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A protein related to splicing factor U2AF^{35} that interacts with U2AF^{65} and SR proteins in splicing of pre-mRNA

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Recognition of a functional 3′ splice site in pre-mRNA splicing requires a heterodimer of the proteins U2AF^{65}/U2AF^{35}. U2AF^{65} binds to RNA at the polypyrimidine tract[1,2], whereas U2AF^{35} is thought to interact through its arginine/serine-rich (RS) domain with other RS-domain-containing factors bound at the 5′ splice site, assembled in splicing enhancer complexes, or associated with the U4/U6.U5 small nuclear ribonucleoprotein complex[3–7]. It is unclear, however, how such network interactions can all be established through the small RS domain in U2AF^{35}. Here we describe the function of a U2AF^{35}-related protein (Urp), which is the human homologue of a mouse imprinted gene. Nuclear extracts depleted of Urp are defective in splicing, but activity can be restored by addition of recombinant Urp. U2AF^{35} could not replace Urp in complementation, indicating that their functions do not overlap. Co-immunodepletion showed that Urp is associated with the U2AF^{65}/U2AF^{35} heterodimer. Binding studies revealed that Urp specifically interacts with U2AF^{65} through a U2AF^{35}-homologous region and with SR proteins (a large family of RS-domain-containing proteins) through its RS domain. Therefore, Urp and U2AF^{35} may independently position RS-domain-containing factors within spliceosomes.

We previously purified and cloned a mammalian serine kinase, SRPK1, which is highly specific for the RS-rich superfamily of splicing factors[8–10]. This stringent substrate specificity enabled us to do a yeast two-hybrid screen to search for new RS-domain-containing proteins. We isolated a positive clone from a HeLa complementary DNA library that contains a typical RS domain at the carboxy terminus (Fig. 1a). The recombinant protein was efficiently phosphorylated in the RS domain, like other RS-domain proteins, by SRPK1 *in vitro* (data not shown). This protein of 483 amino acids does not contain any RNA-recognition motif characteristic of the SR family of splicing factors[11,12]; instead, it contains two stretches of sequences, H1 and H2 (boxed in Fig. 1b), homologous to the human splicing factor U2AF^{35}. We therefore named it Urp, for U2AF^{35}-related protein. Northern blotting analysis indicates that both U2AF^{35} and Urp are ubiquitously expressed in all human tissues and cell lines examined (data not shown). Homology searches revealed that Urp had been independently cloned from humans as U2AF1-RS2 (ref. 13), which is nearly identical (94% amino-acid identity) to another human gene, U2AF1-RS1 (ref. 13) (Fig. 1b). These two human genes are equally homologous (~80% identical) to two mouse genes[14], one of which is imprinted[15], and the other has a high transmission distortion in interspecific backcross progeny[16]. Thus, several U2AF^{35}-related proteins are expressed in mammalian cells and may be members of the superfamily of RS-domain-containing splicing factors[11].

To determine the function of Urp, we raised rabbit polyclonal antibodies against recombinant Urp expressed in bacteria. Immunoblotting of HeLa cell nuclear extracts demonstrated that anti-Urp antibodies specifically detect a single band of relative molecular mass 66K (Fig. 2a), which corresponds to endogeneous Urp as it matches the size of Urp translated in reticulocyte lysate (data not shown). To see whether Urp is essential for splicing, we constructed affinity columns with anti-Urp antibodies or preimmune serum. Control depletion with preimmune serum had no effect, but passing nuclear extract through an anti-Urp affinity column efficiently depleted it of Urp, as evidenced by western blotting and complete inhibition of splicing (data not shown). Although this result is consistent with Urp being a splicing factor, this Urp-depleted extract could not be complemented by recombinant Urp, probably because of co-depletion of other essential splicing factors (see below). We therefore did the immunodepletion in the presence of high salt (0.5 M KCl) to minimize co-depletion. As shown in Fig. 2c, human β-globin pre-mRNA splicing in Urp-depleted nuclear extract was significantly decreased (compare lanes 1 and 2). The second step of the splicing reaction, which generates released lariat intermediate and

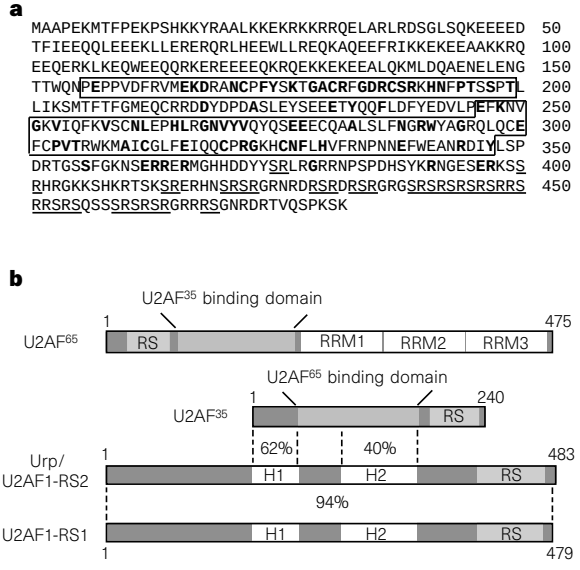

Figure 1 Urp structure and sequence comparison with U2AF^{35}. **a**, Amino-acid sequence of Urp. It is identical to U2AF1-RS2 (GenBank D49677; ref. 13). The two U2AF^{35} homologous domains, H1 and H2, are boxed and amino-acid residues identical to those in U2AF^{35} are shown in bold. Repeats of SR or RS dipeptides are underlined. **b**, Domain structure of the large and small subunits of U2AF and comparison of human U2AF^{35}, Urp/U2AF1-RS2 and U2AF1-RS1. Percentage identity between homologous domains is indicated.

ligated exons, was preferentially affected by Urp depletion under these conditions, indicating that Urp may play a role in this step; however, we could not rule out the possibility that it was required for the first step as we did not detect any accumulation of first-step splicing intermediates. To confirm the essential function of Urp in splicing, we restored Urp activity to a Urp-depleted nuclear extract with a glutathione-*S*-transferase (GST)–Urp fusion protein produced in baculovirus. Because Urp was insoluble in baculovirus-infected cells, we purified recombinant Urp from inclusion bodies, then isolated the protein by SDS–PAGE (Fig. 2b). After denaturation and renaturation in the presence of bovine serum albumin, recombinant Urp restored splicing activity to the Urp-depleted nuclear extract in a concentration-dependent manner (Fig. 2c, lanes 4 to 6). There was no complementing activity associated with BSA (Fig. 2c, lane 3). We conclude that Urp is an essential splicing factor.

SR proteins have redundant functions, at least *in vitro* (for review, see refs 11, 12). U2AF^{35} was originally shown to be non-essential for constitutive splicing because U2AF^{65} alone was sufficient to reconstitute a splicing-deficient nuclear extract from which the U2AF heterodimer was removed by poly(U) in the presence of high salt[17]. However, U2AF^{35} is essential for constitutive and enhancer-dependent splicing by immunodepletion and reconstitution experiments[5]. Although the *Drosophila* homologue of U2AF^{35} is required for viability[18], there may be factors that are partially redundant with U2AF^{35}. Urp is such a candidate because it is related to U2AF^{35} in structure and sequence. To test this possibility, we expressed and purified U2AF^{35} in the same way as Urp (Fig. 2b), and found that U2AF^{35} could not complement the Urp-depleted nuclear extract (Fig. 2c, lanes 7 to 9), indicating that Urp and U2AF^{35} are not functionally redundant. Results were identical when the complementation was repeated with a U2AF^{35} protein preparation that had previously been shown to be functional in restoring splicing activity to a U2AF^{35}-depleted extract (ref. 5, and data not shown). Surprisingly, splicing in the Urp-depleted nuclear extract could be stimulated reproducibly by U2AF^{65} (Fig. 2c, lanes 10 to 12). It has been found that U2AF^{65} can stimulate splicing in a U2AF^{35}-deficient nuclear extract, probably as a result of recruiting the remaining U2AF^{35} in the depleted extract[5]. U2AF^{65} may therefore also interact with Urp, recruiting the remaining Urp in the Urp-depleted extract.

To test this, we examined the abundance of various splicing factors in the Urp-depleted nuclear extract by western blotting. As shown in Fig. 3a, Urp was effectively depleted by the anti-Urp affinity column in comparison to Urp present in untreated as well as preimmune serum-treated nuclear extracts. A fraction of U2AF^{65} was co-depleted, consistent with an interaction between U2AF^{65} and Urp. In contrast, the level of U2AF^{35} was only slightly reduced, indicating that a large fraction of U2AF^{35} is not associated with Urp or complexed with U2AF^{65}, in agreement with previous functional studies on U2AF^{35} (ref. 5). The amount of SR proteins detected by monoclonal antibody 104 or of snRNP-associated proteins detected by anti-Sm antibodies was not affected by Urp depletion. The co-immunodepletion of Urp and U2AF^{65} indicates that Urp forms a functional complex with U2AF^{65} *in vivo*. This conclusion does not contradict the observation that U2AF^{65} purifies only as a heterodimer with U2AF^{35}, because 2M guanidine-HCl was used in a

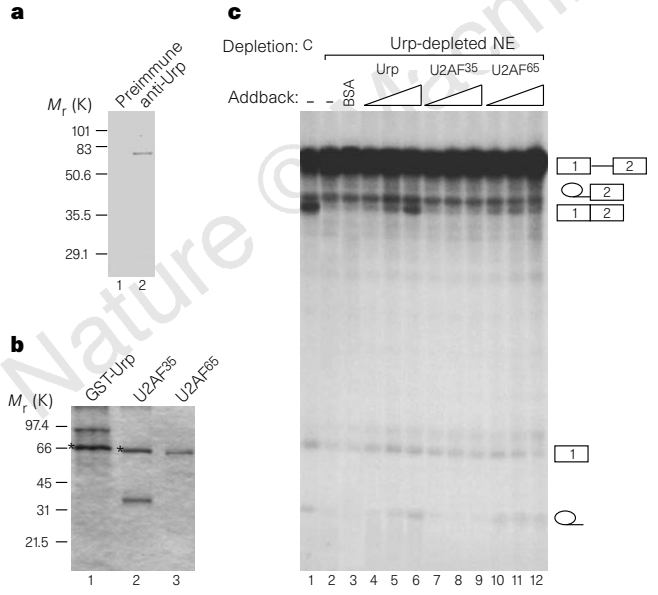

Figure 2 Urp is an essential splicing factor. **a**, Urp was detected in HeLa nuclear extracts (25 μg protein per lane) by anti-Urp antibodies (1 : 3,000) but not by the preimmune serum (1 : 1,000). **b**, Recombinant proteins used for complementation. All proteins were expressed in Sf9 cells. GST–Urp (lane 1) and His–U2AF^{35} (lane 2) were purified by SDS–PAGE. Specific protein concentration was adjusted to 0.1 mg ml^{-1}. BSA (asterisks) was used as a carrier during gel elution and denaturation and renaturation. His–U2AF^{65} (lane 3) was directly purified from sonicated cell lysate on a Ni^{2+} column (Invitrogen). **c**, Splicing of human β-globin pre-mRNA in control depleted (lane 1), or Urp-depleted (lanes 2–12) HeLa cell nuclear extracts (NE). The Urp-depleted nuclear extract was complemented with BSA control (9 μl, lane 2), or with three concentrations (1, 3, 9 μl) of GST–Urp (lanes 4–6), His–U2AF^{35} (lane 7–9), or His–U2AF^{65} (lane 10–12). On the right are the structures of human β-globin splicing substrate, intermediates and products: boxes, exon 1 and exon 2; thin line, intron.

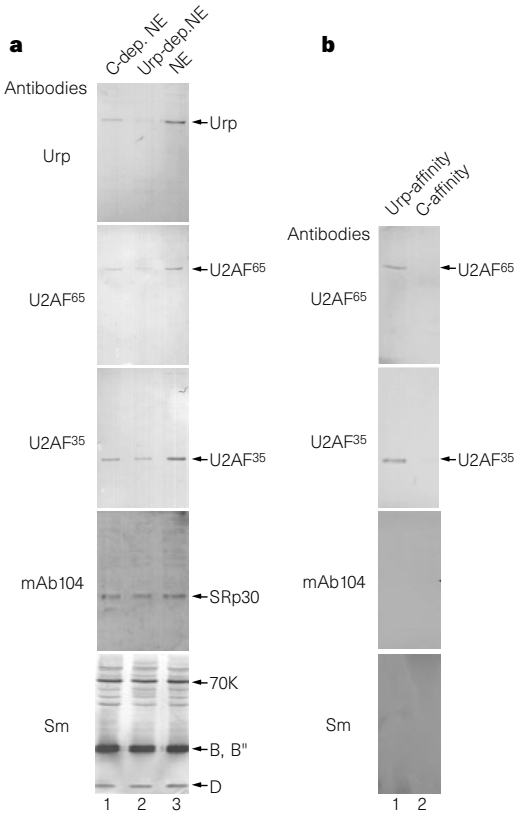

Figure 3 Urp is associated with the U2AF heterodimer. **a**, 2 μl of nuclear extracts that had been depleted with preimmune serum (lane 1), anti-Urp antibodies (lane 2), or untreated (lane 3) were probed by western blotting with anti-Urp antibodies (1 : 3,000), anti-U2AF^{65} (1 : 5,000), anti-U2AF^{35} (1 : 5,000), mAb104 (culture supernatant), or anti-Sm (Y12, culture supernatant), as indicated on the left. Specific proteins detected by these antibodies are indicated on the right. **b**, Anti-Urp and control affinity columns (0.5 ml bed volume) were eluted with 0.5 ml of PBS plus 2M NaCl. 10 μl of eluant from each column was probed with the antibodies indicated on the left; and proteins detected are indicated on the right.

purification step[19]: these extreme conditions were not used in Urp depletion, so a weaker association between Urp and U2AF^{65} was likely to be preserved.

We also tested for any association of Urp with other splicing factors by western blotting analysis of high-salt eluants from the control and anti-Urp affinity columns. As expected, U2AF^{65}, but not SR proteins or Sm antigens, was specifically detected in the eluant of the anti-Urp affinity column (Fig. 3b). U2AF^{35} seemed to be stoichiometrically present in the Urp–U2AF^{65} complex, because similar amounts of U2AF^{65} and U2AF^{35} were detected by specific antibodies of similar titres. This can be explained by the specific association of Urp with the U2AF heterodimer. Alternatively, Urp may be independently associated with equal amounts of U2AF^{65} and U2AF^{35}, but we consider this less likely as only U2AF^{65} binds strongly to Urp *in vitro* and in the yeast two-hybrid system (see below).

We next assayed the binding between Urp and U2AF to investigate the role of Urp in splicing. We expressed GST–U2AF^{35} and GST–U2AF^{65} in bacteria, and carried out a GST-pulldown assay using *in vitro* translated Urp, U2AF^{35} and U2AF^{65} (Fig. 4a). As expected, *in vitro* translated U2AF^{65} bound strongly to GST–U2AF^{35} immobilized on glutathione–Sepharose beads but not to vacant beads, demonstrating the specificity of binding. Under the same conditions (0.4M KCl), both Urp and U2AF^{35} bound efficiently to immobilized U2AF^{65} (Fig. 4a). The interaction between Urp and U2AF^{65} was not mediated by RNA because the extent of binding was similar in the presence of RNase A (data not shown).

Interaction between Urp and U2AF^{65} could be mediated by the RS domain present in both proteins as this domain is a known protein–protein-interaction interface[3,4], or by one or both of the U2AF^{35} homologous domains (H1 and H2). The H2 domain in Urp is a strong candidate because the corresponding region in U2AF^{35} is known to interact with U2AF^{65} (ref. 20). We therefore prepared and tested a series of Urp mutants (Fig. 4b). Several conclusions can be drawn from the data shown in Fig. 4c and quantified in Fig. 4d. First, wild-type Urp binds to U2AF^{65}, but its binding is less efficient than that between U2AF^{65} and U2AF^{35}; the Urp–U2AF^{65} interaction was prevented in the presence of 1 M KCl (data not shown). This explains why Urp is associated with U2AF^{65} only under immunodepletion conditions and not under the more stringent purification conditions[19]. Second, deletion of the RS domain from Urp has no effect on its binding to U2AF^{65}, indicating that the RS domain is not involved in the interaction between the two proteins. Third, although deletion of the H1 domain has no effect, deletion of the H2 domain significantly, but not completely, diminished Urp binding to U2AF^{65}. Thus, U2AF^{35} and Urp seem to interact with U2AF^{65} through homologous sequences. However, the interaction between Urp and U2AF^{65} could not be competed by increasing amounts of unlabelled U2AF^{35} (data not shown), supporting the idea that Urp interacts with the U2AF heterodimer *in vivo*.

To verify the sequence requirements for protein–protein interaction between Urp and U2AF^{65}, and to determine whether Urp, like U2AF^{35}, also interacts with SR proteins through its RS domain, we extended the binding studies to the yeast two-hybrid system. As shown in Fig. 4e, Urp interacts with U2AF^{65} in yeast and deletion of the H2 domain diminishes binding, but deletion of the RS domain in Urp has no effect, consistent with the *in vitro* results. Urp, like U2AF^{35}, interacts with two SR proteins, SC35 and SF2/ASF, but not with the U1-70k protein (data not shown); deletion of the RS domain in Urp reduces its interaction with either SR protein, indicating that the interaction between Urp and SR proteins is mediated by their RS domains (Fig. 4e). Deletion of the H2 domain had no effect on the interaction between Urp and SC35. However, for unknown reasons, the removal of the H2 domains from Urp decreased its interaction with SF2/ASF. We could not detect any significant interaction between U2AF^{35} and SF2/ASF, in contrast to a previous report[3]. Under the same conditions, Urp interacts with SF2/ASF, indicating a preference for SF2/ASF to bind with Urp rather than U2AF^{35} (Fig. 4e).

We have shown that Urp is an essential splicing factor. On the basis of our finding that Urp is associated with the U2AF heterodimer in nuclear extracts and interacts with U2AF^{65} and SR proteins through distinct domains, we propose that Urp may form part of a larger U2AF complex and be engaged in network interactions during spliceosome assembly. Because Urp seems to play a non-redundant function with

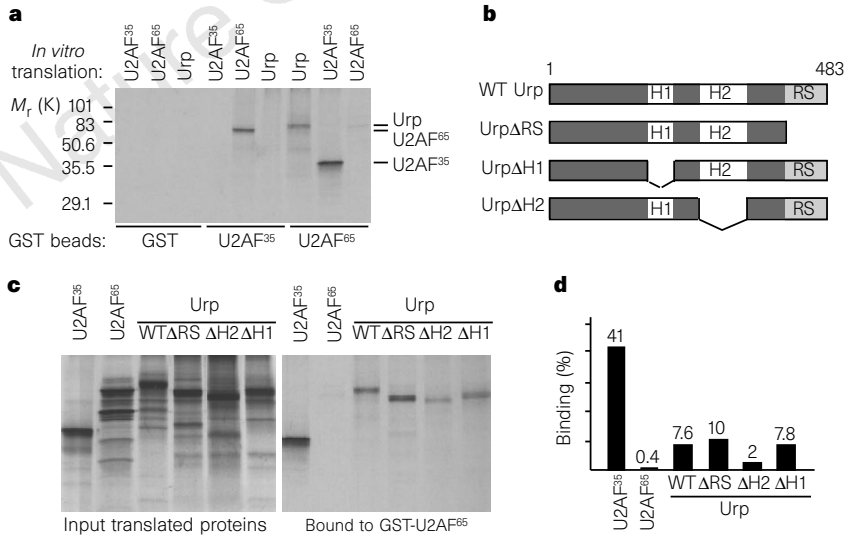

Figure 4 Urp interacts with U2AF^{65} and SR proteins *in vitro* and in yeast. **a**, Identical amounts of *in vitro* translated ^{35}S-labelled Urp, U2AF^{65} and U2AF^{35} (top) were incubated with GST, GST–U2AF^{35} or GST–U2AF^{65} immobilized on glutathione beads (bottom). Bound proteins are indicated on the right. On the left are low-range prestained M_r markers (Bio-Rad). **b**, Organization of wild-type (WT) Urp and three deletion mutants. **c**, Binding of wild-type and mutant Urp to immobilized GST-U2AF^{65}. **d**, Quantification of binding efficiency. **e**, Pairwise protein–protein interaction in the yeast LexA two-hybrid system. Each number of represents the average of data derived from at least three independent transformations and is expressed as fold activation above the background determined using specific bait and the empty prey vector.

e Protein-protein interaction in the yeast two-hybrid system

bait \ prey	U2AF^{35}	Urp	UrpΔRS	UrpΔH2	U1-70K
U2AF^{65}	68.0	43.0	33.6	2.4	1.1
SC35	6.5	12.8	2.4	13.7	24.1
ASF/SF2	1.0	9.2	0.7	3.0	17.5

U2AF[35] and appears preferentially to affect the second step of the splicing reaction, it may interact with a selective set of factors during reaction. The unique behaviour of Urp homologues in mouse indicates that Urp and its family members may be targets for regulation. □

Methods

Cloning, expression and purification. SRPK1 was cloned in pGBT9 as bait to screen a HeLa cDNA library (from G. Hannon) constructed in the pGAD-GH vector as described[21]. The initial Urp clone was lacking 92 amino acids from its N terminus, and full-length Urp cDNA was isolated from a λgt11 HeLa S3 cDNA library (Clontech).

Urp was expressed as GST fusion proteins either in bacteria or by baculovirus. Thrombin-cleaved bacterial Urp was used to raise rabbit antibodies, diluted 1 : 3,000 for western blotting. For splicing complementation, baculovirus-expressed Urp and U2AF were purified from the inclusion bodies of Sf9 cells, followed by isolation from SDS–PAGE. Briefly, 10^8 infected Sf9 cells were sonicated in 10 ml buffer A (20 mM Tris, pH 7.6, 150 mM KCl, 2 mM EDTA, 1 mM DTT) plus standard protease inhibitors. Samples were centrifuged through a 15-ml sucrose cushion (40% sucrose in buffer A) at 12,000 r.p.m. for 30 min in a HB-4 rotor. Pellets were resuspended in 1 ml buffer A plus 8 M urea. An estimated 10 μg of specific protein was loaded per well onto a 10% SDS–polyacrylamide gel. Proteins were eluted from gel slices in buffer B (50 mM Tris, pH 7.5, 0.1% SDS, 0.1 mg ml^{-1} BSA, 1 mM DTT, 0.2 mM EDTA, 0.1 mM PMSF and 2.5% glycerol) overnight at 4 °C. Eluted proteins were precipitated with four volumes of cold acetone overnight at −20 °C. After a 15-min spin, the pellet was washed with cold methanol, dried and resuspended in 2.5 μl 8 M urea. Protein was renatured by adding 125 μl buffer C (20 mM Tris, pH 7.5, 10 mM KCl, 1 mM DTT, 20 mM PMSF and 0.2 mM EDTA), incubating at 4 °C for 24 h, and dialysing for 4 h against 20 mM Tris, pH 7.9, plus 1 mM DTT. Samples were normalized to contain 0.1 mg ml^{-1} protein, aliquoted, and stored at −80 °C.

Immunodepletion and reconstitution. Protein A–Sepharose CL-4B beads (0.5 ml; Pharmacia) were washed with PBS and mixed with an equal volume of anti-Urp serum or preimmune serum. After binding overnight, antibodies were crosslinked to protein A according to ref. 22. Antibody-bound beads were washed with 10 ml 0.1 M glycine (pH 2.5) then 2 × 10 ml buffer D (20 mM HEPES pH 7.6, 100 mM KCl, 1 mM DTT, 10% glycerol). HeLa cell nuclear extracts were used for immunodepletion as previously described[5]. To minimize co-depletion of other essential splicing factors, high-salt conditions were used: 1 ml nuclear extract was adjusted to 0.5 M KCl, then passed through a 0.5-ml (packed volume) antibody-affinity column 5 times at 4 °C. The depletion procedure was repeated once on a fresh 0.5-ml affinity column. Depleted extracts were dialysed against buffer D for 4 h at 4 °C, aliquoted, and stored at −80 °C. Antibody-affinity columns were regenerated by washing with 0.1 M glycine (pH 2.5) and stored in buffer D. Standard splicing reactions were carried out as described[5].

***In vitro* and *in vivo* binding.** $U2AF^{65}$ and $U2AF^{35}$ cDNAs in pSP64 and in pGEX were provided by J. Fleckner and M. Green; anti-$U2AF^{65}$ and $U2AF^{35}$ antisera were gifts from P. Zuo and T. Maniatis, and were used at 1 : 5,000 dilution. Wild-type and mutant Urp cDNAs generated by PCR were inserted downstream of the T7 promoter in pcDNA3 (Invitrogen). *In vitro*-translated proteins were prepared using a TNT kit (Promega) in the presence of [^{35}S]methionine. ^{35}S-labelled proteins were incubated with 1 μg GST–$U2AF^{65}$ or GST–$U2AF^{35}$ immobilized on 5 μl glutathione beads (Pharmacia) in binding buffer (20 mM HEPES, pH 7.6, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Tween, 10% glycerol, 1 mM DTT, 0.4 M KCl) plus 5% BSA at 4 °C for 1 h. After beads were washed 4 times in binding buffer, proteins were resolved on SDS–PAGE, visualized by autoradiography, and quantified on a Phosphoimager.

Two-hybrid pairwise interactions were carried out using the LexA system[23] in the host yeast strain EGY48. Bait plasmids contained individual cDNAs in frame downstream of the LexA gene in pEG202, and prey plasmids had individual cDNAs in pJG4-5. Several bait and prey plasmids ($U2AF^{35}$ and SF2/ASF) were gifts from J. Wu[3]. EGY48 cells were co-transformed by the lithium acetate method and transformants were selected on glucose/CM-ura-his-trp plates. Six randomly picked colonies were restreaked on Gal/Raff/CM-ura-his-trp plates containing X-gal for colour development. Interactions were quantified by liquid β-galactosidase assay[23].

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correction

Expression of *Radical fringe* in limb-bud ectoderm regulates apical ectodermal ridge formation

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