 1b]*. All three transgenes fully complement the *dU2AF⁵⁰* recessive lethal allele, *9-21^{XR15}*. *y, w, 9-21^{XR15}, f/Bins* (*y, w, sn, B*); *ΔE18/Sm6β* (*Cy, RoI*); *P[w⁺; dU2AF⁵⁰]/+* virgin females were crossed to *w/Y; ΔE18/Sm6β* (*Cy, RoI*); *P[w⁺; dU2AF³⁸]/+* males. Rescued *y, w, 9-21^{XR15}, f/Y; ΔE18/ΔE18*; *P[w⁺; dU2AF⁵⁰]/P[w⁺; dU2AF³⁸]* male progeny were scored and percent rescue was determined by comparison with *y, w, 9-21^{XR15}, f/w; ΔE18/Sm6β*; *P[w⁺; dU2AF⁵⁰]/P[w⁺; dU2AF³⁸]* sisters. *y, w, 9-21^{XR15}, f/w; ΔE18/ΔE18*; *P[w⁺; dU2AF³⁸]* and *y, w, 9-21^{XR15}, f/Y; ΔE18/Sm6β*; *P[w⁺; dU2AF⁵⁰]* siblings were also scored to ensure individual transgenes could efficiently complement *ΔE18* and *9-21^{XR15}*. Rescue of *ΔE18* by both *dU2AF³⁸* transgenes was ~100%. Rescue of *9-21^{XR15}* by the *dU2AF⁵⁰* transgene was between 44%–72%. As a further control, complementation of *ΔE18* and *9-21^{XR15}* by *P[w⁺; dU2AF⁵⁰ΔRS 9a]* and *P[w⁺; dU2AF³⁸ 9]* or *P[w⁺; dU2AF⁵⁰ 28.1]* and *P[w⁺; dU2AF³⁸ΔRS 2a]* was analyzed. Rescue of the double mutant by *P[w⁺; dU2AF⁵⁰ΔRS 9a]* and *P[w⁺; dU2AF³⁸ 9]* or *P[w⁺; dU2AF⁵⁰ 28.1]* and *P[w⁺; dU2AF³⁸ΔRS 2a]* was 16% and 43%, respectively.

Immunoblot analysis

Whole-fly extract (1/8 fly equivalent per lane for *dU2AF⁵⁰* and 1/4 fly equivalent per lane for *dU2AF³⁸*) was subjected to electrophoresis on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, blocked and probed with affinity-purified anti-*dU2AF⁵⁰* or anti-*dU2AF³⁸* polyclonal antibodies as described previously (Rudner et al. 1998).

RT-PCR analysis

RNA from 100 adult flies was isolated using guanidinium thiocyanate and a CsCl step gradient (Sambrook et al. 1989). *dsx* splicing was analyzed by RT-PCR. *dsx* primers specific for male and female RNA isoforms described by Amrein et al. (1994) were used. DNase I-treated total RNA (2 µg) was reverse transcribed using 25 pmoles of both male-specific and female-specific 3' primers in the same vessel. Twenty percent of the reverse transcriptase reaction was amplified in a standard PCR reaction containing both 3' primers and the common 5' primer. The PCR reactions included 0.2 µCi of [α - 32 P]dCTP (800 Ci/mmol). Amplification products were analyzed on an 8% native polyacrylamide and visualized by autoradiography. Products were typically analyzed between cycles 20 and 24. To confirm the identity of the male and female products, unlabeled amplification reactions were separated on an 8% native polyacrylamide gel, electroblotted onto Hybond N⁺ membrane (Amersham) and hybridized with a 32 P-labeled probe from the upstream exon common to both RNA isoforms.

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Molecular genetic analysis of the heterodimeric splicing factor U2AF: the RS domain on either the large or small *Drosophila* subunit is dispensable in vivo

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