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The Drosophila U1-70K Protein Is Required for Viability, but Its Arginine-Rich Domain Is Dispensable

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ABSTRACT

The conserved spliceosomal U1-70K protein is thought to play a key role in RNA splicing by linking the U1 snRNP particle to regulatory RNA-binding proteins. Although these protein interactions are mediated by repeating units rich in arginines and serines (RS domains) *in vitro*, tests of this domain's importance in intact multicellular organisms have not been carried out. Here we report a comprehensive genetic analysis of U1-70K function in Drosophila. Consistent with the idea that U1-70K is an essential splicing factor, we find that loss of U1-70K function results in lethality during embryogenesis. Surprisingly, and contrary to the current view of U1-70K function, animals carrying a mutant U1-70K protein lacking the arginine-rich domain, which includes two embedded sets of RS dipeptide repeats, have no discernible mutant phenotype. Through double-mutant studies, however, we show that the U1-70K RS domain deletion no longer supports viability when combined with a viable mutation in another U1 snRNP component. Together our studies demonstrate that while the protein interactions mediated by the U1-70K RS domain are not essential for viability, they nevertheless contribute to an essential U1 snRNP function.

IN metazoans, the majority of precursor messenger RNAs (pre-mRNAs) contain one or more introns that must be removed to generate functional RNA molecules. This processing event, known as RNA splicing, is carried out by the spliceosome, a catalytic RNA-protein machine composed of five snRNAs and ~300 proteins (Jurica and Moore 2003). Assembly of the spliceosome on its pre-mRNA target occurs in several stages from the preassembled U1, U2, U4, U6, and U5 small nuclear ribonucleoprotein particles (snRNPs) and a set of proteins that are loosely associated with the snRNPs. The U1 snRNP, which is recruited to the 5′ splice site early in the spliceosomal assembly pathway, contains the U1 snRNA, whose 5′ sequence is complementary to the 5′ splice site, a set of seven Sm proteins shared with the other spliceosomal U snRNPs and three U1-specific proteins: U1-70K, U1-A, and U1-C. Although in most cases the individual contributions of the U1-specific proteins are still poorly defined, studies from several different organisms have suggested that U1-70K plays a key role in targeting the U1 snRNP to the 5′ splice site through protein/protein interactions with regulatory splicing factors (Kohtz *et al.* 1994; Jamison *et al.* 1995; Cao and Garcia-Blanco 1998).

In extracts from cultured mammalian cells, U1-70K interacts with members of the SR protein family to mediate recruitment of the U1 snRNP to the 5′ splice site. One of the best-studied examples of this type of interaction is with the ASF/SF2 protein (Caceres *et al.* 1993; Eperon *et al.* 1993; Woppmann *et al.* 1993; Wu and Maniatis 1993; Kohtz *et al.* 1994; Jamison *et al.* 1995; Cao and Garcia-Blanco 1998). The interaction between these two proteins is mediated by arginine-/serine-rich (RS) domains located at the C termini of each protein. Because both U1-70K and at least one SR protein are essential for splicing *in vitro*, it was anticipated that the interaction between the two proteins would be essential for recruitment of the U1 snRNP to the 5′ splice site and splicing. However, recent studies have suggested that this is not universally true, as splicing of a number of substrates is not impaired when the RS domain of ASF/SF2 is deleted (Eperon *et al.* 2000; Zhu and Krainer 2000). Given that ASF/SF2 is but one member of the SR protein family and that U1-70K is capable of interacting with several of these splicing factors, it has been postulated that *in vivo* the other SR proteins are capable of providing the necessary connection to U1-70K.

While these and other studies point to a key role for the U1-70K RS domain in linking the U1 snRNP particle

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to regulatory RNA-binding proteins, studies directly testing its importance have not been carried out. Here we report that in Drosophila, the loss of U1-70K function leads to embryonic lethality, consistent with the view that U1-70K is an essential splicing factor. However, contrary to the current view of U1-70K function, we find that animals carrying a mutant U1-70K protein lacking its RS domain, including its two embedded sets of RS dipeptide repeats, have no mutant phenotype. Surprisingly, we find that when the U1-70K RS deletion allele is combined with a viable mutation in another U1 snRNP component, the double-mutant animal dies during embryogenesis. Our finding that the U1-70K RS domain becomes essential in a splicing-compromised background thus demonstrates that the protein links mediated by the U1-70K RS domain are part of a redundant network of interactions necessary to support viability. The unexpected redundancy revealed by these studies underscores the importance of examining protein function directly in the animal.

MATERIALS AND METHODS

Fly strains and the isolation of *U1-70K* mutant alleles: *U1-70K^{2}* was isolated in a standard chemical mutagenesis screen where ~5000 individual second chromosomes were isolated from males fed 0.025 M ethyl methanesulfonate (EMS) and tested for their ability to complement the lethality of the *U1-70K^{1}* allele. *U1-70K^{ON35}* was isolated by transposase-induced male recombination according to the method of Preston and Engels (1996). Briefly, 27 recombinants between *al* and *sp* were collected from the progeny of *U1-70K^{1}/al px sp; ry^{506} Sb P{Δ2-3}/ry^{506}* males. Ten recombinants were selected for molecular analysis because they failed to complement *U1-70K^{1}* and all were found to contain deletions extending from the original site of the *P*-element insertion. Of these, *U1-70K^{ON35}* proved to be the most useful for this study. Descriptions of marker mutations and balancers not listed in the text are described on FlyBase (http://www.flybase.org). All crosses were carried on standard Drosophila medium at room temperature (22°).

***P*-element-mediated transformation and genetic rescue experiments:** The *P{hs::U1-70K}* rescue construct was generated by subcloning a natural *Eco*RI fragment into the pCaSpeRhs transformation vector (Thummel *et al.* 1988). This 1390-bp fragment extends from the transcription start site to a region that lies between the AATAAA polyadenylation signal and the polyadenylation site for the longer transcript described in Mancebo *et al.* (1990). The *P{genomic}*, *P{HCterm}*, and *P{ΔRrich-HCterm}* transgenes were generated in the context of a 9-kb *Xho*I/*Not*I genomic fragment (which contains the *U1-70K* transcription unit and flanking sequences) and then inserted into the pCaSpeR4 transformation vector (Thummel and Pirrotta 1992). The *P*-element transformation vectors used in this study carry a *white*$^{+}$ mini-gene to recognize and follow the transgene. Germline transformants were obtained by standard methods and multiple insertions were obtained for each transgene. Although initially multiple insertion lines were tested for their ability to rescue the *U1-70K* lethal phenotype, when the data for this article were collected, only one representative example of each transgene remained. Thus, the data presented here were all obtained with a single example of each transgene.

Western blot and immunoprecipitation experiments: Immunoprecipitation, Western blot analysis, RNA isolation from the RNA-protein complexes, and Northern blot analysis were carried out as previously described (Stitzinger *et al.* 1999). The antibodies used in this study, anti-SNF (Flickinger and Salz 1994) and anti-70K (Nagengast *et al.* 2003), have been described previously.

RESULTS

The Drosophila U1-70K protein is U1 snRNP specific: The Drosophila genome encodes a set of proteins that are clearly orthologous to each of the human U1 snRNP-specific proteins, except that the U1-A counterpart, encoded by the *sans-fille* (*snf*) gene, also fulfills the function of the U2 snRNP-specific protein, U2-B″ (Mount and Salz 2000). Although the putative U1-70K ortholog was identified on the basis of sequence conservation over 14 years ago (Mancebo *et al.* 1990; Figure 1), its functional identity has been inferred only by the presence of an appropriately sized protein in purified U1 snRNPs (Labourier and Rio 2001). To establish that the Drosophila U1-70K protein is present in U1 snRNPs, we used antisera raised against amino acids 1–213 of the protein to ask whether U1 snRNAs can be co-immunoprecipitated from whole-cell extracts. As shown in previous studies, antibodies directed against SNF immunoprecipitate both U1 snRNAs and U2 snRNAs from whole-cell extracts (Figure 2). In contrast, the U1-70K specific antibody precipitates U1 snRNA without bringing down significant amounts of U2 snRNA. Thus, we conclude that in *Drosophila melanogaster*, as in other organisms, U1-70K is a U1 snRNP-specific protein.

U1-70K is essential for viability: To identify mutations in the *U1-70K* gene, we searched the Drosophila databases for *P*-element insertions located in or near *U1-70K* and identified a recessive lethal called *l(2)02107*, which contained a *P*-element insertion in the 5′ UTR of the U1-70K transcription unit (Spradling *et al.* 1999). Homozygous mutant animals complete embryogenesis with no consistent cuticular phenotype, but never hatch (data not shown). The embryonic lethality is rescued by both a transgene carrying the *U1-70K* coding sequence under control of the *hsp70* promoter and a genomic rescue construct that contains only the *U1-70K* transcription unit (data not shown). Together, these results demonstrate that the loss of *U1-70K* function is lethal and that *U1-70K* is an essential gene. We therefore refer to *l(2)02107* as *U1-70K^{1}*.

Additional *U1-70K* alleles were identified in two independent screens. Screening for EMS-induced mutations that failed to complement the lethality of *U1-70K^{1}* identified one new allele, *U1-70K^{2}*. DNA sequencing revealed a single nucleotide substitution in the coding sequence of the *U1-70K* gene that results in conversion of W88 to a stop codon (UGG to UAG; Figure 1). We note that while some readthrough of Drosophila stop codons has been described (Chao *et al.* 2003), the circumstance most favorable for this (a C residue immediately after the stop) does not apply here.

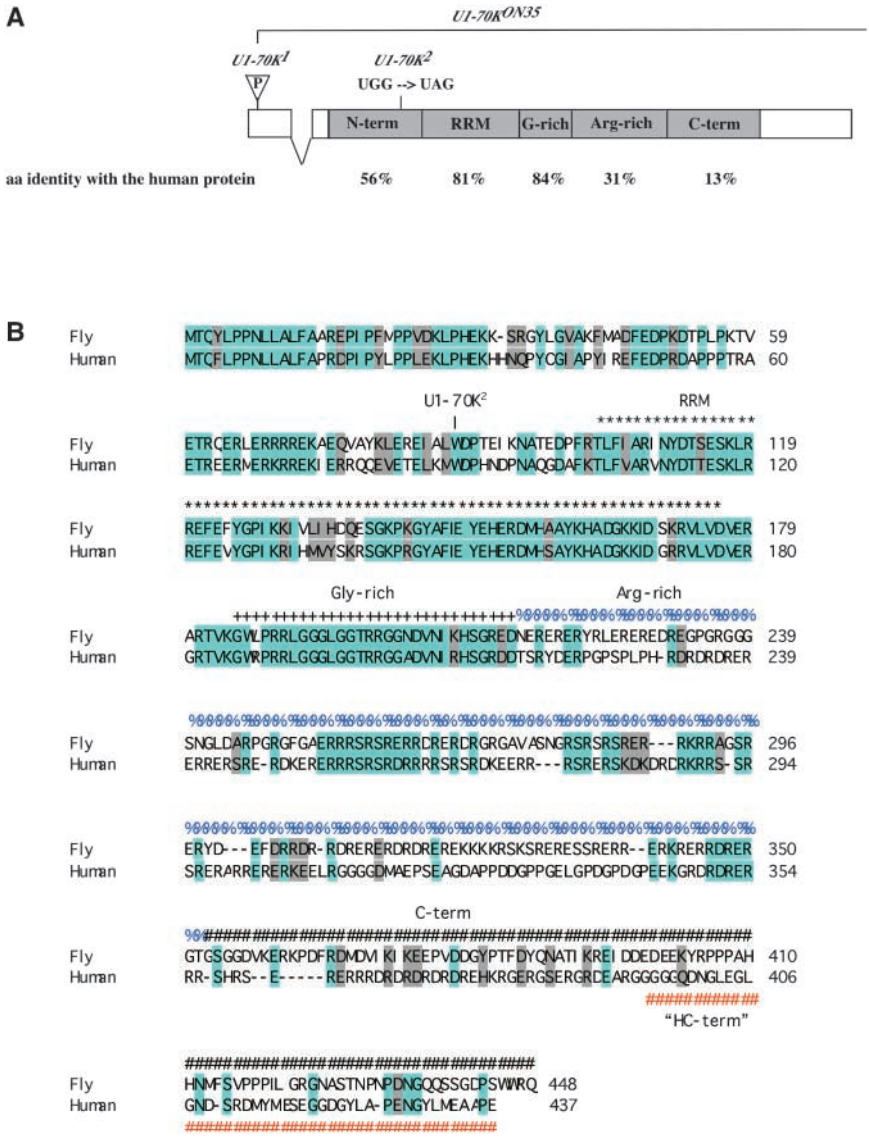

FIGURE 1.—The U1-70K gene: sequence comparison with the human protein, gene structure, and location of mutations. (A) Schematic of the *U1-70K* transcription unit. Solid boxes represent exons and lines represent introns. The position of the *P*-element insertion in *U1-70K*^{1}, the point mutation in *U1-70K*^{2}, and the extent of the deletion in *U1-70K*^{ON35} are indicated at the top of the diagram. As indicated below the diagram, each individual protein domain shares significant amino acid identity with the human U1-70K protein. (B) Alignment of the U1-70K protein from Drosophila and humans. Identical residues are boxed in green and conservative substitutions are boxed in gray. The positions of the different protein domains discussed in the text are indicated either above or below the sequence. An arrow indicates the position of the stop codon in the U1-70K^{2} mutation.

A definitive null allele, *U1-70K*^{ON35}, was isolated by selecting for male-recombination events involving the *P*-element in *U1-70K*^{1}. Sequencing of this allele shows that excision of the *P*-element generated a 1997-nt deletion of genomic DNA extending from the site of the *U1-70K*^{1} insertion in the 5′ UTR through the open reading frame, ending in the 3′ UTR (Figure 1B). As was observed for *U1-70K*^{1}, homozygous *U1-70K*^{ON35} mutant animals complete embryogenesis but do not hatch (data not shown). Similar late embryonic phenotypes are also observed with *U1-70K*^{2}, indicating that all three alleles are genetic nulls.

Functional substitution of the fly C-terminal domain by the human C-terminal domain: The structural organization of U1-70K is conserved between humans and flies, with sequence similarity extending over the entire length of the protein (Figure 1A). The amino-terminal half of U1-70K is highly conserved and consists of a 102-amino-acid N-terminal domain of unknown function (56% identical) followed by a 73-amino-acid RNA recognition motif (81% identical) and a 30-amino-acid glycine-rich region (G-rich, 84% identical). Following these highly conserved motifs is an arginine-rich region, which contains two embedded sets of RS dipeptide repeats called RS domains (Arg-rich, 31% identical) and a poorly conserved C-terminal domain (C-term, 13% identical). Interestingly, in Drosophila, this C-terminal region is expanded at the expense of the arginine-rich region when compared to the human protein.

To determine whether the expanded C-terminal domain is functionally significant, we modified the U1-70K wild-type genomic rescue construct by replacing the endogenous C-terminal domain (amino acids 353–448) with the human C-terminal domain (HC-term, amino acids 395–437; see Figure 3A). Transgenic lines carrying this construct (designated *P{HCterm}*) were generated and tested for function by genetic complementation. Specifically, the ability of the chimeric construct to res-

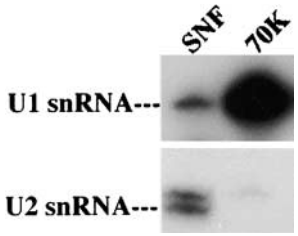

FIGURE 2.—U1-70K is a U1 snRNP-specific protein. snRNP incorporation was tested by immunoprecipitation of either U1-70K or SNF from extracts made from adult flies followed by Northern blotting to detect U1 and U2 snRNAs in the RNA extracted from the precipitated fractions.

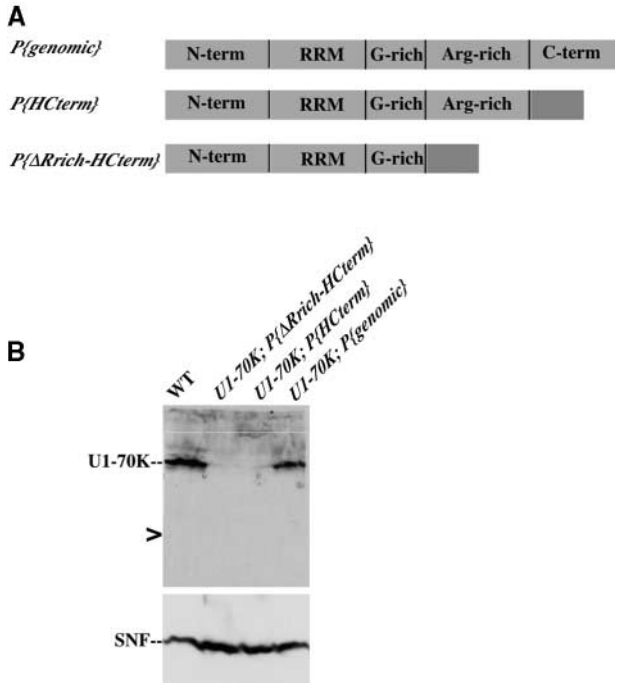

FIGURE 3.—Structure and expression of wild-type and mutant U1-70K proteins. (A) Schematic of the wild-type and mutant constructs used in this study. (B) Expression of the transgenic proteins was assayed by Western blot analysis using a polyclonal antibody specific for U1-70K (top) and reprobed with an antibody specific for SNF as a loading control (bottom). Extracts were made from wild-type (WT) or from *U1-70K^{ON35}/U1-70K^{2}* animals that also carry a copy of the appropriate transgene. A caret indicates the position of the 25-kD molecular weight marker.

cue the lethal phenotype of the *U1-70K^{ON35}/U1-70K^{2} trans*-heterozygotes was compared to that of the wild-type genomic rescue construct *P{genomic}*. In this experiment, we used these *trans*-heterozygotes to avoid complications due to second-site mutations that may be carried on the parental chromosomes, but comparable rescue of *U1-70K^{ON35}* homozygotes is also observed (data not shown). The results indicate that a single copy of *P{HCterm}* provides sufficient activity to fully complement the lethal phenotype of *U1-70K* mutants (Table 1). As described in more detail below, we found that in all of our assays, the rescuing activity of *P{HCterm}* is comparable to *P{genomic}*. Thus we conclude that the C-terminal domain of the human U1-70K protein can substitute for the fly C-terminal domain. These studies, however, do not address what the function of the C-terminal domain is, leaving open the possibility that the C-terminal domain is dispensable altogether.

The arginine-rich domain, with its embedded RS dipeptide repeats, is dispensable: A number of studies have led to the general conclusion that the U1-70K arginine-rich domain, with its embedded RS dipeptide repeats, is critical for linking the U1 snRNP to other splicing factors during the course of spliceosome assembly (see Introduction). We tested the importance of this domain *in vivo* by modifying the *P{HCterm}* construct such that the 138-amino-acid arginine-rich region (amino acids 215–352) is deleted (see Figure 3). Surprisingly, a single copy of the resulting *P{ΔRrich-HCterm}* transgene also provided sufficient activity to complement the lethal phenotype of U1-70K males and females (Table 1). The robust rescue of the lethal phenotype indicates that in an otherwise wild-type background the absence of the arginine-rich domain does not have a major effect on U1-70K function.

To allow a more rigorous interpretation of these genetic data, we assayed the expression of the endogenous and transgenic proteins in the *U1-70K^{ON35}/U1-70K^{2}; P{ΔRrich-HCterm}* surviving animals by Western blot analysis. As expected, we did not detect any endogenous protein, confirming that both *U1-70K^{ON35}* and *U1-70K^{2}* are protein null alleles. To our surprise, we found that the protein produced by *P{ΔRrich-HCterm}* and its progenitor *P{HCterm}* is not detectable on Western blots under conditions where the parental *P{genomic}* transgene could be detected easily (Figure 3B). It is not clear why the antibody, which is made against the N-terminal end of the protein, does not recognize the transgenic proteins. Clearly, the transgenes are functional, as judged by their ability to rescue the lethal phenotype. Perhaps the epitope is masked in the fusion proteins; or perhaps the level of expression changes dramatically during development and drops below the level of detection during adulthood.

The arginine-rich domain becomes essential in a *snf* mutant background: Although our finding that *U1-70K^{ON35}/U1-70K^{2}*; *P{ΔRrich-HCterm}* animals are wild type indicates that the loss of RS-mediated events does not disrupt U1 snRNP function sufficiently to impair viability, a subtle effect on U1 snRNP function cannot be ruled out. In an earlier study, we proposed a similar scenario to explain why SNF, the counterpart of the mammalian U1A protein, is also dispensable for U1 snRNP function (NAGENGAST *et al.* 2003). Although *snf* mutations that make no protein are lethal, one allele, *snf^{148}*, is viable even though it encodes a protein that is not stably associated with the U1 snRNP. Despite the lack of phenotype, it nevertheless seemed likely that U1

TABLE 1

Rescue activity of *U1-70K* transgenes

Genotype	Transgene (rescue activity)		
	P{ΔRrich-HCterm}	*P{HCterm}*	*P{genomic}*
XX; U1-70K^{ON35}/U1-70K^{2}; transgene	268 (80%)	256 (100%)	83 (73%)
XY; U1-70K^{ON35}/U1-70K^{2}; transgene	263 (81%)	260 (100%)	92 (87%)
XX; U1-70K/CyO; transgene	670	454	226
XY; U1-70K/CyO; transgene	648	417	211

To assay for rescue of the *U1-70K^{ON35}/U1-70K^{2}* recessive lethal phenotype *w/w; U1-70K^{2}/CyO*, virgin females were crossed to *w; U1-70K^{ON35}/CyO* males carrying a single copy of the appropriate transgene on the third chromosome, and the resulting progeny were scored. The transgene is recognized and followed by the expression of the *white^{+}* mini-gene included in each construct. Rescue activity was assessed by comparing the number of *w/w; U1-70K^{ON35}/U1-70K^{2}* animals carrying transgene to the number of "expected animals," which is one-half of the number of same-sexed balanced progeny carrying the transgene. We note that we did not recover any *w/w; U1-70K^{ON35}/U1-70K^{2}* animals without the transgene, indicating that *U1-70K^{ON35}/U1-70K^{2}* is a tight lethal.

snRNP function might be compromised in either of these mutants. If this is the case, then simultaneously mutating both components might have a significant effect on U1 snRNP function, perhaps resulting in lethality. To test this idea, we determined whether this *snf* mutation had a significant impact on the rescue activity of each transgene (Table 2). In control crosses, we found that the *snf^{148}* mutation did not have significant effect on the survival rate of *U1-70K^{ON35}/U1-70K^{2}*; *P{HCterm}* or *U1-70K^{ON35}/U1-70K^{2}*; *P{genomic}* animals. In contrast, *snf^{148}*; *U1-70K^{ON35}/U1-70K^{2}*; *P{ΔRrich-HCterm}* males were never recovered, even though their *U1-70K^{ON35}/U1-70K^{2}*; *P{ΔRrich-HCterm}* siblings were viable. The finding that these two alleles have synergistic effects on viability demonstrates that the arginine-rich domain indeed contributes to U1-70K function.

DISCUSSION

Here, we provide evidence that U1-70K is an essential splicing factor by demonstrating that null mutations are embryonic lethal. Our finding that U1-70K is essential for development is in agreement with the conclusions drawn from RNA interference knock-down experiments in both Arabidopsis and *Caenorhabditis elegans* (Golovkin and Reddy 2003; MacMorris *et al.* 2003). While splicing assays have so far not established that the lethality associated with the loss of metazoan U1-70K function is caused by an accumulation of unspliced RNAs, genetic studies in both yeast and Drosophila have suggested that U1-70K is critical for proper splicing of at least some transcripts (Hilleren *et al.* 1995; Nagengast *et al.* 2003). Interestingly, the *Saccharomyces cerevisiae* U1-70K ortholog is not essential for viability, although its absence does cause a slow-growth phenotype (Hilleren *et al.* 1995). In yeast, U1-70K may be dispensable because the yeast U1 snRNP is more complex with seven more protein components than metazoan U1 snRNPs, leaving open the possibility that one or more of these proteins might provide a compensating function in its absence (Gottschalk *et al.* 1998; Rigaut *et al.* 1999; Mount and Salz 2000).

A striking outcome of our studies is the finding that U1-70K can accomplish its vital function in the absence

TABLE 2

Rescue activity of *U1-70K* transgenes in a *snf* mutant background

Genotype	Transgene (rescue activity)		
	P{ΔRrich-HCterm}	*P{HCterm}*	*P{genomic}*
snf^{148};U1-70K^{ON35}/U1-70K^{2}; transgene	0	139 (86%)	97 (66%)
snf^{148}/+; U1-70K^{ON35}/U1-70K^{2}; transgene	203	161	147

To assay for rescue in a *snf* mutant background, *w snf^{148}/FM6;U1-70K^{2}/CyO* virgin females were crossed to *w; U1-70K^{ON35}/CyO* males carrying a single copy of the appropriate transgene on the third chromosome, and the resulting progeny were scored. The transgene is recognized and followed by the expression of the *white^{+}* mini-gene included in each construct. Rescue activity was assessed by comparing the number of *w snf^{148};U1-70K^{ON35}/U1-70K^{2}* males carrying transgene to the number of "expected animals" as determined by the number of sibling *w snf^{148}/w +;U1-70K^{ON35}/U1-70K^{2}* controls carrying the transgene.

of an RS domain. The failure to detect a phenotype in these mutant animals challenges the prevailing view that the U1-70K RS motif provides a vital link between splicing regulators and the U1 snRNP. We suggest instead that either the interactions detected *in vitro* are not essential *in vivo* or there are multiple means by which the U1 snRNP can interact with splicing regulators. Support for the latter view comes from our demonstration that U1 snRNP particles lacking both the U1-70K RS domain and SNF can no longer support viability. Synthetic lethality is attributable to the simultaneous loss of two functions that contribute to the same activity or pathway. Interestingly, in *S. cerevisiae* synthetic lethal interactions have been observed between nonlethal mutations in several different U1 snRNP components (Gottschalk *et al.* 1998). Thus, our *in vivo* results argue that while disruption of the U1-70K RS-mediated protein links has no detectable consequence to the living organism, the simultaneous disruption of multiple connections causes U1 snRNP function to fall below the level needed to support development and viability.

In an interesting parallel to our studies, Rio and colleagues demonstrated that disruption of the RS domain of one, but not both, of the U2AF heterodimer subunits does not result in lethality (Rudner *et al.* 1998a,b,c). The RS domains of both subunits of the U2AF heterodimer have RS domains that were believed, on the basis of *in vitro* studies, to interact with distinct proteins. Using a similar genetic approach to the one described here, the RS domain of the Drosophila large U2AF subunit ortholog was found to be dispensable for viability. Similarly, the RS domain of the small subunit ortholog was also dispensable. However, flies mutant for both RS deletions could not be recovered, indicating that either set of protein links provided by the U2AF heterodimer is sufficient for viability. Together, these studies highlight the importance of looking at the roles of splicing factors in their natural contexts to unambiguously identify which functions are truly essential and which functions are part of the redundant network necessary for accurate and efficient splicing.

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