Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p

Katja Strässer & Ed Hurt

BZH—Biochemie-Zentrum Heidelberg, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany

The yeast nuclear protein Yra1p is an essential export factor for mRNA. Yra1p interacts directly with the mRNA transport factor Mex67p/Mtr2p, which is associated with the nuclear pore[1,2]. Here, we report a genetic interaction between *YRA1* and *SUB2*, the gene for a DEAD box helicase involved in splicing[3–5]. Mutation of *SUB2* as well as its overexpression leads to a defect in mRNA export. Moreover, Yra1p and Sub2p bind directly to each other both *in vivo* and *in vitro*. Significantly, Sub2p and Mex67p/Mtr2p bind to the same domains of Yra1p, and the proteins compete for binding to Yra1p. Together, these data indicate that the spliceosomal component Sub2p is also important in mRNA export and may function to recruit Yra1p to the mRNA. Sub2p may then be displaced from Yra1p by the binding of Mex67p/Mtr2p, which participates in the export of mRNA through the nuclear pores.

The essential yeast mRNA export factors Yra1p and Mex67p/Mtr2p bind to each other *in vivo* as well as *in vitro*[2]. Despite their interaction, Yra1p localizes to the nucleus[2,6], whereas the Mex67p/Mtr2p complex is enriched at the nuclear pores[1]. Thus, the major pools of these two mRNA export factors are separated *in vivo*. To identify potential interaction partners of Yra1p, which could be components of the intranuclear mRNA export machinery, we sought to perform a synthetic lethal screen with mutant alleles of *YRA1*.

To find an appropriate mutant allele for this approach, we carried out a functional analysis of the domains in Yra1p. The highly conserved N1 domain, the variable, RG-rich N2 domain, the RNA recognition (RRM) domain, and the basic C1 domain of Yra1p are indicated in Fig. 1a. Surprisingly, deletion of any one of these domains does not lead to a growth defect (Fig. 1b, top rows; see Fig. 1a for deleted amino acids). However, *yra1-ΔN1* and *yra1-ΔRRM* are synthetically lethal with a mutant allele of *MEX67*, *mex67-5* (Fig. 1b, bottom rows).

In light of this observation and the possibility that the *yra1-ΔRRM* allele may identify RNA-binding proteins, we used this allele for the synthetic lethal screen. In this screen, 30 synthetic lethal mutants were isolated. The wild-type gene that rescued the synthetic lethality of one of the mutants was cloned by complementation and shown to be *SUB2* (data not shown). Interestingly, Sub2p was recently identified as an essential pre-mRNA splicing factor critical in spliceosome assembly[3–5]. This protein and its mammalian homologue UAP56 are members of the DEAD box family of RNA helicases[3–5]. From the remaining 29 synthetically lethal mutants, 13 were complemented by *SUB2*, 6 by *MEX67* and 2 by *MTR2*, indicating that the *yra1-ΔRRM* synthetic lethal screen predominantly yields factors involved in mRNA export, and thus is highly specific for this export pathway.

To test for synthetic lethality between *SUB2* and *YRA1* directly, mutant alleles of *yra1* and *sub2* were combined. As shown in Fig. 1c, *yra1-ΔRRM* and *yra1-ΔN1* are synthetically lethal with a *sub2* mutant allele, *sub2-85* (see below). This finding confirms the synthetically lethal relationship found in the screen. In contrast, *sub2-85* is not synthetically lethal with mutants of *MEX67* (data not shown). This result demonstrates a tight genetic interaction between *YRA1* and *SUB2*.

The genetic interaction between *SUB2* and the mRNA factor *YRA1* prompted us to test whether Sub2p functions not only in splicing, but also in mRNA export. To this end, we monitored nuclear export of poly(A)$^+$ RNA in yeast cells depleted of the essential Sub2p. Expression of a protein A (ProtA)-tagged version of Sub2p under control of the *GAL1* promoter, but not under the control of the constitutive *NOP1* promoter, was terminated by shifting the cells from galactose- to glucose-containing medium (Fig. 2a, left panel). Significantly, cells depleted of Sub2p greatly accumulate mRNA in the nucleus (Fig. 2a, right panel). Furthermore, overexpression of Sub2p by the strong *GAL1* promoter (Fig. 2a, left panel) also causes a nuclear accumulation of poly(A)$^+$ RNA (Fig. 2a, right panel). The latter result suggests that excess Sub2p impairs mRNA export in a dominant negative manner by titrating one or more other mRNA export factors.

To verify a role for Sub2p in mRNA export, we generated a thermosensitive mutant of *SUB2* (Fig. 2b, left panel). Analysis of poly(A)$^+$ RNA localization in *sub2-85* thermosensitive cells revealed normal export of mRNA at the permissive temperature but a significant accumulation inside the nucleus shortly (15 min) after

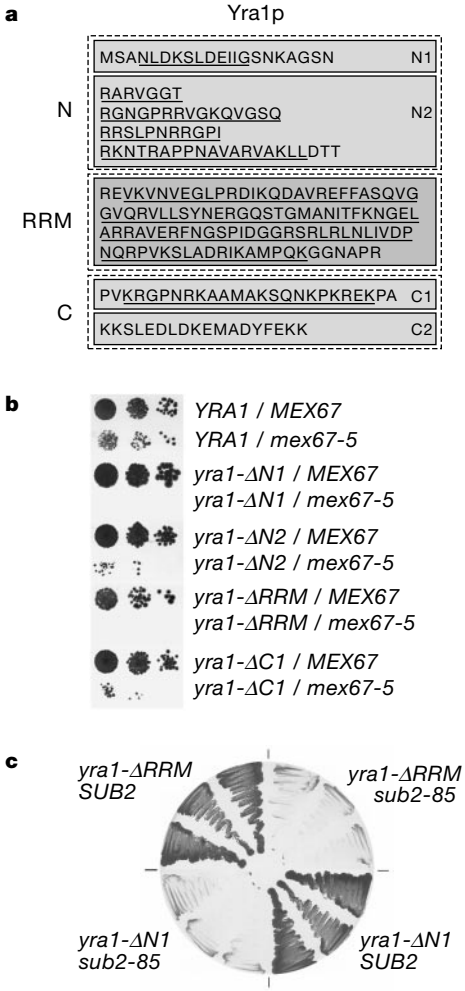

Figure 1 *YRA1* interacts genetically with *MEX67* and *SUB2*. **a**, Domain organization of Yra1p. Deletions are underlined. All deletion mutants are viable in a *yra1Δ* as well as a *yra1Δ/yra2Δ* background. Yra2p (YKL214c) is homologous to Yra1p, and *YRA2* on an *ARS/CEN* plasmid rescues the nonviable *yra1* null mutant (data not shown). **b**, Synthetic lethality of *yra1-ΔN1* and *yra1-ΔRRM* with *mex67-5* assessed on 5-fluoroorotic acid (5-FOA) plates (5 d at 30 °C). **c**, Synthetic lethality of *yra1-ΔN1* and *yra1-ΔRRM* with *sub2-85*. A *yra1/sub2* double-shuffle strain containing the indicated alleles of *YRA1* and *SUB2* was grown on a 5-FOA plate (5 d at 23 °C).

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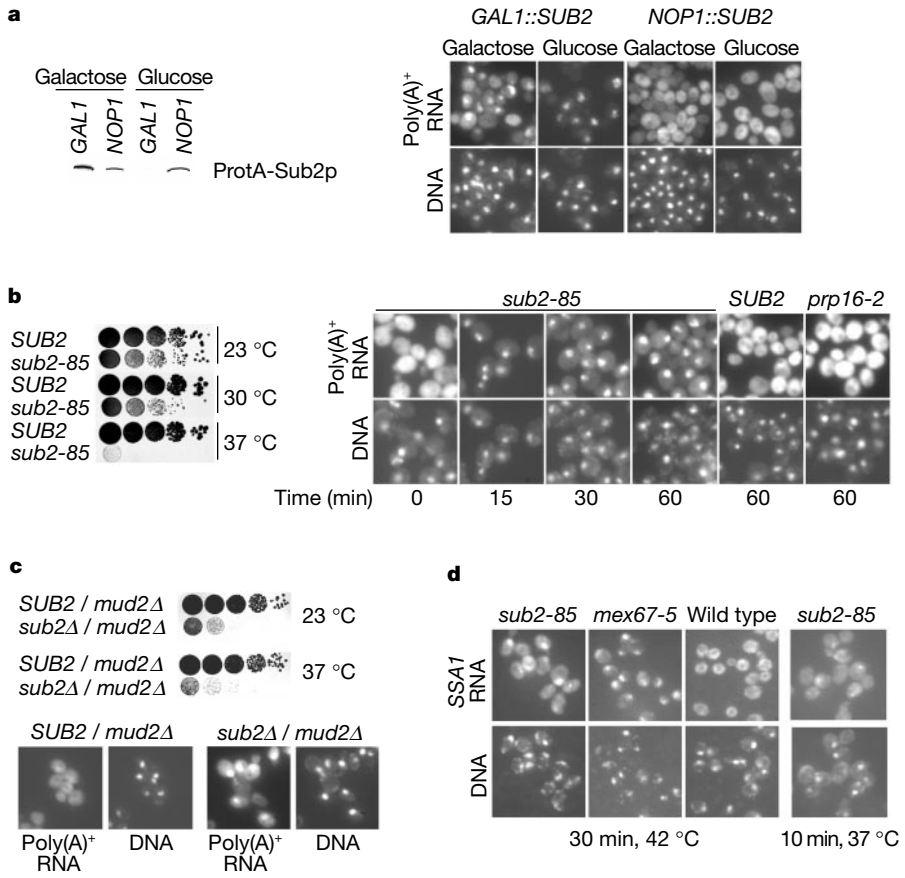

Figure 2 Sub2p is involved in nuclear mRNA export. **a**, Depletion or overexpression of Sub2p leads to an mRNA export defect. Left, western blot showing Sub2p expression. Right, localization of poly(A)$^+$ RNA in *GAL1::SUB2* cells grown in galactose (overexpression) or shifted to glucose medium (10 h depletion). **b**, Left, growth of *SUB2* or *sub2-85* thermosensitive cells at different temperatures. Right, mRNA accumulation in *SUB2*, *sub2-85* and *prp16-2* cells shifted from 23 to 37 °C for the indicated times. **c**, Growth of the *sub2Δ/mud2Δ* and *mud2Δ* strains (top) and localization of poly(A)$^+$ RNA (bottom). **d**, *sub2-85* and *mex67-5* cells were shifted for the indicated periods to the indicated temperatures, and the *SSA1* mRNA (intron-lacking transcript) was localized by *in situ* hybridization.

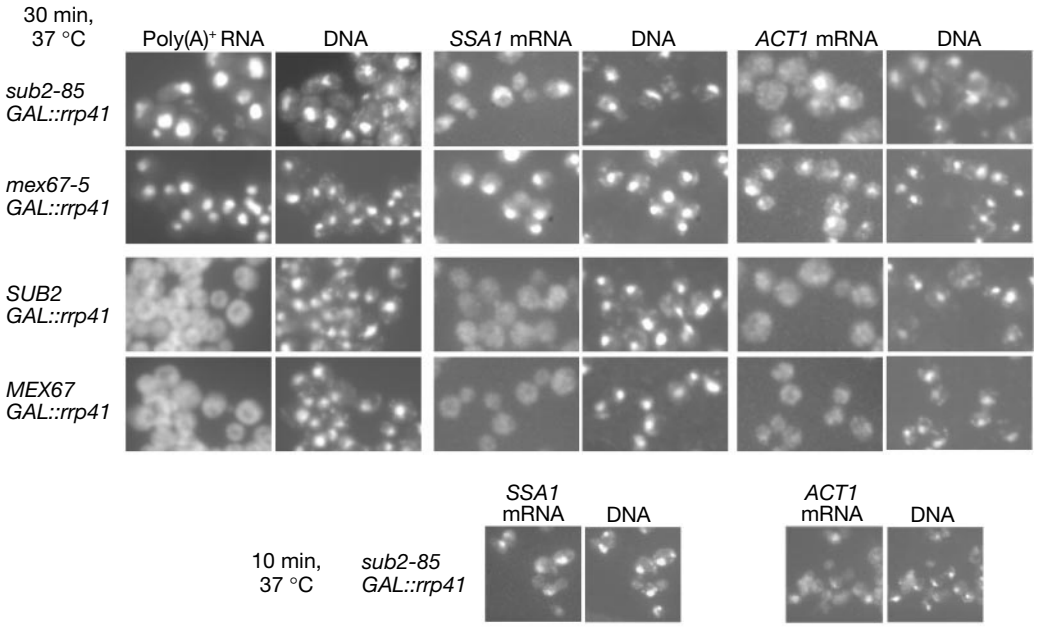

Figure 3 Sub2p is involved in nuclear mRNA export of both intron-containing and intron-lacking mRNAs. *sub2-85/GAL::rrp41*, *mex67-5/GAL::rrp41*, *SUB2/GAL::rrp41* and *MEX67/GAL::rrp41* strains were depleted for Rrp41p (10 h in glucose-containing medium) and shifted for 10 or 30 min to 37 °C. *SSA1* and *ACT1* mRNAs as well as poly(A)$^+$ RNAs were localized by *in situ* hybridization.

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shifting to the restrictive temperature (Fig. 2b, right panel). The fast onset of nuclear accumulation indicates a direct role for Sub2p in mRNA export. In contrast to the *sub2-85* mutant, thermosensitive splicing mutants such as *prp16-1* (Fig. 2b, right panel) or *prp4-2* (data not shown) do not show a significant mRNA export defect. Another nuclear RNA export pathway, transfer RNA export, is not impaired in the *sub2-85* thermosensitive mutant (data not shown). These latter two results, together with the observations that *in vivo* depletion of Sub2p, overexpression of Sub2p, and a thermosensitive mutant of Sub2p all result in nuclear accumulation of mRNA, demonstrate that the splicing factor Sub2p also has a specific function in the export of mRNA.

It was recently shown that deletion of *MUD2* (another splicing factor) can bypass the requirement for Sub2p (ref. 5). Consistent with this study, we found that a *sub2Δ/mud2Δ* double mutant is viable, but grows extremely slowly at physiological temperatures (for example, at 23 and 30 °C) (Fig. 2c, top panel). These data indicate that Sub2p has functions other than releasing Mud2p from the spliceosome. Consistent with the slow-growing phenotype, poly(A)$^+$ RNA export is still impaired in the *subΔ2/mud2Δ* double mutant, although the degree of nuclear mRNA accumulation is slightly less than in the *sub2* thermosensitive mutant (Fig. 2c, bottom panel). We conclude that Sub2p is an important mRNA export factor, the function of which is only partly restored through deletion of *MUD2*.

We next investigated whether Sub2p is preferentially involved in the nuclear export of intron-containing mRNAs, which require splicing before export, or whether it also participates in the export of mRNAs naturally lacking introns. As an example of an mRNA naturally lacking introns, we used the heat shock mRNA (*SSA1*)[7,8]. Notably, nuclear export of *SSA1* mRNA is not only impaired in the *mex67-5* mutant (see also ref. 7), but also in the *sub2-85* thermosensitive mutant (Fig. 2d). We conclude that Sub2p is important in the export of mRNAs lacking introns.

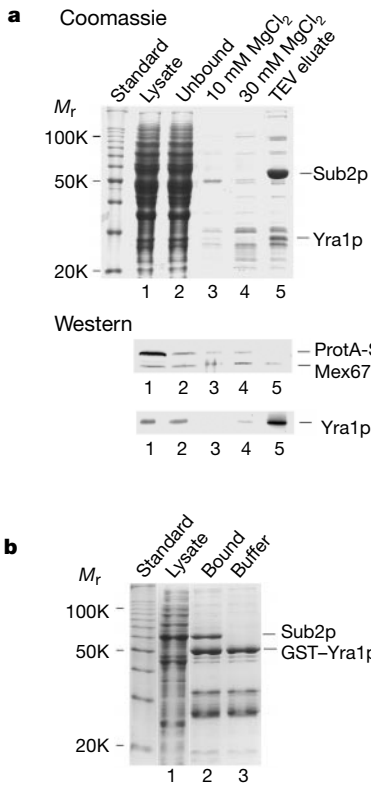

Figure 4 Yra1p interacts with Sub2p *in vivo* and *in vitro*. **a**, ProtA-TEV-Sub2p was affinity purified on IgG Sepharose. Total cell lysate (lane 1), unbound fraction (lane 2), washes with 10 mM $MgCl_2$ (lane 3) and 30 mM $MgCl_2$ (lane 4), and elution with TEV protease (lane 5) were analysed by SDS–PAGE. Coomassie staining (top) and western blotting with the indicated antibodies (bottom) are shown. **b**, *E. coli* lysate containing Sub2p (lane 1) or buffer was incubated with GST–Yra1p. Bound fractions were analysed by SDS–PAGE and Coomassie staining.

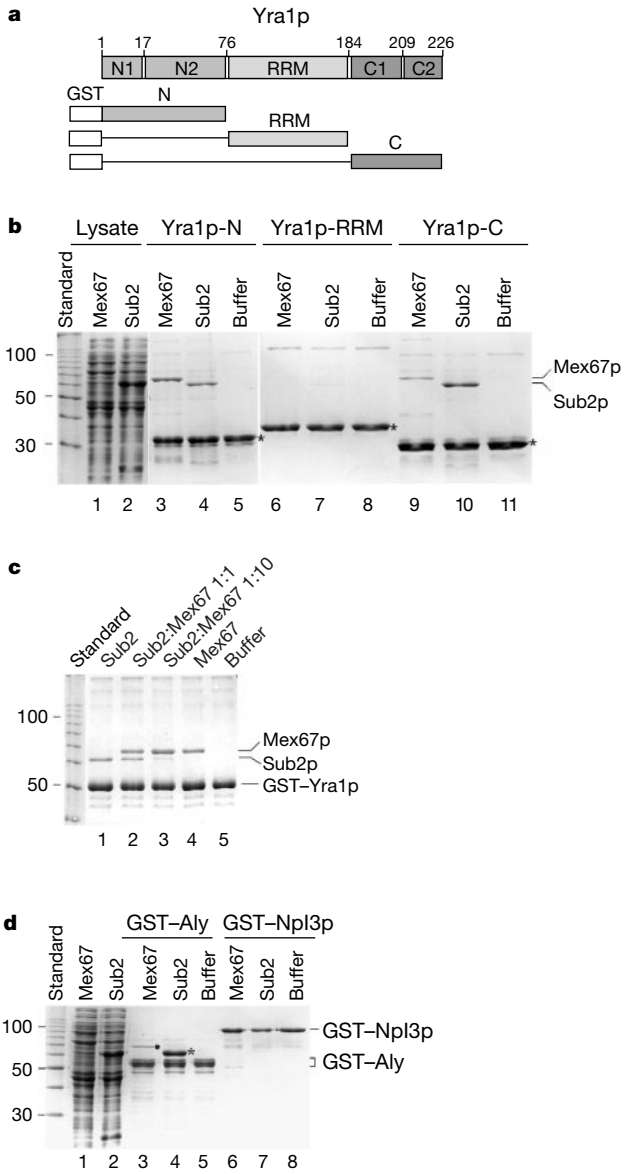

Figure 5 Mex67p/Mtr2p competes with Sub2p for binding to Yra1p. **a**, Domain organization of Yra1p and GST-fusion proteins used for *in vitro* binding. **b**, GST–N-domain (lanes 3–5), GST–RRM-domain (lanes 6–8), or GST–C-domain (lanes 9–11) of Yra1p immobilized on glutathione sepharose beads were incubated with *E. coli* lysate containing Mex67p/Mtr2p (lane 1, input; lanes 3, 6, 9, pull-downs), Sub2p (lane 2, input; lanes 4,7,10, pull-downs), or buffer (lanes 5, 8,11, pull-downs). Asterisks indicate the position of the GST–Yra1p domains. **c**, GST–Yra1p was incubated with *E. coli* lysates containing Sub2p (lane 1), Sub2p plus equimolar (lane 2) or tenfold molar excess of Mex67p/Mtr2p (lane 3), Mex67p/Mtr2p only (lane 4), or buffer only (lane 5). **d**, Human Aly binds to Mex67p/Mtr2p (filled circle) and Sub2p (asterisk) *in vitro*. GST–Aly (lanes 3–5) or GST–Npl3p (lanes 6–8) were incubated with *E. coli* lysate containing Mex67p/Mtr2p (lane 1, input; lanes 3, 6, pull-downs), Sub2p (lane 2, input; lanes 4, 7, pull-downs) or buffer (lanes 5, 8, pull-downs).

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We could not detect nuclear accumulation of an intron-containing mRNA (for example, actin mRNA) using *in situ* hybridization, in either the *mex67-5* or the *sub2-85* thermosensitive mutant (data not shown). This may be due to the lower abundance of actin transcripts compared with *SSA1* mRNAs, which thus remain under the *in situ* detection limit. To assess whether Sub2p and Mex67p are involved in the nuclear export of intron-containing mRNAs, we sought to stabilize intranuclear actin mRNA by slowing down pre-mRNA degradation. Previous studies showed that nuclear turnover of pre-mRNA is delayed in the exosomal *GAL::RRP41* mutant[9]. We therefore repressed expression of the *RRP41* gene (by growth in glucose-containing medium) in the *mex67-5* and *sub2-85* thermosensitive mutants, before shifting to the restrictive temperature. Importantly, wild-type *MEX67* or *SUB2* cells with repressed *GAL::RRP41* do not exhibit nuclear accumulation of poly(A)$^+$, *SSA1* or *ACT1* mRNAs at 37 °C (Fig. 3). In contrast, *ACT1* mRNA, *SSA1* mRNA and poly(A)$^+$ RNA accumulate in the nucleus of *mex67-5* and *sub2-85* thermosensitive cells on shifting to the restrictive temperature with repressed *RRP41* (Fig. 3). We conclude that Mex67p and Sub2p are involved in the nuclear export of both intron-containing and intron-lacking mRNA.

That *YRA1* and *SUB2* interact genetically and are both essential for mRNA export prompted us to look for a physical interaction between these two proteins. First, we tested whether Yra1p is in a complex with Sub2p *in vivo*. We affinity purified functional ProtA-tagged Sub2p from yeast. As shown in Fig. 4a, Sub2p is efficiently purified from yeast cells together with Yra1p. In contrast, Mex67p is not enriched in the eluate (Fig. 4a, Mex67p, compare lanes 1 and 5). We conclude that Yra1p specifically interacts with Sub2p *in vivo*.

We next tested whether the interaction between Sub2p and Yra1p is direct. Glutathione *S*-transferase (GST)-tagged full-length Yra1p was immobilized on beads and incubated with an *Escherichia coli* lysate containing Sub2p. As shown in Fig. 4b (lane 2), Sub2p binds to GST–Yra1p, whereas the other *E. coli* proteins in the lysate do not. Furthermore, this interaction between Sub2p and Yra1p is not RNA dependent, as the *E. coli* lysate was incubated with RNase before being added to GST–Yra1p. Thus, Yra1p and Sub2p interact directly both *in vivo* and *in vitro*.

Previous studies showed that Yra1p also binds directly to the Mex67p/Mtr2p complex[2,10]. Therefore, we sought to identify the domains of Yra1p that interact with Sub2p or Mex67p/Mtr2p. We expressed GST-fusion proteins containing the N domain, the RRM domain, or the C domain of Yra1p (see Fig. 5a) and tested them for binding to either the Mex67p/Mtr2p complex or Sub2p. Significantly, both Mex67p/Mtr2p and Sub2p bind to the N domain (Fig. 5b, lanes 3 and 4) and the C domain of Yra1p (Fig. 5b, lanes 9 and 10). In contrast, the RRM domain does not bind to either protein (Fig. 5b, lanes 6 and 7). We conclude that Sub2p and Mex67p/Mtr2p bind to the same regions of Yra1p.

That Sub2p and Mex67p interact with the same regions of Yra1p raised the possibility that there is a temporal order of binding to Yra1p. Specifically, Sub2p may bind to Yra1p first and then be replaced by Mex67p. To test this possibility, we carried out a competition assay. An *E. coli* lysate containing Sub2p was mixed with an equimolar amount or a tenfold excess of purified Mex67p/Mtr2p complex and added to GST–Yra1p on beads. Mex67p competed with Sub2p for binding to Yra1p when Mex67p and Sub2p were present in equimolar amounts (Fig. 5c, lane 2). When Mex67p is present in a tenfold excess over Sub2p, Mex67p efficiently displaces Sub2p from Yra1p (Fig. 5c, lane 3). These competition data, together with the finding that Sub2p and Mex67p interact with the same regions of Yra1p, suggest that Sub2p and Mex67p/Mtr2p may bind to Yra1p sequentially. A possible implication of these data is that binding of the Mex67p/Mtr2p complex to Yra1p might release Sub2p from Yra1p before mRNA export.

To determine whether the interaction of Yra1p with Mex67p/Mtr2p or with Sub2p is conserved, we used GST-tagged Aly, the metazoan homologue of Yra1p, for interaction assays (Fig. 5d). Significantly, GST–Aly binds to both Mex67p/Mtr2p and Sub2p (Fig. 5d, lanes 3 and 4). In contrast, GST–Npl3p, which was used as a negative control, does not bind to either protein (Fig. 5d, lanes 6 and 7). We conclude that the interaction between Yra1p and Sub2p, as well as the interaction between Yra1p and Mex67p/Mtr2p, is conserved.

We have shown that Yra1p, a conserved mRNA export factor, interacts with the conserved splicing factor Sub2p both genetically and physically. Thus, a splicing-coupled mechanism of mRNA export may also exist in *Saccharomyces cerevisiae*, in which about 40% of the total amount of transcripts derived from RNA polymerase II contain introns[11]. In an accompanying paper[18], evidence is provided that the mammalian homologue of Sub2p, UAP56, recruits the Yra1p counterpart Aly to the spliced mRNP, the complex that targets mRNA for export[12]. Our findings show that Sub2p has a specific role in nuclear mRNA export. In addition, Sub2p and Mex67p compete for binding to Yra1p. Thus, Mex67p may replace Sub2p and then target the mRNP to the nuclear pores by direct interaction of the Mex67p/Mtr2p complex with nucleoporins. Together, our data suggest a model in which Sub2p is involved in recruiting Yra1p to mRNPs. Apparently, Sub2p functions in nuclear export of both intron-containing and intron-lacking mRNAs. There is a precedent for splicing factors being involved in nuclear export of mRNAs lacking introns in metazoans[13]. In this case, members of the SR protein family are recruited by a specific element in the exon. The mechanism by which Sub2p is recruited to mRNAs lacking introns remains to be determined. However, Aly was implicated in transcriptional co-activation[14] and suggested to act as a nuclear protein chaperone that stimulates transcriptional activity of bZIP proteins[15]. Therefore, the interaction between Sub2p/UAP56 and Yra1p/Aly could likewise mediate the export of intron-lacking transcripts. □

Methods

Yeast strains

Yeast strains used in this study are provided in the Supplementary Information. Microbiological and yeast techniques were done essentially as described[2]. Plasmids pUN100-*MEX67*, pUN100-*mex67-5*, pRS314-*YRA1* and L20GST-*YRA1* were reported previously[1,2]. The *sub2Δ/yra1Δ* and *sub2Δ/mud2Δ* double-disruption strains were constructed by mating the *sub2* shuffle strain to the *yra1* shuffle or the *mud2* disruption strain, respectively, and dissecting the tetrads. The *sub2Δ/GAL::RRP41* and *mex67Δ/GAL::RRP41* double-mutant strains were constructed by mating the *sub2* or *mex67* shuffle strain to the *GAL::RRP41* strain[9] and dissecting the tetrads.

Plasmid construction

Plasmids pRS314-*yra1-ΔN1* and pRS314-*yra1-ΔRRM* were constructed by mutagenic polymerase chain reaction (PCR). Plasmid pHT4467-*YRA1*, used for the synthetically lethal screen, was cloned by subcloning intron-lacking *YRA1* into plasmid pHT4467 (ref. 1). Plasmids L20GST-*YRA1-N*, *-M* and *-C* were cloned by amplification of the corresponding base pairs by PCR and insertion into vector L20GST. For recombinant, HIS-tagged Sub2p the open reading frame of *SUB2* was amplified by PCR and cloned into pET9d-HIS6-TB and expressed in BL21. The ProtA-TEV-tagged version of Sub2p was constructed by amplification of the *SUB2* open reading frame and cloning into pNOPPATA1L (ref. 16) (*NOP1* promoter) or pGALPATG1L (*GAL1* promoter). The *sub2-85* mutant was obtained by mutagenic PCR as described[2].

Protein and mRNA analyses

For affinity purification of ProtA-TEV-Sub2p, cells were lysed with glass beads, proteins extracted with buffer (20 mM HEPES at pH 7.0, 100 mM potassium acetate, 2 mM magnesium acetate, 0.1% Tween 20, 5 mM β-mercaptoethanol, protease inhibitors), and the supernatant incubated with immunoglobulin-γ (IgG) Sepharose. The beads were washed with buffer, buffer containing 10 mM and 30 mM $MgCl_2$, and bound proteins eluted by cleavage with TEV protease in a final concentration of 100 mM $MgCl_2$. The synthetically lethal screen, the poly(A)$^+$ *in situ* hybridization, protein expression in *S. pombe*, and the *in vitro* binding assay were performed as described[2]. *In situ* hybridization using oligonucleotides specific for *SSA1* and *ACT1* mRNAs was performed according to ref. 7. Oligonucleotides specific for *SSA1* RNA were previously described[7]. *ACT1* mRNA was detected with a mixture of 5′-Cy3-labelled oligonucleotides as indicated in the Supplementary Information. SDS–PAGE and western blot analyses were performed as described[17].

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Correspondence and requests for materials should be addressed to E.H. (e-mail: cg5@ix.urz.uni-heidelberg.de).

corrections

A mouse knock-in model exposes sequential proteolytic pathways that regulate p27^{Kip1} in G1 and S phase

Nisar P. Malek, Holly Sundberg, Seth McGrew, Keiko Nakayama, Themis R. Kyriakides & James M. Roberts

Nature **413,** 323–327 (2001).

In this Letter, the last name of Themis R. Kyriakides was misspelled as 'Kyriakidis'. □

CREB regulates hepatic gluconeogenesis through the coactivator PGC-1

Stephan Herzig, Fanxin Long, Ulupi S. Jhala, Susan Hedrick, Rebecca Quinn, Anton Bauer, Dorothea Rudolph, Gunther Schutz, Cliff Yoon‖, Pere Puigserver, Bruce Spiegelman & Marc Montminy

Nature **413,** 179–183 (2001).

In the third sentence, "hyperglycaemia" should have read "hypoglycaemia". The corrected sentence is: "Here we show that mice carrying a targeted disruption of the cyclic AMP (cAMP) response element binding (CREB) protein gene, or overexpressing a dominant-negative CREB inhibitor, exhibit fasting hypoglycaemia and reduced expression of gluconeogenic enzymes." □

erratum

A titanosilicate molecular sieve with adjustable pores for size-selective adsorption of molecules

Steven M. Kuznicki, Valerie A. Bell, Sankar Nair, Hugh W. Hillhouse, Richard M. Jacubinas, Carola M. Braunbarth, Brian H. Toby & Michael Tsapatsis

Nature **412,** 720–724 (2001).

Correspondence and requests for materials relating to the structural analysis in this Letter should be addressed to Michael Tsapatsis (e-mail: tsapatsi@ecs.umass.edu). □

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