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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