

Supplementary Information

Cwc2 and its human homolog RBM22 promote an active conformation of the spliceosome catalytic center

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Inventory of Supplementary Information

Supplementary Figures: Fig. S1
Fig. S2
Fig. S3
Fig. S4

Supplementary Methods:

- Chemical modification experiments
- Immunodepletions
- Preparation of purified yeast and human spliceosomal complexes
- Production and characterization of anti-RBM22 antibodies
- *In vitro* splicing reactions
- Northern blotting
- Cwc2 and RBM22 expression and purification
- List of yeast strains used in this work

Supplementary References

Supplementary Figure 1

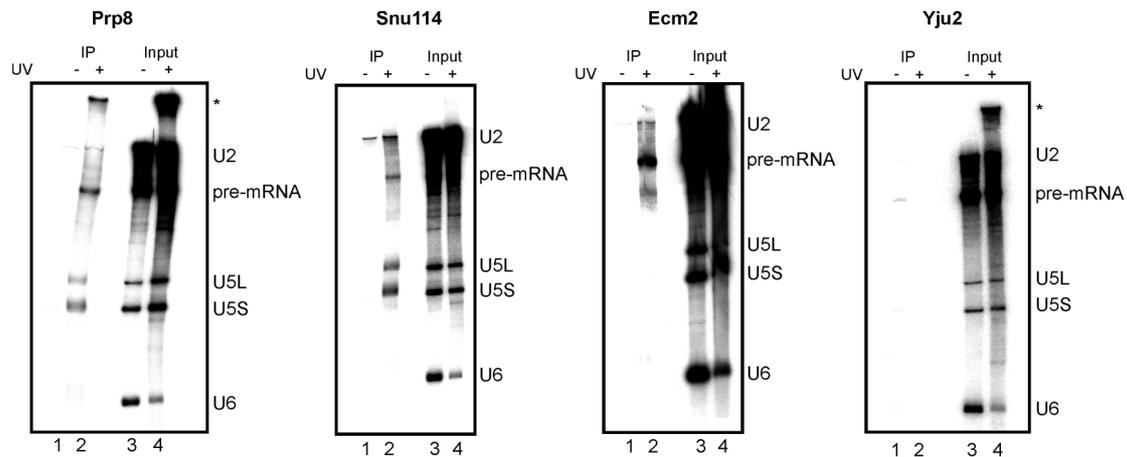


Figure S1. Crosslinking of Prp8, Snu114, Ecm2 and Yju2 to pre-mRNA and snRNAs within affinity-purified activated B^{act} spliceosomes. Northern analysis of the snRNA derived from UV-irradiated B^{act} complexes carrying Prp8 or Snu114 or Ecm2 or Yju2 tagged with the TAP tag (lanes 4) and after immunoprecipitation of denatured B^{act} complexes with IgG Sepharose beads (IP, lanes 2). Lanes 1 and 3 are controls without UV irradiation. RNAs were analyzed on an 8% polyacrylamide gel and visualized by autoradiography. The positions of the snRNAs and M3Act Δ 6 pre-mRNA are indicated on the right. Prp8 crosslinks to U5 snRNA and to pre-mRNA. Snu114 crosslinks to U5 snRNA, to the pre-mRNA and also to U2 snRNA. Ecm2 crosslinks to the pre-mRNA, while Yju2 showed no RNA–protein crosslinks at all in the B^{act} complex. Asterisks: high-molecular-weight crosslinked product.

Supplementary Figure 2

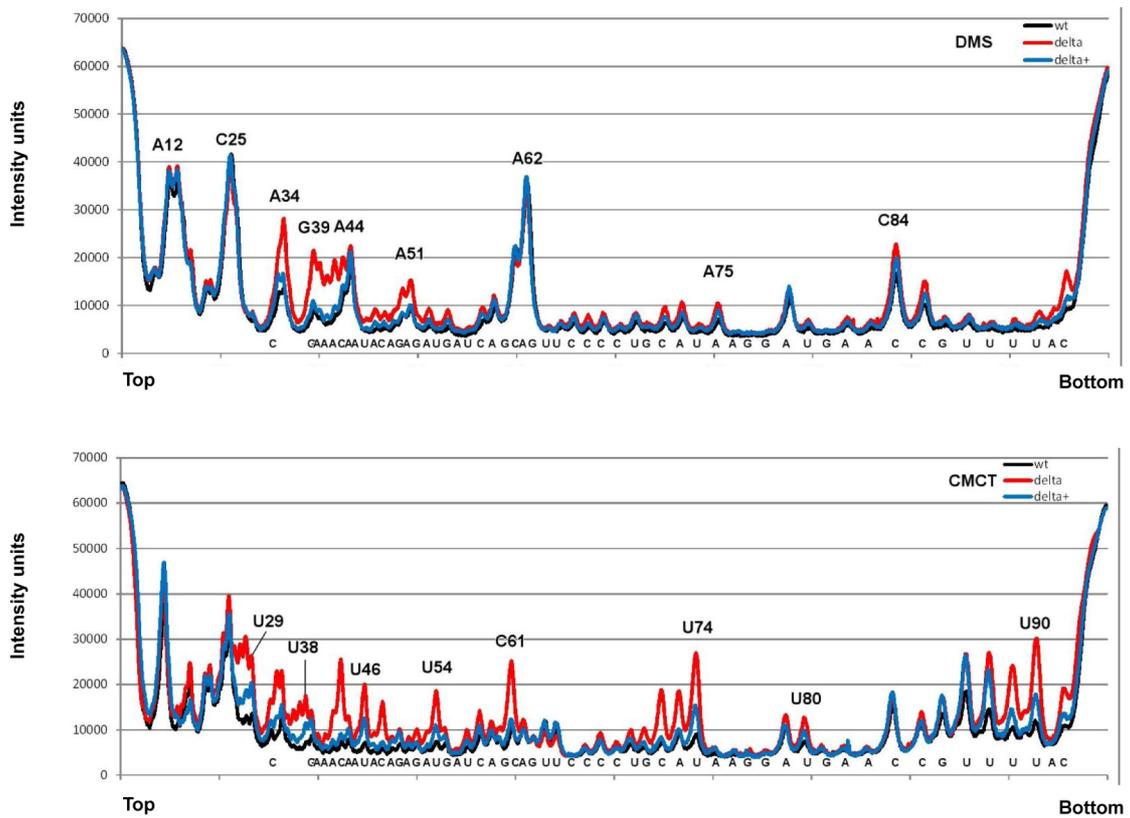


Figure S2. Densitometric quantitative analysis of yeast U6 snRNA nucleotides accessible towards chemical probes. Densitometry from Figure 4, lanes 5, 7 and 9 (DMS) and from lanes 5, 7 and 9 (CMCT). The wildtype lane (wt), before Cwc2 depletion, is shown in black; the Δ Cwc2 lane (delta) is shown in red and the Δ Cwc2 plus addition of recombinant Cwc2 lane (delta +) is shown in blue. A number of the nucleotides of U6 snRNA modified by chemical probes, following depletion of Cwc2, show higher peak values. The identity of nucleotides is indicated.

Supplementary Figure 3

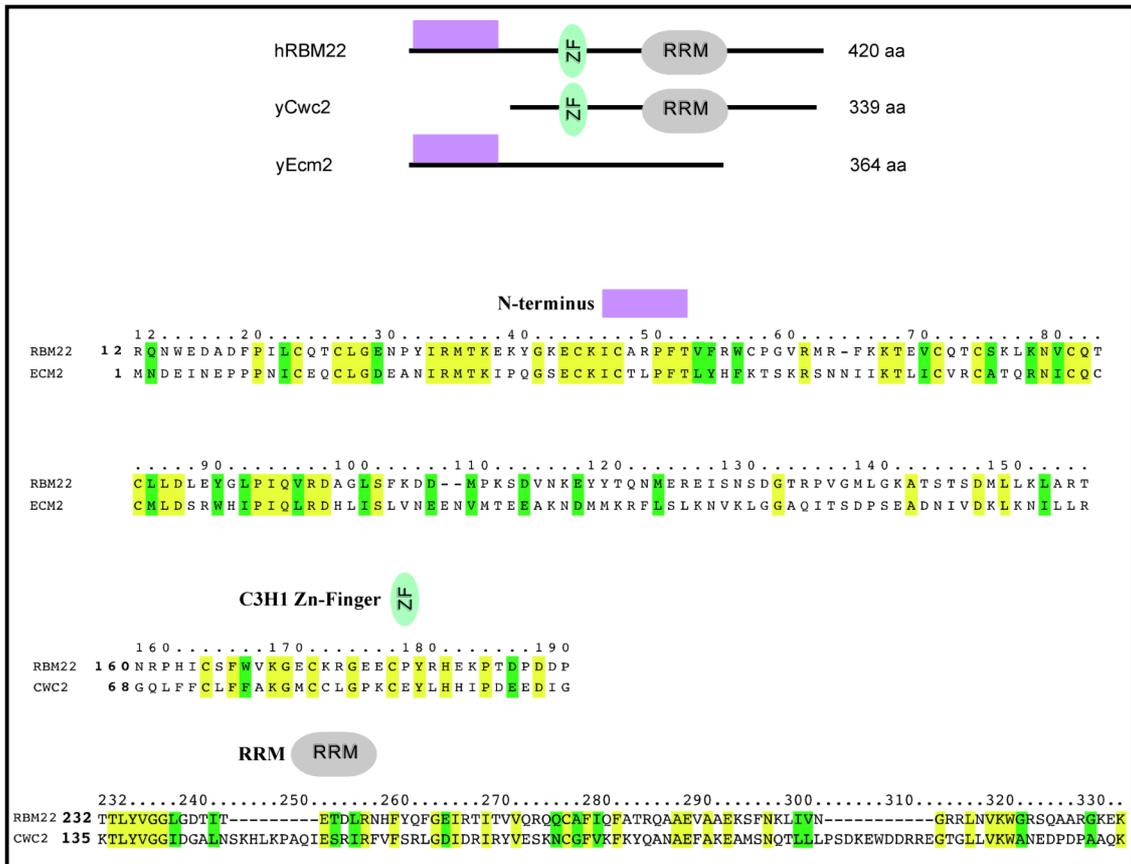


Figure S3. The N-terminal portion of RBM22, the human homolog of Cwc2, shares homology with yeast *S. cerevisiae* Ecm2. A database search with the human protein RBM22 sequence retrieved both Ecm2 and Cwc2 from *S. cerevisiae* and revealed that the N-terminal part of RBM22 (amino acids (aa) 21-145) is highly evolutionarily conserved (51% similarity, 37% identity) to that of Ecm2 (aa 10-128). The central and C-terminal part of RBM22 (aa 164-303), which includes Zn-fingers and RRM, is in turn homologous to Cwc2 (aa 72-226) (39% similarity, 26% identity). Identical amino acids are shown in yellow and similar amino acids are shown in green.

Supplementary Figure 4

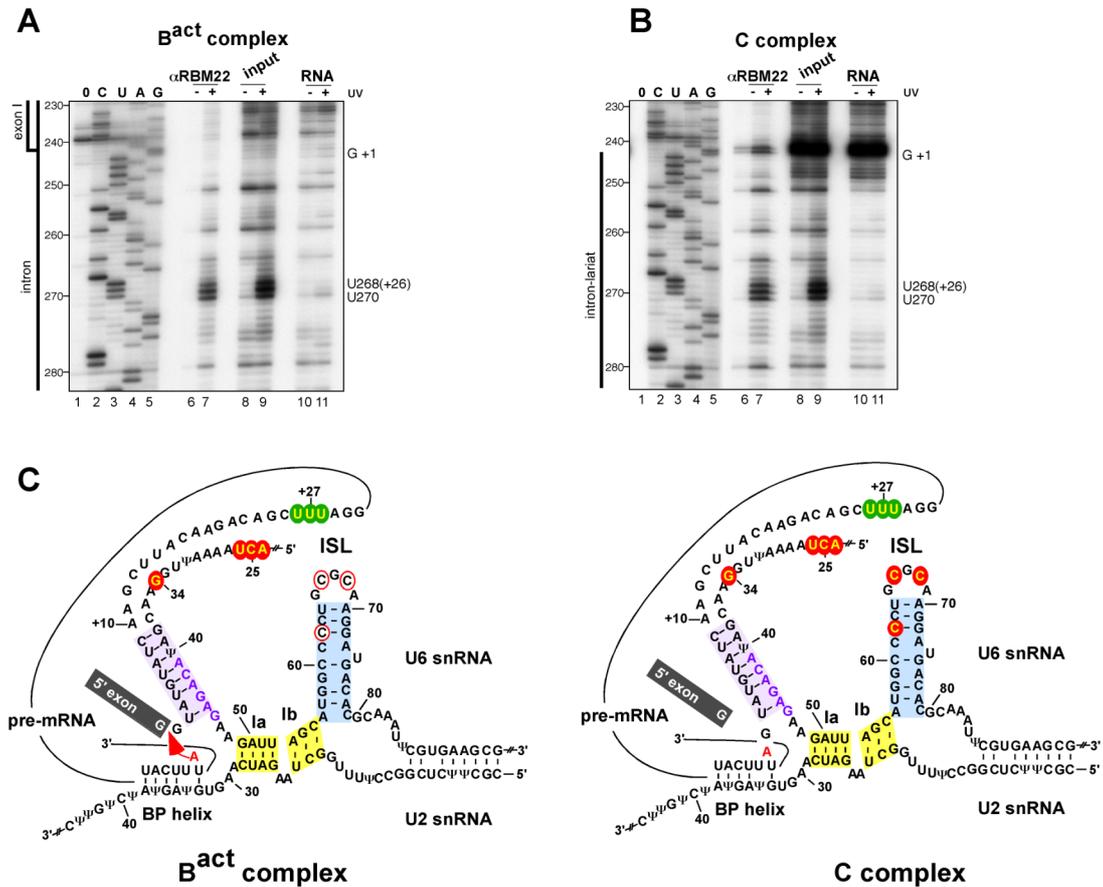


Figure S4. Mapping RBM22 pre-mRNA interaction sites in purified human B^{act} and C complexes. (A) (B) Primer extension analysis of PM5 pre-mRNA derived from UV-irradiated B^{act}, and C complexes, after immunoprecipitation with anti-RBM22 antibody (αRBM22, lanes 7) or without immunoprecipitation (input, lanes 9). Primer extension analysis of UV-irradiated naked RNA isolated from the B^{act} and C complexes, respectively (lanes 11). The primer used was complementary to nucleotides 317–336 of human PM5 pre-mRNA. Lanes 6, 8 and 10 are controls without UV irradiation. C, U, A, and G are dideoxy sequence markers prepared with the same oligonucleotide ("0", no ddNTP). Nucleotides within the PM5 intron, exon1 and intron-lariat sequences are listed on the left. Reverse transcriptase stops that are due to RNA–protein crosslinks are denoted on the right. (C) Secondary-structure models of human U2/U6/pre-mRNA before (B^{act} complex) and after (C complex) the first step of splicing. The attack of the branch point A at the 5'SS is indicated by an arrow in the B^{act} complex. Sites in U6 (see text) and PM5 pre-mRNA crosslinked to RBM22 are indicated by red and green circles, respectively.

Supplementary Materials and Methods

Chemical modification experiments

The modification reagents used were dimethylsulfate (DMS; Riedel-de Haën), and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate (CMCT; Fluka). For the modification reactions, 500 fmol of B^{act} complex were incubated with modification reagents in the presence of 10 μ g *E. coli* tRNA, essentially as described by others (Ehresmann et al., 1987). In brief, 1 μ l DMS was added per 200 μ l reaction and incubated at 20 °C for 8 min. The reaction was stopped by the addition of 50 μ l DMS stop buffer (1 M Tris-HOAc pH 7.5, 2 M β -mercaptoethanol, 12.5 mM EDTA). CMCT modifications were performed at 20 °C for 30 min in the presence of 8 mg of CMCT per 200 μ l reaction. To recover the RNA, samples were first ethanol-precipitated and then digested with proteinase K and PCI-extracted. Following ethanol precipitation, samples were diluted with water and used for primer extension analysis.

Immunodepletions

For depletion of TAP tagged Cwc2, yeast extracts in AGK buffer (20 mM Hepes-KOH pH 7.9, 200 mM KCl, 1.5 mM MgCl₂, 10% glycerol), were incubated either with IgG Sepharose, or (for mock depletion) with Protein A Sepharose (PAS), for 2 hours at 4 °C. After incubation, the Sepharose beads were sedimented by brief centrifugation and the supernatant (depleted extract) was dialyzed against buffer D (20 mM Hepes-KOH pH 7.9, 50 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA pH 8.0, 0.5 mM DTT, 0.5 mM PMSF and 2 mM benzamidine) and then analyzed by Western blotting. For immunodepletion of RBM22, anti-RBM22 antibodies coupled to PAS beads were incubated with HeLa nuclear extract containing 200 mM NaCl.

Splicing efficiency (Figure 5B) was calculated as the ratio of the mRNA signal (at the 20 min time point) to the initial pre-mRNA signal (at time 0).

Preparation of purified yeast and human spliceosomal complexes

For MS2-MBP affinity purification, three MS2 binding sites were added at the 5' ends of various yeast actin pre-mRNA constructs (M3Act). To isolate biochemically homogenous yeast B^{act} and C complexes (Figure 3A-D) we employed modified ³²P-labeled actin pre-mRNA substrates, M3Act Δ 6 and M3Act Δ 31, respectively (Fabrizio et al., 2009) and splicing extracts prepared from strain SC1887. To obtain yeast B* complexes, ³²P-labeled wild-type M3Act pre-mRNA was used instead. First, the extract from strain YNR1, was depleted of Cwc2 (Δ Cwc2) and then the temperature-sensitive Prp2 mutant was heat-inactivated (B^{act} Δ Prp2 Δ Cwc2). Each stalled complex was isolated by centrifugation, followed by MS2-MBP affinity-selection (Fabrizio et al., 2009; Warkocki et al., 2009). Complexes were converted into B* complexes upon addition of recombinant Prp2 and Spp2 in the presence of ATP and they were converted into C complexes upon addition of Cwc25 (Figure 1E, lane 4). The B complex (assembled at 0.05 mM ATP, shown in Figure 1C), was purified from the temperature-sensitive Prp2 mutant extract which was not heat-inactivated. Human B^{act} and C complexes were assembled on PM5-20 and PM5 pre-mRNAs respectively, and isolated by MS2-MBP affinity-selection, essentially as described previously (Bessonov et al., 2008; Bessonov et al., 2010).

Production and characterization of anti-RBM22 antibodies

Two anti-RBM22 peptide antibodies were raised and affinity-purified using the respective cognate peptides. Both antibodies were produced by immunizing rabbits

with a 17-amino-acid peptide, selected from the central sequence of RBM22 and spanning amino acids 179–195 (peptide M: CPYRHAKPTDPDDPLAD) or from the C-terminal sequence spanning amino acids 405–420 plus a cysteine (peptide C: CQDPQRMGAHAGKHSSP). Anti-RBM22 antibodies were affinity-purified from immunosera by using a Sulfolink column charged with the cognate peptide, following the manufacture's protocol (Pierce). The specificity of the purified anti-RBM22 antibodies was tested on HeLa nuclear extract by Western blotting using anti-RBM22 antibodies diluted 1:1000. Both purified antibodies reacted specifically on a Western blot with a protein in HeLa nuclear extract matching the size of RBM22 and recognized recombinant RBM22.

***In vitro* splicing reactions**

Splicing was performed *in vitro* with yeast whole-cell extract, essentially as described previously (Fabrizio et al., 1989), by using 0.4 nM uniformly ³²P-labelled actin pre-mRNA and 40-20% yeast whole-cell extract. RNA was separated by electrophoresis in an 8% polyacrylamide gel containing 8.0 M urea. For complementation of Cwc2-depleted extracts with recombinant Cwc2, the depleted extract was supplemented with recombinant protein to a final concentration of 0.5–3 μM. Conditions for a standard HeLa splicing reaction were as follows: 30% HeLa cell nuclear extract in buffer D (20 mM Hepes-KOH pH 7.9, 0.1 M KCl, 1.5 mM MgCl₂, 10% (v/v) glycerol, 0.2 mM EDTA pH 8.0, 0.5 mM DTT and 0.5 mM PMSF) supplemented with 25 mM KCl, 20 mM creatine phosphate, 2 mM ATP, 3 mM MgCl₂ and ³²P-labelled MINX pre-mRNA substrate. The mixture was incubated at 30 °C for the desired time. RNA was recovered and analyzed on a 10% polyacrylamide gel. For complementation of

RBM22-depleted extract, recombinant RBM22 was added at a final concentration of 1 or 2 μ M.

Northern blotting

For Northern blots, RNA was separated by electrophoresis on an 8% polyacrylamide gel containing 8M urea, transferred to nylon membrane (Amersham) and fixed by UV irradiation. Probes of the full-length yeast or human genes coding for U1, U2, U4, U5, and U6 snRNAs were made by the random priming method with the Prime-It kit (Stratagene). Hybridization and washing of blots were as previously described (Fabrizio et al., 1989).

Cwc2 and RBM22 expression and purification

The full-length *CWC2* and *RBM22* genes were PCR-amplified from genomic DNA of *S. cerevisiae* (Strain W303A) and MegaMan cDNA library (Stratagene), respectively, and ligated into the vector pETM11 (EMBL), generating fusion proteins with an N-terminal hexahistidine tag, cleavable with tobacco etch virus (TEV) protease. The primers for PCR amplification of the genes are available upon request. The constructs were verified by sequencing and transformed into *E. coli* strain Rosetta II (Novagen). Cultures were grown in auto-inducing media (Studier, 2005) or in 2xYT containing antibiotics for the selection of a specific vector and induced with 0.2 mM IPTG. Cultures were incubated at 17 °C for 48 hours. Cells were harvested by centrifugation, washed and the pellet stored at –80 °C. Protein purification was performed at 4 °C. The cell pellets were suspended by vortexing in 5 ml of lysis buffer (50 mM HEPES-NaOH pH 7.5, 600 mM NaCl, 2 mM β -mercaptoethanol (β -ME), 20 mM imidazole and 10% glycerol) per 1 g cells and lysed with a fluidiser

system (Microfluidics) six times at 80 p.s.i. Insoluble material was removed by centrifugation for 40 min at 10,000 rpm in a Sorvall SA-300 rotor. Proteins were purified on a HisTrap HP FF crude column (GE Healthcare), using the Äkta Prime system (GE Healthcare), essentially as described previously (Warkocki et al., 2009).

List of yeast strains used in this work

SC1887	(<i>MATa</i> ; <i>ade2</i> ; <i>arg4</i> ; <i>leu2-3</i> ; <i>112</i> ; <i>trp1-289</i> ; <i>ura3-52</i> ; <i>CWC2::TAP-K.I.URA3</i> C-terminus); purchased from Euroscarf (Frankfurt am Main)
SC0546	(<i>MATa</i> ; <i>ade2</i> ; <i>arg4</i> ; <i>leu2-3</i> ; <i>112</i> ; <i>trp1-289</i> ; <i>ura3-52</i> ; <i>ECM2::TAP-K.I.URA3</i> C-terminus); purchased from Euroscarf (Frankfurt am Main)
SC1215	(<i>MATa</i> ; <i>ade2</i> ; <i>arg4</i> ; <i>leu2-3</i> ; <i>112</i> ; <i>trp1-289</i> ; <i>ura3-52</i> ; <i>YJU2::TAP-K.I.URA3</i> C-terminus); purchased from Euroscarf (Frankfurt am Main)
YRK1	<i>MATa</i> , <i>trp1Δ1</i> ; <i>his3Δ</i> ; <i>ura3-52</i> ; <i>lys2-801</i> ; <i>ade2-101</i> ; <i>SNU114::TAP-K.I.TRP1</i> C-terminus (Häcker et al., 2008)
YRK5	<i>MATa</i> , <i>trp1Δ1</i> ; <i>his3Δ</i> ; <i>ura3-52</i> ; <i>lys2-801</i> ; <i>ade2-101</i> ; <i>PRP8::TAP-K.I.TRP1</i> C-terminus (Häcker et al., 2008)
YNR1	(<i>MATalpha</i> ; <i>prp2-1</i> ; <i>ade2</i> ; <i>his3</i> ; <i>lys2-801</i> ; <i>ura3</i> ; <i>CWC2::TAP-K.I.URA3</i> C-terminus) (This study).
3.2.AID/CRL2101	(<i>MATalpha</i> ; <i>prp2-1</i> ; <i>ade2</i> ; <i>his3</i> ; <i>lys2-801</i> ; <i>ura3</i> , carrying a G360D substitution in the helicase domain of Prp2)(Yean and Lin, 1991)

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