MATERIALS AND METHODS

1. Strains and oligonucleotides

Tab. 1: *E. coli* strains

Name	Genotype	Usage
MC1066	galU galK strAr $hsdR^-$ delta(lacIPOZYA)X74 trpC9830 leuB6 pyrF74::Tn5(km^r)	general cloning and storage
SC305	thr^- leu^- dcm^- his^- $galK^-$ $lacY^-$ $xy1^-$ $mt1^-$ araC+B+A+D+ str	dcm^- strain

Tab. 2: *S. cerevisiae* strains

Name	Genotype	Usage / Reference
BSY320	$MATa/\alpha$ trp_{1-289}/trp_{1-289} ura_{3-52}/ura_{3-52} arg_4/arg_4 $leu_{2-3,112}/leu_{2-3,112}$ ade_2/ade_2	general
MGD453-13D	$MATa$ trp_{1-289} ura_{3-52} arg_4 $leu_{2-3,112}$ ade_2	general; (Séraphin *et al.*, 1988)
YDL401	$MATa$ $his_{3\Delta 200}$ gal_2 $gal_{\Delta log}$ $leu_{2\Delta 1}$ trp_1 ura_{3-52}	N-terminal tagging; (Lafontaine and Tollervey, 1996)
FY1679	$MATa/\alpha$ ura_{3-52}/ura_{3-52} $leu_{2\Delta 1}/+$ $trp_{1\Delta 63}/+$ $his_{3\Delta 200}/+$	genomic DNA for PCR, genome sequenced (Goffeau *et al.*, 1996)

Tab. 3: Oligonucleotides

Name	Sequence (5'-3')
BR1	TTTTATTTGTGTTGAAGG
BR2	TGAAATTACCGATGAGAA
BR3	ACCTACACCTCCGGGACTGCAAGGGCCTCCCGGATTAAAGCTGGAGCTCAAAAC
BR7	AAGCGAAAATGTGGATGAAGA
BR8	TTTCACCAAGCGGAAATTTTCAAAGCAGAATTTTTTCCCTAATTTCTCTTGGCCTCC TCTAGT
BR12	CGTATGCTTCAAAAAGTTG
BR28	AACAAACCTACACCTCCGGGACTGCAAGGGCCTCCCGGATTATCCATGGAAAAGAGA AG
BR29	GATGAGGACGACTTTGACATGATGGAAGCAACCCAACTTTCCATGGAAAAGAGAAG
BR31	CTTTTCTATTTTCAAAGTATCTAGAGTTAATCCTTCTAAAACTCATATTCGCGTCTA CTTTCGG

BR33	GCTACAAGTGCTATTCTATCCCACAATAAAAGGAAATTGTTGATGAAGCTGGAGCTCAAAAC
BR34	TACTGGGCCCTCGAGCCTTCTTGTATACAG
BR35	ACGTACGTCGACAAATTAGGGAAAAAATTC
BR36	TGACTAGTCGACAAGTGCTATTCTATC
BR37	ATACGGATCCAAGCTTTGAATAAAGGTAAG
BR39	TTAAAGGAGGTTCCGTCG
BR41	GTTGAGATAGCCCTAAAG
BR46	CGCCAATACAGAGCAATG
BR48	TTTCTACAGCCATACCTT
BR49	CAAGTGCTATTCTATCCC
BR50	TGTTAAACTTGGCTTGTG
BR51	CCACTCAGCTATTAGATT
BR52	GATGCTATTGTGAAGGAG
BR57	AAGACCGAATATACTTTTTATATTACATCAATCATTGTCATTATCAAATGAAGCTGGAGCTCAAAAC
BR64	AGTGGGGCCCTAACTAGGGAAC
BR65	AGTGGGGCCCTAACTGGGGAAC
BR66	TTTACCTTGGGCATCATAAACTGGTGGCGGAGAAGGTGACCTATTTTTCCT
BR67	TTTACCTTGGGCATCATAAACTGGTGGCGGAGAAGGTGACCTACTTTTCCT
BR77	AGTGGGGCCCTAACTGGGGAACAAATATACTCCTATCAAGTCATGTT
BR78	AGTGGGGCCCTAACTGGGGAACAAATATACTCCTATCAAGCCATGTT
ML50	AACTGCAGTAGAGCGAAATTAAGTA
ML51	GAAGCACCCAATTTAAGAC
ML52	GACGACTTTGACATGATGGAAGCAACCCAACTTTCCGAGCTCAAAACCGCGGCTC
GR4	GATCCATGGAAAAGAGAAGATGGAAAAAG
EM133	AUCUCUGUAUUGUUUCAAAUUGACCAA
OF2	GTCATTCATCCAGCAGG
FC6.2	TCGGTATATACGTGCTGCTATGAATAGAGCGTTAAGCCAGGAACGATCATTCACTA

2. Molecular biology techniques

2.1. General methods

Standard recombinant DNA techniques (growth and handling of *E. coli*, preparation and transformation of competent *E. coli* cells, restriction enzyme digestions, ligations) were performed as described previously (Sambrook *et al*., 1989). Medium scale preparations of plasmid DNA were performed with the Plasmid Midi Kit (Qiagen). Restriction enzymes

were purchased from New England Biolabs if not indicated otherwise. PCR was performed using either the Perkin Elmer AmpliTaq DNA polymerase or the New England Biolabs Vent DNA polymerase under conditions recommended by the manufacturers. Sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977), on double stranded plasmids, using either the Pharmacia T7 sequencing kit (^{35}S labeling) or the Pharmacia AutoRead^{TM} sequencing kit (fluorescent labeling). Automated sequencing was done on the ALF-system (Pharmacia).

2.2. Construction of the TAP-cassette for chromosomal tagging

The CBP-tag fused to a TEV-cleavage site was PCR amplified from plasmid pGR1 (gift of G. Rigault) with the primer GR4 and the standard T7 primer using Vent polymerase (NEB). The resulting PCR product was cleaved with *Sac*I at a site present immediately downstream of the TEV cleavage site and inserted together with a *Sac*I/*Hind*III fragment containing two IgG binding modules of the *Staphylococcus aureus* protein A from plasmid pBS1365 (Puig *et al.*, 1998) into the *Ecl136*II (blunt) and *Hind*III sites of plasmid pBS1408 (gift of E. Bragado-Nilsson). The resulting plasmid (pBS1479) was checked by restriction digest and sequencing of the inserted PCR fragment. The protein tag present in this plasmid (CBP-TEV-ProtA) was named the Tandem affinity purification (TAP)-tag (Figure 45; Rigaut *et al.*, 1999).

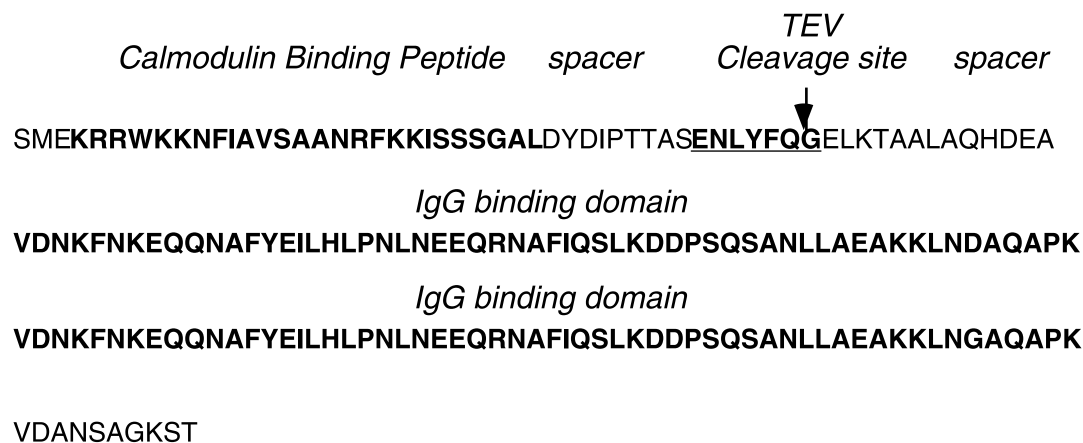

Figure 45. Sequence and structure of the TAP tag (Rigaut *et al.*, 1999)

The various domains constituting the TAP tag are indicated.

2.3. Construction of *MSL5* yeast expression vectors

The *MSL5* gene (promoter and open-reading frame) was amplified from genomic DNA by PCR using the primers BR34 and BR37. The PCR product was digested with *Xho*I and *BamH*I and cloned into the corresponding sites of the yeast shuttle vector pRS416 (Sikorski and Hieter, 1989), which contains a *URA3* marker. The resulting plasmid was named pBS1703.

2.4. Construction of *msl5* mutant library

The open-reading frame of *MSL5* was amplified by PCR from genomic DNA with the primers BR36 and BR37. The amplified fragment was cloned into the *Sal*I and *Hind*III sites of pBluescript SK(-) (Stratagene) and used as a template for the generation of the mutant library (pBS1743). The mutagenic PCR was performed on plasmid pBS1743 with the T3 and T7 primers adapting a method described previously (Cadwell and Joyce, 1992). The dNTP concentration was changed to 2 mM dGTP, 2 mM dATP, 10 mM dTTP, 10 mM dCTP, $MnCl_2$ was added to a final concentration of 0.5 mM and the $MgCl_2$ concentration was increased to 4 mM. The concentration of enzyme (Amplitaq, Perkin-Elmer) was increased to 5 U per 25 µl reaction and 30 amplification cycles were performed with an annealing temperature of 50 °C.

The *MSL5* promoter was amplified with the primers BR34 and BR35 from genomic DNA and cloned into the *Bsp120*I and *Sal*I sites of yeast shuttle vector pRS415 which contains a *LEU2* marker (Sikorski and Hieter, 1989) resulting in plasmid pBS1702. The mutagenic PCR was digested with *Sal*I and *BamH*I and inserted behind the *MSL5* promoter in pBS1702. Eight independent clones were partially sequenced with an automated sequencer (ALF-system, Pharmacia) to estimate the mutation frequency. About 13000 independent clones were collected and used for the preparation of the mutant library pBS1780. The wild type *MSL5* ORF was amplified by PCR with the primers BR36 and BR37 from genomic yeast DNA, digested with *Sal*I and *Hind*III and inserted behind the *MSL5* promoter in pBS1702, yielding plasmid pBS1759.

Tab. 4: Plasmids for generation of conditional msl5-mutants

Plasmid	Name	Yeast Marker
pBS1743	*MSL5*-ORF in pBluescript SK(-)	-
pBS1702	*MSL5*-promoter	*LEU2*
pBS1703	*MSL5*-promoter+*MSL5*-ORF	*URA3*
pBS1759	*MSL5*-promoter+ *MSL5*-ORF	*LEU2*
pBS1780	*MSL5*-promoter+mutant-*msl5*-ORF-library	*LEU2*
pBS1810	*MSL5*-promoter+*msl5-1*-ORF	*LEU2*
pBS1811	*MSL5*-promoter+*msl5-2*-ORF	*LEU2*

pBS1812	*MSL5*-promoter+*msl5-3*-ORF	*LEU2*
pBS1813	*MSL5*-promoter+*msl5-4*-ORF	*LEU2*
pBS1814	*MSL5*-promoter+*msl5-5*-ORF	*LEU2*
pBS1815	*MSL5*-promoter+*msl5-7*-ORF	*LEU2*
pBS1816	*MSL5*-promoter+*msl5-8*-ORF	*LEU2*
pBS1817	*MSL5*-promoter+*msl5-9*-ORF	*LEU2*
pBS1818	*MSL5*-promoter+*msl5-10*-ORF	*LEU2*
pBS1819	*MSL5*-promoter+*msl5-11*-ORF	*LEU2*
pBS1820	*MSL5*-promoter+*msl5-12*-ORF	*LEU2*
pBS1821	*MSL5*-promoter+*msl5-13*-ORF	*LEU2*
pBS1822	*MSL5*-promoter+*msl5-14*-ORF	*LEU2*

2.5. Mapping relevant mutations in thermo- and cold-sensitive *msl5* mutants

The plasmids harbouring the mutants *msl5-2* and *msl5-5* were sequenced with four primers (BR39, BR41, BR46, BR49) on an automatic sequencer (ALF-system, Pharmacia). The restriction enzymes *Sal*I, *BsmA*I, *Mva1269*I (MBI), *Bgl*II and *BamH*I were used to split the ORF into 4 domains labeled N, KH, Zn and C (in this order from C-terminus to N-terminus of the corresponding protein). Standard cloning techniques were used to exchange each of these domains or combinations thereof with the corresponding domains of a plasmid containing the *MSL5* wild type ORF (pBS1759). The resulting plasmids were tested by the shuffling strategy described below (see 3.12.) for their ability to confer the temperature sensitive phenotype to yeast strain BSY809. The smallest mutant fragment still showing a mutant phenotype was used as basis for the next mapping step. For *msl5-5* the two mutations present in the Zn-domain were separated using the restriction enzyme *BspE*I resulting in a final construct that contained one mutation in the KH-domain and one mutation in the Zn-domain. For *msl5-2* the N-domain was further dissected by the use of the restriction enzymes *Bsp120*I (MBI) and *Sty*I. This resulted in a construct containing 4 mutations in the N-domain (between *Bsp120*I and *Sty*I) and one mutation in the KH-domain. The 4 mutations were further dissected by insertion of products of PCR with mutagenic (BR65, BR67, BR77, BR78) or wild type (BR64, BR66) primers on mutant (*msl5-2*) or wild type templates between the *Bsp120*I (MBI) and *Sty*I sites. This yielded a final construct containing 2 mutations in the N-domain and one mutation in the KH-domain. Constructs were checked by restriction sites overlapping with mutations to ensure the presence or absence of a mutant fragment. The PCR generated constructs were checked by sequencing. A point mutation (T437→K) was detected which was present in all mutants and the original wild type construct. To remove this point mutation the corresponding region was exchanged with the corresponding region from a correct wild type construct

(pBS1703). Only the most relevant constructs were repaired in the same manner (indicated by “rep.” behind the name) and tested again for temperature sensitive phenotypes.

Tab. 5: Plasmids for mapping of relevant mutations

Plasmid	Name	Construction and Remarks
pBS1883	msl5-5 N+KH	#1814 (SalI-Mva1269I) in #1759 (SalI-Mva1269I)
pBS1884	msl5-5 N+KH+Zn	#1814 (SalI-BglII) in #1759 (SalI-BglII)
pBS1891	msl5-2 N+KH	#1811 (SalI-Mva1269I) in #1759 (SalI-Mva1269I)
pBS1892	msl5-2 N+KH+Zn	#1811 (SalI-BglII) in #1759 (SalI-BglII)
pBS1895	msl5-2 Zn+C	#1811 (Mva1269I-BamHI) in #1759 (Mva1269I-BamHI)
pBS1896	msl5-5 KH	#1759 (SalI-BsmAI) + #1814 (BsmAI-Mva1269I) in #1759 (SalI-Mva1269I)
pBS1910	msl5-2 N	#1811 (SalI-BsmAI) + #1759 (BsmAI-Mva1269I) in #1759 (SalI-Mva1269I)
pBS1911	msl5-2 KH	#1759 (SalI-BsmAI) + #1811 (BsmAI-Mva1269I) in #1759 (SalI-Mva1269I)
pBS1912	msl5-5 Zn	#1814 (Mva1269I-BglII) in #1759 (Mva1269I-BglII)
pBS1975	msl5-5 KH+Zn	#1912 (Mva1269I-BglII) in #1896 (Mva1269I-BglII)
pBS1976	msl5-5 N+Zn	#1814 (SalI-BsmAI) + #1759 (BsmAI-Mva1269I) in #1912 (SalI-Mva1269I)
pBS1988	msl5-5 KH+Zn(n1)	#1814 (Mva1269I-BspEI) + #1759 (BspEI-BglII) in #1896 (Mva1269I-BglII)
pBS1989	msl5-5 KH+Zn(c1)	#1759 (Mva1269I-BspEI) + #1814 (BspEI-BglII) in #1896 (Mva1269I-BglII)
pBS1990	msl5-2 N(n2)+KH	#1811 (SalI-Bsp120I) + #1911 (Bsp120I-Mva1269I) in #1759 (SalI-Mva1269I)
pBS1991	msl5-2 N(c6)+KH	#1759 (SalI-Bsp120I) + #1811 (Bsp120I-Mva1269I) in #1759 (SalI-Mva1269I)
pBS1992	msl5-2 N(n6)+KH	#1811 (SalI-StyI) + #1911 (StyI-Mva1269I) in #1759 (SalI-Mva1269I)
pBS1993	msl5-2 N(c2)+KH	#1759 (SalI-StyI) + #1811 (StyI-Mva1269I) in #1759 (SalI-Mva1269I)
pBS1995	MSL5-WT variant.	#1759 (partial digest Bsp120I, Klenow, religated), Bsp120I site upstream of ORF destroyed
no stock	msl5-2 N(#1)+KH	#1911 (StyI+SacI) + PCR (BR65 + BR66 on #1759, Bsp120I+StyI) in #1995 (Bsp120I+SacI)
no stock	msl5-2 N(#2+3)+KH	#1911 (StyI+SacI) + PCR (BR64 + BR66 on #1811, Bsp120I+StyI) in #1995 (Bsp120I+SacI)
no stock	msl5-2 N(#2+3+4) +KH	#1911 (StyI+SacI) + PCR (BR64 + BR67 on #1811, Bsp120I+StyI) in #1995 (Bsp120I+SacI)
no stock	msl5-2 N(#1+4)+KH	#1911 (StyI+SacI) + PCR (BR65 + BR67 on #1759, Bsp120I+StyI) in #1995 (Bsp120I+SacI)
no stock	msl5-2 N(#4)+KH	#1911 (StyI+SacI) + PCR (BR64 + BR67 on #1759, Bsp120I+StyI) in #1995 (Bsp120I+SacI)
pBS2012	msl5-2 N(#1+2+3) +KH	#1911 (StyI+SacI) + PCR (BR65 + BR66 on #1811, Bsp120I+StyI) in #1995 (Bsp120I+SacI)

pBS2013	msl5-2 N(m4)+KH	#1992 (Bsp120I+SacI) in #1995 (Bsp120I+SacI)
no stock	msl5-2 N(#1+2)+KH	#1911 (StyI+SacI) + PCR (BR66 + BR78 on #1759, Bsp120I+StyI) in #1995 (Bsp120I+SacI)
pBS2078	msl5-2 N(#1+3)+KH	#1911 (StyI+SacI) + PCR (BR66 + BR77 on #1811, Bsp120I+StyI) in #1995 (Bsp120I+SacI)
pBS2082	MSL5-WT rep.	#1703 (BglII+SacI) in #1759 (BglII+SacI)
pBS2083	msl5-2 N+KH rep.	#1811 (SalI-Mva1269I) in #1759 (SalI-Mva1269I)
pBS2084	msl5-2 N(n6)+KH rep.	#1811 (SalI-StyI) + #1911 (StyI-Mva1269I) in #1759 (SalI-Mva1269I)
pBS2085	msl5-2 N(#1+2+3) +KH rep.	#1911 (StyI+SacI) + PCR (BR65 + BR66 on #1811, Bsp120I+StyI) in #1995 (Bsp120I+SacI)
pBS2086	msl5-2 N(#1+3)+KH rep.	PCR (BR66+BR77 on #1811, Bsp120I-StyI) + #1911 (StyI-SacI) in #1759 (SalI-SacI)
pBS2087	msl5-5 N+KH+Zn rep.	#1814 (SalI-BglII) in #1759 (SalI-BglII)
pBS2088	msl5-5 KH+Zn(c1) rep.	#1759 (Mva1269I-BspEI) + #1814 (BspEI-BglII) in #1896 (Mva1269I-BglII)

3. Yeast techniques

3.1. General methods

Standard yeast techniques (growth, handling, mating, sporulation, dissection of tetrads etc.) were performed essentially as described previously (Sherman *et al.*, 1983; Guthrie and Fink, 1991). YP (2 % bactopeptone, 1 % yeast extract), supplemented with 2 % of the appropriate sugar (glucose, galactose, raffinose etc.) was used as complete medium. Yeast selective media contained 0.67 % yeast nitrogen base without amino acids (Difco) and 2 % of the appropriate sugar and were supplemented with the required amino acids.

3.2. Growth analysis

Strains were grown in the appropriate medium at the desired temperature. The OD_{600} was measured in regular intervalls and medium was replaced when necessary to keep the cells growing in log phase. A cumulative OD_{calc} was calculated by an iterative procedure: $OD_{calct2}=k*(OD_{600t2}/OD_{600t1})*OD_{calct1}$. OD_{600t1} was measured at time t_1, before dilution of the culture, OD_{600t2} was measured at time t_2, k is the factor by which the culture was diluted

at the beginning of the growth period and OD_{calct1} is the cumulative OD_{calc} calculated for the previous growth period.

3.3. Gene disruption

MSL5 and *UPF1* were disrupted as described previously following strategy B (Figure 46, Puig *et al*., 1998). The gene of interest was replaced by the *Kluyveromyces lactis TRP1*-marker and the two IgG binding modules of the *Staphylococcus aureus* protein A (ProtA-KL-*TRP1*) in either a haploid (MGD453-13D for *UPF1*) or a diploid (BSY320 for *MSL5*) wild-type strain. A region of about 500 nt immediately downstream of the open reading frame of the *MSL5* or the *UPF1* ORF were amplified from genomic DNA using primers BR1 and BR2 (*MSL5*) or primers BR48 and BR50 (*UPF1*). This PCR fragment was ligated to the *Xho*I cut and Klenow-fragment treated vector pBS1408 (Tab. 5) by blunt end ligation. The ligation product was directly used in a second PCR with primers BR2 and BR3 (*MSL5*) or BR48 and BR57 (*UPF1*). The resulting fragment was transformed into strain MGD453-13D or BSY320 and transformants were selected on SD-Trp plates. Colonies were screened by PCR (see 3.6.). The diploid strain containing the *MSL5* disruption (BSY883) was transformed with a plasmid containing the wild type *MSL5* gene under its endogenous promoter (pBS1703) followed by sporulation and dissection of tetrads. Cells derived from single spores were tested for the disruption by PCR. Markers (auxotroph and mating type) were tested by replica plating. This yielded the strains BSY809 (Mat**a**) and BSY810 (Matα).

Tab. 6: Plasmids used for disruption and tagging

Plasmid	Tag-marker-cassette	Construction/Reference
pBS1173	ProtA-*TRP1*	Puig *et al*., 1998
pBS1365	ProtA-*KL-URA3*	Puig *et al*., 1998
pBS1408	ProtA-*KL-TRP1*	gift of E. Bragado-Nilsson
pBS1479	CBP-TEV-ProtA-*KL-TRP1* (TAP-tag)	see 2.2.
pBS1539	CBP-TEV-ProtA-*KL-URA3* (TAP-tag)	gift of E. Bragado-Nilsson
pTL27	GAL-ProtA-*HIS3*	Lafontaine and Tollervey, 1996

Tab. 7: Strains containing gene disruptions

Name	Number	Mating type	Marker for disruption	Construction	Analysis
*upf1*Δ	BSY879	Mat**a**	*K.l. TRP1*	Strategy B (BR48, BR50, BR57, pBS1408) in MGD453-13D	PCR (OF2+BR52; FC6.2+BR51)
msl5-Δ diploid	BSY806	Matα /**a**	*K.l. TRP1*	Strategy B (BR1, BR2, BR3, pBS1408) in BSY320	PCR (BR34+OF2; BR34+BR7)

msl5-Δ **a**	BSY809	Mat**a**	*K.l. TRP1*	dissection of BSY806	PCR (BR34+OF2; BR34+BR7)
msl5-Δ α	BSY810	Matα	*K.l. TRP1*	dissection of BSY806	PCR (BR34+OF2; BR34+BR7)
msl5-Δ+*upf1*-Δ diploid	BSY883	Matα /**a**	*K.l. TRP1*	mating of BSY879 and BSY810	Markers
msl5-Δ+ *upf1*-Δ	BSY884	Matα	*K.l. TRP1*	sporulation and dissection of BSY883	PCR (BR34+OF2; BR52+OF2)
mud2-Δ	BSY518	Mat**a**	*TRP1*	in MGD453-13D (gift of B. G. M. Luukkonen)	-
lea1-Δ	BSY625	Mat**a**	*K.l. URA3*	in MGD453-13D (gift of F. Caspary)	-
nam8-Δ	BSY644	Mat**a**	*K.l. URA*	in MGD453-13D (gift of O. Puig)	-
lea1-Δ+*upf1*-Δ	BSY971	Mat**a**	*K.l. URA + K.l. TRP1*	mating of BSY886 and BSY625, sporulation, dissection	Markers + PCR (BR52+OF2)
nam8-Δ+*upf1*-Δ	BSY973	Mat**a**	*K.l. URA + K.l. TRP1*	mating of BSY886 and BSY644, sporulation, dissection	Markers + PCR (BR52+OF2)
mud2-Δ+*upf1*-Δ	BSY969	Mat**a**	*K.l. TRP1 + TRP1*	mating of BSY886 and BSY518, sporulation, dissection	Markers + PCR (BR52+OF2; BR11+BR12)

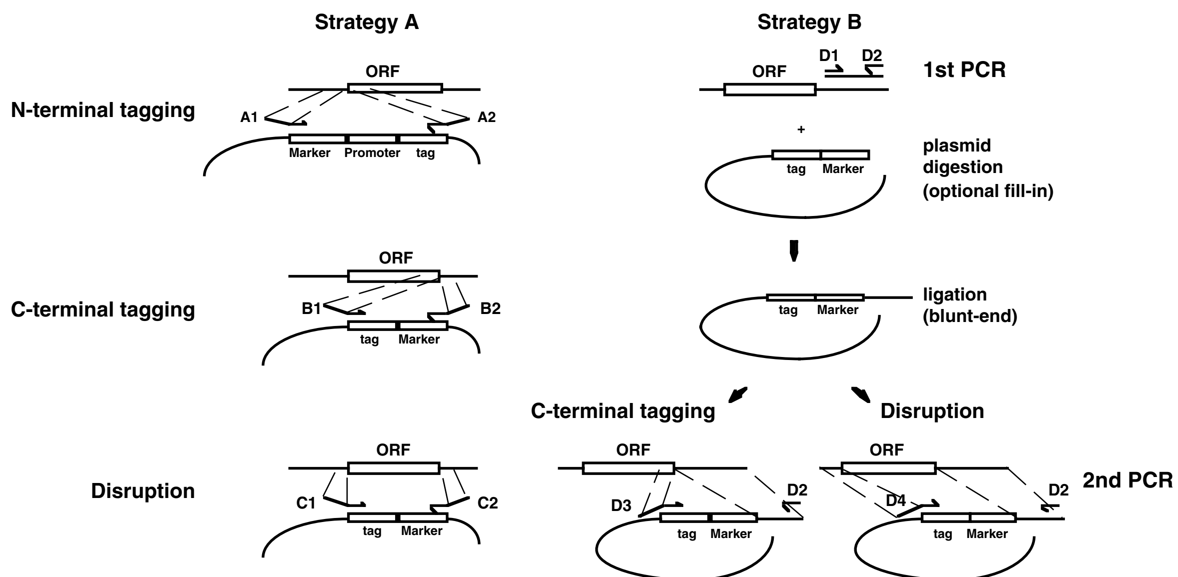

Figure 46. Genomic tagging and disruption of ORFs in the yeast genome

The two strategies used for tagging and disruption are depicted (modified from Puig *et al*., 1998). Primers are denoted as A1, A2, B1, B2, C1, C2, D1, D2, D3, D4. Note that strategy B is more efficient due to the longer region of homology.

3.4. C-terminal tagging of proteins

The BBP-ProtA strain (BSY576) was obtained by transformation with a DNA-fragment resulting from a three step PCR-ligation strategy using 3 primers (Strategy B, Figure 46, Puig *et al*., 1998). A region of about 500 nt immediately downstream of the open reading frame of the gene coding for BBP was amplified from genomic DNA using primers BR1 and BR2. This PCR fragment was ligated to the *Xho*I cut and Klenow-fragment treated vector pBS1173 by blunt end ligation. The ligation product was directly used in a second PCR with primers BR2 and BR3. The resulting fragment was transformed into strain MGD453-13D following the procedure of Ito et al. (Ito *et al*., 1983). The Mud2-ProtA strain (BSY573) was provided by B. G. M. Luukkonen. The Lea1-CBP/SmB-ProtA strain (BSY723) was provided by F. Caspary (Caspary *et al*., 1999). The Snu71-ProtA strain (BSY619) was provided by O. Puig. The TAP-tagged Snu71 strain (BSY701) was provided by G. Rigaut (Rigaut *et al*., 1999). The BBP-TAP containing strains were generated as described for BBP-ProtA, but using primers BR1, BR2, BR28 and plasmid pBS1479. The Mud2-TAP strain was generated in the same way, but using primers ML50, ML51 and BR29.

3.5. N-terminal tagging of proteins

To generate the GAL-ProtA-BBP strain the haploid strain YDL401 was used. The construction of the transforming fragment was as described (Strategy A, Figure 46; Lafontaine and Tollervey, 1996) by amplification with primers BR8 and BR31 from plasmid pTL27. Prior to transformation the PCR fragment was gel-purified to separate it from the original plasmid. The constructed strain was checked for correct expression of the ProtA-fusion protein by western blotting.

Tab. 8: Strains containing tagged proteins and/or regulatable promoters

Name	Number	Marker	Construction	Analysis
BBP-ProtA	BSY576	*TRP1*	Strategy B (BR1, BR2, BR3, pBS1173) in MGD453-13D	Western, PCR (BR3+BR7)
Mud2-ProtA	BSY573	*TRP1*	in MGD453-13D (gift of B. G. M. Luukkonen)	Western, PCR (ML52+BR12)
GAL-ProtA-BBP	BSY734	*HIS3*	Strategy A (BR8, BR31, pTL27) in YDL401	Western
GAL-U2 snRNA	BSY88	*URA3*	in MGD453-13D (gift of B. Séraphin)	-
Lea1-CBP+ SmB-TEV-ProtA	BSY723	*K.l.TRP1*+ *K.l.URA3*	in MGD453-13D (gift of F. Caspary)	-

Snu71-ProtA	BSY619	*K.l.URA3*	in MGD453-13D (gift of O. Puig)	-
Snu71-TAP	BSY701	*K.l.TRP1*	in MGD453-13D (gift of G. Rigaut)	-
BBP-TAP	BSY682	*K.l. TRP1*	Strategy B (BR1, BR2, BR28, pBS1479) in MGD453-13D	Western, PCR (GR4+BR7)
Mud2-TAP	BSY683	*K.l. TRP1*	Strategy B (ML50, ML51, BR29, pBS1479) in MGD453-13D	Western, PCR (GR4+BR12)
mud2-Δ+BBP-TAP	BSY740	*TRP1*+ *K.l.URA3*	Strategy B (BR1, BR2, BR28, pBS1539) in BSY518	Western

3.6. Yeast transformation and screening for integrations by PCR

Yeast transformations were done as described (Ito *et al.*, 1983; Soni and Carmichael, 1993). For the latter method cells were grown o/n at 30 °C to an OD_{600} of ≈ 1. 1.4 ml culture was centrifuged for 20 s. Pellets were gently vortexed prior to addition of 10 µl carrier DNA (from fish sperm, Boehringer) and the DNA to be transformed (≈ 1 µg of DNA for tagging and disruption). After gentle vortexing 500 µl of LP buffer were added and thoroughly mixed. Then 55 µl of DMSO were added and again everything was mixed. After incubation for ≈ 15 min at RT the cells were heat shocked for ≤ 15 min at 42 °C. Immediately cells were diluted with 500 µl of sterile TE buffer, gently mixed and centrifuged for 1 min at 4000 rpm. The pellets were washed with 1 ml of TE buffer and again centrifuged as before. Pellets were resuspended in 100 µl TE buffer and plated on the appropriate selective medium.

To screen for integrations by PCR some material from colonies (derived from subclones) was resuspended in 12.5 µl PCR reaction mix I. Alternatively DNA was extracted prior to amplification following the protocol by Polaina and Adam (see 3.7.). PCR reaction mix I contained 0.125 µl of primer one (100 µM) and 0.125 µl of primer two (100 µM), 1.25 µl of 10 x buffer (Perkin Elmer) and ddH_20. After mixing, 12.5 µl of mix II were added. This contained 1.25 µl of 10 x buffer (Perkin Elmer), 1.0 µl of a dNTP mix (2.5 mM of each dATP, dCTP, dGTP and dTTP) 0.1 µl of AmpliTaq DNA polymerase (Perkin Elmer) and ddH_20. The PCR cycles were usually as follows: 1 x 95 °C for 5 min; 30 x (95 °C for 30 s / 48 °C for 30 s / 72 °C for 1 min (1 min per 1000 bp)); 1 x 72 °C for 10 min). Primers were designed so that one was outside and one inside of the insertion and that the obtained product was 0.5-1 kb.

LP buffer: 40 % polyethylenglycol 4000; 0.1 M LiAc; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA

3.7. Extraction of plasmid DNA

For use in PCR reactions DNA was extracted as described previously (Polaina and Adam, 1991). Cells from 1.5 ml of liquid yeast culture were harvested in a microfuge tube and resuspended in 100 µl TE buffer containing 3 % SDS. After 15 min gentle shaking at room temperature 0.5 ml of TE buffer were added and nucleic acids were extracted with 1 volume of phenol equilibrated with TE. To the aqueous phase 1/10 volume of 3 M Na-Acetate and 1 volume of isopropanol were added and precipitation was carried out for 1 h at -20°C. After a single wash with 80% ethanol the pellet was resuspended in 20 µl TE buffer. For a PCR reaction 1 µl of this material was used.

For transformation of plasmid DNA into *E. coli* 1.5 ml of liquid yeast culture were harvested in a microfuge tube. The pellet was washed with 1 ml of H_2O and resuspended in 150 µl of Lysis buffer. After incubation for 1 h at 37 °C 500 µl of SDS-buffer were added. This mixture was incubated for 30 min at 65 °C and after addition of 150 µl KAc for 1 h on ice. The cell debris was pelleted by centrifugation for 10 min in the cold room and the liquid phase was mixed by inversion with 700 µl isopropanol and left 10 min at room temperature. The nucleic acids were precipitated by centrifugation for 10 s at room temperature and the pellet was resuspended in 200 µl TE containing 10 µg RNase (DNase-free). After incubation for 30 min at 37 °C the DNA was extracted with phenol/chloroform/isoamylalcohol (PCI) and precipitated again with 1/10 volume of 3 M NaAc and 2 volumes of ethanol for 1 h at -20 °C. The pellet was washed with 80 % ethanol and resuspended in 50 µl TE buffer. The amount of DNA for transformation of *E. coli* was titrated to obtain optimal efficiencies.

Lysis-buffer:	1.2 M SCE, 50 µl β-mercaptoethanol, 2 mg Zymolyase 100T (Seikagaku)
SDS-buffer:	0.1 M Tris-Cl pH 9.7, 0.05 M EDTA pH 8.0, 0.5% SDS
1.2M SCE:	1.2 M Sorbitol, 0.1 M Na-Citrate, 75 mM EDTA, adjust to pH 7.5 with 5M NaOH
PCI:	200 g melted phenol (65 °C), 200 ml chloroform, 8 ml isoamylalcohol, 0.2 g 8-hydroxyquinoline

3.8. Small scale preparation of yeast extracts with glass beads

Yeast cells were grown in 100 ml YPD to late log phase (OD_{600} = 1-2). Cells were harvested by centrifugation, washed once with water and once with 5 ml buffer A. The cell pellet was resuspended in 1 ml buffer A and transferred to a 15 ml corex tube. Approximately 750 µl of acid-washed and siliconised glass beads were added and the cells were lysed by vortexing 4 times 30 s in the coldroom. In between they were stored on ice. The lysate was centrifuged 5 min at 5000 rpm to pellet the glass beads and unbroken cells. To the supernatant, KCl was added from a 2 M stock solution to a final concentration of 0.2 M. Remaining aggregates

were pelleted in a micro-centrifuge and the supernatant was transferred to a polycarbonate tube. After centrifugation at 56000 rpm for 20 min in a TLA 100-2 rotor (Beckman), the cleared extract was removed under the floating lipid layer and transferred to dialysis tubing. Dialysis against buffer D was carried out for 60 min at 4 °C. The dialyzed extract was aliquoted, frozen in dry ice and stored at -80 °C.

Buffer A: 10 mM K-Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 2 mM benzamidine, 0.5 µg/ml leupeptin, 1.4 µg/ml pepstatin A, 2.4 µg/ml chymostatin, 17 µg/ml aprotinin.
Buffer D: 20 mM K-Hepes pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20 % glycerol, 0.5 mM PMSF, 2 mM benzamidine

3.9. Medium scale preparation of yeast extracts with zymolyase

One liter of yeast culture (OD_{600} = 2-3) was harvested by centrifugation and washed once with water. The pellet was resuspended in 15 ml of SB30 buffer and incubated for 15 min at RT (25 °C). Cells were centrifuged again (3250 rpm for 5 min) and pellets were resuspended in 15 ml of SB3 buffer containing 100 µl Zymolyase 100T (Seikagaku) solution (20 mg/ml in 50 mM K_2HPO_4/KH_2PO_4, pH 7.5). The suspension was gently shaken for 1 h at 30 °C until spheroplasts were formed (checked under the microscope). Spheroplasts were centrifuged (3250 rpm for 5 min), washed gently with 15 ml SB3 buffer and pellets were resuspended in 8 ml of buffer A (see 3.8.). Spheroplasts were broken by douncing the suspension 5 times with a tight 15 ml douncer (Kontes). KCl was added to a final concentration of 0.2 M. After 20 min stirring on ice the suspension was centrifuged (13000 rpm for 30 min). The supernatant was centrifuged at 33500 rpm for 1 h at 4 °C (T150-2; Beckman) and the clear upper phase (without the lipid layer on the top) was dialyzed for 3 h in buffer D (see 3.8.).

SB30 buffer: 50 mM Tris-HCl, pH 7.5; 1M sorbitol; 10 mM $MgCl_2$; 30 mM DTT
SB3 buffer: 50 mM Tris-HCl, pH 7.5; 1M sorbitol; 10 mM $MgCl_2$; 3 mM DTT

3.10. Large scale preparation of yeast extracts with French Press

Strains were grown at 30°C in 2liter of YPD to OD600 =2-3. Cells were harvested by centrifugation and washed once with cold water. Pellets were resuspended in an equal volume of buffer A (see 3.8.). Cells were broken by pressing the suspensions 3 times through the French Press at a pressure of approximately 1000psi. KCl was added from a 2M stock solution to 0.2M final concentration before the lysates were centrifuged for

30min at 17000rpm (SS34). The supernatant was centrifuged again for 1h at 33500rpm (T150-2; Beckman) and the cleared extract was recovered between the lipid phase and the pellet. The extract was dialyzed for 4h at 4°C in buffer D (see 3.8.).

3.11. *In vivo* depletion of splicing factors (BBP/ScSF1 and U2 snRNA)

The strains BSY734 (GAL-ProtA-BBP) and BSY526 (WT) were grown over night in 100 ml YP-medium containing 2 % galactose, 2 % lactate pH 5.5, 2 % glycerole and 0.05 % glucose. Cells were harvested and resuspended in 100 ml YP-medium containing 2 % glucose. Growth was continued at 30°C, fresh medium was added to keep the growth in log-phase and aliquots of the culture were removed at different times for extract preparation (see 3.8.).

The strain BSY88 (GAL-U2snRNA) was grown to OD_{600}=1 in 50 ml YP-medium containing 3 % galactose, 1 % sucrose and 0.05 % glucose. Cells were harvested and resuspended in 100 ml YP-medium containing 4 % glucose. Growth was continued for 16 h at 30 °C before harvesting the cells. Extracts were prepared according to the small scale extract protocol (see 3.8.) and tested for their block in pre-spliceosome formation in native gels (see 4.3.).

3.12. Screening for conditional *msl5* mutants

The mutant *msl5* library (pBS1780, see 2.4.) and a wild type control (pBS1759) were transformed into the yeast strain BSY809 and transformants were grown at 23°C on SD-Leu plates. About 2000 independent clones were streaked onto SD-Leu plates, grown at 23°C and replica plated onto 5-fluorotic acid (5FOA) containing plates. The surviving clones (about 400) were streaked on YPD plates, grown again at 23°C and replica plated onto YPD plates that were incubated at 16, 23, 30 and 37°C. In parallel the *LEU2*, *TRP1* and *URA3* markers were checked on the appropriate plates at 23°C. Clones that showed a temperature sensitive growth phenotype were subcloned, checked again for their growth defect and DNA was extracted using the method described above (see 3.7.). The DNA was transformed into *E. coli*, amplified and retransformed into the yeast strain BSY809 followed by the shuffling strategy described above. The *msl5* ORF of three of the mutants selected was sequenced entirely and five other mutants were sequenced partially.

3.13. *In vivo* splicing and pre-mRNA retention analysis

The mutant yeast strains *msl5-2*, *msl5-3*, *msl5-5*, *msl5-9* and an isogenic wild type strain were transformed with reporter plasmids. The reporters for splicing analysis were: RP51A wild type intron (HZ18, Teem and Rosbash, 1983), mut5'SS (HZ12, Jacquier *et al*., 1985),

mutBP (HZ8, Jacquier *et al*., 1985), mut5'SS+mutBP (HZ8+HZ12, Jacquier *et al*., 1985). The reporters for pre-mRNA retention analysis: mRNA in frame (pLG-Acc°), pre-mRNA in frame (pLG-Nde°Acc°) and no intron (pLG-SD5) were described previously (Legrain and Rosbash, 1989). As a control for background activity plasmid YEp24 (Botstein *et al*., 1979) was used. The thermosensitive strains (*msl5-2*, *msl5-3*, and *msl5-9*) and the wild type control were grown at 23°C in synthetic medium without uracil containing 2 % lactate (pH 5.5), 2 % glycerole and 0.05 % glucose to an OD_{600} of 0.5-0.8. Then the cells were heat-shocked for 30 min at 37 °C before transcription of the reporters was induced by addition of galactose to a final concentration of 2 %. Induction was continued for 2 h at 37 °C. Cells were harvested, the OD_{600} was measured and two 1 ml aliquots were taken for the analysis of β-galactosidase activity (see 3.14.). Three independent clones of each strain were analyzed in duplicate, errors were calculated as the cumulative maximal error from the standard deviations of the measured values. For RNA analysis RNA was extracted (see 4.8.) and normalized by measuring OD_{260}. Primer extension was performed using ^{32}P labeled primer EM38 (see 4.9.). The extension products were migrated in a 6 % polyacrylamide/7 M Urea gel, dried and exposed to a Fluor Image Analyzer screen (Fuji). Signals corresponding to pre-mRNA and mRNA were quantified.
Analysis of β-galactosidase activity of the cold sensitive mutant (*msl5-5*) was as described above except that cells were grown at 30 °C before a 30 min cold-shock at 16 °C followed by a 4 h induction at 16 °C.

3.14. β-galactosidase assay

An aliquot of 1 ml of yeast culture was centrifuged. To the pellet 0.5 ml of buffer Z and 200 µl of water saturated ether were added and the mixture was vortexed for 20 s. The suspension was centrifuged for 1 min. After evaporation of the ether (10 min under the hood) the suspension was incubated for 5 min at 30 °C. The reaction was started by adding 100 µl of ONPG and further incubation at 30 °C (4 mg/ml in buffer Z; freshly prepared). When the colour turned yellow the reaction was stopped by adding 250 µl of 1 M Na_2CO_3. The sample was centrifuged for 5 min. 200 µl of the supernatant were transferred into a 96-well-plate for the measurement of the OD_{420} in a plate reader (Tecan). β-galactosidase activity was calculated as follows: Activity = $(1000 \times OD_{420}) / (volume \times OD_{600} \times time_{min})$. To standarize the experiments all activities were expressed as the percentage of the activity of a reporter containing no intron (pGL-SD5) in the same yeast strain processed in parallel.

Buffer Z: 100 mM Na_2HPO_4/NaH_2PO_4 pH 7.2; 10 mM KCl; 1 mM $MgSO_4$;
360 µl β-mercaptoethanol (freshly added)

3.15. Synthetic phenotype analysis

The *UPF1* disrupted strain (BSY879) was transformed with plasmid pRS415 (Sikorski and Hieter, 1989) containing a *LEU2* marker and mated with strain BSY810 which contained the *MSL5* disruption complemented with a plasmid copy of *MSL5*. The mated diploid strain was selected by the simultaneous presence of the *LEU2* and *URA3* markers and named BSY883. It was sporulated, dissected and the disruption of both genes, *UPF1* and *MSL5* was checked by the presence of the *TRP1* and *URA3* markers and by PCR The resulting strain was called BSY884 (MATα). It was transformed with the *LEU2* marker plasmids containing the mutants *msl5-2*, *msl5-3* or *msl5-9* or the wild type *MSL5* gene followed by the shuffling strategy described above to eliminate the *URA3* marker plasmid carrying a wild type copy of *MSL5*. The resulting strains were replica plated on YPD plates that were incubated at 23, 30, 33 and 37°C.

The strains BSY971 (*lea1*-Δ+*upf1*-Δ), BSY973 (*nam8*-Δ+*upf1*-Δ) and BSY976 (*mud2*-Δ+*upf1*-Δ) were generated by mating of the appropriate single disrupted strains, sporulation and dissection (Tab. 6). Spores were analyzed for the presence of markers and for the disruptions by PCR. The growth was anlayzed as described above.

4. RNA techniques

4.1. General methods

For the handling of RNA care was taken to prevent contamination with RNases. All solutions were sterile filtered and Millipore-filtered and autoclaved water was used. All materials were handled with clean gloves. Prepacked pipette tips (Gilson) were used. RNA or cell pellets for RNA extraction were stored at -80 °C.

Denaturating acrylamide gels contained 420 g/l of Urea and x% of an acrylamide mix (acrylamide (BDH):bis-acrylamide (BDH); 19:1) in 1xTBE (pH 8.3).

4.2. *In vitro* transcription of RNA

A short pre-mRNA (pBS195) derived from the RP51A intron, the same pre-mRNA with a deleted BP region (pBS199) or a deleted BP and a mutated 5' splice site (pBS345) (Séraphin and Rosbash, 1991) were *in vitro* transcribed for native gel analyses. A pre-mRNA containing the full length RP51A intron (pBS7) was *in vitro* transcribed for the *in vitro* splicing assays. The RNAs were transcribed from 1 μg of linearized plasmid (digested

with *Dde*I) in a 10 µl reaction containing 1 x TSC transcription buffer (Promega), 10 mM DTT, 1 mM GpppG, 1 mM ATP, 1 mM CTP, 0.2 mM GTP, 0.1 mM UTP, 8 U Rnasin (Promega), 1.25 µl α^{32}P UTP (20 mCi/ml, 500-800 Ci/mmole, Amersham) and 10 U T7 RNA polymerase (Promega).
For the generation of biotinylated pre-mRNA, plasmids were digested with *Pvu*II and the transcription reaction was carried out as above, except that 1 mM UTP, 0.5 mM bio-16-UTP (Boehringer Mannheim) and 0.625 µl of 12.5-fold diluted α^{32}P UTP (20 mCi/ml, 500-800 Ci/mmole, Amersham) were used. For the generation of "unlabeled" pre-mRNA the reaction was carried out as above, except that 1 mM UTP and 1.25 µl of 50 -fold diluted α^{32}P UTP (20 mCi/ml, 500-800 Ci/mmole, Amersham) were used.
After incubation for 1 h at 37 °C, an equal volume of RNA loading dyes was added to the reaction, the samples were heated to 65 °C for 3-5 min and loaded on a 5 % acrylamide gel. Following autoradiography of the gel, the band corresponding to the full length transcript was excised and the RNA was eluted from the gel o/n at RT, with 300 µl of 1 x PK buffer and 4 µg of carrier *E. coli* tRNA. The aqueous phase was subsequently extracted twice with PCI and the RNA was precipitated by adding half a volume of 7.5 M NH_4Ac and 2.5 volumes of ethanol and precipitating for 20 min at -80 °C. After centrifugation, the pellet was washed twice with 80 % ethanol, dried and resuspended in 50 µl water. The concentration of the synthesized RNA was determined by measuring Tscherenkow counts of an aliquot in a liquid szintillation counter.

RNA loading dyes:	90 % deionized formamide; 100 mM Tris-HCl, pH 7.5; 0.025 % xylene cyanol; 0.025 % bromophenol blue; 1 mM EDTA, pH 8; 0.05 % SDS
1 x PK buffer:	100 mM Tris-Cl, pH 7.5; 12.5 mM EDTA, pH 8.0; 150 mM NaCl; 1 % SDS

4.3. Native gel analysis

Native gel analysis was performed essentially as described previously (Séraphin and Rosbash, 1989). The native gel was prepared by pouring first 1 cm of a gel plug (2 ml of 30 % Protogel: 37.5:1 acrylamide-mix (National Diagnostic); 0.5 ml of 10 x TBE; 7.4 ml of water; 0.1 ml of 10 % APS; 10 µl of TEMED). After 10 min the non-polymerized gel was removed with a Whatman 3MM paper. The running gel was prepared in a vacuum flask. 0.2 g of agarose (Gibco BRL), 2 ml 10 xTBE, 2.0 g glycerol (100 %) and 31.6 ml water were mixed, weighted and boiled. The weight was adjusted with water and the mixture was briefly boiled again. After cooling at RT for 4 min, 4 ml of 30 % Acrylamide/Bisacrylamide mix (60:1) were added and the solution was degazed before adding 0.4 ml of 10 % APS and

40 µl of TEMED. The solution was quickly poured and the comb was inserted. After polymerizing for > 1 h the comb was carefully removed under buffer or H_2O. The gel was pre-run at 4 °C for 30 min at 70-80 V.
For the complex assembly reaction 4 µl of yeast extract were mixed with 4 µl of Splicing buffer, 1 µl of 20 mM ATP and 1 µl of labeled pre-mRNA (20000 cpm). After incubation for 30 min at 30 °C the reactions were stopped by transferring them on ice and by adding 10 µl of stop buffer. Samples were incubated on ice for 10 min. Then 5 µl loading dye was added and 5-10 µl of the reaction were loaded on the gel. Electrophoresis was carried out for 13-14 h at 60-80 V. The gel was transferred to a Whatman 3MM paper, dried for 1 h and exposed at -80 °C with an enhancer screen.
To deplete ATP, the reactions were incubated for 10 min at RT with 2 mM glucose (instead of ATP) prior to addition of labeled pre-mRNA (Liao *et al*., 1992). For the shift of protein A containing complexes the splicing extracts were incubated for an additional 10 min at room temperature with 1 µg rabbit IgG (Sigma) before they were stopped on ice.

Running buffer:	0.5 x TBE; 5 % glycerol
Buffer R:	50 mM Hepes-KOH, pH 7.9; 2 mM MgAc
Stop buffer:	10 µl of buffer R; 1 µl of total yeast RNA (10 µg/µl)
Splicing buffer:	7.5 % PEG 6000; 150 mM K_2HPO_4/KH_2PO_4, pH 7.0; 6.25 mM $MgCl_2$
5 x Loading dye:	50 % glycerol; 2.5 xTBE; 0.05 % brome-phenol blue

4.4. *In vitro* splicing

Splicing reactions were as described above, except that the incubation was performed for 30 min at 25 °C and in the indicated reactions 20 mM cordycepin (Sigma) was used instead of ATP. Pre-mRNA was generated by *in vitro* transcription of plasmid pBS7 cut with *Dde*1 (Séraphin *et al*., 1988). The reaction was stopped by addition of 200 µl PK-buffer (see 4.2.) containing 80 µg Proteinase K (Sigma) and 10 µg *E. coli* tRNA and incubation for 20 min at 37 °C. RNA was extracted (see 4.7.) and analyzed in a 5 % polyacrylamide/7 M urea gel.

4.5. Affinity selection of splicing complexes on biotinylated RNA

Reactions of 50 µl containing 20 µl extract with the ProtA-tagged protein of interest and 20 µl splice salts were incubated with 2 kcpm of biotinylated pre-mRNA (see 4.2.) in the presence or absence (depletion as in 5.8.) of 2 mM ATP for 30 min at 30 °C. Then 50 µl of WB150 and 1 µl of *E. coli* tRNA (10 µg/µl) were added and the reaction was placed on ice for 10 min. To this mixture 300 µl WB150 containing 20 µl equilibrated streptavidin-agarose beads (Sigma) were added and binding was performed by rotation of the tubes at 4 °C for

2 h. The supernatant was removed and the beads were washed four times with 500 µl WB150. Input, supernatant and beads were analyzed for their RNA and protein content as described in chapters 4.7., 4.9., 5.2. and 5.3.

WB150: 50 mM Tris-Cl pH 8.0, 150 mM KCl, 0.05 % NP40, 1 mM DTT

4.6. Co-precipitation of splicing complexes

50 µl extract from strain BSY723 harboring Lea1-CBP and SmB-ProtA were mixed on ice with 50 µl extract from strain BSY576 (BBP-ProtA), BSY573 (Mud2-ProtA) or BSY619 (Snu71-ProtA). For the co-precipitation of pre-spliceosomes the mixed extracts were incubated with 0.5 nmol of oligo EM133 under splicing conditions (Pikielny *et al*., 1986) in the absence of ATP for 15 min at 30 °C (Fabrizio *et al*., 1989). Then 2 mM ATP and 0.5 µg unlabeled pre-mRNA (WT or 5'SS + ΔBP, see 4.2.) were added. After further incubation for 30 min at 30 °C the reactions were diluted with 3 volumes of CBP buffer 300 containing approximately 10-15 µl calmodulin-agarose beads (Stratagene). The mixture was rotated for 1.5 h at 4 °C, supernatant was removed and the beads were washed 3 times 10 min with CBP buffer 300. The beads were eluted with 20 µl Calmodulin elution buffer. Inputs, supernatants and eluates were split and analyzed for proteins or RNA. Proteins were resolved on a 10 % SDS-polyacrylamide gel, transferred to nitrocellulose membrane (Schleicher and Schuell) and detected using peroxidase-antiperoxidase complex (Sigma) and the ECL detection system (Amersham) or the Renaissance system (NEN). RNA was extracted and analyzed by primer extension as in described in 4.7. and 4.9.

As a control, the same experiment was performed using extract from the strain BSY701 (Snu71-TAP) mixed with extract from the strain BSY576 (BBP-ProtA). The conditions used were the same as above except that the oligonucleotide complimentary to U6 snRNA was omitted and the extracts were incubated with 2 mM glucose at 25°C for 10 min before incubation with the RNA in the absence of ATP.

CBP buffer 300:	10 mM Tris-Cl pH 8.0, 300 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1 % NP40, 10 mM β-mercaptoethanol
Calmodulin elution buffer:	10 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 4 mM EGTA pH 8.0, 0.1 % NP40, 10 mM β-mercaptoethanol

4.7. RNA extraction from yeast extracts, extract fractions or affinity beads

To the sample 200 µl PK-buffer (see 4.2.) containing 80 µg Proteinase K (Sigma) and 10 µg *E. coli* tRNA were addedand the mixture was incubated for 60 min at 50 °C. The supernatant (in the case of beads extraction) or the whole mixture was extracted twice with PCI. RNA was precipitated by adding 1/2 volume of 7.5 M $(NH_4)_2OAc$ and 2.5 volumes of ethanol and placing the mixture at -80°C. The pellet was washed with 80 % ethanol and resuspende in H_2O.

4.8. Total RNA extraction

Yeast cells corresponding to 5-30 OD_{600} were centrifuged and pellets were washed with 1 ml of extraction buffer. Pellets were frozen in dry ice and stored at -80 °C or immediately processed. To the pellets 400 µl of PCI, 400 µl of extraction buffer, 10 µl of 10 % SDS and 400 µl of pre-cooled glass beads (acid washed, siliconized and baked) were added. Up to 8 tubes were vortexed in parallel (twice for 45 s) in a pre-cooled cell homogeniser (Braun). After centrifugation (13000 rpm for 10 min) RNA was extracted from supernatants twice with PCI and precipitated with 30 µl of 3 M NaAc and 800 µl Ethanol at -20 °C for 1h. The pellets were washed with 80 % ethanol, dried and resuspended in 180 µl of water and once more extracted with PCI. Supernatants were precipitated with half a volume 7.5 M $(NH_4)_2Ac$ and 2.5 volumes of ethanol for 1 h at -20 °C. After centrifugation, washing (80 % ethanol) and drying the pellets were resuspended in 11 µl of water. 1 µl was mixed with 500 µl water and OD_{260} was measured to determine the RNA concentration (1 OD_{260} = 40 µg of RNA). 3-5 µg of total RNA were further analyzed by primer extension.

Extraction buffer: 100 mM LiCl; 1 mM EDTA; 100 mM Tris-HCl, pH 7.5

4.9. Primer Extension

Primers used for primer extension are listed in the table below. To label a primer, 10 pmol of the oligonucleotide were incubated with 5 µl $\gamma^{32}P$ ATP (10 mCi/ml; >5000 Ci/mmol, Amersham) in a 10 µl reaction containing 1 xkinase buffer (100 mM Tris-Cl pH 8.0, 10 mM $MgCl_2$), 7 mM DTT and 5 U T4 polynucleotide kinase (New England Biolabs) for 30 min at 37 °C. After the incubation, 40 µl TE buffer were added and the enzyme was inactivated by incubating for 5 min at 95-100 °C.

To anneal the primers to their RNA target, 0.5 µl of each of the labeled primers were mixed with the RNA (1-5 µg of total RNA) and 1x reverse transcriptase buffer in a total volume of 6 µl. The tubes were placed in a heat block at 60 °C and let gradually cool down to 52 °C for

1 h (for the RP51A pre-mRNA the temperature was kept at 42 °C for 1 h). Per tube, 4 µl of extension mix were added which contained 0.5 µl of a 2.5 mM stock solution of each dATP, dGTP, dCTP and dTTP, 0.5 µl of 200 mM $MgCl_2$, 0.5 µl of 20 mM DTT, 0.25 µl of 5 mg/ml actinomycin D (in ethanol), 0.5 µl of 5x reverse transcriptase buffer and 2-6 U AMV reverse transcriptase (Stratagene). The tubes were then placed in a 48 °C heatblock which was switched to 45 °C and reverse transcription was allowed to proceed for 30 min (for the RP51A pre-mRNA the temperature was kept at 37 °C for 30 min). To stop the reaction, 10 µl of loading dyes were added. Samples were heated to 95-100 °C for 5 min prior to loading on a 6-8 % poyacrylamide gel.
Quantification of bands was done with a Fluorescent Image Analyzer (Fuji).

5x Reverse transcriptase buffer:	250 mM Tris-Cl, pH 8.0; 200 mM KCl; 2.5 mM EDTA
Loading dyes:	946 µl deionized formamide; 2 µl 5M NaOH; 2 µl 0.5 M EDTA, pH 8.0; 25 µl 1 % bromephenol blue; 25 µl 1 % xylene cyanol

Tab. 9: Sequences of oligonucleotides used for primer extension

Name	Sequence (5'-3')	Target
BR14	CAGTAGGACTTCTTGATC	U1 snRNA
GR3	GCCAAAAAATGTGTATTGTAA(C)	U2 snRNA
Oligo6	GGTATTCCAAAAATTCCCTACATAGTC	U4 snRNA
EM19	GGCCCACAGTTCTTGATGTTG	U5 snRNA
Oligo5	TCATCCTTATGCAGGG	U6 snRNA
EM38[a]	CACGCTTGACGGTCTTGGT	RP51A
BR32	ACCACCTACTTGAGATGC	YRA1 (exon 2)

[a] exon 2-specific oligonucleotide has been described previously (Pikielny and Rosbash, 1985)

5. Protein techniques

5.1. Rapid extraction of proteins from yeast cells

Strains were grown to OD_{600}=2-3 in the appropriate medium and 1 ml of culture was harvested by centrifugation. To the cell pellet 100 µl of SDS loading buffer and 25 µl of glass beads (0.45-0.5 mm, Braun Melsungen) were added. The mixture was vortexed three times for 30 s (placing the tubes on ice in between), boiled for 5 min at 95 °C, vortexed again and analyzed in an SDS-PAGE.

5.2. SDS-polyacrylamide gel electrophoresis

Analysis of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie brilliant blue were performed as described (Sambrook *et al.*, 1989). Protein concentrations were determined with the BioRad protein assay kit, according to the manufacturers instructions. Silver staining was performed as described previously (Shevchenko *et al.*, 1996). Briefly, the gel was fixed for 30 min (MeOH:HAc:water in the ratio 45:5:45 v/v/v). After washing the gel in water (≥ 60 min) it was sensitized with 0.02 % thiosulfate (1-2 min at RT). The gel was rinsed with water (2 times 1 min) prior to incubation with chilled 0.1 % $AgNO_3$ solution (20-40 min at 4 °C). After rinsing the gel again with water (2 times 1 min) it was developed by incubation in developing solution (0.04 % formaldehyde, 2 % Na_2CO_3). The developing solution was immediately replaced when it turned yellow. Development was quenched by 1 % HAc solution and the gel was stored in this solution or in water at 4 °C.

5.3. Western blotting

After electrophoresis, proteins were transferred to a nitrocellulose membrane by electroblotting in a Trans-Blot Cell (BioRad) in transfer buffer at 250 mA for 1-2 h at 4 °C. Proteins were visualized on the membrane by staining with Ponceau S (Serva), to control the transfer quality. The filter was pre-blocked in blotto by shaking gently for 1 h at RT. The filter was then washed briefly with wash buffer. For detection of the ProtA fusion proteins, horseradish peroxidase-anti-peroxidase (PAP, Sigma) was used in a 1:2000 dilution in blotto. Binding was performed for 1 h at RT followed by 5 washes with wash buffer (each 5 min). Membrane bound horseradish peroxidase-labeled antibodies were detected with the enhanced chemiluminescence (ECL) kit (Amersham), following the manufacturers instructions.

For detection of BBP and Mud2p the pre-blocked filter was incubated with the appropriate rabbit sera (1:500 dilution) in blotto for 1 h at RT. The filter was then washed (3 times 10 min) before it was incubated with Anti-Rabbit IgG linked to horseradish peroxidase in blotto (1: 2000 dilution; 1 h at RT). After extensive washing (5 times 5 min), membrane bound horseradish peroxidase-labeled antibodies were detected with the ECL detection kit.

Transfer buffer:	25 mM Tris base; 40 mM glycine; 0.05 % SDS; 20 % glycerol
Blotto:	1 x PBS; 0.2 % Tween-20; 5 % low-fat dried milk
Wash buffer:	1 x PBS; 0.2 % Tween-20

5.4. *In vitro* depletion of BBP

Extract from a strain containing BBP tagged with ProtA (usually 200 µl) was adjusted to 150 mM KCl and 0.1 % NP-40 prior to incubation with 110 µl of pre-washed (with IPP150, see 5.5.) IgG-sepharose or glutathione-sepharose beads (Pharmacia) for 2 h at 4 °C. Supernatants and pellets were analyzed by western blot to determine the level of depletion.

5.5. Tandem Affinity Purification (TAP)

Two-step native affinity purification was performed with strains BSY682 and BSY683 as previously described (Rigaut *et al.*, 1999). Purifications were started from 2 l of cell culture grown to an OD_{600} between 2 and 3. The large scale yeast extract (≈ 10 ml) was adjusted to 10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 0.1 % NP-40 and transferred to a Poly-Prep Chromatography Column (10 ml volume; BioRad) which contained 100 µl of pre-washed (with IPP150) IgG sepharose beads (Amersham). The closed column was slowly rotated for 2 h at 4 °C. After three washes with 10 ml of IPP150 and one wash with 10 ml of TEV cleavage buffer, 1 ml TEV cleavage buffer and 30-50 µl of TEV enzyme (provided by G. Rigaut) were added and the column was rotated for 2 h at 16 °C. The eluate was transferred to a new column, containing three volumes of calmodulin binding buffer (3 ml), 3 µl of 1 M $CaCl_2$ and 200 µl calmodulin affinity resin (Stratagene) which had been washed with calmodulin binding buffer. After rotation for 1 h at 4 °C the beads were washed three times with 10 ml calmodulin wash buffer. Finally complexes were eluted in five fractions of 200 µl with calmodulin elution buffer.

To concentrate the proteins eluates were mixed with 1/100 volume of 16 mg/ml sodium deoxycholate, pH >8.0. After vortexing samples were incubated on ice for 30 min. 112.2 µl of trichlor-acetic-acid (TCA) per ml of protein extract (final concentration 10 %) were added and the mixture was vortexed and incubated on ice for at least 10 min. Then samples were centrifuged for 15 min at 13000 rpm and pellets were washed twice with TCA wash buffer. The protein pellet was dried at room temperature for 10 min before it was dissolved in 8 M urea. SDS-loading dye was added (Sambrook *et al.*, 1989) and samples were loaded on a 7 % to 25 % SDS-acrylamide gradient gel. Proteins were silver stained and analyzed by mass spectrometry in the lab of M. Wilm, EMBL Heidelberg.

IPP150:	10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1 % NP-40
TEV cleavage buffer:	10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1 % NP-40; 0.5 mM EDTA; 1 mM DTT (freshly added)
Calmodulin binding buffer:	10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1 % NP-40 10 mM β-mercaptoethanol; 1 mM MgAc; 1 mM imidazol 3 mM $CaCl_2$
Calmodulin wash buffer:	same as calmodulin binding buffer but 1 mM $CaCl_2$

Calmodulin elution buffer:	same as calmodulin binding buffer but 2 mM EGTA instead of $CaCl_2$
TCA wash buffer:	70 ml acetone; 20 ml ethanol; 2.5 ml of 2 M Tris-HCl, pH 8.0; 7.5 ml water

6. Biocomputing

6.1. Database searches

Sequence databases were searched using BLAST (Altschul *et al*., 1990), BLAST2 (Yan *et al*., 1998) or the Smith-Waterman algorithm on the BIOCCELERATOR web server at EMBL. Database searches using sequence profiles (see 6.2.) were done using the program SearchWise (Birney *et al*., 1996).

6.2. Sequence alignments

Sequence alignments were made with ClustalX (Thompson *et al*., 1997) or DNAMAN (Lynnon BioSoft). Profiles from alignments were made with PROFILEWEIGHT (Thompson *et al*., 1994). Identification of exon/intron structures in genomic DNA was performed using the program PairWise (Birney *et al*., 1996). Sequences from automated sequencing were assembled and analyzed using the GCG package (Devereux *et al*., 1984).

6.3. Secondary structure prediction

Secondary structure prediction was performed on the basis of a sequence alignment (see 6.2.) using the PredictProtein server at EMBL Heidelberg (Rost and Sander, 1993; Rost and Sander, 1994).