Identification and characterization of yUAP/Sub2p, a yeast homolog of the essential human pre-mRNA splicing factor hUAP56

Meng Zhang and Michael R. Green[1]

Howard Hughes Medical Institute, Programs in Gene
Function and Expression and Molecular Medicine, University
of Massachusetts Medical School, Worcester,
Massachusetts 01605, USA

**The human 56-kD U2AF[65]-associated protein (hUAP56),
a member of the DExD/H box protein family of RNA-
dependent ATPases, is required for the stable binding of
U2 snRNP to the pre-mRNA branchpoint. Here we iden-
tify a highly conserved *Saccharomyces cerevisiae* homo-
log of hUAP56, yUAP/Sub2p. yUAP/Sub2p can be func-
tionally substituted for by hUAP56 and, like its human
counterpart, is an essential pre-mRNA splicing factor.
yUAP/Sub2p is required for formation of the commit-
ment complex, the precursor for U2 snRNP addition. In
conjunction with previous studies, we conclude that at
least two DExD/H box proteins, Prp5p and yUAP/Sub2p,
mediate the U2 snRNP-branchpoint interaction.**

Pre-mRNA splicing occurs in a large and structurally
dynamic complex, the spliceosome, within which the
two-step transesterification reactions occur. To as-
semble the spliceosome, four ribonucleoprotein particles
(U1, U2, U5, and U4/U6), together with a large number
of non-snRNP proteins, interact with the pre-mRNA in a
highly ordered and stepwise pathway (Will and Luhr-
mann 1997; Burge et al. 1999). The earliest event that
specifically targets the pre-mRNA to the splicing path-
way is formation of the U1 snRNP/pre-mRNA commit-
ment complex both in yeast (Seraphin and Rosbash 1991)
and in mammals (Michaud and Reed 1991; Jamison et al.
1992). In yeast, two distinct commitment complexes,
CC1 and CC2, have been identified: CC1 requires only
the 5′ splice site, whereas formation of CC2 requires
both the 5′ splice site and the branchpoint (Seraphin and
Rosbash 1989). The likely mammalian counterpart of
CC2 is the E complex, whose formation requires both
the 5′ splice site and the polypyrimidine (Py) tract adja-
cent to the branchpoint.

Although commitment complex assembly is ATP in-
dependent, many of the subsequent steps in spliceosome
assembly and splicing require ATP-hydrolysis, the first
of which is the stable binding of U2 snRNP to the pre-
mRNA branchpoint. These ATP-dependent events are
mediated by a series of splicing factors that are members
of the DExD/H box protein family, the founding member
of which is eIF-4A, a known RNA helicase (Kramer 1996;
Staley and Guthrie 1998). Several of these DExD/H box
splicing factors have been shown to possess an ATP-de-
pendent RNA unwinding activity (Laggerbauer et al.
1998; Raghunathan and Guthrie 1998; Wagner et al.
1998; Wang et al. 1998) and are thought to use ATP hy-
drolysis as a driving force to modulate specific RNA
structural rearrangements during spliceosome assembly.
Among yeast protein splicing factors, seven belong to the
DExD/H box protein family, and four of these have known
human orthologs (for review, see Staley and Guthrie 1998).

To date, Prp5p is the only yeast DExD/H box protein
shown to be required for the stable binding of U2 snRNP
to the branchpoint (Dalbadie-McFarland and Abelson
1990; Ruby et al. 1993). In mammals, hUAP56 is the
only known DExD/H box protein required for stable U2
snRNP binding (Fleckner et al. 1997). hUAP56 is an es-
sential splicing factor, recruited to the pre-mRNA depen-
dent on the Py-tract and U2AF[65]. hUAP56 interacts with
U2AF[65] directly, which presumably directs hUAP56 to
the vicinity of the branchpoint. A variety of metazoan
homologs of hUAP56 have been identified, from *C. el-
egans* through man, all of which contain a DECD motif
as opposed to the canonical DEAD/H motif.

In this article, we identify yUAP/Sub2p, the *S. cerevi-
siae* homolog of hUAP56, and analyze its role in pre-
mRNA splicing and spliceosome assembly. Our results
indicate that hUAP56 is both structurally and function-
ally conserved from yeast to man and have mechanistic
implications regarding early events of spliceosome as-
sembly.

Results

Identification of a highly conserved yeast homolog of hUAP56

hUAP56 is required for the U2 snRNP-branchpoint in-
teraction (Fleckner et al. 1997). In yeast, Prp5p is the
only DExD/H box protein known to be required for this
step of spliceosome assembly, raising the possibility that
Prp5p may be the hUAP56 homolog. However, a search
of the complete *S. cerevisiae* genome revealed an unchar-
acterized open reading frame whose protein product is
far more similar to hUAP56 than Prp5p. Figure 1A shows
an alignment between hUAP56, the *S. cerevisiae* protein
and a putative *Schizosaccharomyces pombe* homolog.
The *S. cerevisiae* protein is 65% identical and 83% simi-
lar to hUAP56, whereas Prp5p is only 30% identical and
55% similar to hUAP56. Moreover, the similarity be-
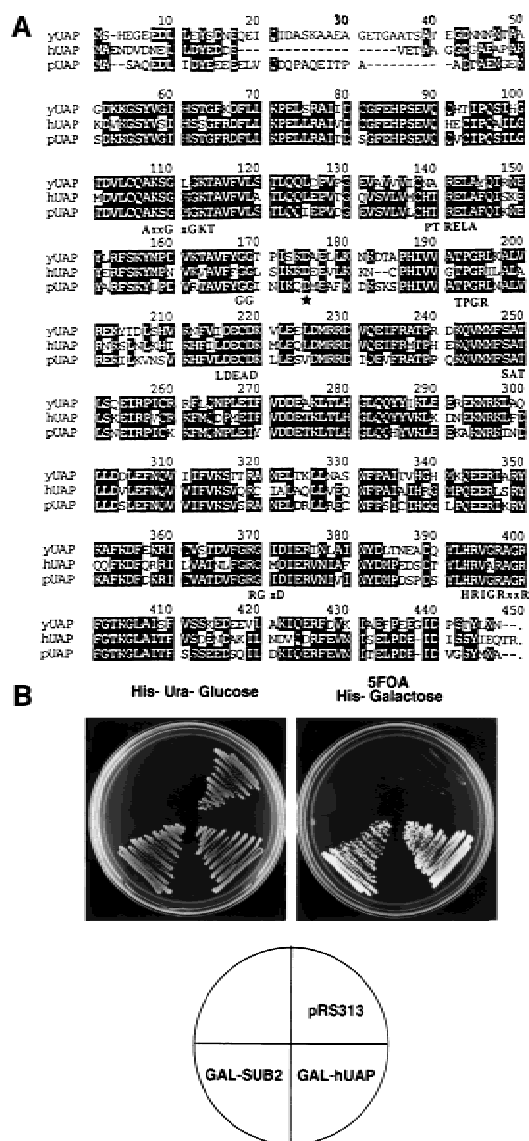tween Prp5p and hUAP56 is confined to the seven sig-

Figure 1. A highly conserved *Saccharomyces cerevisiae* homolog of hUAP56. (*A*) Amino acid alignment. The *S. cerevisiae* yUAP/Sub2p amino acid sequence was aligned to human UAP56 (hUAP) and the hypothetical *Schizosaccharomyces pombe* UAP (pUAP) found in database searches. Identical amino acids in all three homologs are shaded. Conserved motifs characteristic of DExD/H box protein family are indicated at the bottom of the alignment. Star indicates the mutated aspartic acid residue in *sub2-100*, a temperature-sensitive allele of *SUB2*. (*B*) hUAP56 can functionally substitute for yUAP/Sub2p. Growth of cells on 5FOA, galactose plate is shown.

nature motifs of DExD/H box proteins, whereas the similarity between the yeast protein and human UAP56 occurs throughout the entire protein. Finally, hUAP56 possesses a DECD sequence, as does the yeast protein, whereas Prp5p contains a DEAD sequence. On the basis of this unusually high homology and the experiments presented below, we conclude that this *S. cerevisiae* protein is the true homolog of hUAP56. Significantly, the gene encoding yUAP has been implicated previously in

splicing as a high-copy suppressor of an snRNP biogenesis mutant, *brr1*-1, and referred to as *SUB2* (Noble and Guthrie 1996; Kistler and Guthrie 2001). We therefore will refer to the gene as *SUB2* and to its protein product as yUAP/Sub2p.

Using standard gene disruption procedures (Guthrie and Fink 1991; see Materials and Methods), we found that *SUB2* was essential for viability (data not shown). The significant amino acid similarity between hUAP56 and yUAP/Sub2p (Fig. 1A) prompted us to ask whether hUAP56 can functionally substitute for yUAP/Sub2p. Plasmids in which hUAP56 or yUAP/Sub2p were expressed from the *GAL1* promoter were transformed, in parallel, into a *SUB2* deletion strain. Figure 1B shows that both hUAP56 and yUAP/Sub2p transformants grew on 5FOA plates, which selects against retention of the *URA3*-marked wild-type yUAP/Sub2p expression plasmid. Thus, hUAP56 can functionally substitute for yUAP/Sub2p, indicating that yUAP/Sub2p and hUAP56 are structurally and functionally conserved.

yUAP/Sub2p is an essential pre-mRNA splicing factor

To investigate the role of yUAP/Sub2p in splicing, we constructed a yeast strain conditionally expressing yUAP/Sub2p from the *GAL1* promoter. The strain grew on both glucose- and galactose-containing media (data not shown), indicating that the low level of uninduced expression from the *GAL1* promoter was sufficient to support cell growth. To circumvent this problem, we expressed yUAP/Sub2p from a more tightly controlled promoter, *SPO13–GAL* (Fig. 2A). The *SPO13* promoter is normally repressed by a cis-acting upstream element (URS1; Wang et al. 1987). Repression can be overridden by a strong transcriptional activator, such as GAL4 (Vidal et al. 1996). Figure 2B shows that the growth of this strain halted ~12 h after shift from galactose- to glucose-containing media.

To assess the role of yUAP/Sub2p in splicing, extracts were prepared from the *SPO13–GAL–SUB2*-containing strain after growth in glucose-containing media, which represses expression of yUAP/Sub2p, or galactose-containing media, which induces expression of yUAP/Sub2p. Figure 2C shows that splicing extracts prepared from cells expressing yUAP/Sub2p were active (lane 1), whereas the yUAP/Sub2p-depleted extract failed to support splicing (lane 2). Addition of yeast-derived GST-yUAP/Sub2p to the yUAP/Sub2p-depleted extract restored splicing (lanes 3–5). Thus, like its human counterpart, yUAP/Sub2p is an essential splicing factor.

The direct role of yUAP/Sub2p in pre-mRNA splicing was confirmed by isolation and characterization of a conditional (temperature-sensitive) allele of yUAP, *sub2-100*. A yeast strain carrying *sub2-100* grew normally at 30°C, whereas at 37°C, growth of this strain was severely inhibited (Fig. 3A). Sequence analysis revealed that the temperature-sensitive phenotype of *sub2-100* was conferred by a single point mutation (adenine to guanine) resulting in the replacement of the aspartic acid at position 175 with glycine (see Fig. 1A). We note that D175 is
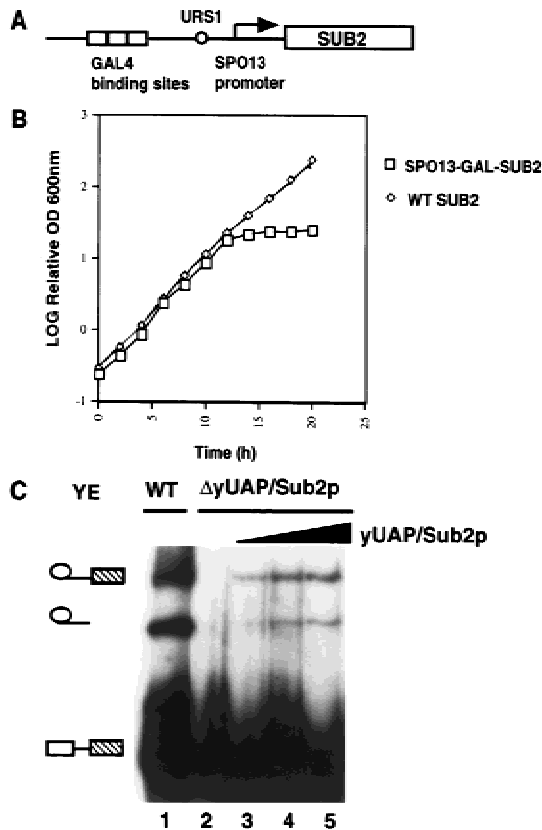
Figure 2. yUAP/Sub2p is an essential pre-mRNA splicing factor. (*A*) Schematic diagram of a conditional yUAP/Sub2p expression construct. (*B*) Growth curve of strain MZ105, which conditionally expresses yUAP/Sub2p. Strain MZ102a, which expresses yUAP/Sub2p constitutively, was used as a control. Cultures of MZ105 and MZ102a in 4% galactose containing medium were centrifuged and the cells resuspended in rich medium containing 4% glucose at time zero. Every 2 h, the OD_{600} of the cultures was measured and the concentration of cells adjusted to remain between OD_{600} 1.0 and 3.0. (*C*) Splicing analysis. Splicing reactions were carried out at 25°C for 30 min. In lane *1*, splicing extract was prepared from strain MZ105 grown in galactose-containing media. In lanes *2–5*, splicing extract was prepared from strain MZ105 16 h following shift from galactose- to glucose-containing media. In lanes *3–5*, 0.1 μL, 0.5 μ, and 1 μL of GST-yUAP/Sub2p (0.1 mg/mL) was added to the reaction mixture.

not in any of the DExD/H box protein consensus motifs but is highly conserved from yeast to man.

Figure 3B shows that extracts prepared from the *sub2-100* expression strain, grown at the permissive temperature, exhibited splicing activity comparable to that of the wild-type extract (cf. lanes 1 and 3). However, this extract was unable to support splicing following preincubation at 37°C for 15 min (lane 5). In contrast, the splicing activity of the wild-type extract was unaffected by the 37°C preincubation (cf. lanes 1 and 2).

yUAP/Sub2p is required for formation of CC2

To assess the role of yUAP/Sub2p in complex assembly, we first used a native gel assay to determine the splicing

complexes formed in a yUAP/Sub2p-depleted extract. As controls, U1- and U2-depleted extracts were analyzed in parallel. Figure 4A shows, as reported previously, that U1 snRNA depletion abolished formation of all splicing complexes (lane 1), U2 snRNA depletion resulted in arrest at the CC2 complex (lane 3), and a combination of U1- and U2-depleted extracts fully supported spliceosome assembly (lane 5). The yUAP/Sub2p-depleted extract (lane 2) was unable to form any ATP-dependent spliceosome, analogous to results in HeLa cell extracts (Fleckner et al. 1997). Unexpectedly, the branchpoint-dependent commitment complex, CC2, was also absent, whereas formation of the CC1 complex, which requires only the 5′ splice site, was not significantly affected. The yUAP/Sub2p-depleted extract efficiently complemented both U1 snRNA- and U2 snRNA-depleted extracts (lanes 4 and 6).

The direct role of yUAP/Sub2p in splicing complex assembly was further verified by analyzing the *sub2-100* extract. Figure 4B shows that extract prepared from the *sub2-100* expression strain, grown at the permissive temperature, supported spliceosome assembly (lane 3). However, following preincubation at 37°C for 15 min, the *sub2100* extract was unable to support spliceosome assembly (lane 4). By contrast, in the wild-type extract, spliceosome assembly was unaffected by the 37°C preincubation (cf. lanes 1 and 2). Significantly, in the heat-inactivated *sub2-100* extract, the level of CC2 was also substantially reduced (cf. lanes 2 and 4), confirming a role for yUAP/Sub2p in CC2 formation.

Role of the yUAP/Sub2p DExD/H box consensus motifs

The fact that yUAP/Sub2p is essential for the formation of the CC2 complex, which is ATP independent, prompted us to examine the role of the DExD/H box family consensus motifs, several of which are required for ATP utilization. We designed a series of point mutations in yUAP/Sub2p based on the known role of these motifs in other DExD/H box proteins, in particular, of eIF-4A (Schmid and Linder 1991; Pause and Sonenberg 1992; Pause et al. 1993). The GKT motif was mutated to GNT to eliminate ATP binding; the DECD motif was changed to EECD or the SAT motif was mutated to LAT to eliminate putative RNA helicase activity (see Fig. 5A). Mutant or wild-type yUAP/Sub2p derivatives were placed under control of the *GAL1* promoter and introduced into a *SUB2* deletion strain. To test the ability of these yUAP/Sub2p mutants to support cell growth, transformants were restreaked onto 5-FOA, galactose-containing plates to shuffle out the wild-type *SUB2*-containing, *URA3*-marked plasmid. Figure 5B shows that cells expressing wild-type yUAP/Sub2p grew on 5-FOA, galactose plates, whereas cells transformed with mutant yUAP/Sub2p expression vector, or vector alone, did not. Thus, the GKT, DECD, and SAT motifs are essential for yUAP/Sub2p function, indicating that in addition to the ATP-independent role in commitment complex formation, yUAP/Sub2p has an ATP-dependent function (see Kistler and Guthrie 2001; Libri et al. 2001).

To monitor the effects of overexpression of mutant yUAP/Sub2p on cell growth, mutant yUAP/Sub2p ex-

Figure 3. A temperature-sensitive mutant of yUAP/Sub2p. (*A*) Growth phenotype of *sub2-100* containing strain, MZ306. (WT yUAP/Sub2), yeast strain MZ102b carrying *SUB2*. (Sub2-100), yeast strain MZ306 carrying a mutant allele of *SUB2, sub2–100*. (*B*) Splicing analysis. Splicing extracts were derived from strain MZ102b (lanes *1,2*) and MZ306 grown at 30°C (lanes *3,4,5*), respectively. Splicing reactions were carried out at 25°C for 30 min. In lane *4*, the splicing extract was preincubated at 37°C for 5 min. In lanes *2* and *5*, splicing extracts were preincubated at 37°C for 15 min.
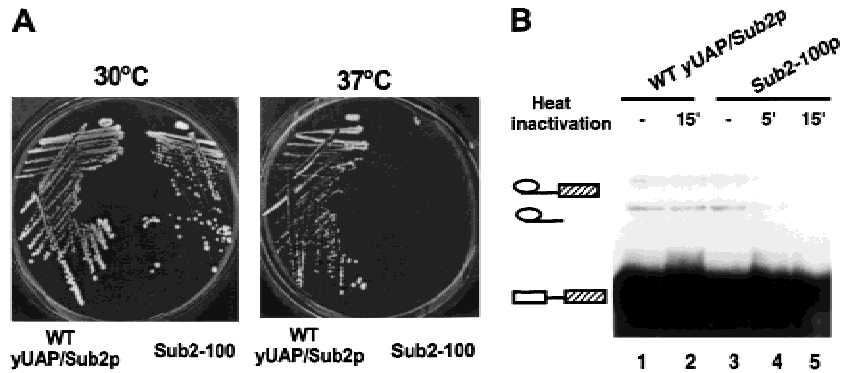
pression vectors were introduced into a wild-type yeast strain and the resulting transformants restreaked onto galactose-containing plates to induce mutant yUAP/Sub2p expression. Figure 5B shows that yUAP/Sub2p(LAT) transformants failed to grow; in contrast, yUAP/Sub2p(GNT) and yUAP/Sub2p(EECD) had little impact on cell growth. These results indicate that the SAT to LAT mutation in yUAP/Sub2p conferred a dominant-negative phenotype, analogous to the results with Prp2p (Plumpton et al. 1994).

A characteristic feature of all known hUAP56 homologs is the presence of a DECD motif (Fleckner et al. 1997; see Fig. 1A). This high conservation suggested that the cysteine residue may be required for UAP function. We mutated the DECD motif in yUAP/Sub2p to DEAD, and Figure 5B shows that yeast harboring yUAP/Sub2p (DEAD) were inviable. We conclude that complete yUAP/Sub2p function requires the cysteine residue of the DECD motif.

Discussion

In this article, we have identified and characterized yUAP/Sub2p, a yeast homolog of hUAP56. yUAP/Sub2p is essential for viability and can be replaced by hUAP56. Consistent with our previous work on hUAP56 (Fleck-

ner et al. 1997), yUAP/Sub2p is an essential splicing factor required for stable binding of U2 snRNP to the pre-mRNA branchpoint. Surprisingly, we found that yUAP/Sub2p was required for the formation of the branchpoint-dependent CC2 commitment complex, whereas formation of branchpoint-independent CC1 commitment complex was unaffected by yUAP/Sub2p depletion or inactivation. This result suggests a role for yUAP/Sub2p in early 3′ splice site/branchpoint recognition or in 5′–3′ splice sites communication. Consistent with this idea, in the accompanying manuscripts *SUB2* is shown to genetically interact with MUD2 (Kistler and Guthrie 2001) and PRP40/Namp8 (Libri et al. 2001). MUD2, PRP40, and Namp8 have all been implicated in commitment complex formation. Because CC2 complex formation occurs in the absence of ATP, the role of yUAP/Sub2p in this step must be unrelated to its putative ATP-dependent RNA helicase or ATPase activity. However, the DExD/H box motifs required for ATP utilization are essential for yUAP/Sub2p function, indicating that yUAP/Sub2p must also be required for the subsequent ATP-consuming steps. Accordingly, the accompanying manuscripts (Kistler and Guthrie 2001; Libri et al. 2001) demonstrate a role for yUAP/Sub2p subsequent to the

Figure 4. yUAP/Sub2p is required for commitment complex formation. (*A*) Analysis of splicing complex formation using U1 snRNA-, U2 snRNA-, or yUAP/Sub2p-depleted extracts. yUAP/Sub2p-, U1 snRNA-, or U2 snRNA-depleted splicing extract was derived from strain MZ105, BS-Y82, or BS-Y88, respectively, 16 h after shift from galactose- to glucose-containing media. Splicing reactions (40% extract) were carried out and analyzed on a native gel. In lanes *4–6*, 20% of the indicated extracts were used. In lanes *7* and *8*, extracts were prepared from strain W303 and MZ105 grown in galactose-containing media. Commitment (CC1 and CC2) and spliceosomal (SP) complexes are indicated. (*B*) Analysis of splicing complex formation

using *sub2-100* containing extracts. Splicing extracts were derived from strain MZ102b (lanes *1,2*), and MZ306 grown at 30°C (lanes *3,4*), respectively. Splicing reactions (40% extract) were carried out for 20 min and analyzed on a native gel. In lanes *2* and *4*, splicing extracts were preincubated at 37°C for 15 min before assembling the splicing reaction mixture.
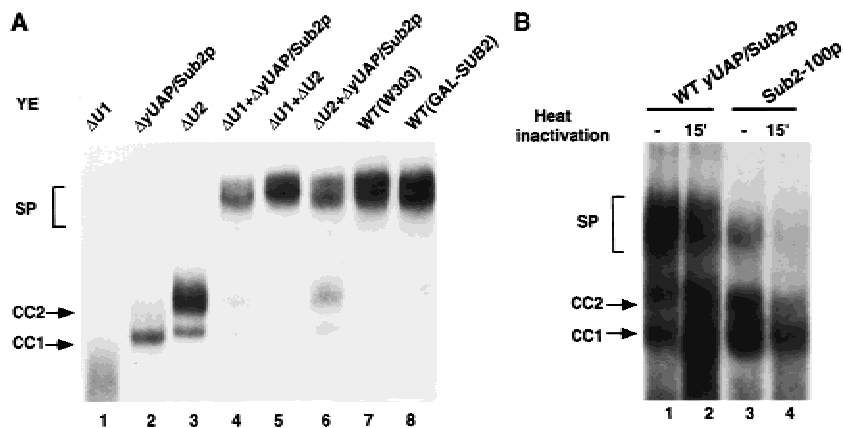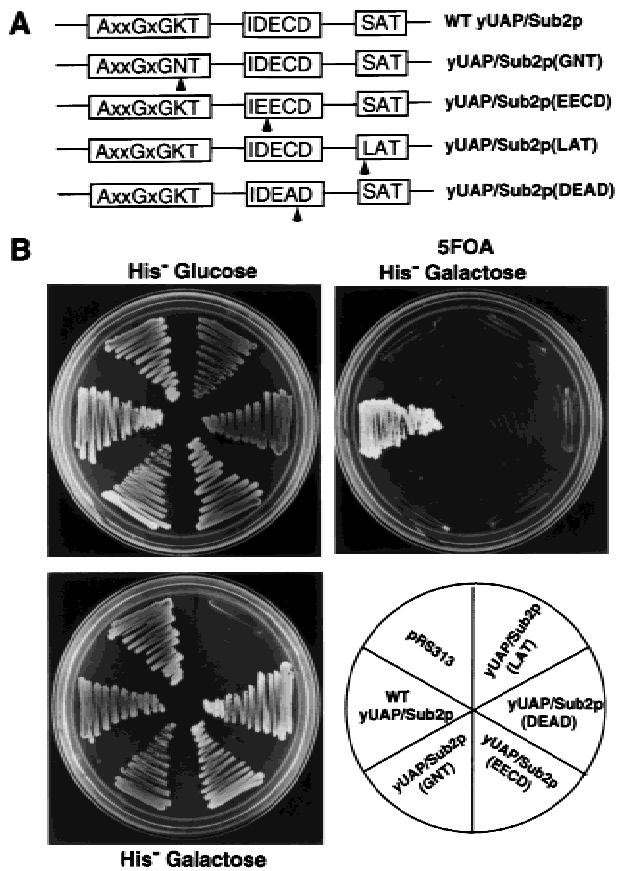
Figure 5. Role of DExD/H box protein family consensus motifs in yUAP/Sub2p function. (*A*) Amino acid sequences of the mutated motifs. Arrows indicate the mutated residues. (*B*) Effects of yUAP/Sub2p mutations on cell growth. Strains used in top panel were derived from strain MZ102b and contain plasmid pMZ11 (*SUB2*, *URA3*, CEN/ARS) and an integrated copy of wild-type yUAP/Sub2p (MZ104) or various yUAP/Sub2p mutants (MZ106, MZ107, MZ108, and MZ109) expressed from the G*AL1* promoter. Growth of cells carrying the indicated genes on His⁻ glucose or 5FOA His⁻ galactose is shown. In the bottom panel, W303 was used as the parental strain. Growth of cells on a His⁻ galactose plate is shown.

ATP-dependent U2 snRNP-branchpoint interaction. The different complex assembly defects observed in the three studies are most likely explained by the fact that distinct yUAP/Sub2p mutants were analyzed. Taken together, the results of the three studies indicate that yUAP/Sub2p acts at multiple steps and in both an ATP-dependent and ATP-independent manner during spliceosome assembly.

Prp5p is another yeast DExD/H box protein required for entry of U2 snRNP into the spliceosome (Dalbadie-McFarland and Abelson 1990). Both genetic and biochemical studies have suggested that U2 snRNA is the target of Prp5p (Ruby et al. 1993; Wells and Ares 1994; O'Day et al. 1996; Wells et al. 1996; Wiest et al. 1996) and that Prp5p may facilitate U2 snRNP binding by exposing the U2 snRNA branchpoint recognition region (O'Day et al. 1996). It will be important to determine how the two DExD/H box proteins, yUAP/Sub2p and

Prp5p, act in concert to promote the U2 snRNP-branchpoint interaction.

Materials and methods

Plasmids

The yeast *SUB2* gene was cloned by PCR amplification of yeast genomic DNA and inserted into plasmid pRS416 (*URA3*, CEN/ARS) and pRS313 (*HIS*, CEN/ARS) at the *Bam*HI site to generate plasmid pMZ11 and pMZ12. For hUAP56 or yUAP/Sub2p expression, pMZ13 (*GAL*–hUAP *HIS3*) or pMZ14 (*GAL*–yUAP/Sub2p *HIS3*) were constructed by inserting the human UAP56 or yUAP/Sub2p coding sequence into pSW107 (Walker et al. 1997) under control of the *GAL1* promoter. Plasmid pMZ19 (*SPO13*–*GAL*–yUAP/Sub2p *HIS3*), used for conditional yUAP/Sub2p expression, contains the *SPO13*–*GAL* promoter derived from pMV253-13 (Vidal et al. 1996) and the yUAP/Sub2p coding sequence in the pRS313 backbone.

Synthetic oligonucleotides were designed to introduce point mutations in the *SUB2* gene by a two-round PCR strategy (Higuchi et al. 1988). Mutated yUAP/Sub2p fragments were subcloned into the wild-type yUAP/Sub2p plasmid pMZ14, generating plasmids pMZ15 (*GAL*–yUAP/Sub2p–GNT), pMZ16 (*GAL*–yUAP/Subp2–EECD), pMZ17 (*GAL*–yUAP/Sub2p–LAT), and pMZ18 (*GAL*–yUAP/Sub2p–DEAD), respectively. All mutations were confirmed by sequencing.

Yeast strains

Stains for conditional U1 or U2 snRNA expression, BS-Y82 (*GAL*-U1) and BS-Y88 (*GAL*-U2), were obtained from M. Rosbash (Seraphin and Rosbash 1989). All strains constructed in this study were derived from W303 (*ade2-1 his3-11 his3-15 leu2-3 leu2-112 trp1-1 ura3-1 can-100*). Growth of yeast cells in rich or synthetic medium was performed by standard procedures (Guthrie and Fink 1991).

To disrupt the *SUB2* gene, a 2073-bp *Hpa*I–*Stu*I fragment containing the entire yUAP/Sub2p coding sequence was replaced with *TRP1* gene. This null *SUB2* allele was introduced into diploid W303 by one-step gene replacement (Guthrie and Fink 1991). Correct integration in the resulting strain MZ101 (*SUB2/sub2::TRP1*) was verified by Southern blotting.

Strain MZ101 (*SUB2/sub2::TRP1*) was transformed with pMZ11, and four isogenic haploids, MZ102a, MZ102b, MZ102c, and MZ102d, were obtained. MZ102b (*sub2::TRP1/pMZ11*) was used for all the subsequent gene-shuffling experiments.

hUAP56 and yUAP/Sub2p expression strain MZ103 (*sub2::TRP1*, *trp3::GAL*–hUAP56 *HIS3*, pMZ11) and MZ104 (*sub2::TRP1*, *trp3::GAL*–yUAP/Sub2p *HIS*, pMZ11) were constructed by integration of linearized pMZ13 and pMZ14 into MZ102b at the *TRP3* locus as described (Walker et al. 1997). For conditional yUAP/Sub2p expression, plasmid pMZ19 was transformed into MZ102b, and pMZ11 was shuffled out to generate MZ105 (*sub2::TRP1*, pMZ19). To construct strains conditionally expressing yUAP/Sub2p mutants, linearized plasmids pMZ14, pMZ15, pMZ16, pMZ17, and pMZ18 were integrated into strain MZ102b or W303a at the *TRP3* locus. The resulting strains are: MZ104, MZ106 (*sub2::TRP1*, *trp3::GAL*–yUAP/Sub2p–GNT *HIS3*, pMZ11), MZ107 (*sub2::TRP1*, *trp3::GAL*–yUAP/Sub2p–EECD *HIS3*, pMZ11), MZ108 (*sub2::TRP1*, *trp3::GAL*–yUAP/Sub2p–LAT *HIS3*, pMZ11), MZ109 (*sub2::TRP1*, *trp3::GAL*–yUAP/Sub2p–DEAD *HIS3*, pMZ11), MZ117 (*trp3::GAL*–yUAP/Sub2p *HIS3*), MZ110 (*trp3::GAL*–yUAP/Sub2p–GNT *HIS3*), MZ111 (*trp3::GAL*-yUAP/Sub2p–EECD *HIS3*), MZ112 (*trp3::GAL*–yUAP/Sub2p–LAT *HIS3*), and MZ113 (*trp3::GAL*–yUAP/Sub2p–DEAD *HIS3*).

Splicing and complex assembly assay

Splicing extracts were prepared according to Liao et al. (1992). For preparation of depleted extracts, cells were harvested after shifting from galactose- to glucose-containing media for 16 h. The pre-mRNA substrate WT-Δ2 (Liao et al. 1992) was used in all experiments. Splicing and native gel analysis of splicing complex formation was carried out as described previously (Seraphin and Rosbash 1989).

Preparation of GST–yUAP/Sub2p

yUAP/Sub2p coding sequence was cloned into plasmid pPS892 (a gift from P. Silver, Harvard Medical School) in frame, and the resulting plasmid pMZ20 (*GAL1*–GST–yUAP/Sub2p, *URA3*) was transformed into strain MZ105 to generate strain MZ114 (*sub2::TRP1*, pMZ20). Strain MZ114 was grown in galacotose-containing media, which induces GST–

yUAP/Sub2p expression, to OD_{600} 2–3, and whole-cell extracts were pre-pared. GST–yUAP/Sub2p was purified on glutathione-agrose beads and dialyzed against buffer D.

Isolation of a yUAP/Sub2p temperature-sensitive mutant

Mutagenized yUAP/Sub2p fragments were created using error-prone PCR (Leung et al. 1989) and subcloned into plasmid pMZ12 at *Hpa*I and *Xba*I to replace the wild-type yUAP/Sub2p fragment. All the *Escherichia coli* transformants (~10,000) were collected to generate a library of yUAP/Sub2p mutants. This library was subsequently transformed into yeast strain MZ102b. To screen temperature-sensitive yUAP/Sub2p mutants, ~4000 transformants were patched onto His⁻ plates and then replica-plated to 5FOA media at 30°C and 37°C. Approximately 30% of the transformants did not grow on 5FOA plates, indicating lethal mutations in yUAP/Sub2p. Colonies that grew at 30°C but not at 37°C were re-streaked to verify the temperature-sensitive phenotype. Plasmids were recovered from temperature-sensitive strains and retransformed into a *SUB2* deletion strain to confirm that the temperature-sensitive phenotype was caused by mutated yUAP/Sub2p.

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