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Genome-Wide Analysis Reveals an Unexpected Function for the *Drosophila* Splicing Factor U2AF^{50} in the Nuclear Export of Intronless mRNAs

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Summary

The protein factor U2AF is an essential component required for pre-mRNA splicing. Mutations identified in the *S. pombe* large U2AF subunit were used to engineer transgenic *Drosophila* carrying temperature-sensitive U2AF large subunit alleles. Mutant recombinant U2AF heterodimers showed reduced polypyrimidine tract RNA binding at elevated temperatures. Genome-wide RNA profiling comparing wild-type and mutant strains identified more than 400 genes differentially expressed in the dU2AF^{50} mutant flies grown at the restrictive temperature. Surprisingly, almost 40% of the downregulated genes lack introns. Microarray analyses revealed that nuclear export of a large number of intronless mRNAs is impaired in *Drosophila*-cultured cells RNAi knocked down for dU2AF^{50}. Immunopurification of nuclear RNP complexes showed that dU2AF^{50} associates with intronless mRNAs. These results reveal an unexpected role for the splicing factor dU2AF^{50} in the nuclear export of intronless mRNAs.

Introduction

Surprisingly, the human genome sequence has revealed that the number of genes contained in our genome is smaller than originally anticipated. It is now estimated that the human genome contains approximately 30,000 genes, about two times more than the number of genes found in the nematode, *C. elegans*, and the fruit fly, *D. melanogaster* (Adams et al., 2000; Lander et al., 2001; *C. elegans* Sequencing Consortium, 1998). One strategy employed in all higher eukaryotes to increase protein diversity is the capacity of the messenger RNAs to be alternatively spliced to generate multiple protein isoforms from a single gene. In the fruit fly *Drosophila melanogaster*, one gene in particular illustrates the extensive coding potential generated by alternative splicing. The *Drosophila* *D*own's syndrome *c*ell *a*dhesion *m*olecule (Dscam) gene contains 95 alternative exons and has the potential to code for more than 38,000 different proteins (Schmucker et al., 2000).

In eukaryotes, intron removal results from the stepwise assembly of the U1, U2, U4/U6, and U5 snRNPs on the pre-mRNA through an extensive network of RNA-RNA, RNA-protein, and protein-protein interactions (Reed, 2000; Staley and Guthrie, 1998). In the initial steps of spliceosome assembly, U2AF, together with the SF1/BBP protein, facilitates U2 snRNP binding to the branchpoint sequence, which lies in the intron upstream of the 3′ splice site (Berglund et al., 1997; Krämer, 1992; Zamore and Green, 1989; Zamore et al., 1992). U2AF is a highly conserved heterodimer composed of large and small subunits (Zamore and Green, 1989). Although the human small U2AF subunit (U2AF^{35}) is dispensable for in vitro splicing of simple model pre-mRNA substrates (Zamore and Green, 1991), both subunits are essential for viability in flies, worms, and yeast (Kanaar et al., 1993; Potashkin et al., 1993; Rudner et al., 1996; Zorio and Blumenthal, 1999b). The large U2AF subunit recognizes the intron polypyrimidine tract upstream of the 3′ splice site (Singh et al., 1995), while the small subunit directly binds to the 3′ splice site AG dinucleotide (Merendino et al., 1999; Wu et al., 1999; Zorio and Blumenthal, 1999a).

The large U2AF subunit is a modular protein with an N-terminal RS domain rich in arginine and serine (RS dipeptides). Adjacent to the RS domain on the large U2AF subunit, a proline-rich segment mediates protein-protein interactions with the pseudo-RNA binding domain (RRM for *R*NA *r*ecognition *m*otif) of the small U2AF subunit (Kielkopf et al., 2001; Rudner et al., 1998c). The C terminus of the large U2AF subunit contains three RRMs. Although all three RRMs were initially described as required for the U2AF-RNA interaction (Zamore et al., 1992), RRM1 and RRM2 were subsequently shown to be sufficient for specific RNA binding (Ito et al., 1999), while RRM3 is responsible for protein-protein interactions with the branchpoint binding protein SF1/BBP (Berglund et al., 1998; Rain et al., 1998; Selenko et al., 2003). In addition to interacting with SF1/BBP, U2AF interacts with members of the SR family of splicing factors (McKinney et al., 1997; Page-McCaw et al., 1999; Potashkin et al., 1993; Romfo et al., 1999; Tronchere et al., 1997; Wu and Maniatis, 1993), with the U2 snRNP-associated protein, SAP155 (Gozani et al., 1998), UAP56, a putative RNA helicase required for splicing (Fleckner et al., 1997) and nuclear export of mRNA, (Gatfield et al., 2001; Herold et al., 2003), and the transport factor TAP/NXF1 (Zolotukhin et al., 2002).

To better understand the mechanisms underlying splice site selection and the control of alternative splicing, we used genome-wide approaches to pursue our genetic and biochemical investigation of the *Drosophila* large U2AF subunit (dU2AF^{50}). Several groups have reported the identification of temperature-sensitive mutations in the yeast *S. pombe* large U2AF subunit homolog that reside in conserved amino acids common to all known U2AF large subunits (Figure 1). We transferred the *S. pombe* mutations to *Drosophila* and created new temperature-sensitive dU2AF^{50} transgenic strains. In vitro, these mutant recombinant U2AF heterodimers show a dramatic temperature-dependent reduction in RNA polypyrimidine tract binding, without exhibiting any defect on in vitro splicing of model pre-mRNAs. Genome-wide

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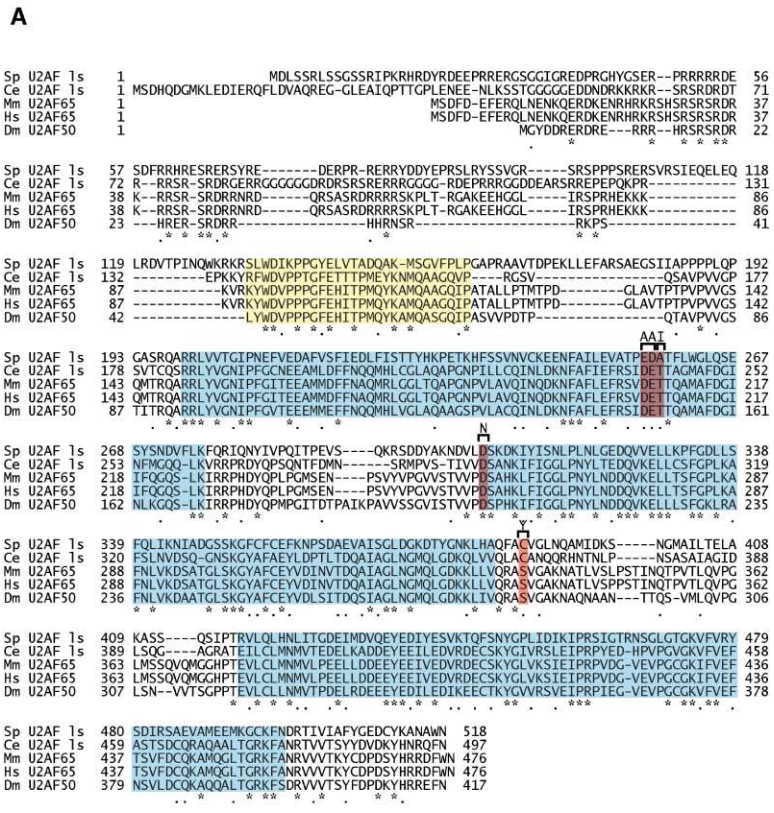
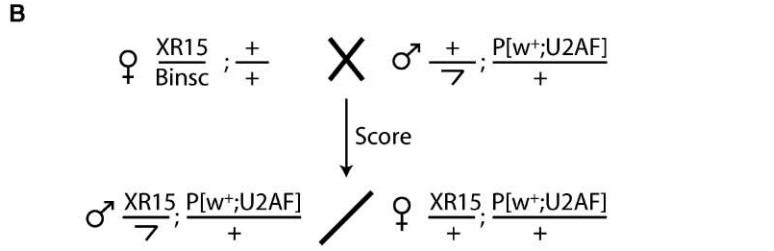
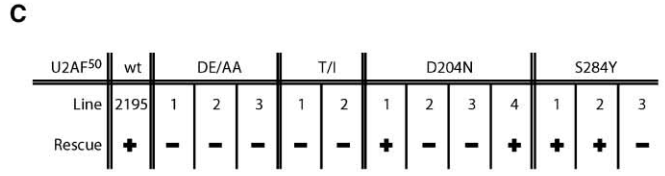
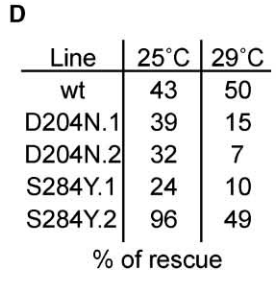








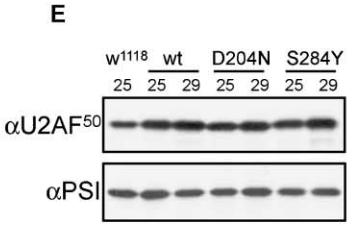


Figure 1. Identification of New dU2AF^{50} Temperature-Sensitive Alleles

(A) Sequence alignments of the *Schizosaccharomyces pombe* (Sp), *Caenorhabditis elegans* (Ce), mouse (Mm), human (Hs), and *Drosophila melanogaster* (Dm) U2AF large subunits. Yellow box, interaction domain with the small U2AF subunit; blue boxes, RNA binding domains (RRM) 1 to 3; red boxes, point mutations conferring a temperature-sensitive phenotype in *S. pombe*.

(B) Outline of the genetic crosses used to test transgenic flies for complementation of a null allele of the dU2AF^{50} gene.

(C) Results from rescue crosses from 12 independent transgenic lines.

(D) The D204N and S284Y mutations confer a temperature-sensitive viability phenotype. The crosses depicted in (A) were repeated at either 25°C or 29°C. The percentage rescue was calculated from the number of rescued males over the nonbalanced females carrying the transgene in the sibling progeny.

(E) dU2AF^{50} protein expression was similar in wild-type and mutant strains grown at either the permissive or restrictive temperature. Total protein was extracted from adult flies from the rescue crosses and immunoblot with polyclonal antibody specific for dU2AF^{50} or, as a control, with antibodies against the splicing.

expression profiles of the mutant flies identify genes that are specifically differentially expressed at the restrictive temperature. These results have allowed the identification of U2AF-sensitive target mRNAs and specific RT-PCR analysis has confirmed that splicing is impaired in the mutant flies. Most interestingly, a high proportion of intronless genes were downregulated in the mutant flies when grown at the restrictive temperature. High-density microarrays and dU2AF^{50} knockdown in cultured cells were used to survey the nucleo-cytoplasmic distribution of all expressed genes. This analysis revealed that more that 28% of mRNAs accumulated in the nucleus upon dU2AF^{50} knockdown, regardless of their intron number. A genome-wide approach analyzing RNAs bound in nuclear RNPs as well as a bioinformatic analysis confirmed that dU2AF^{50} associates with intronless RNAs. These results reveal a previously unknown function for dU2AF^{50} in the nuclear export of intronless mRNAs.

Results

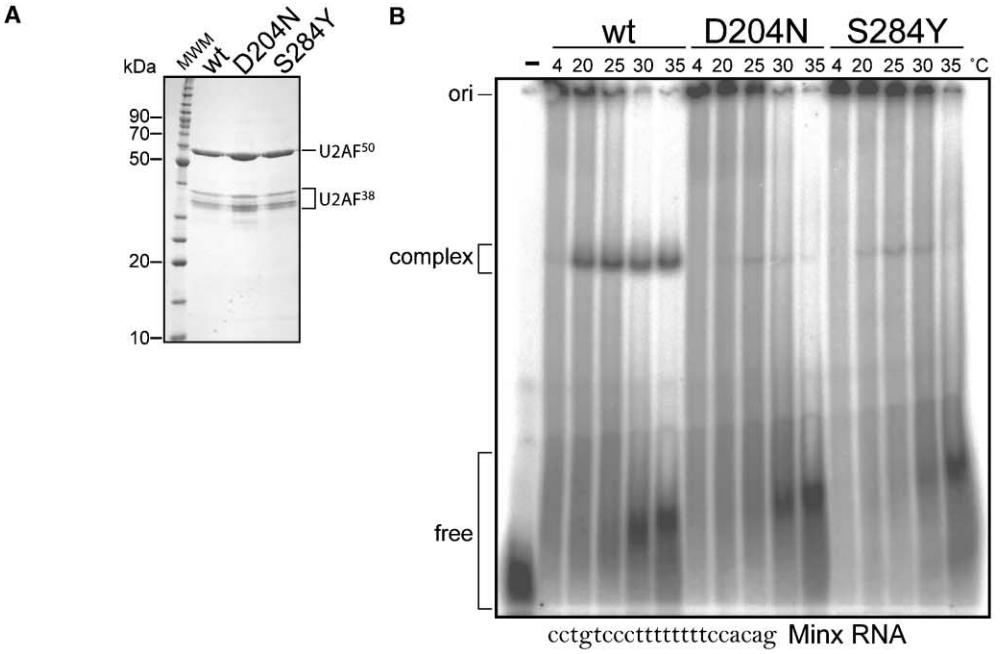
Mutations in the Large U2AF Subunit Show Temperature-Dependent Rescue of Viability of a dU2AF^{50} Deletion

A fully penetrant recessive lethal deletion of the gene coding for dU2AF^{50} has been characterized and can be rescued by dU2AF^{50} cDNA transgenes under the control of the dU2AF^{50} genomic promoter. *S. pombe* temperature-sensitive alleles in highly conserved residues of the large U2AF subunit have been identified and documented in vivo (Figure 1A; Potashkin et al., 1993; Romfo et al., 1999). The yeast mutations were individually introduced into the cDNA coding for dU2AF^{50} (Rudner et al., 1998b, 1998c), and transgenic flies were successfully recovered for all four mutations tested. The transgenes were then assayed for their ability to genetically complement a deletion of dU2AF^{50} (XR15). Females carrying the XR15 deletion over a balancer chromosome were mated with the different transgenic males (Figure 1B). A functional rescue allele was scored by the presence of non-Binsinscy males (males without the balancer chromosome) in the progeny resulting from flies carrying the XR15 deletion allele and rescued by the transgene. Of the four mutations tested only two, D204N and S284Y, successfully rescued the dU2AF^{50} XR15 deletion (Figure 1C).

The two mutant dU2AF^{50} alleles were then tested for a temperature-sensitive phenotype by repeating the crosses at 25°C and 29°C and by counting the ratio of rescued males to the nonbalanced females that eclosed at the two temperatures. The wild-type dU2AF^{50} transgene efficiently rescued the dU2AF^{50} deletion with a similar rescue percentage at both 25°C and 29°C (Figure 1D). By contrast, flies carrying the mutant transgenes efficiently rescued the dU2AF^{50} deletion at 25°C, but a marked reduction in rescued male viability was observed upon growth of the two mutant transgenic strains at 29°C (Figure 1D). This strong temperature-sensitive phenotype was observed for each mutation in two independent transgenic strains (Figure 1D). The temperature-dependent phenotype was not due to differences in either dU2AF^{50} expression or stability in the mutant transgenic strains since similar levels of dU2AF^{50} protein were observed in rescued males from the wild-type and from the two mutant transgenic strains at all temperatures tested (Figure 1E). Homozygous males and females carrying the dU2AF^{50} XR15 deletion rescued by the S284Y transgene were successfully engineered. This homozygous mutant strain displayed the same temperature-sensitive phenotype when grown at the restrictive temperature (30°C), the homozygous females laid eggs with abnormal eggshell morphology, and the mutant embryos failed to developed into larvae (data not shown). Taken together, these results confirm that the *S. pombe* temperature-sensitive mutations are transferable to the *Drosophila* dU2AF^{50} gene to generate new temperature-sensitive alleles.

The Temperature-Sensitive Mutations Reduce RNA Binding Affinity

dU2AF^{50} binds to the intron polypyrimidine tract upstream of the 3′ splice site and U2AF is required for 3′ splice site recognition of all characterized pre-mRNAs. The identified temperature-sensitive mutations D204N and S284Y flank the second RRM and thus might affect dU2AF^{50}/dU2AF^{38} RNA binding. Using a bicistronic system to simultaneously express both dU2AF subunits in bacteria, soluble wild-type and mutant recombinant dU2AF^{50}/dU2AF^{38} heterodimers (hereafter referred to as U2AF) were efficiently produced and tested for their ability to bind RNA (Rudner et al., 1998a). All three recombinant protein preparations were similar in composition, with dU2AF^{50} being expressed as a single species and dU2AF^{38} being slightly proteolysed, as observed previously (Figure 2A; Rudner et al., 1998a). Wild-type dU2AF efficiently formed specific RNA-protein complexes with an RNA oligonucleotide carrying the polypyrimidine tract and AG of the 3′ splice site of the first intron of the adenovirus major late pre-mRNA (MINX) when incubated at different temperatures, ranging from 20°C to 35°C (Figure 2B). However, both the D204N and S284Y mutants were defective in forming specific RNA-protein complexes at all temperatures tested (Figure 2B). Interestingly, both dU2AF mutants formed apparent protein-RNA aggregates at the top of the gel similar to what was seen with the wild-type protein at 4°C (Figure 2B). We attribute those large complexes to nonspecific RNA-protein complexes which are displaced upon specific interaction between the RNA and dU2AF. At 20°C, small amounts of specific RNA-protein complexes can be seen with the D204N and S284Y U2AF mutants (Figure 2B). By titrating the amount of protein, specific complexes can be formed at 20°C with the D204N and S284Y dU2AF, although less efficiently than with the wild-type dU2AF (apparent K_D of 0.6 μM, 2.3 μM, and 7.8 μM for the wild-type, D204N and S284Y dU2AF, respectively; see Experimental Procedures and Figure 2C). Incubating the binding reaction at 35°C does not affect wild-type dU2AF (apparent K_D of 1.0 μM), but completely abrogated binding of both the D204N and S284Y mutant dU2AFs (apparent $K_D > 50$ μM, Figure 2C). These results demonstrate that the D204N and S284Y temperature-sensitive dU2AF mutations affect RNA binding in a temperature-dependent manner.



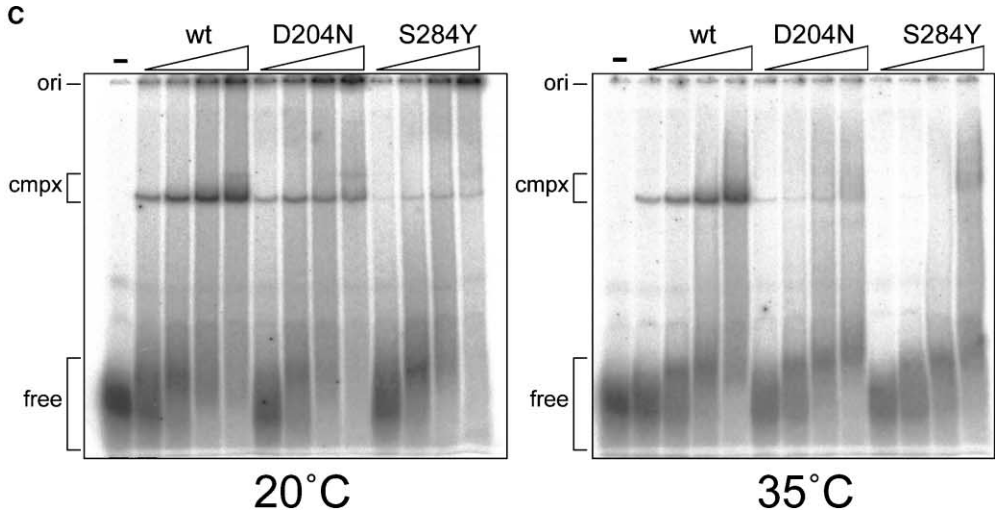


Figure 2. Mutant U2AF Heterodimers Display Reduced Temperature-Dependent RNA Binding

(A) A Coomassie blue-stained gel of the purified wild-type, D204N, and S284Y recombinant dU2AF^{50}/dU2AF^{38} heterodimers.

(B) RNA-protein complexes were assembled on a ^{32}P-5′ end-labeled RNA oligonucleotide corresponding to the polypyrimidine tract and 3′ splice site of the first intron of adenovirus 1 major late pre-mRNA (Minx). The recombinant proteins (3.3 μM) were mixed with RNA and incubated at the indicated temperature for 60 min, and then the complexes were resolved by native gel electrophoresis.

(C) Titration of the different recombinant proteins (0.03, 0.13, 0.67, and 3.33 μM) performed at either 20°C or 35°C.

dU2AF^{50} Temperature-Sensitive Mutants Specifically Affect Expression of a Subset of Genes

Although the RNA-affinity of the mutant protein is greatly reduced, in vitro splicing using a U2AF depletion-reconstitution system failed to show any defect of the mutant dU2AF recombinant proteins (data not shown). Different pre-mRNAs at all temperatures tested were spliced as efficiently in the presence of the mutant proteins as they were spliced in the presence of the wild-type dU2AF (data not shown). The use of efficiently spliced in vitro models might have impaired our capacity to observe splicing defect with the mutant dU2AF^{50} proteins. However, in vivo, more sensitive dU2AF^{50} targets might be less efficiently spliced, retained in the nucleus because they still contain an intron, and then degraded in the mutant dU2AF^{50} flies at the restrictive temperature. Individual gene expression using high-density microarrays was quantified from RNA extracted from both wild-type and mutant homozygous adult grown at the permissive and restrictive temperatures. Temperature-dependent

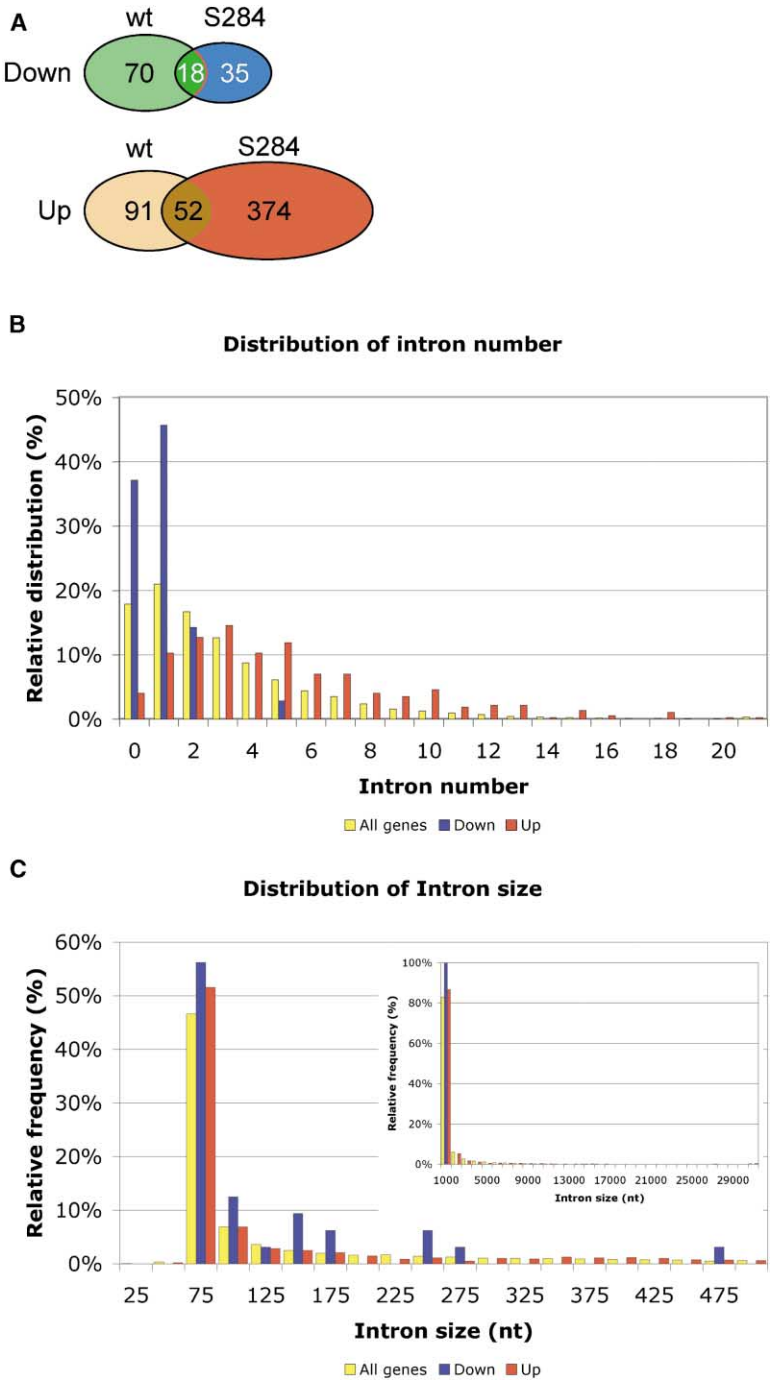


Figure 3. Specific Genes Are Affected upon Growth of S284Y dU2AF50 Mutant Flies at the Restrictive Temperature

(A) Venn diagram of the number of up- and downregulated genes in S284Y and wild-type flies grown at 30°C. Each circle represents the number of genes specifically affected in the flies grown at the restrictive temperature.

(B) The average number of introns per gene varies significantly in the genes up- and downregulated when compared to the overall genomic distribution. To describe the number of introns per gene, the average number was calculated to take into account genes with multiple transcripts containing different numbers of introns.

(C) The distribution of the intron lengths does not vary significantly in the up- and downregulated genes when compared to the average genomic distribution. The inset represents the distribution of intron length per 1000 nt intervals (from 1000 to 29,000).

variations in expression of individual genes were obtained by pairwise comparison of the triplicate experimental samples (nine comparisons). Significant variations in gene expression were determined using the Affymetrix statistical algorithm (MAS vers 5.01), and a positive score was attributed to genes that were significantly affected in at least seven out of nine pairwise comparisons (highly stringent). The analysis revealed that, at the restrictive temperature, 88 and 53 genes were downregulated in the wild-type and the mutant flies, respectively (Figure 3A) and that 35 genes were specifically downregulated only in the mutant flies (Figure 3A). By contrast, 143 and 426 genes were significantly upregulated in the wild-type and mutant flies grown at the restrictive temperature, out of which 374 were specifically upregulated in the S284Y mutant flies (Figure 3A). The differential expression of some genes were confirmed by semiquantitative RT-PCR (see Supplemental Figure S1B at http://www.molecule.org/cgi/content/full/14/6/775/DC1).

While the upregulated genes from the mutant flies do not display any striking functional clustering (see Supplemental Table S1), the downregulated genes cluster into two major categories. Eight genes (23% of the

Table 1. Some Genes Are Specifically Downregulated in the S284Y dU2AF^{50} Mutant Flies Grown at the Restrictive Temperature

Trypsin-like Protease			
Symbol	Name	Average	SD
CG18030	–	−1.4	0.7
CG10475	–	−1.4	0.9
λTry	λ Trypsin	−1.2	0.6
CG17571	–	−1.2	0.8
δTry	δ Trypsin	−0.8	0.4
CG2229	–	−0.8	0.4
βTry	β Trypsin	−0.6	0.5
yip7	–	−0.5	0.6
Oogenesis			
Cp19	Chorion protein 19	−1.0	0.6
Cp16	Chorion protein 16	−1.0	0.6
Cp18	Chorion protein 18	−0.9	0.6
Vm32E	Vitelline membrane 32E	−0.9	0.4
Cp38	Chorion protein 38	−0.8	0.5
Yp3	Yolk protein 3	−0.7	0.9
Cp36	Chorion protein 36	−0.6	0.4
Unknown or Unrelated Function			
	18S ribosomal RNA	−4.1	2.5
Ag5r2	Antigen 5-related 2	−1.7	0.9
CG18404	–	−1.6	0.9
CG4783	–	−1.5	1.0
CG5097	–	−1.5	1.1
CG13323	–	−1.5	0.9
CG13323	–	−1.4	1.0
PGRP-SC1a	–	−1.4	0.7
Cys	Cystatin-like	−1.4	0.8
CG8628	–	−1.3	0.5
CG16712	–	−1.2	0.9
CG6503	–	−1.2	0.8
CG11051	–	−1.2	0.6
CG7630	–	−1.1	0.7
Obp99c	Odorant-binding protein 99c	−1.1	0.7
PGRP-SC1b	–	−1.1	0.6
CoVa	Cytochrome c oxidase subunit Va	−1.1	0.7
CG5932	–	−1.1	0.5
CG15304	–	−0.9	0.6
EG:140G11.3	–	−0.8	0.4
cype	–	−0.7	0.3

The genes specifically downregulated in the S284Y mutant flies are grouped on the basis of their functional relationships. Averages correspond to the base 2 logarithm of the average signal of individual genes in at least seven to nine pairwise analyses between the S284Y flies grown at 30°C (restrictive temperature) over the same flies grown at 25°C (permissive temperature). SD, standard deviations.

downregulated genes) are known or predicted to be trypsin-like proteases, while seven genes (20% of the downregulated genes) are known to be involved in oogenesis (Table 1). The observation that several genes involved in oogenesis were downregulated fits well with the observed abnormal eggshell phenotype in the mutant strains.

Up- and Downregulated Genes in the U2AF Mutant Flies Show Drastically Different Intron Distributions

Since U2AF is known to be a splicing factor, we looked at the intron characteristics in the up- and downregulated genes in the S284Y mutant flies shifted to the restrictive temperature. In the latest release of the *Drosophila* genome annotation (v. 3.1), the average number of introns per gene (see Experimental Procedures) ranges from 0 introns (17.9% of all genes) to 49 introns (CG17150) and the distribution of the average number of introns per gene is centered around 1 intron per gene (21%; Figure 3B). Interestingly, the upregulated genes from the S284Y mutant flies grown at the restrictive temperature showed a consistent overrepresentation for genes containing multiple introns with a distribution centered around three introns per gene (Figure 3B). By contrast, the identified downregulated genes showed predominantly zero or one intron per gene (37% and 45.7%, respectively; Figure 3B). The differences in the intron number distributions were specific for the differentially expressed genes in the mutant flies since no significant differences were observed for the genes that were up- and downregulated in the wild-type flies grown at 30°C (data not shown). The observation that the distribution of introns for the specifically affected genes in the S284Y dU2AF^{50} mutant flies is significantly different from the average genomic distribution suggests that the dU2AF^{50} mutation might have a direct effect on those genes. In *Drosophila*, the modal intron length is 75 nt (Figure 3C) and does not vary significantly for the genes up- and downregulated in the dU2AF^{50} mutant S284Y flies grown at the restrictive temperature (Figure 3C). These results indicate that a mutation which reduces the RNA binding affinity of dU2AF^{50} can directly affect the expression of both intronless, as well as, intron-containing genes.

Up- and Downregulated Intron-Containing Genes in the U2AF Mutant Flies Show Differential Splicing Behavior

We next tested whether splicing was affected for some of the genes downregulated in the S284Y dU2AF^{50} mutant flies grown at the restrictive temperature. Using oligonucleotide primer pairs flanking the single intron of two genes (CG4783 and Ag5r2, which are the fourth and second most downregulated genes in the S284Y flies, respectively), RT-PCR was performed on RNA extracted from wild-type and mutant flies grown at both the permissive and restrictive temperatures. Splicing of CG4783 is efficient and similar in wild-type flies grown at both 25°C and 30°C temperatures (Figure 4A, top). However, in the S284Y mutant flies at 25°C, the splicing efficiency of CG4783 is reduced (Figure 4A, top) and, moreover, is even more reduced in the S284Y flies grown at 30°C (Figure 4A, top). Additionally, splicing of Ag5r2 is efficient and similar in both wild-type and mutant flies grown at the permissive temperature (25°C; Figure 4A, bottom). However, when the flies are shifted to the restrictive temperature (30°C), the splicing efficiency of Ag5r2 is reduced in the wild-type and the reduction in splicing is even more evident in the S284Y mutant flies (Figure 4B). This confirms that the S284Y dU2AF^{50} mutation affects, in a temperature-sensitive fashion, the splicing efficiency of some target genes in vivo.

RT-PCR assays were also performed on two genes that were upregulated in the dU2AF^{50} mutant flies shifted to the restrictive temperature (30°C). Oligonucleotide primer pairs flanking their multiple introns, two and six introns in CG7036 and transportin, respectively, were used to analyze splicing defects. The splicing efficiency of both pre-mRNAs was similar in both wild-type and

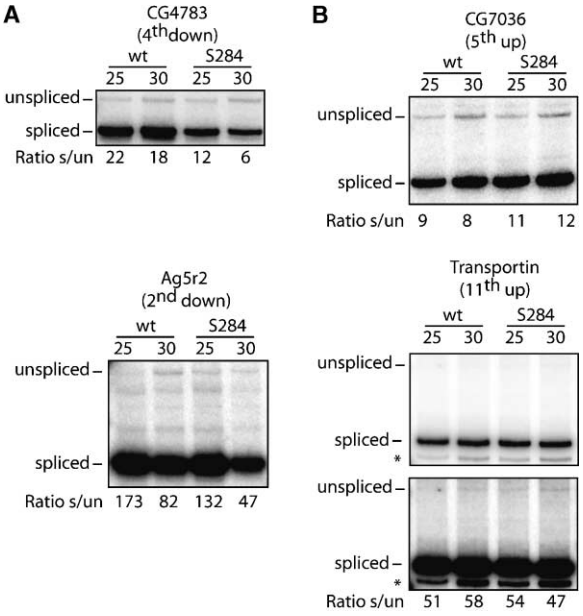


Figure 4. Downregulated Genes Show a Reduced Splicing Efficiency

(A) Splicing efficiency of downregulated genes is reduced in S284Y flies grown at the restrictive temperature. RT-PCR assays with primer pairs flanking the single intron of CG4783 and Ag5r2 were performed on RNA samples extracted from wild-type and S284Y mutant flies grown at either 25°C or 30°C.

(B) Splicing efficiency of upregulated genes is unaffected in S284Y flies grown at the restrictive temperature. RT-PCR assays with primer pairs flanking the multiple introns of CG7036 and Transportin were performed on RNA extracted from wild-type and S284Y mutant flies grown at either 25°C or 30°C. The calculated ratios of spliced to unspliced mRNA are indicated below the panels. The position of unspliced and spliced products are identified next to each panel. The * labeled transportin product is a PCR artifact contaminant derived from the fully spliced mRNA. Two pictures, under- and over-exposed, of the transportin RT-PCR assays are shown.

mutant flies grown at either the permissive or restrictive temperatures (Figure 4B). Taken together, these results suggest that the temperature-sensitive S284Y mutation in dU2AF50 reduces splicing of some of the downregulated pre-mRNAs in vivo in the mutant flies grown at the restrictive temperature but does not appear to affect the splicing of the upregulated genes, which contains multiple introns.

dU2AF50 Is Involved in Nuclear Export of Intronless mRNAs

We reasoned that the reduction in the level of some intronless mRNA in the dU2AF50 mutant flies might result from nuclear retention and degradation as has been observed when RNA export factor expression is knocked down (Herold et al., 2003). The expression of dU2AF50 was efficiently knocked down by RNAi in cultured *Drosophila* SL2 cells to less than 20% of the endogenous level (Figure 5A), and nuclear and cytoplasmic RNA was isolated from control and dU2AF50 knocked-down cells (Figure 5B). High-density oligonucleotide microarrays were used to measure the RNA level of 13,738 genes in both the nuclear and cytoplasmic fractions isolated from control and dU2AF50 knocked-down cells. Four thousand, seven hundred, and thirty-six genes show consistent expression in L2 cells, and for each expressed genes, the nucleo-cytoplasmic ratio from the control and dU2AF50- knocked-down sample was used to calculated a nuclear retention index describing the effect of the dU2AF50 RNAi. Using an arbitrary retention index greater than 0.1, more than 28% of the analyzed genes (1334 genes) were predicted to be retained in the nucleus in the dU2AF50 knocked-down cells with 12% of the retained mRNAs (166 genes) being intronless (see Supplemental Tables S2 and S3, respectively). The microarray analysis was validated by RT-PCR (Figure 5C) with eight individual genes showing variable retention indices. The RpL32 pre-mRNA contains two introns while the other genes are intronless (Figure 5D). The nucleo-cytoplasmic distribution predicted by the microarray and measured by RT-PCR nicely correlated for RpL32, as well as the intronless genes CG15784, CyCB3, Gip, Mpp6, and slp1, and confirms their nuclear retention in the dU2AF50 knocked-down cells (Figure 5D). In addition, the number of genes predicted to be retained in the nucleus by the microarray analyses is likely to be an underestimate of the real number due to the normalization process. The normalization is done using the calculated population average, and this normalization process is based on the ad hoc assumption that only a small proportion of genes are significantly different between the compared samples. As an example, the mRNAs coding for CG30342 and noi, which have slightly negative retention index (−0.39 and −0.07 respectively, see Supplemental Table S2) and thus were not predicted to be retained in the nucleus, nonetheless showed nuclear retention when assayed by RT-PCR (Figures 5C and 5D). These results indicate that the essential pre-mRNA splicing factor dU2AF50 also plays a significant and unexpected role in the nuclear export of a large number of intronless mRNAs.

dU2AF50 Can Be Found Associated with Intronless Genes

The previous results predict that dU2AF50 should associate with intronless mRNAs, either directly or indirectly, as part of a multicomponent RNP complex in order to promote their nuclear export. In order to test this prediction, we performed a specific immunopurification of nuclear RNP complexes from a 0–12 hr *Drosophila* embryonic RNP preparation, using affinity-purified anti-dU2AF50 antibody (Rudner et al., 1998c). Genome-wide identification of the associated RNAs was performed using spotted *Drosophila* cDNA microarrays containing approximately 6000 different EST PCR fragments. Five independent experiments were performed, and RNAs consistently present in at least three assays were used for further analyses (see Supplemental Table S3). In the top 200 RNAs, the dU2AF50-associated genes show an overrepresentation for pre-mRNAs with multiple introns, with a distribution centered around two introns per gene (Figure 6A). Surprisingly, but consistent with our hypothesis, four of the first 200 dU2AF50-bound RNAs were intronless (Figure 6B), confirming that dU2AF50 can be found stably associated with intronless RNAs. Semi-quantitative RT-PCR was used to confirm that those RNAs

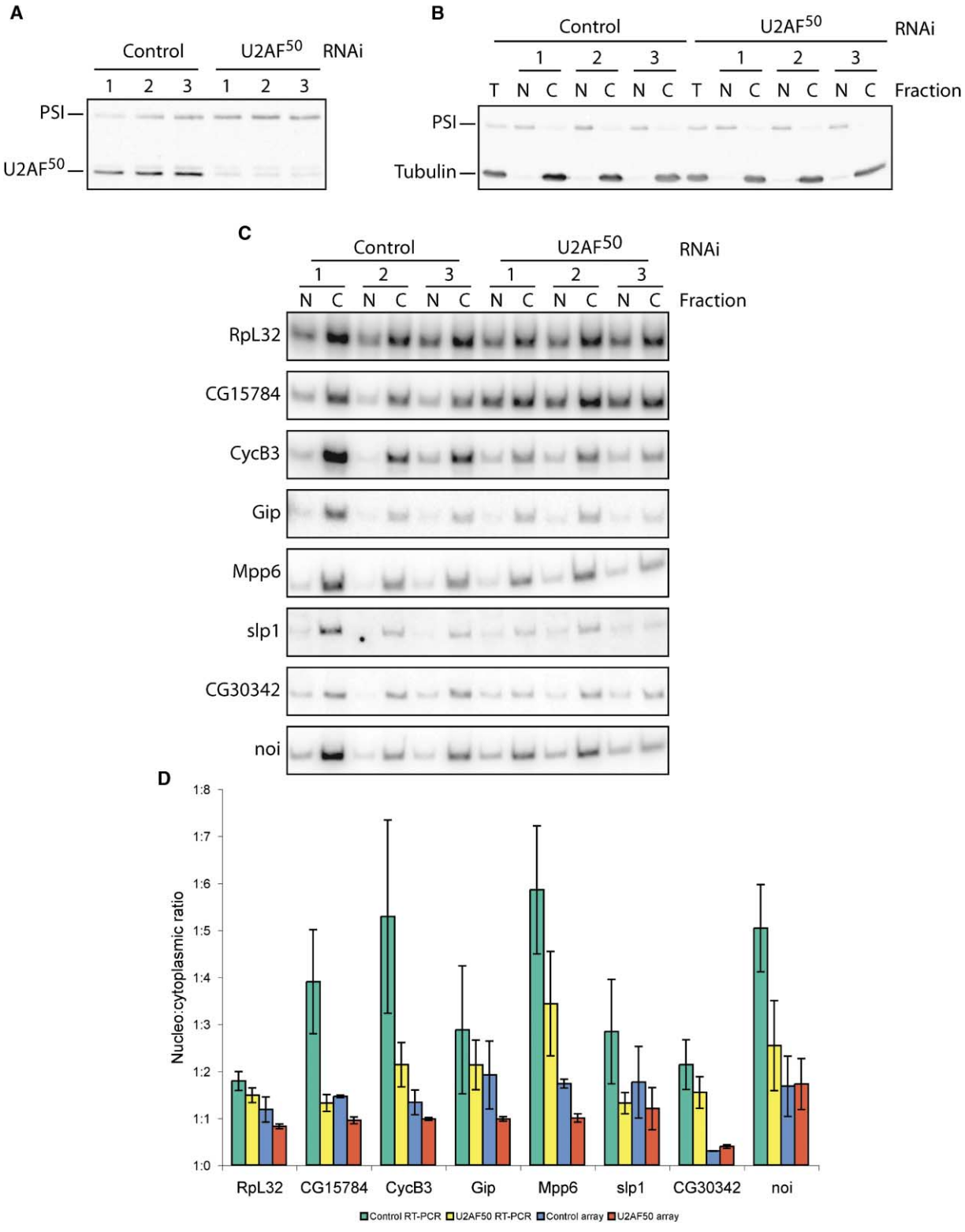
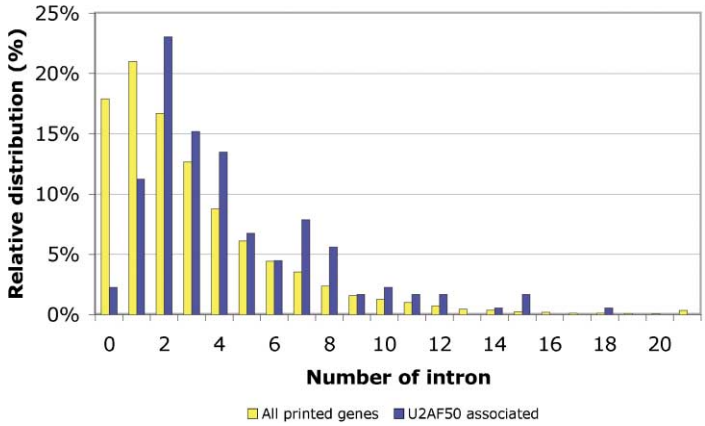


Figure 5. Export of Intronless mRNAs Is Compromised in dU2AF^{50} RNAi Knocked-Down Cells

(A) RNAi in SL2 cells efficiently knocks down dU2AF^{50} expression. Immunoblot of control or dU2AF^{50} RNAi knocked-down cells detected with an antibody against dU2AF^{50} and, as a control, against PSI.

(B) Nuclear-cytoplasmic fractionation of *Drosophila* SL2 cells. Control or dU2AF^{50} RNAi knocked-down SL2 cells were lysed in hypotonic buffer (see Experimental Procedures). Protein and RNA were isolated from the total lysate (T) or from the fractionated nuclei (N) and cytoplasm (C). The fractionation was verified by immunoblot of the isolated protein sample using PSI and α-tubulin as a nuclear and cytoplasmic marker, respectively.

A



B

CG ID	Symbol	Name
CG30342	CG30342	---
CG2925	noi	noisette
CG16738	Slp1	sloppy paired I
CG9653	brk	brinker

Figure 6. Intronless mRNAs Are Found in Immunoaffinity-Purified Nuclear RNP Complexes Containing dU2AF50 and Contain Putative U2AF Binding Sites

(A) High-throughput identification of RNAs associated with dU2AF50 in nuclear RNP complexes. Zero to twelve hour *Drosophila* RNP preparations were used to specifically immunoaffinity purify RNA-protein complexes (RNPs) containing dU2AF50. The isolated RNAs were identified by hybridization to a *Drosophila* cDNA microarray. The distribution of the average number of introns per gene in the first 200 dU2AF50-associated genes (blue) and for all genes in the annotated fly genome (yellow) is shown.

(B) In the first 200 dU2AF50-associated genes, four were intronless.

were immunoaffinity purified together with dU2AF50 (see Supplemental Figure S1A). Moreover, three of them (CG30342, Slp1, and noi) are expressed in L2 cells and show nuclear retention when the expression of dU2AF50 is knocked down (Figures 5C and 5D).

A statistical model of all known U2AF binding sites (3′ splice sites) was generated and used to search for binding sites in intronless genes (see Supplemental Figure S2). This approach found that more than one third of all intronless mRNAs contain at least one site that matches this U2AF model as well as the average 3′ splice site. However, no enrichment for strong U2AF binding sites was observed in the set of intronless genes as a whole. The four intronless mRNAs found by microarray analysis of affinity-selected dU2AF50-containing RNP complexes all contain putative U2AF binding sites that match the model as well as the average splice site.

The binding assay supports the notion that dU2AF50 can associate with intronless RNAs, and the bioinformatic analyses is consistent with this observation. Together this suggests that dU2AF50 participates in the export of a large number of intronless genes. While only a small fraction of the top mRNAs found in stable U2AF-containing nuclear RNP particles were intronless, the vast majority of intronless mRNAs are predicted to contain putative U2AF binding sites. Thus, even transient association of U2AF with these transcripts for the recruitment of RNA export factors could account for the effects of dU2AF50 mutations or depletion of the nucleo-cytoplasmic transport of intronless mRNAs.

Discussion

In this study, we have characterized two *Drosophila* dU2AF50 temperature-sensitive alleles and have shown that they directly impair U2AF RNA binding to an intron polypyrimidine tract. In addition, the mutations reduced the splicing efficiency of some target genes in vivo. Surprisingly, although U2AF is a known splicing factor, a large number of intronless genes were found to be downregulated in the mutant flies at the restrictive temperature. By looking at the nucleo-cytoplasmic distribution of all expressed genes, we found that reducing dU2AF50 expression had a dramatic and widespread effect on the nucleo-cytoplasmic mRNA localization irrespective of the intron number of the affected genes. Finally, some intronless RNAs were found to be associated with dU2AF50 in nuclear RNP complexes. Thus, in addition to the well-known role of U2AF in defining 3′ splice sites in pre-mRNAs, the results presented here reveal an unexpected function for dU2AF50 in nuclear export of intronless mRNAs.

As previously reported, the large U2AF subunit is highly conserved from *S. pombe* to humans (Kanaar et al., 1993; Potashkin et al., 1993; Zamore et al., 1992; Zorio et al., 1997) and has been shown to be highly refractory to mutations (Romfo et al., 1999). Here, we have shown that two mutations identified in *S. pombe* generate new temperature-sensitive dU2AF50 alleles in *Drosophila*. Both mutations, D204N and S284Y, lie on each side of the second RNA binding domain (RRM2)

(C) The distribution of specific mRNAs was determined by RT-PCR from RNA isolated from cytoplasmic and nuclear fractions of control and dU2AF50 RNAi knocked-down cells.

(D) Quantification of the nuclear:cytoplasmic distribution observed by RT-PCR (control, green bar; dU2AF50 RNAi, yellow bars) or by the microarray experiment (control, blue bar; dU2AF50 RNAi, red bars). The error bar is the calculated standard deviation of the average signal ($n = 3$).

without being part of it. The structures of several RRM-RNA complexes have recently been solved and shown to conform to a canonical $\beta1\alpha1\beta2\beta3\alpha2\beta4$ fold (Handa et al., 1999; Oubridge et al., 1994; Price et al., 1998) In the structure of the human RRM2, asparatic acid 204 is located four amino acids upstream of the first β sheet ($\beta1$) of RRM2, which is predicted to be part of the RNA interaction platform. Although the D204 residue was not in the structure of RRM2, it is conceivable that asparatic acid 204 is involved in a salt bridge and in absence of this putative salt bridge, the RNA-RRM2 interaction might be less stable leading to the observed temperature-sensitive reduction in RNA binding. The dU2AF^{50} serine 284 residue is conserved in mouse and human U2AF^{65} and is substituted by a cysteine in *S. pombe* and *C. elegans* (Figure 1A). Interestingly, in the U1A:RNA structure, the C-terminal region next to $\beta4$ contacts the RNA (Oubridge et al., 1994), and, similarly, the same region in U2AF^{65} also appears to contact RNA (Ito et al., 1999). Thus, changing a serine for a bulky aromatic tyrosine may cause steric effects that might result in reduced RNA affinity.

Surprisingly, although the temperature-sensitive mutations dramatically affect dU2AF^{50} RNA binding affinity, in vitro splicing of model substrates was not affected. This probably reflects the highly cooperative nature of spliceosome assembly. Reduction in U2AF RNA binding affinity might be compensated for by interaction with other spliceosomal factors. For instance, interaction of the small U2AF subunit with the 3′ splice site together with interaction of the large U2AF subunit with SF1/BBP, which binds to the branchpoint sequence, could be involved in stabilizing U2AF binding to the polypyrimidine tract (Berglund et al., 1997).

The most striking observation we have found is that a very high proportion of intronless genes are downregulated in the dU2AF^{50} mutant flies grown at the restrictive temperature. Although this observation could be interpreted as an indirect effect, the fact that this enrichment for intronless RNAs in the dU2AF^{50} mutants is very different from the average genomic intron distribution suggests a direct role for dU2AF^{50} in the expression of intronless genes. Moreover, the observation that RNAi knockdown of dU2AF^{50} expression results in the nuclear accumulation of a large number of intronless mRNAs, that dU2AF^{50} is found to be associated with intronless mRNAs in purified nuclear RNP complexes, and that the vast majority of intronless genes possess putative U2AF binding sites (R.E.G. et al., unpublished data) support a direct role for dU2AF^{50} in the nuclear export of intronless mRNAs. In mammals, U2AF has been shown to directly interact with the protein factor UAP56, a putative DEAD box RNA helicase essential for splicing (Fleckner et al., 1997), and the essential transport receptor TAP/NXF1 (Zolotukhin et al., 2002). It has been proposed that UAP56 is recruited to the spliceosome through an interaction with RRM1 of U2AF^{65} (Fleckner et al., 1997). In *Drosophila* and yeast, UAP56 is an essential export factor that functions to bridge the mRNA to the export machinery (Gatfield et al., 2001; Herold et al., 2003; Jensen et al., 2001). Interestingly, UAP56 was shown to be required not only for export of spliced mRNAs but also for export of intronless mRNAs (Gatfield et al., 2001; Jensen et al., 2001; Strasser and Hurt, 2001; Strasser et al., 2002). One attractive possibility is that U2AF, as with intron-containing genes, is involved in the recruitment of UAP56, or other members of the RNA export machinery, for instance, TAP/NXF1, to intronless mRNAs prior to their nuclear export.

Recently, a subset of the SR family of splicing factors has been shown to be involved in the export of a class of intronless mRNAs (Huang et al., 2003; Huang and Steitz, 2001). The SR proteins 9G8, SRp20, and SF2/ASF are proteins that shuttle between the nucleus and cytoplasm (Cáceres et al., 1998) and serve as adaptors between the intronless histone mRNA and the export factor TAP (Huang et al., 2003; Huang and Steitz, 2001). Interestingly, mammalian U2AF has been shown to continuously shuttle between the nucleus and cytoplasm (Gama-Carvalho et al., 2001; Zolotukhin et al., 2002), and although there is no known function for U2AF in the cytoplasm, our results are suggestive of a general and direct role of U2AF in the export of intronless, as well as intron-containing mRNAs.

We have also shown that the genes that are upregulated upon growth of the mutant dU2AF^{50} strain at the restrictive temperature generally contain multiple introns. In addition, their pre-mRNAs are generally longer than the average genomic pre-mRNA length (data not shown). Intriguingly, no splicing defect was found for any of several individual upregulated genes tested. One possible explanation for this observation could reside in the recent report that the splicing machinery can stimulate the transcription apparatus (Fong and Zhou, 2001). One might envision that mRNA maturation in the mutant flies might be slower on long pre-mRNAs or on pre-mRNAs containing multiple introns because of reduced dU2AF^{50} RNA binding affinity. This could increase the time during which partially spliced mRNA-containing snRNPs or partially assembled spliceosomes would colocalize on nascent transcripts with the transcriptional machinery. Those spliceosomal components could feed back on the transcription machinery releasing potentially paused RNA polymerase II complexes. This would lead to an overall increase in transcriptional rate on some genes that were more prone to RNA polymerase II pausing. Although it is not known, we speculate that genes upregulated in the mutant flies might be part of such a class.

Over the past few years it has become evident that what were originally thought to be distinct steps in the gene expression pathway are tightly coupled through an extensive network of interactions between the transcriptional RNA processing and RNA export machineries (Maniatis and Reed, 2002; Manley, 2002). However, most of our knowledge comes from intron-containing genes, and this, in part, accounts for our relatively poor understanding of the mechanisms controlling expression of intronless genes. The observation that the splicing factor dU2AF^{50} can influence nuclear export of intronless genes suggests that common mechanisms and RNA-protein interactions are probably shared between these two classes of genes.

Experimental Procedures

Drosophila Stocks and Complementation Analysis

Point mutations in the dU2AF^{50} cDNA were introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene) following

the manufacturer's protocol (oligonucleotide sequences available upon request), using pDR154 as the starting DNA (Rudner et al., 1998a) to generate pEL1 (DE150-1AA), pEL2 (T160I), pEL3 (D204N), and pEL4 (D204N). The integrity of the constructs was confirmed by DNA sequencing. The mutant dU2AF^{50} cDNAs were subcloned under the control of the dU2AF^{50} genomic promoter, 5′ and 3′ UTRs by transferring the XhoI/BstEII fragment from pEL1, pEL2, pEL3, and pEL4 into pDR141 (Rudner et al., 1998b) to generate pEL5, pEL6, pEL7, and pEL8, respectively. The transformation vectors were generated by transferring NotI fragments from pEL5, pEL6, pEL7, and pEL8 into pw8 to generate pEL9, pEL10, pEL11, and pEL12, respectively. Germline transformations of w^{1118} embryos were carried out using standard microinjection methods.

RNA-Protein Interaction Assay

The mutant heterodimer expression constructs were generated by transferring the XbaI/BamHI fragment from pEL3 and pEL4 into pdr154 (Rudner et al., 1998a) cleaved with XbaI and BamHI to generate pEL29and pEL30, respectively. Expression of the wild-type and mutant D204N and S284Y proteins was performed by transforming pdr154, pEL29, and pEL30, respectively, into *E. coli* HMS174 (DE3), pLys S. Expression, purification, and RNA-protein interaction were as described (Rudner et al., 1998a).

Gene Expression Profiling and RT-PCR

Wild-type and S284Y flies were grown in standard noncrowded conditions at either 25°C or 30°C for 4 days. Total RNA from five males and five females was extracted with Trizol (Invitrogen) in triplicate and repurified using the mini-RNAeasy kit (Qiagen) following the manufacturer's protocol. The RNAs were then treated following the Affymetrix labeling protocol and individually hybridized to *Drosophila* GeneChip arrays (Affymetrix). The scanned arrays were analyzed using the MAS v. 5.0.1 statistical algorithm, and the data were mined using FileMaker Pro 6.0 v. 4 and the *Drosophila* genome annotation v. 3.1. Specific RT-PCR reactions were performed using dT-primed cDNA reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's protocol and amplified with Taq polymerase in standard PCR conditions (1× New England Biolab thermo pol buffer, 200 μM each dNTP, 0.3 μM forward and reverse oligonucleotides, 100 ng of cDNA from total RNA, 5 μCi α^{32}P-dCTP, 2.5 U Taq/reaction). The amplified products were resolved on a 5% native polyacrylamide (19:1 acrylamide:bis-acrylamide) in 1× TBE buffer. The analysis of intensity was performed on unsaturated phosphor imager exposures using ImageQuant software.

Identification of U2AF Nuclear RNP-Associated RNAs

U2AF-interacting RNAs were identified as previously described (Labourier et al., 2002) with four independent immunoaffinity-purified RNA samples. Raw data from individual microarrays were filtered with the following parameters: signal at 635 or 532 nm, >2500 quanta, signal/background ratio >3, and spot diameter >100 μm. The calculated 635 nm/532 nm ratios (log2[Cy5/Cy3]) were normalized with a mean of 0 and a variance of 1 (N[0;1]) to allow comparison between experiments. Only the spots that were reproducibly detected in at least three out of four independent experiments were selected for analysis.

Nucleo-Cytoplasmic Fractionation of RNAi Knocked-Down Cells

Four hundred nucleotide-long PCR fragments containing a T7 promoter at each end were amplified from a vector containing the dU2AF^{50} open reading frame or, as a control, from empty pBluescript KS II vector. Production of double-stranded RNA and RNAi were as described by Clemens et al. (2000). After 4 days, cytoplasm and nuclei were isolated as described by Herold et al. (2003). The efficiency of the fractionation was verified by immunoblot of the different fractions using affinity-purified anti-PSI (Siebel et al., 1995) and a mouse monoclonal anti-α-tubulin (Sigma-Aldrich). The cytoplasmic and nuclear RNA were hybridized in triplicates on Affymetrix *Drosophila* genome array following the manufacturer's protocol and analyzed using MAS v. 5.0.1. The retention index was calculated using the $\log_2$[average RNAi(nuclear/cytoplasmic)] − $\log_2$[average control(nuclear/cytoplasmic)].

Supplemental Data can be downloaded from http://www.riolab.net/SupplementalData.pdf.

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Accession Numbers

The microarrays data are accessible through http://www.ncbi.nlm.nih.gov/geo/ with accession numbers GSE1342, GSE1344, and GSE1345.