

immunoprecipitated with anti-flag beads, eluted with flag peptide and analysed by western blot with anti-flag (total LRP6) or HRP-streptavidin (cell surface LRP6).

Luciferase reporter and BRET assays

Luciferase Wnt reporter assays in 293T cells were done in 96-well plates at least in triplicate as described¹⁹. Luciferase activity was normalized against Renilla activity. For BRET assays, 293T cells in 24-well plate were transfected with 200 ng pCS-LRP6-RL and pCS-mkrm-YFP constructs with or without co-transfection of 100 ng *dkks*. 24 hours post-transfection, cells were collected in Hank's buffer (with Ca²⁺ and Mg²⁺) and transferred in 96-well luminometer plates. Coelenterazine h (Molecular Probe) was added to a final concentration of 5 μM, and light emission (E) was measured at 470 nm and 530 nm sequentially using an Ascent FL luminometer (Labsystems). Data are represented as BRET ratio, which is defined as (E₅₃₀/E₄₇₀)_{sample} - (E₅₃₀/E₄₇₀)_{LRP6-RL only}. Expression of all constructs was controlled by luminescence or fluorescence. For real time BRET assay, cells were grown and transfected with 50 ng pCS-LRP6-RL and 20 ng pCS-mkrm1-YFP directly in 96-well luminometer plates, and cell growth and transfection were controlled in parallel in a regular 96-well plate. 30 hours post-transfection, medium was removed and Coelenterazine h (10 μM in Hank's buffer) was added and incubated for 1 min before Dkk proteins (5 nM final) were added. Light emission at 470 nm and 530 nm was measured continuously with 1 s integration per data point at 37 °C. Data were collected and processed with Microsoft Excel by averaging ten data points, and are represented as the absolute changes of the BRET ratios from time t = 0.

Drosophila transgenesis

Oregon R flies carrying *UAS-krm2* and *UAS-dkk1* were generated by P-element transformation of P{ry⁺; Δ2-3}99B flies with the pUAST-Vector containing cDNA insert of pCS2-Xdkk1-flag and pCS2-mkrm2-V5, and bred with *sd-GAL4*-P{GAL4}SD^{SG29.1} and *hsp70-GAL4* (Bloomington stock 1799) flies. Protein extracts were prepared from third-instar larvae carrying the *UAS-dkk1* transgene driven by either by *sd-GAL4* or *hsp70-GAL4*. Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE), and Dkk1 protein was detected with anti-15 Dkk1 antibody⁷.

Immunofluorescence microscopy

293T cells on cover slips were transfected with LRP6-GFP (300 ng per well) or LRP6-GFP/mkrm2 (300/100 ng per well). 30 hours after transfection, the cells were incubated with Dkk1-flag conditioned medium for 2 h at 4 °C, washed with cold PBS and changed to fresh medium, then one group was kept at 4 °C as control while the other group was incubated at 37 °C for 30 min. Cells were fixed, permeabilized and proceeded for staining with anti-flag antibody and confocal microscopy.

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Competing interests statement

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New components of the spliced leader RNP required for nematode *trans*-splicing

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Pre-messenger-RNA maturation in nematodes and in several other lower eukaryotic phyla involves spliced leader (SL) addition *trans*-splicing^{1,2}. In this unusual RNA processing reaction, a short common 5' exon, the SL, is affixed to the 5'-most exon of multiple pre-mRNAs. The nematode SL is derived from a *trans*-splicing-specific ~100-nucleotide RNA (SL RNA) that bears striking similarities to the *cis*-spliceosomal U small nuclear RNAs U1, U2, U4 and U5 (refs 3, 4); for example, the SL RNA functions only if it is assembled into an Sm small nuclear ribonucleoprotein (snRNP)⁵. Here we have purified and characterized the SL RNP and show that it contains two proteins (relative molecular masses 175,000 and 30,000 (M_r 175K and 30K)) in addition to core Sm proteins. Immunodepletion and reconstitution with recombinant proteins demonstrates that both proteins are essential for SL *trans*-splicing; however, neither protein is required either for conventional *cis*-splicing or for bimolecular (*trans*-) splicing of fragmented *cis* constructs. The M_r 175K and 30K SL RNP proteins are the first factors identified that are involved uniquely in SL *trans*-splicing. Several lines of evidence indicate that the SL RNP proteins function by participating in a *trans*-splicing specific network of protein-protein interactions analogous to the U1 snRNP-SF1/BBP-U2AF complex that comprises the cross-intron bridge in *cis*-splicing.

Previously, we identified two candidate SL RNP-specific proteins of M_r ~30K and 175K whose association with the *Ascaris* SL RNP was correlated with function in *trans*-splicing⁶. To characterize these proteins further, the SL RNP was purified in amounts sufficient for protein sequencing. This analysis yielded several unique peptides from both the M_r 30K and 175K proteins. Using degenerate oligonucleotides and a variety of polymerase chain reaction (PCR) approaches, we obtained what seemed to be full-length complementary DNA clones encoding both proteins; both cDNAs contained all of the expected peptides and each had a single long open reading frame that began with a methionine codon and ended with multiple stop codons (see Supplementary Information). Other than presumptive orthologues in *Caenorhabditis elegans* (see Supplementary Information), computer searches did not reveal any proteins with significant similarity. Furthermore, neither protein

contained any characterized motif suggestive of function. Although the larger protein had an M_r of 175K, conceptual translation of its corresponding cDNA clone predicted a protein of only ~95K. To ensure that we had indeed obtained full-length clones, synthetic mRNAs corresponding to the cDNA clone were translated in rabbit reticulocyte lysate. The translation product migrated at M_r ~175K (data not shown). We do not yet know why the protein migrates

aberrantly on denaturing gels.

To study the proteins further, polyclonal antisera were raised against bacterially produced fragments of each; M_r 30K-specific antisera recognized a single *Ascaris* protein of appropriate size on Western blots, as did the 175K-specific antisera (see Fig. 1b). To determine whether either or both proteins were associated with one or more RNAs, immunoprecipitation analyses were performed. Strikingly, both antisera precipitated a single RNA, the SL RNA, indicating that we had indeed cloned proteins associated with the SL RNP and that the proteins were SL RNP-specific (Fig. 1a). We then

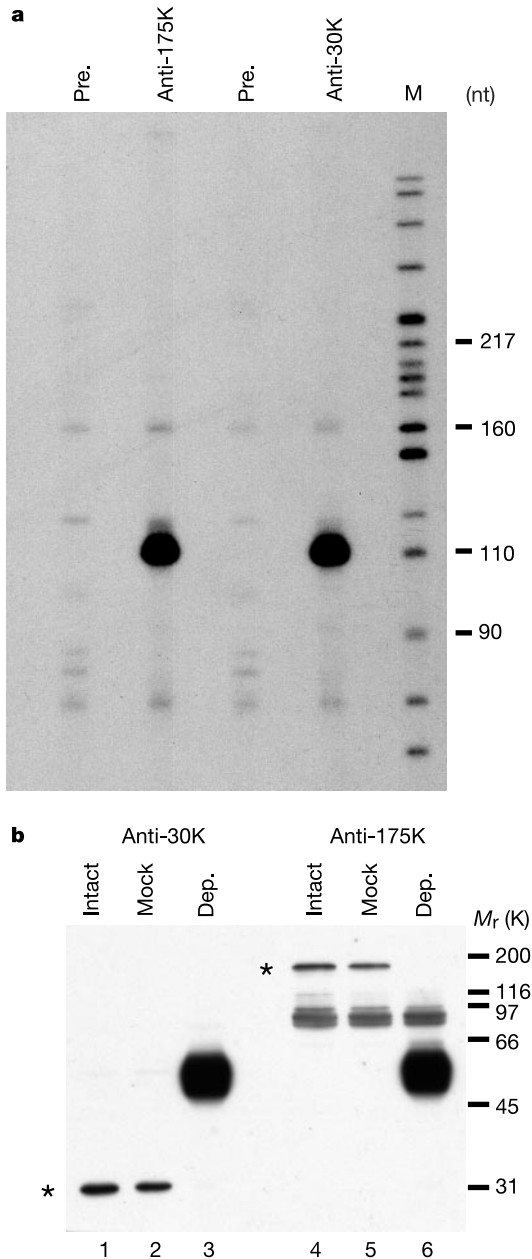


Figure 1 The M_r 30K and 175K proteins are SL RNP-specific. **a**, RNAs present in immunoprecipitates of *Ascaris* whole-cell extract were labelled and fractionated on denaturing gels (see Methods); the antisera used are indicated. The single-labelled RNA present in the anti-175K and anti-30K lanes was identified as SL RNA by digestion with RNase H in the presence of SL-specific oligodeoxynucleotides (data not shown). Pre., preimmune serum; M, DNA markers. **b**, Immunoblot probed with the indicated antisera; lanes 1 and 4, intact extract; lanes 2 and 5, mock-depleted extract; lanes 3 and 6, depleted extract (Dep.) (see Methods). The asterisks denote the 30K and 175K proteins. In lanes 3 and 6, the dark signal arises from residual antibody after immunodepletion. In lanes 4–6, the signal of M_r ~80K is unrelated to the 175K protein and is observed with preimmune sera.

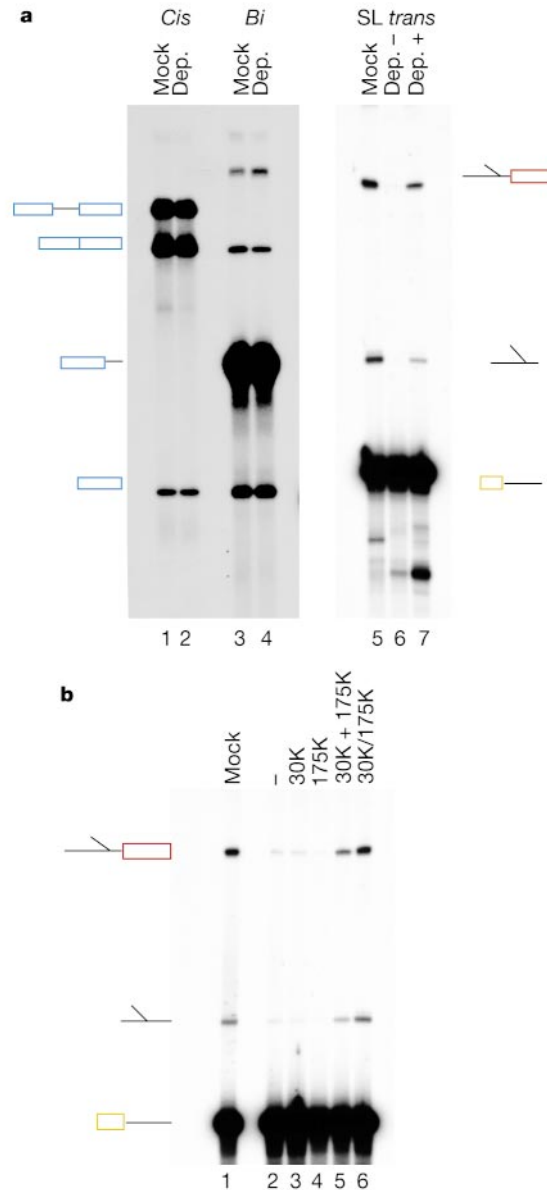


Figure 2 Both SL-RNP-specific proteins are required for *trans*-splicing. **a**, *Cis*, bimolecular (Bi) and SL *trans*-splicing were assayed in mock-depleted extract or extract sequentially depleted of the M_r 30K and 175K proteins (see Methods). In lane 7, the depleted extract was supplemented with 20 ng of preformed 30K/175K heterodimer. The slowly migrating labelled RNA in lanes 3 and 4 is the Y-branched intermediate generated by the first step of bimolecular splicing; other labelled RNAs are indicated schematically. **b**, Splicing reactions performed as in lanes 5–7 in **a**, except in a different depleted extract. Lane 2 contained no additional protein. Lane 3 was supplemented with 20 ng of 30K protein; lane 4 was supplemented with 20 ng of 175K protein; lane 5 was supplemented with 20 ng each of the 30K and 175K proteins; lane 6 was supplemented with 20 ng of preformed 30K/175K heterodimer.

prepared recombinant proteins for functional analyses. An amino-terminal glutathione *S*-transferase (GST)-tagged version of the M_r 30K protein was well expressed in bacteria and could be purified to apparent homogeneity with glutathione–Sepharose (data not shown). We were unable to express full-length M_r 175K protein

in bacteria but obtained reasonable yields of N-terminal His-tagged protein from baculovirus-infected insect cells. Immunoprecipitation assays indicated that both recombinant proteins were functional for SL RNP assembly; as expected from previous studies⁶, binding of the SL RNP-specific proteins depended on prior association of Sm proteins (data not shown). Additional analyses, including reciprocal pulldown and co-immunoprecipitation, showed that the M_r 175K and 30K proteins interact physically, suggesting that the SL-specific proteins bind to the Sm SL RNP as a preformed heterodimer (see below).

Having established that the M_r 175K and 30K proteins were SL RNP-specific, we examined whether they were necessary for SL RNP function in *trans*-splicing. In extracts immunodepleted for the SL-specific proteins, SL *trans*-splicing with either endogenous or, more importantly, exogenously supplied SL RNA was markedly inhibited (data not shown, and Fig. 2a). Remarkably, in the same extracts, *cis*-splicing activity was not affected (Fig. 2a). As a further control for specificity, we examined whether immunodepleted extracts could catalyse the bimolecular (*trans*-) splicing of a '*cis*' construct in which 5' and 3' exons and their accompanying splicing signals were physically separated (for example, refs. 7, 8). Figure 2a (*Bi*) shows that the joining of such separated exons was not affected by immunodepletion. This result provides the first direct evidence that SL *trans*-splicing and the *trans*-splicing of '*cis* constructs in pieces' are mechanistically distinct processes.

To ensure that the inhibition of SL *trans*-splicing observed in immunodepleted extracts was due to the depletion of the SL-specific proteins and not the fortuitous and simultaneous depletion of one or more other factors, reconstitution studies were performed. Supplementation with either the M_r 175K protein or the 30K protein alone did not restore SL *trans*-splicing; however, the addition of both proteins simultaneously or supplementation with preformed 175K/30K heterodimer restored SL *trans*-splicing to levels comparable to those in mock-depleted extracts (Fig. 2a, b). We conclude that both SL-specific proteins are required for SL *trans*-splicing.

Because it seemed possible that the function of the SL-specific proteins was to establish interactions between the SL RNP and other components of the splicing apparatus, we performed yeast two-hybrid analyses to identify potential interacting partners. Clones (1.5×10^6) were screened with the M_r 175K protein as bait and 31 confirmed positives were obtained, all of which corresponded to the 30K protein. When the 30K protein was used as bait, 35 confirmed positives were obtained from 3.0×10^6 clones screened. Of these, 32 corresponded to the 175K protein; intriguingly, the remaining 3 corresponded to the *Ascaris* orthologue of the splicing factor SF1/BBP (Fig. 3a). SF1/BBP orthogonally binds to the branch-point recognition sequence of the pre-mRNA early in spliceosome assembly, and several lines of evidence indicate that this protein is of central importance in the formation of a network of protein–protein interactions that bridges the two ends of *cis* introns (Fig. 3e, *cis*; and see ref. 9). In this regard, in yeast, SF1/BBP can establish simultaneous contacts with a U1 snRNP-specific protein (Prp40p) bound at the 5' splice site and Mud2p (a U2AF-like protein) bound to the 3' splice site⁹. A similar cross-intron bridge is thought to exist in higher eukaryotes; however, a definitive Prp40p homologue has yet to be identified⁹. The same bridge cannot be formed in SL *trans*-splicing because U1 snRNP does not participate in this reaction¹⁰. Indeed, a fundamental unanswered question in SL *trans*-splicing is how the 5' splice site present in the SL RNA associates efficiently with the 3' splice site present in the target pre-mRNA.

The two hybrid results indicated that the interaction of M_r 30K protein with SF1/BBP could mediate such an association. To investigate this possibility we first confirmed the two-hybrid interaction by reciprocal pulldown analyses (data not shown). We then examined whether the interaction could occur in the context of a fully assembled SL RNP. As shown in Fig. 3b, an immobilized

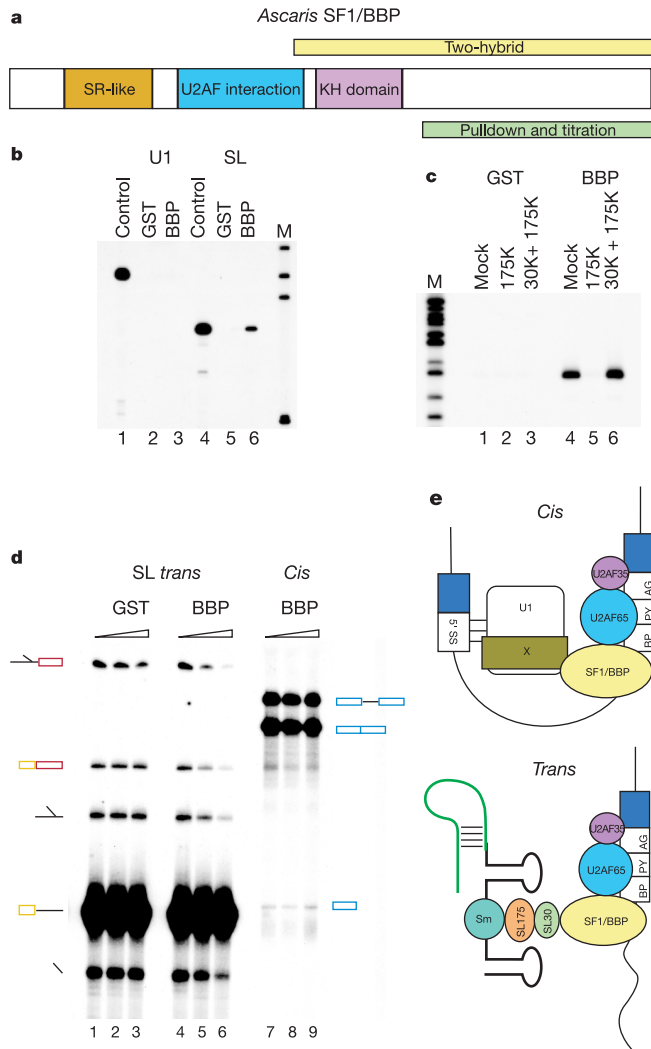


Figure 3 The M_r 30K SL RNP-specific protein interacts functionally with the splicing factor SF1/BBP. **a**, Schematic of *Ascaris* SF1/BBP, which shares 65% identity and 75% similarity with its *C. elegans* homologue; domains are depicted as described previously¹⁷. The portion of the protein recovered by two-hybrid screening is indicated, as is the fragment used for pulldown assays (**b**, **c**) and competition/titration experiments (**d**). **b**, *Ascaris* extract was passed over immobilized GST or GST–BBP (indicated). Bound RNAs were recovered from the reduced glutathione eluate and subjected to primer extension analysis with oligonucleotides complementary to either *Ascaris* U1 small nuclear RNA (lanes 1–3) or SL RNA (lanes 4–6). In lanes 1 and 4, RNA from 1 μ l intact extract was primer-extended. **c**, *Ascaris* extract either mock-depleted (lanes 1 and 4) or depleted of the SL-specific proteins (lanes 2, 3, 5 and 6) (see Methods). The depleted extract was supplemented with either recombinant M_r 175K protein alone (lanes 2 and 4) or both the 175K and 30K proteins (lanes 3 and 6) as in Fig. 2b. Labelled SL RNA was added to each extract and, after assembly, extracts were diluted and passed over columns containing immobilized GST or GST–BBP as indicated. Bound material was recovered from the reduced glutathione eluate and then fractionated on denaturing gels. **d**, SL *trans*-splicing and *cis*-splicing assayed as in Fig. 2b except that the SL RNA was uniformly labelled. Lanes 1–3 were supplemented with 0, 2 and 6 μ g of GST respectively, whereas lanes 4–6 and 7–9 were supplemented with equivalent amounts of GST–BBP. **e**, Schematic representation of *cis* and *trans* splice site bridging complexes. The *cis* model is adapted from ref. 9; for details see the text.

fragment of SF1/BBP specifically selected the SL RNP from unfractionated extract; reconstitution experiments demonstrated that the interaction of the SL RNP with SF1/BBP required the M_r 30K protein (Fig. 3c). We then used a competition approach designed to disrupt the 30K–SF1/BBP interaction in intact extracts. As shown in Fig. 3d, the inclusion of excess polypeptide corresponding to the 30K interaction region of SF1/BBP specifically inhibited SL *trans*-splicing; under these conditions, the polypeptide prevents interaction of the 30K protein with full-length SF1/BBP as assayed by pulldown analysis (data not shown). The same fragment of SF1/BBP had no effect on *cis*-splicing, a result consistent with the fact that this region of SF1/BBP is not required for *cis*-splicing¹¹. Collectively, these analyses indicate that the 30K–SF1/BBP interaction is functionally relevant to SL *trans*-splicing. The striking parallels between our results and those obtained in *cis*-splicing systems strongly suggests that the 30K–SF1/BBP interaction fulfils, in *trans*-splicing, a bridging function analogous to the U1 snRNP–SF1/BBP cross-intron interaction (Fig. 3e).

We have thus identified and characterized two SL RNP intrinsic proteins that are essential for SL *trans*-splicing but not for *cis*-splicing. The apparent absence of similar proteins from human, fly and plant genomes is consistent with the fact that there is no evidence for SL *trans*-splicing in these organisms. It will be of considerable interest to determine whether orthologues of the SL-specific proteins are present in other organisms that do process their mRNAs by *trans*-splicing (for example, *Hydra* and trypanosomes (reviewed in ref. 12)). The presence of such proteins in divergent phyla would indicate that SL *trans*-splicing is an ancestral trait and would thus answer the long-standing debate of whether the process arose once or several times in the course of eukaryotic evolution^{12,13}. Finally, SL *trans*-splicing, although not confined to parasitic organisms, is present in a wide range of medically and economically important parasites¹⁴. The presence of *trans*-splicing-specific factors indicates that this process is a possible target for therapeutic intervention. □

Methods

Extracts, splicing assays and RNA analysis

Ascaris lumbricoides whole-cell embryo extracts were prepared as described¹⁰. Mock depletion or immunodepletion was performed as described¹⁵ with antisera as indicated in figure legends. The *cis* splice substrate used was uniformly labelled with [α -³²P]GTP and corresponded to the wild-type *cis* substrate described in ref. 16. To assay bimolecular splicing, the same *cis* substrate was bisected within the intron; uniformly labelled 5' half-molecules were incubated with unlabelled 3' half-molecules. The 3' exon of the 3' half-molecule contains an exonic splicing enhancer, which promotes 3' splice site recognition in the absence of a 5' splice site, which is a prerequisite for bimolecular splicing¹⁶ (see also refs 7, 8).

To assay SL *trans*-splicing, 3' end-labelled (Fig. 2) or uniformly labelled synthetic SL RNA (Fig. 3) was incubated with the same 3' half-molecule used to assay bimolecular splicing. All splicing reactions were assembled, incubated and analysed as described¹⁶.

To identify RNAs associated with the M_r 30K and/or 175K proteins, immunoprecipitates of whole-cell extract obtained with either anti-175K or anti-30K polyclonal sera were deproteinized. Nucleic acids were recovered and end-labelled with [³²P]pCp as described¹⁶ before electrophoresis on denaturing gels. Digestion by RNase H with oligonucleotides complementary to the SL RNA was performed as described¹⁰.

Recombinant proteins

A full-length cDNA clone encoding the M_r 30K protein was fused in-frame with GST; the fusion protein was expressed in *Escherichia coli* and purified on glutathione–Sephadex. In some experiments (for example, that shown in Fig. 3c), the N-terminal GST tag was removed by cleavage of the fusion protein with thrombin. Similarly, fragments of SF1/BBP were expressed in *E. coli* as N-terminal GST fusion proteins and purified as above. For expression of the M_r 175K protein, full-length cDNA clones were cloned into a baculovirus vector such that the expressed protein contained a His₆ tag at its N terminus. The tagged protein was purified from whole-cell sonicates with Talon (Clontech) beads. To prepare 30K/175K heterodimers, the 175K protein was immobilized on Talon beads and exposed to a 10-fold molar excess of purified 30K protein. After being washed, the heterodimer was eluted with imidazole. Optimal amounts of recombinant proteins used for reconstitution were determined empirically by titration.

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