J. Biochem. 125, 574-585 (1999)

Disruption of the *YRB2* Gene Retards Nuclear Protein Export, Causing a Profound Mitotic Delay, and Can Be Rescued by Overexpression of *XPO1/CRM1*[1]

Eishi Noguchi,* Yoh-hei Saitoh,* Shelley Sazer,† and Takeharu Nishimoto*,[2]

*Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582; and †Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA

Received November 18, 1998; accepted December 9, 1998

Disruption of the *YRB2* gene encoding a nuclear Ran-binding protein homologous to Yrb1p/RanBP1 makes *Saccharomyces cerevisiae* cold sensitive for colony-formation, but not for growth in liquid medium. *Schizosaccharomyces pombe* Hba1p, which is homologous to *Saccharomyces cerevisiae* Yrb2p, rescued the cold sensitivity of *Δyrb2* cells. When released from an α factor block, *Δyrb2* cells underwent a prolonged delay at the short spindle stage of mitosis with a normal level of Clb/p34^{CDC28} kinase activity, but there was no chromosome loss, this being consistent with the finding that *Δyrb2* was synthetic lethal with neither *Δmad1* nor *Δmad3*. The cold sensitive colony-formation of *Δyrb2* cells was rescued by both *XPO1/CRM1* and *GSP1*, but not *CDC5*, carried on a multicopy vector. *XPO1/CRM1* rescued *Δyrb2* even in a single copy. Consistent with such a tight functional interaction, Xpo1p/Crm1p directly bound to Yrb2p, but not Yrb1p, and *Δyrb2* cells were found to have a defect in nuclear export signal (NES)-dependent nuclear protein export. From these results together, the ability of Xpo1/Crm1p to export NES-proteins is suggested to be enhanced by both Yrb2p and Gsp1p, and thereby disruption of *YRB2* retards nuclear protein export, resulting in the mitotic delay.

Key words: mitotic delay, nuclear export, XPO1/CRM1, YRB1/2.

Gsp1p is the *Saccharomyces cerevisiae* Ran-homologue (*1*). Its intrinsic GTPase activity is enhanced by Rna1p (*2, 3*), and Gsp1p nucleotide exchange is stimulated by Prp20p, the *S. cerevisiae* RCC1-homologue (*4*). While RCC1, its *Drosophila* homologue, BJ1, and *Schizosaccharomyces pombe* homologue pim1 have been reported to be localized on the chromatin (*5-7*), the majority of Rna1p is localized in the cytoplasm (*8, 9*). Thus, Gsp1p/Ran shuttles between the nucleus and cytoplasm in order to complete the GTPase cycle. Concurrently with nucleocytoplasmic shuttling, Gsp1p/Ran carries macromolecules into, and out of, the nucleus (*10-14*).

Thus far, two families of RanGTP binding proteins have been reported to be required for the nucleocytoplasmic exchange of macromolecules, *i.e.* the RanBP1 family (*15, 16*), and the Importin β family (*12, 17, 18*). The RanBP1 family consists of RanBP1 (*19-21*), RanBP2/NUP358 (*22-24*), and RanBP3 (*25*). The sumo-modified form of RanGAP1, the mammalian homologue of Rna1p, is localized on RanBP2 and participates in nuclear protein import (*9*). Yrb1p and Sbp1 are the *S. cerevisiae* and *S. pombe* RanBP1 homologues, respectively (*20, 26-28*). In contrast to the cytoplasmic localization of RanBP1 (*29*), RanBP3, which is homologous to *S. cerevisiae* Yrb2p and *S. pombe* Hba1p, is localized in the nucleus (*25-27, 30, 31*). Yrb2p, with a molecular mass of 36 kDa, specifically binds to GTP-Gsp1p, although its Gsp1p binding ability is weak compared to that of Yrb1p (*27*). However, *Δyrb2*, which is cold sensitive for colony formation, is synthetically lethal with *rna1-1* or *prp20-1* (*27, 30*), indicating that Yrb2p is indeed involved in the Gsp1p GTPase cycle.

The Importin β family (*17*) is involved in either nuclear protein import or nuclear protein export (*12, 18*). Each member is thought to bind to a specific cargo. Members of the Importin β family thus far examined bind to RanGTP, and inhibit both nucleotide exchange and GTP-hydrolysis (*17, 32*). RanBP1 binds to several members of the Importin β family, forming trimeric complexes, such as Importin β/RanGTP/RanBP1 (*17, 32-35*). By binding to Importin β, RanBP1 stimulates nuclear protein import. Therefore, RanBP1 is thought to function as a coactivator of Importins, and thereby *yrb1* has a defect in nuclear protein import (*26*). In this study, we found that *Δyrb2* cells show a profound mitotic delay which can be rescued by *XPO1/CRM1* and Gsp1p. Consistent with this finding, *Δyrb2* was found to have a defect in nuclear protein export. Since Crm1p directly bound to Yrb2p, Yrb2p was suggested to regulate nuclear protein export through Crm1p.

[1] This work was supported by Grants-in-Aid for Specially Promoted Research from the Ministry of Education, Science, Sports and Culture of Japan, and by a grant from the HFSP.

[2] To whom correspondence should be addressed. Tel: +81-92-642-6175, Fax: +81-92-642-6183, E-mail: tnishi@molbiol.med.kyushu-u.ac.jp

MATERIALS AND METHODS

Yeast Strains and Media—All *S. cerevisiae* strains and plasmids used in this study are described in Tables I and II. Genetic manipulations and transformation of *S. cerevisiae* were carried out as described (*27*).

Synchronization with α-Factor—*S. cerevisiae* was cultivated at 26°C in YPD medium to the early log phase, and then given 10 μg/ml of α-factor. Two hours later, the cells were given another 10 μg/ml of α-factor and incubated for 1 h at 26°C, and then for 1 h at 14°C. Subsequently, the α-factor was washed out with distilled water to start the cell cycle.

Flow Cytomeric Analysis—Cells were collected and processed for flow cytometric analysis as described by Hutter and Eipel (*36*). These cells were stained with propidium iodide and then analyzed with a Becton-Dickinson FACScan flow cytometer.

Immunofluorescence Microscopy—*S. cerevisiae* strains were processed and fixed by the method described by Hagan and Hyams (*37*). Cellular DNA was stained with Hoechst 33342 or propidium iodide, while tubulin was stained with a monoclonal antibody (mAb) to tubulin, TAT1 (*38*), and then FITC-conjugated goat anti-mouse antibodies (BIO SOURCE International). Confocal images were obtained with a Zeiss Laser Scan Microscope LSM310.

To assay protein export, *S. cerevisiae* strains expressing NES-GFP2-NLS reporter proteins (*39*) were grown in synthetic medium lacking uracil to the mid-log phase, fixed and then processed as described (*27*). Cellular DNA was stained with Hoechst 33342.

Histone H1 Kinase Assay—Cultures of *S. cerevisiae* were collected by centrifugation, washed with yeast stopping buffer (150 mM NaCl, 1 mM NaN_3, 10 mM EDTA pH 8.0, 50 mM NaF) and then frozen at −80°C. Subsequently, the cells were vortexed with glass beads in lysis buffer (25 mM MOPS, pH 7.1, 15 mM $MgCl_2$, 15 mM EGTA, 60 mM β-glycerophosphate, 1.5 mM *p*-nitrophenylphosphate, 1 mM DTT, 1 mM *p*-APMSF) and then centrifuged at 45,000 rpm for 15 min. The supernatant was used as the enzyme source. Four hundred microliters of the supernatant (protein concentration, 0.2 mg/ml) was mixed with 10 μl of $p13^{suc1}$-agarose beads (Oncogene Science). After incubation at 4°C for 2 h, the beads were spun down, and washed twice with lysis buffer and then twice with washing buffer (25 mM MOPS, pH 7.1, 15 mM $MgCl_2$, 15 mM EGTA, 1 mM

TABLE I. **Yeast strains used in this study.**

Strain	Genotype	Source or reference
NBW5	*MAT α ade2 his3-352 leu2-3,112 trp1-289 ura3-1,2*	*63*
NBW6	*MATa ade2 his3-352 leu2-3,112 trp1-289 ura3-1,2*	*63*
N70-4B	*MATa ade2 ade3 leu2 trp1ura3*	Our stock
AM33-1	*MAT α yrb2-Δ2::LEU2 ade2 his3-352 leu2-3,112 trp1-289 ura3-1,2*	NBW5 disrupted with pTKSyrb2-Δ2::LEU2 (*30*)
AM43-1	*MATa yrb2-Δ2::LEU2 ade2 his3-352 leu2-3,112 trp1-289 ura3-1,2*	NBW6 disrupted with pTKSyrb2-Δ2::LEU2
AM50-1	*MAT α yrb2-Δ2::HIS3 ade2 his3-352 leu2-3,112 trp1-289 ura3-1,2*	NBW5 disrupted with pTKSyrB2-Δ2::HIS3
AM38-4	*MATa yrb2-Δ2 ade2 ade3 leu2 trp1 ura3*	N70-4B disrupted with pTKSyrb2-Δ2::LEU2
AM40	*MATa/α yrb2-Δ2/yrb2-Δ2 ade2/ade2+/ade3 ura3/ura3 leu2/leu2 trp1/trp1 +his3*	AM33-1×AM38-4 diploid
AM41	*MATa/α ade2/ade2+/ade3 ura3/ura3 leu2/leu2 trp1/trp1+/his3*	NBW5×N70-4B diploid

TABLE II. **Plasmids used in this study.**

Plasmid	Relevant marker	Description	Source or reference
pGAPhba1	*2μ TRP1* P_{TDH3}*-hba1*	pGAP[a] with *S. pombe hba1* ORF inserted downstream of *TDH3* promoter	This study
p195YRB2	*2μ URA3 YRB2*	YEplac195[b] with 1.6-kb *YRB2* fragment at *Bam*HI/*Hin*dIII site	*30*
p195GSP1	*2μ URA3 GSP1*	YEplac195 with 1.9-kb *GSP1* fragment at *Kpn*I/*Pst*I site	*65*
p195-23	*2μ URA3 GCD7 SEC72 GSP1*	YEplac195 with 3.2-kb genomic fragment containing *GCD7, SEC72,* and *GSP1*	This study
p195CRM1	*2μ URA3 CRM1*	YEplac195 with 5.5-kb *CRM1* fragment at *Sph*I/*Xba*I site	This study
YEp13-sc40	*2μ LEU2 CRM1*	YEp13[c] with 5.5-kb genomic fragment containing *CRM1*	This study
p316CRM1	*CEN URA3 CRM1*	pRS316[d] with 5.1-kb *CRM1* fragment at *Eco*RI/*Sal*I site	This study
pTKSyrb2-Δ2::LEU2	*yrb2-Δ2::LEU2*	Disruption of *YRB2* by replacement of 0.5-kb *YRB2* ORF C-terminal region with 2.9-kb *LEU2* fragment	*30*
pTKSyrb2-Δ2::HIS3	*yrb2-Δ2::HIS3*	Disruption of *YRB2* by replacement of 0.5-kb *YRB2* ORF C-terminal region with 1.75-kb *HIS3* fragment	This study
pKW430	*2μ URA3* P_{ADH1}*-NES-GFP2-NLS*	*NES-GEP2-NLS* fusion cDNA under control of *ADH1* promoter	*39*
pGEX-YRB1	*GST-YRB1*	GST-fused *YRB1* cDNA for *E. coli* expression	*30*
pGEX-YRB2	*GST-YRB2*	GST-fused *YRB2* cDNA for *E. coli* expression	*30*
pGEX-PRP20	*GST-PRP20*	GST-fused *PRP20* cDNA for *E. coli* expression	*30*
pGEX-GSP1	*GST-GSP1*	GST-fused *GSP1* cDNA for *E. coli* expression	*30*
pGEX-CRM1	*GST-CRM1*	GST-fused *CRM1* cDNA for *E. coli* expression	This study
pGEX-KAP95	*GST-KAP95*	GST-fused *KAP95* cDNA for *E. coli* expression	This study

[a]pGAP in Ref. *64*. [b]YEplac195 in Ref. *51*. [c]YEp13 in Ref. *52*. [d]pRS316 in Ref. *53*.

DTT). The washed beads were suspended in 30 μl of H1 kinase buffer {25 mM MOPS, pH 7.1, 15 mM $MgCl_2$, 15 mM EGTA, 1 mM DTT, 4 mg of histone H1, 50 mM γ^{32}p-ATP (1,000-2,000 cpm/pmol)} and then incubated at 30°C for 15 min. The reaction mixtures were mixed with 15 μl of 3×SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 20% β-mercaptoethanol, 9.2% SDS), boiled for 5 min and then subjected to SDS-PAGE electrophoresis. After staining with Coomassie Brilliant Blue (CBB), the gel was dried and the band of histone H1 was cut out and the radioactivity was counted with a liquid scintillation counter.

Construction of a Genomic Library—The genomic DNA of the AM33-1 strain was partially digested with *Sau*3AI, and then ligated into the *Bam*HI site of YEplac195, resulting in a yeast p195yrb2-Δ2 library. A YEp13 genomic library was prepared as described (*40*).

Construction of XPO1/CRM1 Plasmids—p195CRM1 and p316CRM1: From YEp13-sc40 derived from the genomic library, either 5.5 kb of a *Sph*I-*Nhe*I or 5.1 kb of an *Eco*RI-*Sal*I fragment containing the *XPO1/CRM1* genome was cut out with appropriate enzymes, and then inserted into the *Sph*I/*Xba*I site of the YEplac195 vector (p195CRM1), or the *Eco*RI/*Sal*I site of the pRS316 vector (p316CRM1), respectively.

pGEX-CRM1: In order to create an *Nco*I site at the N-terminus of the *XPO1/CRM1*-open reading frame (ORF), 1.0 kb of the N-terminal of *XPO1/CRM1*-ORF (NT) was amplified by PCR using as primers, ACTTCAAAAATAGG-ATAAAAACATACCATGGAAGGAATTTTGGATTTTTC-TAAC possessing an *Nco*I site, and AACAACAACTCTCT-TAACGA. The amplified DNA fragments were digested with the *Nco*I and *Apa*I enzymes, and then inserted into the *Nco*I/*Apa*I site of pUC28, resulting in pUC-CRM1NT. The C-terminal *Apa*I fragment (2.6 kb) of *XPO1/CRM1*-ORF was cut out from YEp13-sc40, and then inserted into the *Apa*I site of pUC-CRM1NT, resulting in pUC-CRM1. The whole ORF of *XPO1/CRM1* was removed from pUC-CRM1 by digestion with the *Nco*I and *Xho*I enzymes, and then inserted into the *Nco*I/*Xho*I site of pGEX-KG, resulting in pGEX-CRM1.

Cloning of KAP95 and Construction of GST-KAP95—KAP95: Total genomic DNA was obtained by the eviction method (*41*) as follows. The genomic DNA of the NBW6 strain was amplified by PCR using as primers, ACATCTG-CTGCAGTTACCAACGCA and CTGCTAGGAGCCCTAT-TGATTC, and then inserted into the *Pst*I/*Sac*II site, resulting in p404KAP95·3′. Plasmid p404KAP95·3′ was digested with the *Nsi*I enzyme, and then introduced into the NBW6 strain through homologous recombination. Genomic DNA of the resulting transformants was prepared, digested with the *Nde*I enzyme and then transformed into *Escherichia coli* DH5α after self-ligation of the digested fragments, resulting in pENY1-1KAP95 possessing a full-length *KAP95* gene.

GST-KAP95: The insert of pENY1-1KAP95 was amplified by PCR using as primers, AAGGAGCCATGGCCA-CCGCTGAA and TTTCATCAACTCTGGCCATGC. The resulting amplified DNA was digested with the *Nco*I enzyme and then inserted into the *Nco*I site of pGEX·KG, resulting in pGEX·KAP95NT. The *Spe*I-*Sac*I fragment of pENY1-1KAP95 was digested out with appropriate enzymes and then inserted into the *Spe*I/*Sac*I site of pGEX·KAP95NT, resulting in pGEX·KAP95.

Purification of GST-Xpo1p/Crm1p, GST-Kap95, Yrb2p, and Yrb1p—E. coli BL21 (DE3)/pGEX-CRM1 cells were cultured at 30°C up to $O.D._{600}$ 0.4, treated with IPTG (final conc., 0.2 mM) for 4 h and then collected by centrifugation. The cell pellets were washed with PBS and then suspended in lysis buffer (40 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 10% glycerol, 1% CHAPS, 1 mM DTT, 1 mM *p*APMSF). After sonication, cell debris was spun down at 28,000 rpm for 30 min. The collected supernatant was incubated with glutathione-Sepharose 4B beads (Pharmacia) for 1 h and then centrifuged to collect the beads. The beads were washed with lysis buffer and then incubated in lysis buffer containing 2 mM ATP at 30°C for 30 min as described (*42*). After washing with wash buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT), GST-Xpo1p/Crm1p was eluted from the beads by incubating them in the wash buffer containing 10 mM reduced glutathione.

GST-Xpo1p/Crm1p adsorbed on glutathione-Sepharose 4B beads was treated with 2 mM (final conc.) ATP as described above, washed with thrombin cleavage buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10% gylcerol, 1 mM DTT), and then digested with thrombin (final 1% w/w GST-Xpo1p/Crm1p) for 2 h at 25°C. The beads were filtered out and the filtered supernatant was stored as the Xpo1p/Crm1p-solution. All procedures were carried out at 4°C except when indicated otherwise. Protein concentrations were determined after SDS-PAGE by staining with CBB.

GST-Kap95p, Yrb1p and Yrb2p were prepared using *E. coli*-produced GST-fusion proteins in a manner similar in the case of Xpo1p/Crm1p.

Purification of Gsp1p—E. coli BL21 (DE3)/pGEX-GSP1 cells were cultured at 30°C, treated with IPTG, and then lysed by sonication as described above except for use as the lysis buffer, the Mg^{2+}-free lysis buffer containing 0.5 mM EDTA and 1 mM GDP. After centrifugation at 28,000 rpm for 30 min, the supernatant was supplemented with $MgCl_2$ (final concentration, 10 mM), held at 4°C for 30 min, and then glutathione-Sepharose 4B beads (Pharmacia) were added. After rotation for 1 h, the beads were washed with the wash buffer described above, incubated in wash buffer containing 2 mM ATP, and then treated with thrombin. To this mixture, a final concentration of 10 mM potassium phosphate buffer (pH 7.5) and 10 mM EDTA were added, and then GDP was added. After holding on ice for 30 min, 40 mM $MgCl_2$ was added to the Gsp1p solution, which was diluted with buffer A (25 mM PIPES-NaOH, pH 6.5, 10 mM potassium phosphate, pH 6.5, 1 mM $MgCl_2$, 1 mM DTT) and then charged onto a Fractogel EMD SO_3^- 650 column equilibrated with buffer A containing 50 mM NaCl. GDP-Gsp1p was eluted from the SO_3^- column with a linear gradient of NaCl (50-1,000 mM) at the flow rate of 1 ml/min. Similarly, GMPPNP (guanylylimidodiphosphate)-Gsp1p was prepared by adding GMPPNP instead of GDP.

The preparation of GDP-Gsp1p and GMPPNP-Gsp1p contained 100% of GDP- and GMPPNP-Gsp1p, respectively, that were analyzed by high-performance liquid chromatography as described (*43*).

Biosensor Analysis—Real-time protein-protein interaction analysis was performed with a BIACORE biosensor instrument (BIACORE) as described (*44*). The anti-GST

antibody (Pharmacia) was immobilized on the CM5 sensor chip as recommended by the manufacturer, and then 1 μM of GST-Xpo1p/Crm1p, GST-Kap95 or, as a control, GST alone was injected to be trapped on the sensor chip through the anti-GST antibody.

Binding experiments were carried out by injecting the indicated concentrations of purified recombinant proteins in buffer containing 20 mM potassium phosphate, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20. Evaluation and calculation of the binding parameters were carried out according to the manual BIA evaluation software type 1 model provided by Pharmacia Biosensor AB.

RESULTS

Hba1p, S. pombe Yrb2p Homologue, Rescues the Cold Sensitive Colony Formation of Δyrb2—S. pombe Hba1p was identified as a protein which confers brefeldin A (BFA) resistance to *S. pombe* through overexpression (*31*). It is 22.1% identical (47.4% similar) to Yrb2p (Fig. 1A). Yrb2p contains several amino acid repeats characteristic of NPC proteins (*15*), but Hba1p has only one FG sequence. However, Hba1p is localized in the nucleus (*31*), similar to Yrb2p. In order to address the question of whether or not Hba1p is a functional homologue of Yrb2p, the *hba1*$^+$ ORF was inserted downstream of the *TDH3* promoter of the pGAP vector and then introduced into the AM33-1 (*yrb2-Δ2*) strain. Trp$^+$ transformants grew well on synthetic medium (trp$^-$) plates at both 14 and 26°C, nonpermissive and permissive temperatures for colony formation by *Δyrb2* cells, respectively (Fig. 1B). Thus, Hba1p is a functional homologue of Yrb2p.

Disruption of the YRB2 Gene Causes a Delay in Mitosis—Although the function of Yrb2p has been conserved throughout evolution, as shown above, *YRB2* is not essential for survival. However, *Δyrb2* cells were found to possess aberrantly elongated spindles and large buds, even at 26°C, a permissive temperature for the colony formation by *Δyrb2*, suggesting that Yrb2p is required for normal cell proliferation. Based on this observation, we determined the point of the cell cycle which was affected by disruption of the *YRB2* gene.

Cultures of the AM43-1 (*yrb2-Δ2::LEU2*) strain and, as a control, the isogenic wild-type NBW6 strain, the growth of both of which had been arrested by the addition of the mating pheromone, α-factor, were transferred to fresh medium at 14°C, a nonpermissive temperature for colony formation by *Δyrb2* cells. Samples were taken every 30 min, and the budding index and Clb/p34^{CDC28} kinase activity were determined. In order to detect total Clb/p34^{CDC28} kinase activity, we used Suc1p beads as reported (*45*). Compared with wild-type cells, both bud-formation and Clb/p34^{CDC28} kinase-activation were delayed in *Δyrb2* cells (Fig. 2A). The most striking difference between wild-type and *Δyrb2* cells, however, was the length of mitosis. After activation of Clb/p34^{CDC28} kinase, the decrease in the level of Clb/p34^{CDC28} kinase activity was delayed in *Δyrb2* cells for almost one generation.

In order to avoid the delay at the start of the cell cycle caused by α-factor arrest, cultures of the AM43-1 and NBW6 strains were arrested in the S phase with hydroxyurea (HU). After release from the HU block, cells were

A

```
Sc.Yrb2p  MSETNGGNAARENSEVKQTAVENPIDKLDGTPKRPREKDQDE--------QAEETSDKSEAPNKN-DEEKKEEGKKDQEPSHKKIKVDDGKTVESGIVED
Sp.Hba1p  MTSKMENNKD-ESISTKNALEEKSNETKDETSKRKHDPAEEESAVSTKVSKSEPLEDKGNAEVKEFKETTKSNGVKPEVEITESTKIQKESNTEPCISTG
          *.    *  *.  *.. *.. .  * *.** ..  ..*         ..*   **..*  *.  *  * .* * .       *..     *. *  .

Sc.Yrb2p  DKKEDK-FVFGAASKFGTGFGVAKKDTKDGDATTSTESLPASD----SKTKKPF---------------AFGSGLSFGSGFNI-------LKNKTENNSE
Sp.Hba1p  GKVEEKELKVNKDVDENEGHVAVETGKKESAAKPAASVSPFSQFASFSNASSPFSNVSTASSEPKEEKSAFGAFASSKSAFTMKSVKDSPFKKFAAGTAV
          .* *.* .          *      . *.. *  ..  * *.   *..  **                ***.  *  *.* .      .*. .   .

Sc.Yrb2p  SEKKATDVDKDKVHSGSEQLAN---------------ASEDTKDKPKPL-------------KLQKQEVKSGEESEECIYQVNAKLYQLSNIKEGWKERG
Sp.Hba1p  ETESGSGKEKENDKKSSENFDELLANTSAKAFENQKGSAGETKSEPKEADKGSGDSTKSTMHQLSDSEIITGEEEEESIFSVRARLYVVADEKKTWKERG
             ... .*..  .**.. .                .. .**  **               .*   *. .*** ** *. * *.** ... *  *****

Sc.Yrb2p  VGIIKINKSKD-DVEKTRIVMRSRGILKVILNIQLVKGFT---VQKGFTGSLQSEKFIRLLAVDDNGDPAQYAIKTGKKETTDELYNIIVKSVPK------
Sp.Hba1p  QGILKVNVPKQRGSGSGRLLMRNDAVHRVIMNVPLFQGMSKKSLQIASASSGGSANYLKIFVIEN-GKSVLYAVRVKDNSLAEQLRNHVLEAIPKGGREDA
           **.*.* .*. .    *..**. .. .**.*. * .* .   .* . ..*  * ..... ... * .  **..   .  ...* * .. ..**
```

B

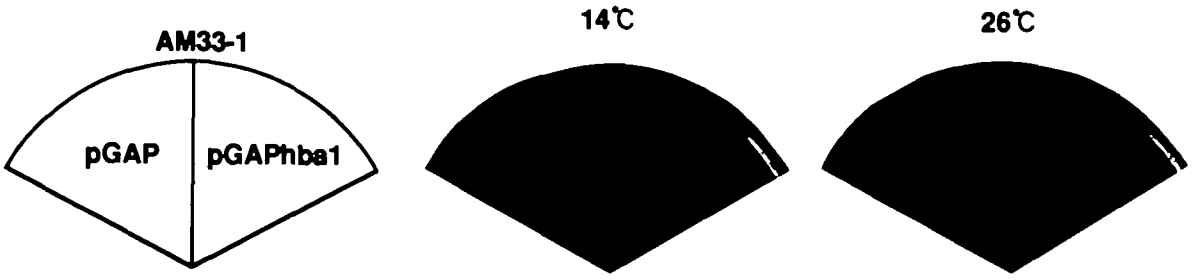
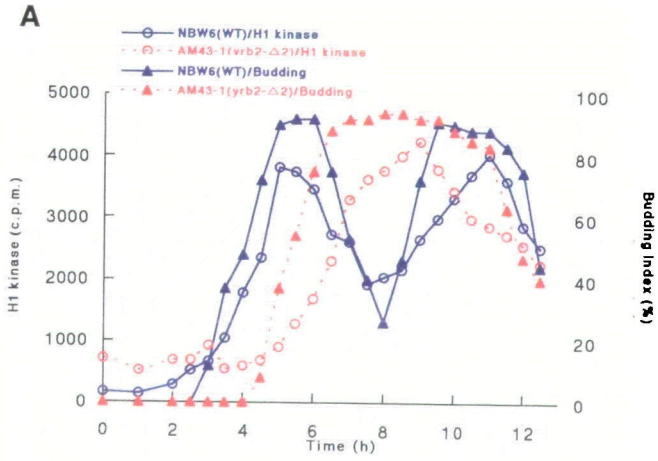


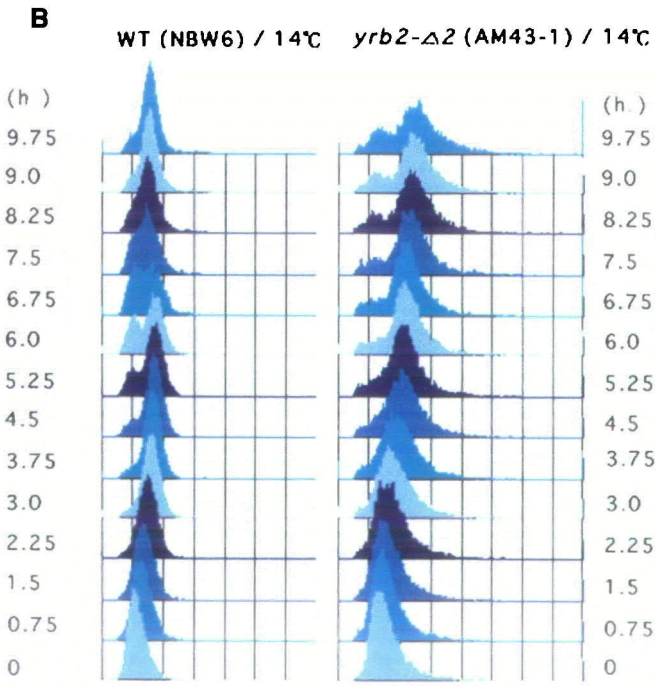
Fig. 1. ***S. pombe hba1*$^+$ rescues the cold sensitive colony formation of *yrb2-Δ2* cells.** A: Amino acid sequence comparison between Yrb2p and Hba1p. Stars and dots indicate the positions of identical and chemically conserved amino acid residues, respectively. B: pGAPhba1 and, as a control, the pGAP vector were introduced into the AM33-1 (*yrb2-Δ2*) strain. Trp$^+$ Transformants were streaked on synthetic medium (trp$^-$) plates, and incubated at either 14 or 26°C.

incubated in fresh YPD medium at 14°C. Samples were taken every 45 min, and assayed for DNA content and Clb/p34^{CDC28} kinase activity. FACS analysis indicated that DNA replication proceeded with the same kinetics in both wild-type and *Δyrb2* cells (Fig. 2B), but cells possessing 2C DNA content accumulated in *Δyrb2* cells afterwards. Consistent with the accumulation of cells possessing 2C DNA content, the activity of Clb/p34^{CDC28} kinase did not decrease in *Δyrb2* cells for the equivalent of about one generation of wild-type cells (Fig. 2C). Thus, disruption of the *YRB2* gene was concluded to cause a profound delay in mitosis.

Δyrb2 Cells Are Restrained at the Short Spindle Stage—In order to determine which stage of mitosis is affected by disruption of the *YRB2* gene, cultures of the AM43-1 strain and, as a control, the wild-type NBW6 strain were arrested with α-factor, and then transferred to fresh medium at 14°C. Cells were doubly stained, every 30 min, with a mAb to tubulin and Hoechst 33342 or propidium iodide. Representative images of each cell cycle phase are aligned in Fig. 3.

In cultures of *S. cerevisiae* released from α-factor arrest, the bud can be easily distinguished from the mother cell, which retains the "shmoo" form (*46*), and the nucleus with a short bipolar spindle migrates toward the neck ahead of the cytoplasmic microtubules. As previously reported (*47*), in wild-type cells, the nucleus was inserted into the neck (Fig. 3, WT). Subsequently, chromosomal DNA spanned the neck and then segregated equally to each pole (Fig. 3, WT, c to f). In contrast to in wild-type cells, the nucleus of *Δyrb2* cells was not trapped in the neck, but completely entered into the bud (Fig. 3, *yrb2-Δ2*). The nucleus in the bud was colocalized with the short bipolar spindle which was associated with aberrantly elongated cytoplasmic microtubules that emanated from one pole of the mother cell (Fig. 3, *yrb2-Δ2*, e). In the bud of *Δyrb2* cells, the nucleus divided (Fig. 3, *yrb2-Δ2*, f), and one of the daughter nuclei migrated back toward the mother cell along the mother-bud axis (Fig. 3, *yrb2-Δ2*, g to h), consistent with the ability of *Δyrb2* cells to proliferate in a liquid medium.





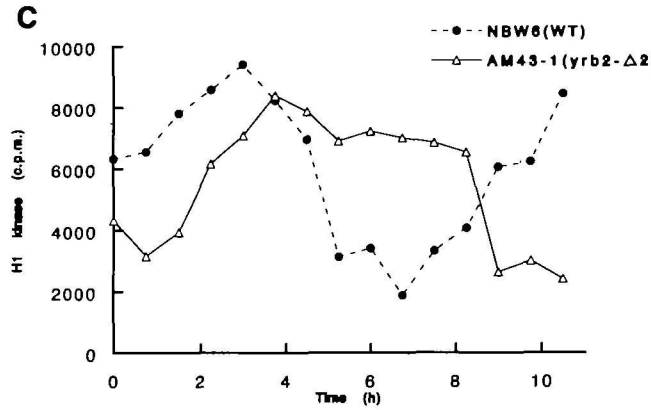


Fig. 2. **Cell-cycle progression of *yrb2-Δ2*.** A: Cultures of the AM43-1 strain and the wild-type NBW6 strain were treated with α-factor. After washing with distilled water, the cells were incubated in fresh YPD medium at 14°C. At the indicated time, cells were taken and processed for determination of the budding index and the histone H1 kinase activity. B and C: Cultures of the AM43-1 and NBW6 strains were treated with hydroxyurea for 4 h at 26°C and then for a further 1 h at 14°C. After washing with distilled water, the cells were incubated in fresh YPD medium at 14°C. At the indicated time, cells were analyzed for the DNA content with FACS (B), and assayed for histone H1 kinase activity (C).

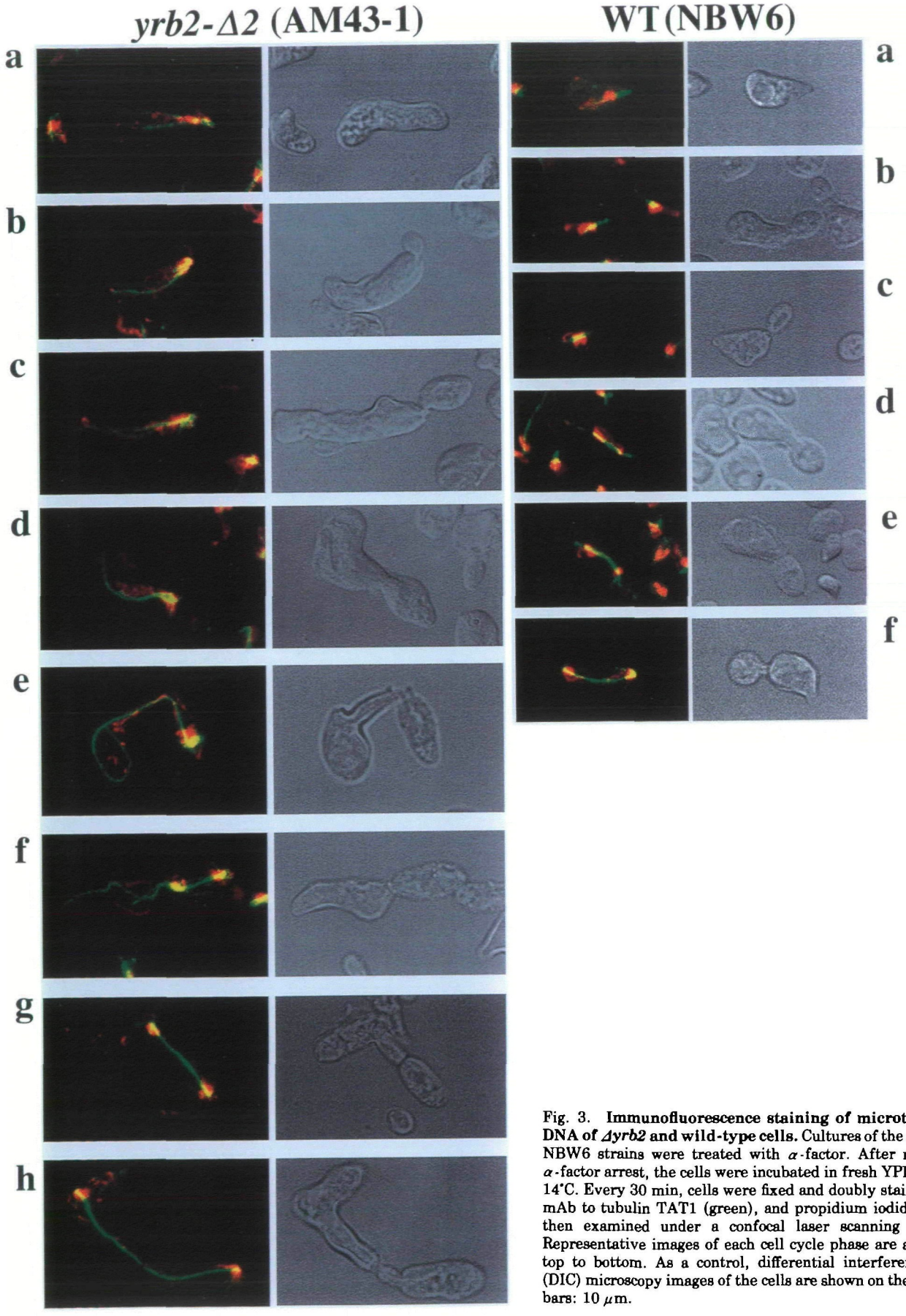


Fig. 3. **Immunofluorescence staining of microtubules and DNA of *Δyrb2* and wild-type cells.** Cultures of the AM43-1 and NBW6 strains were treated with α-factor. After release from α-factor arrest, the cells were incubated in fresh YPD medium at 14°C. Every 30 min, cells were fixed and doubly stained with the mAb to tubulin TAT1 (green), and propidium iodide (red), and then examined under a confocal laser scanning microscope. Representative images of each cell cycle phase are aligned from top to bottom. As a control, differential interference contrast (DIC) microscopy images of the cells are shown on the right. Scale bars: 10 μm.

At each time point following the release from α-factor arrest, the percentage of cells possessing either a short bipolar spindle or an elongated anaphase spindle was determined. Over the course of the experiment, wild-type cells synchronously formed short bipolar spindles which were subsequently converted into elongated anaphase spindles (Fig. 4A). In contrast to wild-type cells, *Δyrb2* cells were found to exhibit a considerable delay in the formation of short bipolar spindles and cells possessing a short spindle accumulated (Fig. 4B). By the time the wild-type cells had passed through the next interphase and started mitosis, a significant fraction of *Δyrb2* cells still possessed a short bipolar spindle which was associated with the cytoplasmic microtubules as shown in Fig. 3 *yrb2-Δ2*, d to e. The time-integrated number of cells which contained a short bipolar spindle was 2.8-fold higher for *Δyrb2* cells compared with for wild-type cells. On the other hand, the time-integrated number of cells which contained a long bipolar spindle was comparable for wild-type and *Δyrb2* cells (1.2-fold higher in *Δyrb2* cells). Therefore, it was concluded that the length of the short bipolar spindle stage was greatly increased by disruption of the *YRB2* gene, whereas the long bipolar spindle stage was not affected.

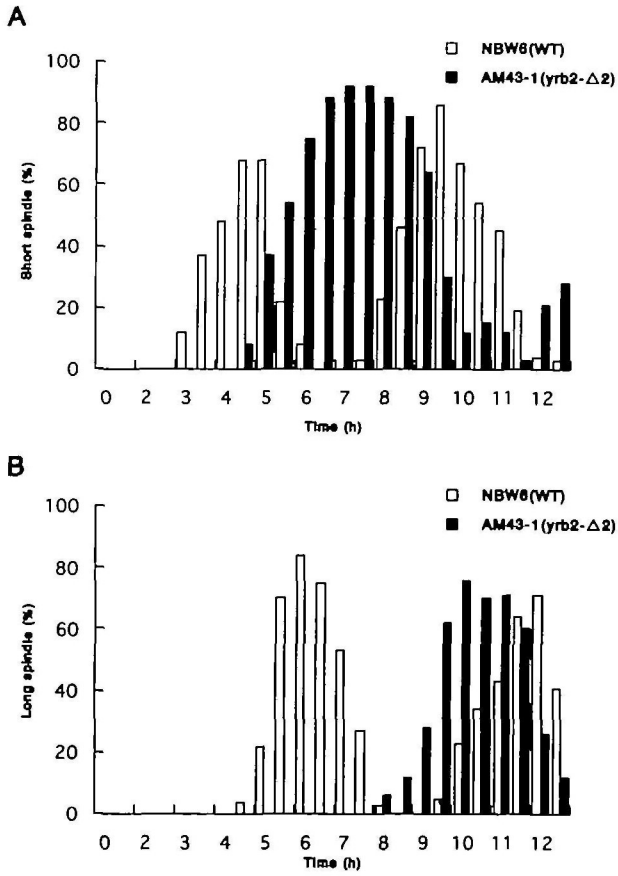


Fig. 4. **The progress of mitotic spindle assembly in *Δyrb2* and wild-type cells.** Cultures of the AM43-1 and NBW6 strains were treated with α-factor. After release from α-factor arrest, the cells were incubated in fresh YPD medium at 14°C. Every 30 min, cells were fixed, and then doubly stained with the mAb to tubulin and Hoechst 33342. Short spindles (A) were defined as spindles associated with non-divided nuclei, and long spindles (B) as spindles associated with divided nuclei.

No Chromosome Loss in Δyrb2 Cells—In order to address the question of whether or not the chromosomes were equally distributed into the daughter nuclei of *Δyrb2* cells, we prepared the AM40 strain, a homozygous diploid of *Δyrb2* (*yrb2-Δ2/yrb2-D2, ade2/ade2, +/ade3*), and then the frequency of chromosome loss was examined by sector formation on YPD medium plates containing 5% glucose. Cultures of the AM40 strain and, as a control, the wild-type diploid strain, AM41 (*YRB2/YRB2, ade2/ade2, +/ade3*), were grown in YPD medium at 30°C, the permissive temperature for colony formation, and then plated onto three YPD medium plates containing 5% glucose. One set of plates was continuously incubated at 30°C. The other two sets of plates were incubated for either 1 or 2 days at 14°C, and then incubated at 30°C. After 3 or 4 days incubation, colonies were examined for sector formation. Out of 1×10^4 colonies, none showed sectoring on plates incubated at 14 or 30°C.

In order to confirm that the mitotic checkpoint control is normal in *Δyrb2* cells, we examined the functional relation-

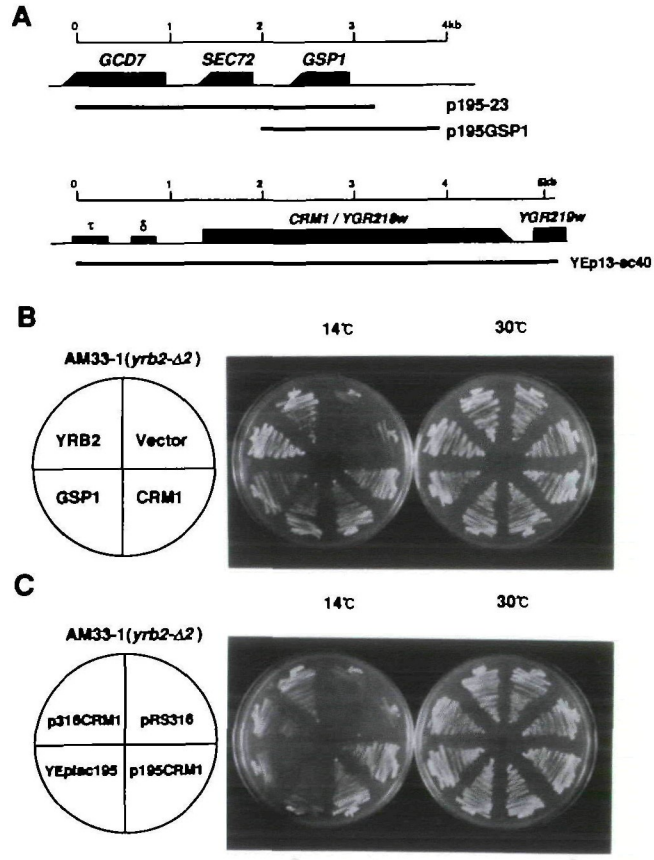


Fig. 5. ***XPO1/CRM1* and *GSP1* rescue the cold sensitive colony-formation of *Δyrb2*.** A: Maps of *S. cerevisiae* genomic DNA fragments. The thick bars indicate the isolated genomic fragments that rescued the cold-sensitivity of the AM43-1 strain. B: Suppression of *Δyrb2*. YRB2, GSP1, and CRM1 carried on the YEplac195 vector, and the vector alone were introduced into the AM33-1 strain. Ura$^+$ transformants were incubated on synthetic medium (ura$^-$) plates for 3 days at 26°C or for 10 days at 14°C. C: Suppression of *Δyrb2* with a single-copy of *CRM1*. p316CRM1 and, as controls, p195CRM1 and the vector alone were introduced into the AM33-1 strain. Ura$^+$ transformants were incubated on synthetic medium (ura$^-$) plates for 3 days at 26°C or for 10 days at 14°C.

ship of *yrb2Δ* with the *MAD* and *CDC5* genes, both of which regulate mitotic progression (*48-50*). A double disruptant of either *mad1Δ yrb2-Δ2* or *mad3Δ yrb2-Δ2* grew at 26°C, and over expression of Cdc5p did not rescue the cold sensitive growth of *Δyrb2* cells (data not shown).

XPO1/CRM1 and GSP1 Rescue the Cold Sensitivity of yrb2-Δ2 Cells—In order to identify the genes which functionally interact with *YRB2*, a genomic DNA-library of the AM33-1 strain, which was constructed using a multicopy vector, YEplac195 (*51*), was introduced into the AM33-1 (*yrb2-Δ2::LEU2*) strain. Out of 1×10^4 ura$^+$ transformants, two colonies became papillated at 14°C. The plasmids obtained from these colonies were found to have the same DNA insert containing three ORFs: *GCD7*, *SEC72*, and *GSP1* (Fig. 5A). Of these, *GSP1* was subcloned into the YEplac195 vector, resulting in p195GSP1. Independently, a genomic library of the wild-type *S. cerevisiae* which was constructed using the multicopy vector, YEp13 (*52*), was introduced into the AM50-1 strain (*yrb2-Δ2::HIS3*). Out of 1×10^5 leu$^+$ transformants, 38 colonies became papillated at 14°C. Two plasmids recovered from these colonies were found to contain a single ORF covering the whole *XPO1/CRM1*, the other 36 plasmids possessing *YRB2* alone. The obtained *XPO1/CRM1* gene carried on the YEp13 vector was subcloned into the YEplac195 vector, resulting in p195CRM1. Both p195GSP1 and p195CRM1,

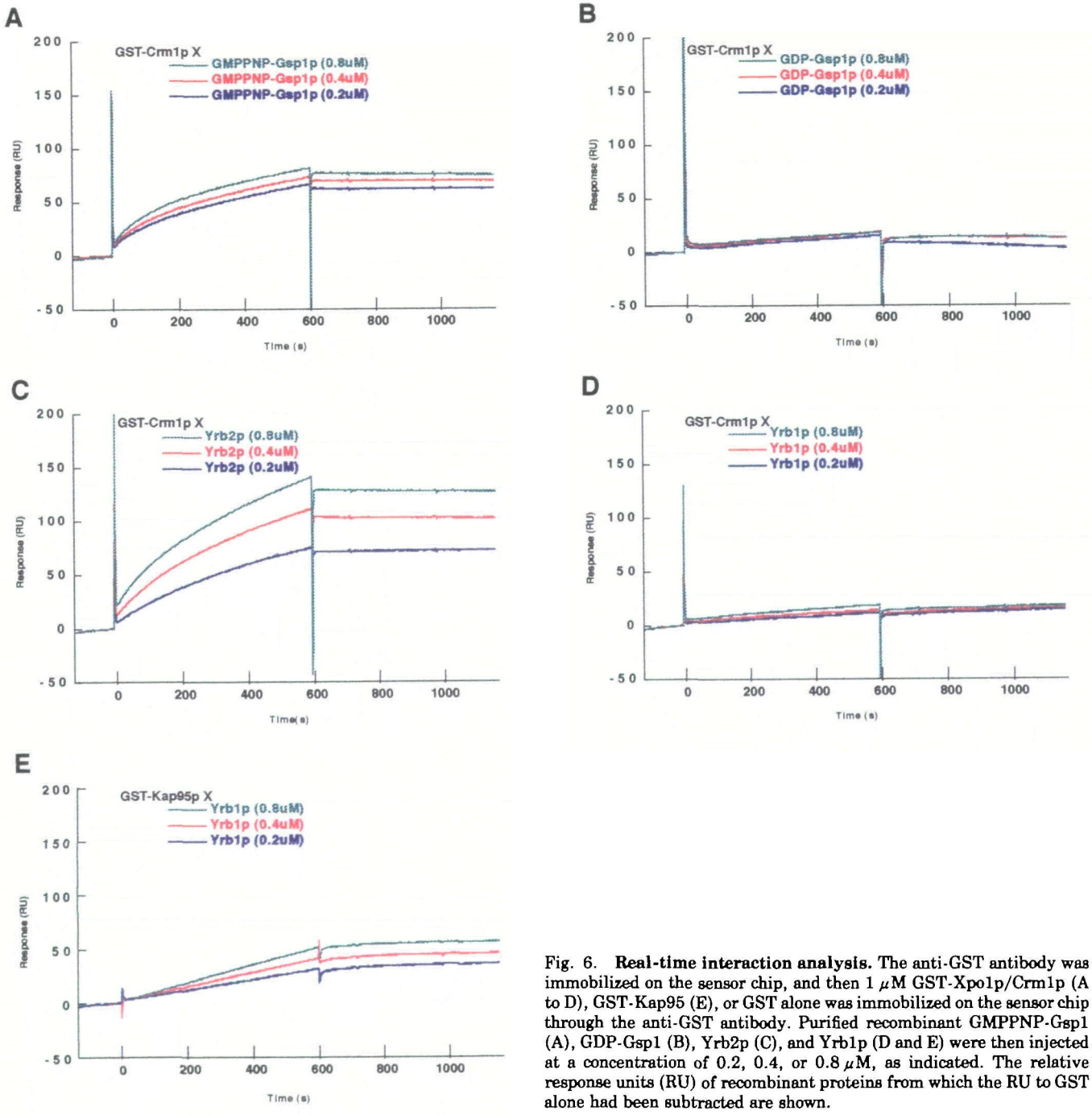


Fig. 6. **Real-time interaction analysis.** The anti-GST antibody was immobilized on the sensor chip, and then 1 μM GST-Xpo1p/Crm1p (A to D), GST-Kap95 (E), or GST alone was immobilized on the sensor chip through the anti-GST antibody. Purified recombinant GMPPNP-Gsp1 (A), GDP-Gsp1 (B), Yrb2p (C), and Yrb1p (D and E) were then injected at a concentration of 0.2, 0.4, or 0.8 μM, as indicated. The relative response units (RU) of recombinant proteins from which the RU to GST alone had been subtracted are shown.

and as controls, p195YRB2 and the vector alone were introduced into the AM33-1 strain. Ura$^+$ transformants were incubated on synthetic medium (ura$^-$) plates at 14 or 30°C (Fig. 5B). Both p195GSP1 and p195CRM1 rescued the cold sensitive colony-formation of *Δyrb2* cells.

Yrb2p Directly Binds to Xpo1p/Crm1p—The *XPO1/CRM1* gene carried on a single copy plasmid, pRS316 (*53*), also rescued the cold sensitive colony-formation of the AM33-1 strain (Fig. 5C). The finding that *Δyrb2* cells can be suppressed by a single copy of *CRM1* revealed the close functional interaction between Xpo1/Crm1p and Yrb2p. In order to address the question of whether or not Xpo1p/Crm1p directly interacts with Yrb2p, both Xpo1p/Crm1p and Yrb2p, and as controls, Yrb1p, Gsp1p, and Kap95p (*S. cerevisiae* Importin β), were each produced in *E. coli* as a GST-fusion protein and purified on a glutathione column. GST-fused Xpo1p/Crm1p and as controls, GST-Kap95p and GST alone were fixed on the sensor chips through the mAb to GST immobilized on the sensor chips. Subsequently, either Yrb2p or Yrb1p was injected onto Xpo1p/Crm1p, Kap95p, or GST alone. As a control, GMPPNP- and GDP-bound Gsp1p were independently injected onto Xpo1p/Crm1p, or GST alone. From the obtained response units, the RU of GST alone was subtracted as the background.

We found that *E. coli* produced *S. cerevisiae* Xpo1p/Crm1p bound to GMPPNP-, but not to GDP-Gsp1p (Fig. 6).

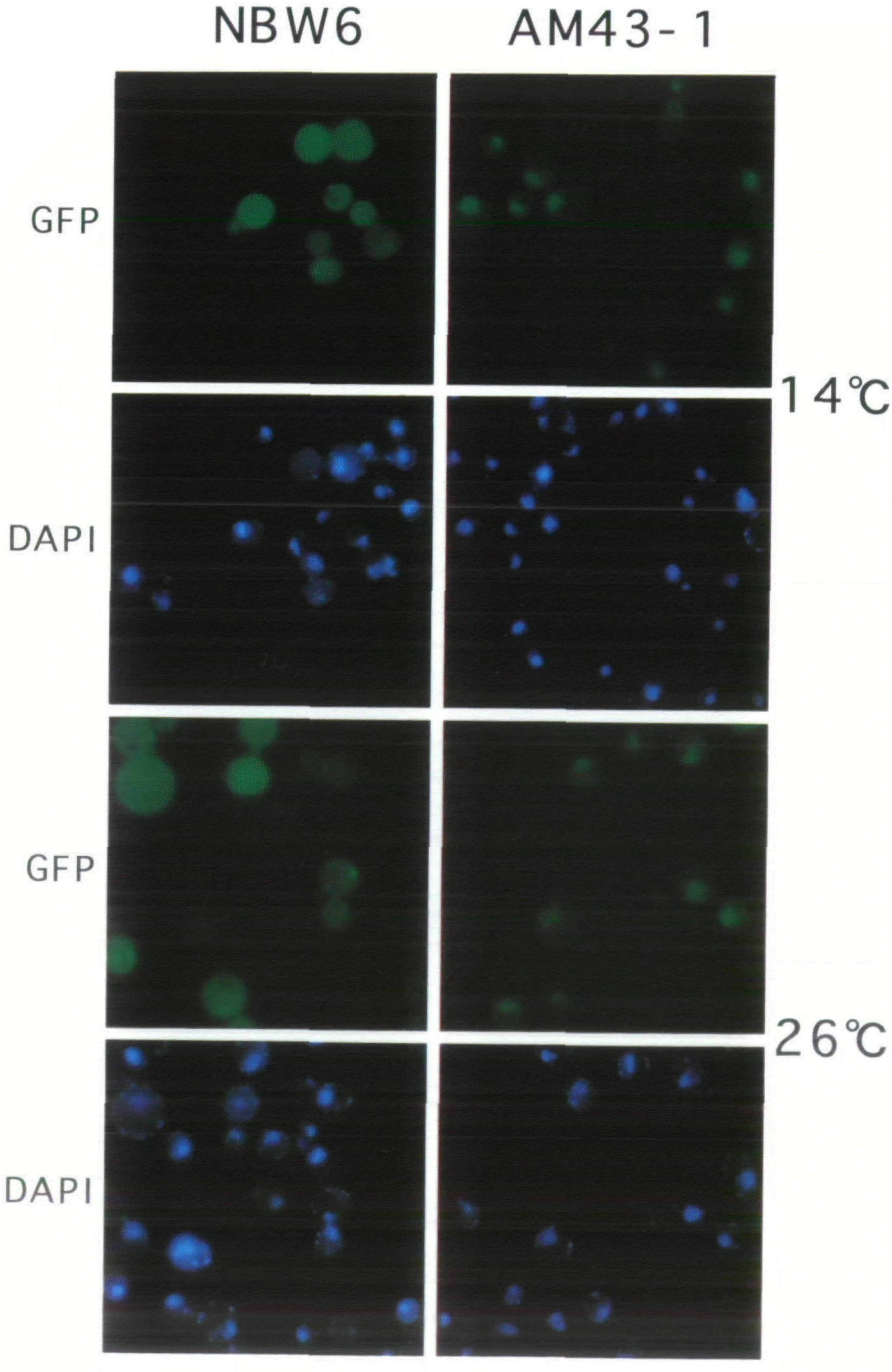


Fig. 7. ***Δyrb2* has a defect in nuclear protein export.** pKW430 carrying the ORF of the NES-GFP2-NLS reporter was introduced into the AM43-1 and, as a control, NBW6 strains. Ura$^+$ transformants were cultivated in synthetic medium (ura$^-$) to the mid-log phase and fixed, and then GFP was excited with blue light.

Under the same buffer conditions, Yrb2p bound to Xpo1p/Crm1p in a dose-dependent manner, but Yrb1p did not. On the other hand, GST-Kap95p bound to Yrb1p in a dose-dependent manner. These results indicate that Xpo1p/Crm1p could directly interact with Yrb2p. Furthermore, our data indicated a direct interaction between GTP-Gsp1p and Xpo1p/Crm1p.

Δyrb2 Cells Have a Defect in Nuclear Protein Export—The direct interaction between Xpo1p/Crm1p and Yrb2p suggested that Crm1p might be an immediate downstream effector of Yrb2p. Since Xpo1p/Crm1p is essential for NES-mediated protein export (*39, 54*), we examined whether or not *Δyrb2* cells have a defect in nuclear protein export. In order to address this issue, plasmid pKW430 possessing the NES-GFP-NLS reporter (*39*) was introduced into the AM43-1 strain and, as a control, the NBW6 strain. Ura^+ transformants were incubated at 14°C for 12 h and then GFP was excited with blue light. As previously reported (*39*), the NES-GFP-NLS reporter was localized in the cytoplasm of wild-type cells (Fig. 7). In all the AM43-1 cells which expressed the transfected NES-GFP-NLS reporter, however, the GFP signal was detected in the nucleus. Even at 26°C, a permissive temperature for colony formation, NES-GFP-NLS reporter-proteins were localized in the nucleus of AM43-1 cells. Thus, nuclear protein export was defective in *Δyrb2* cells.

DISCUSSION

Yrb2p is structurally conserved from yeast to man (*25–27, 30, 31*). We found that *S. pombe* Hba1p can be exchanged for Yrb2p, indicating that Yrb2p has been functionally conserved as well. In *Δyrb2* cells, mitosis is profoundly delayed at the short spindle stage. Cells lacking Nap1p, a protein that specifically binds to Clb2p (*56*) show a similar mitotic delay (*57*). However, the effect of cyclin expression is different between *Δyrb2* and *Δnap1*. In the presence of a complete set of *CLB* genes, *Δnap1* cells do not show a mitotic delay (*57*), but *Δyrb2* cells show a profound mitotic delay. Nap1p is specifically involved in the regulation of $Clb2/p34^{CDC28}$ kinase, and the decrease of this kinase activity is defective in *Δnap1* cells. In contrast to in *Δnap1* cells, total $Clb/p34^{CDC28}$ kinase activity is affected by disruption of the *YRB2* gene, resulting in the prolonged activation of $Clb/p34^{CDC28}$ kinase. In *S. cerevisiae* mitosis, the nucleus is inserted into the neck between the mother and the bud, and then divided. In *Δyrb2* cells, however, the nucleus was not inserted into the neck, but moved entirely into the bud. The movement and positioning of the nucleus is dependent on the balance of microtubule polymerization and the actions of motor proteins, in addition to the functions of the factors tethering the nucleus to the neck (*47*). Overproduction of *Xenopus* cyclin A1 in *S. cerevisiae* has been reported to force the nucleus to migrate into the bud (*58*), this being consistent with our present finding that the prolonged activation of $Clb/p34^{CDC28}$ kinase causes the bud migration of the nucleus.

Interestingly, the nucleus which entirely moved into the bud normally divided in the bud, and one of the daughter nuclei migrated back toward the mother cell. Thus, the short spindle stage was elongated, but not the long spindle stage. Since we detected no chromosome loss, the mitotic check point control does not appear to be affected by disruption of the *YRB2* gene. Consistently, *Δyrb2* cells are not synthetic lethal with a defect of the *MAD* genes. The inactivation of $Clb/p34^{CDC28}$ kinase primarily occurs through ubiquitin-dependent cyclin proteolysis. Indeed, overexpression of Clb2p inhibits the growth of *Δyrb2* cells (E. Noguchi, unpublished results). However, overexpression of the *CDC5* gene, that has been reported to suppress the *cdc15*, *cdc20*, and *dbf2* mutations, which affect cyclin destruction (*50*), did not rescue the cold sensitive growth of *yrb2Δ* cells. In contrast, overexpression of Crm1p/Xpo1 rescued the cold sensitive colony-formation of *Δyrb2* cells. Since Crm1p/Xpo1 is essential for nuclear protein export (*39, 54*), this finding indicates that *Δyrb2* cells may have a defect in nuclear protein export, whereas they have no defect in either nuclear protein import or mRNA export (*27, 30*). While this manuscript was in preparation, it was reported that Yrb2p is involved in nuclear protein export (*55*). Consistently, we found that nuclear protein export is retarded in *Δyrb2* cells. Therefore, it is quite possible that the retarded inactivation of $Clb/p34^{CDC28}$ kinase was caused by a defect in nuclear protein export. In mammalian cells, MPF has been reported to shuttle between the nucleus and the cytoplasm (*59*). This may also be true for yeast. *S. cerevisiae srp1-31*, a ts mutant of the NLS-receptor, Importin α, which has a defect in nuclear protein import, shows a defect in cyclin proteolysis, resulting in G2/M arrest (*60*). Similarly, *cse1-22*, an allele of *CSE1*, the *Xenopus* homologue of which, CAS, is required for the nuclear export of Importin α (*61*), shows a defect in cyclin B destruction (*62*). Taken together, the interruption of either the import or export of nuclear proteins could affect MPF activity. The finding that overexpression of Gsp1p rescued the cold sensitive colony-formation of *Δyrb2* cells also supports this conclusion.

Xpo1p/Crm1p directly binds to Yrb2p, but not to Yrb1p. As reported here, Xpo1p/Crm1p also binds to GTP-Gsp1p, but not to GDP-Gsp1p. RanBP1 functions as a coactivator of Importin β by forming trimeric complexes with RanGTP and Importin β, as described in the introduction. In this context, it is notable that Yrb1p does not bind to Crm1p. Since our Yrb1p preparation bound to Kap95, the *S. cerevisiae* homologue of Importin β, the inability of Yrb1p to bind to Crm1p is not due to inactivation of our Yrb1p preparation. In the nucleus, Yrb2p may take over the function of Yrb1p, and therefore Yrb2p may form a trimeric complex with GTP-Gsp1p and Xpo1/Crm1p to stimulate the nuclear protein export. This notion is consistent with the fact that both Gsp1p and Crm1p rescue the cold sensitive growth character of *Δyrb2* cells, and with the report that *Δyrb2* cells are synthetic lethal with the *xpo1-1* mutation (*55*).

We wish to thank Dr. A. Murray for the *mad* gene disruption plasmids, Dr. A. Sugino for yeast strain and plasmid containing *Cdc5*, Dr. K. Gull for the anti-tubulin antibody TAT1, Dr. K. Weis for pKW430, and our colleague at Kyushu University, M. Oki for the *GSP1* plasmid.

REFERENCES

1. Belhumeur, P., Lee, A., Tam, R., DiPaolo, T., Fortin, N., and Clark, M.W. (1993) *GSP1* and *GSP2*, genetic suppressors of the *prp20-1* mutant in *Saccharomyces cerevisiae*: GTP-binding proteins involved in the maintenance of nuclear organization.

Mol. Cell. Biol. **13**, 2152-2161
2. Bischoff, F.R., Krebber, H., Kempf, K., Hermes, I., and Ponstingl, H. (1995) Human RanGTPase-activating protein RanGAP1 is homologue of yeast Rna1p involved in mRNA processing and transport. *Proc. Natl. Acad. Sci. USA* **92**, 1749-1753
3. Becker, J., Melchior, F., Gerke, V., Bischoff, F.R., Ponstingl, H., and Wittinghofer, A. (1995) RNA1 encodes a GTPase-activating protein specific for Gsp1, the Ran/TC4 homologue of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **270**, 11860-11865
4. Bischoff, F.R. and Ponstingl, H. (1991) Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature* **354**, 80-82
5. Ohtsubo, M., Ozaki, H., and Nishimoto, T. (1989) The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and bind to DNA. *J. Cell Biol.* **109**, 1389-1397
6. Frasch, M. (1991) The maternally expressed *Drosophila* gene encoding the chromatin-binding protein BJ1 is a homolog of the vertebrate gene regulator of chromatin condensation, RCC1. *EMBO J.* **10**, 1225-1236
7. Matynia, A., Dimitrov, K., Mueller, U., He, X., and Sazer, S. (1996) Perturbations in the spi1p GTPase cycle of *Schizosaccharomyces pombe* through its GTPase-activating protein and guanine nucleotide exchange factor components result in similar phenotypic consequences. *Mol. Cell Biol.* **16**, 6352-6362
8. Matunis, M.J., Coutavas, E., and Blobel, G. (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J. Cell Biol.* **135**, 1457-1470
9. Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997) A small ubiquitin related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* **88**, 97-107
10. Görlich, D. and Mattaj, I.W. (1996) Nucleocytoplasmic transport. *Science* **271**, 1513-1518
11. Nigg, E.A. (1997) Nucleocytoplasmic transport: signals, mechanism and regulation. *Nature* **386**, 779-787
12. Weis, K. (1998) Importins and exportins: how to get in and out of the nucleus. *Trends Biochem. Sci.* **23**, 185-189
13. Melchior, F. and Gerace, L. (1998) Two-way trafficking with Ran. *Trends Cell Biol.* **8**, 175-179
14. Wozniak, R., Rout, M.P., and Aitchison, J.D. (1998) Karyopherins and Kissing cousins. *Trends Cell Biol.* **8**, 184-188
15. Dingwall, C.D., Kandels-Lewis, S., and Seraphin, B. (1995) A family of Ran binding proteins that includes nucleoporins. *Proc. Natl. Acad. Sci. USA* **92**, 7525-7529
16. Hartman, E. and Görlich, D. (1995) A Ran-binding motif in nuclear pore proteins. *Trends Cell Biol.* **5**, 192-193
17. Görlich, D., Dabrowski, M., Bischoff, F.R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997) A novel class of RanGTP binding proteins. *J. Cell Biol.* **138**, 65-80
18. Ullman, K.S., Powers, M.A., and Forbes, D.J. (1997) Nuclear export receptors: from Importin to exportin. *Cell* **90**, 967-970
19. Coutavas, E., Ren, M., Oppenheim, J.D., D'Eustachio, P., and Rush, M.G. (1993) Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. *Nature* **366**, 585-587
20. Ouspenski, I.L., Mueller, U.W., Matynia, A., Sazer, S., Elledge, S.J., and Brinkley, B.R. (1995) Ran-binding protein-1 is an essential component of the Ran/RCC1 molecular switch system in budding yeast. *J. Biol. Chem.* **270**, 1975-1978
21. Hayashi, N., Yokoyama, N., Seki, T., Azuma, Y., Ohba, T., and Nishimoto, T. (1995) RanBP1, a Ras-like nuclear G-protein binding to Ran/TC4, inhibits RCC1 via Ran/TC4. *Mol. Gen. Genet.* **247**, 661-669
22. Yokoyama, N., Hayashi, N., Seki, T., Pante, N., Ohba, T., Nishii, K., Kuma, K., Hayashida, T., Miyata, T., Aebi, U., Fukui, M., and Nishimoto, T. (1995) A giant nucleopore protein that binds Ran/TC4. *Nature* **376**, 184-188
23. Wu, J., Matunis, M.J., Kraemer, D., Blobel, G., and Coutavas, E. (1995) Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. *J. Biol. Chem.* **270**, 14209-14213
24. Melchior, F., Guan, T., Yokoyama, N., Nishimoto, T., and Gerace, L. (1995) GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. *J. Cell Biol.* **131**, 571-581
25. Mueller, L., Cordes, V.C., Bischoff, F.R., and Ponstingl, H. (1998) Human RanBP3, a group of nuclear RanGTP binding proteins. *FEBS Lett.* **427**, 330-336
26. Schlenstedt, G., Wong, D.H., Koepp, D., and Silver, P.A. (1995) Mutants in a yeast Ran binding protein are defective in nuclear transport. *EMBO J.* **14**, 5367-5378
27. Noguchi, E., Hayashi, N., Nakashima, N., and Nishimoto, T. (1997) Yrb2p, Nup2p-related yeast protein has functional overlap with Rna1p, yeast RanGAP protein. *Mol. Cell. Biol.* **17**, 2235-2246
28. He, X., Hayashi, N., Walcott, N.G., Azuma, Y., Patterson, T.E., Bischoff, F.R., Nishimoto, T., and Sazer, S. (1998) The identification of cDNAs that affect the mitosis-to-interphase transition in *Schizosaccharomyces pombe*, including sbp1, which encodes a spi1p-GTP-binding protein. *Genetics* **148**, 645-656
29. Richards, S., Lounsbury, K.M., Carey, K.L., and Macara, I.G. (1996) A nuclear export signal is essential for the cytosolic localization of the Ran binding protein, RanBP1. *J. Cell Biol.* **134**, 1157-1168
30. Taura, T., Schlenstedt, G., and Silver, P.A. (1997) Yrb2p is a nuclear protein that interacts with Prp20p, a yeast Rcc1 homologue. *J. Biol. Chem.* **272**, 31877-31884
31. Turi, T.G., Mueller, U.W., Sazer, S., and Rose, J.K. (1996) Characterization of a nuclear protein conferring brefeldin A resistance in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **271**, 9166-9171
32. Schlenstedt, G., Smirnova, E., Deane, R., Solsbacher, J., Kutay, U., Gorlich, D., Ponstingl, H., and Bischoff, F.R. (1997) Yrb4p, a yeast Ran-GTP-binding protein involved in import of ribosomal protein L25 into the nucleus. *EMBO J.* **16**, 6237-6249
33. Chi, N., Adam, J.H., Visser, G.D., and Adam, S.A. (1996) RanBP1 stabilizes the interaction of Ran with p97 in nuclear protein import. *J. Cell Biol.* **135**, 559-569
34. Lounsbury, K. and Macara, I.G. (1997) Ran-binding protein 1 (RanBP1) form a ternary complex with Ran and Karyopherin β and reduces Ran GTPase-activating protein (RanGAP) inhibition by Karyopherin β. *J. Biol. Chem.* **272**, 551-555
35. Bischoff, F.R. and Görlich, D. (1997) RanBP1 is crucial for the release of RanGTP from importin β-related nuclear transport factors. *FEBS Lett.* **419**, 249-254
36. Hutter, K.J. and Eipel, H.E. (1979) Microbial determinations by flow cytometry. *J. Gen. Microbiol.* **113**, 369-375
37. Hagan, I.M. and Hyams, J.S. (1988) The use of cell division cycle mutants to investigate the control of microtubule distribution in fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **89**, 343-357
38. Woods, A., Sherwin, T., Sasse, R., MacRAE, T.H., Baines, A.J., and Gull, K. (1989) Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J. Cell Sci.* **93**, 491-500
39. Stade, K., Ford, C.S., Guthrie, C., and Weis, K. (1997) Exportin (Xpo1p/XPO1/CRM1p) is an essential nuclear export factor. *Cell* **90**, 1041-1050
40. Hayashi, N., Seino, H., Irie, K., Watanabe, M., Clark, K.L., Matsumoto, K., and Nishimoto, T. (1996) Genetical interaction of *DED1* encoding a putative ATP-dependent RNA helicase with *SRM1* encoding a mammalian RCC1 homologue in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **253**, 149-156
41. Winston, F., Chumley, F., and Fink, G.R. (1983) Eviction and transplacement of mutant genes in yeast. *Methods Enzymol.* **101**, 211-228
42. Yu-Sherman, M. and Goldberg, A.L. (1992) Involvement of the chaperonin dnaK in the rapid degradation of a mutant protein in *Escherichia coli*. *EMBO J.* **11**, 71-77
43. Dasso, M., Seki, T., Azuma, Y., Ohba, T., and Nishimoto, T. (1994) A mutant form of the Ran/TC4 protein disrupts nuclear function in *Xenopus laevis* egg extracts by inhibiting the RCC1

protein, a regulator of chromosome condensation. *EMBO J.* **13**, 5732-5744
44. Azuma, Y., Hachiya, T., and Nishimoto, T. (1997) Inhibition by anti-Rcc1 monoclonal antibodies of Rcc1-stimulated guanine nucleotide exchange on Ran GTPase. *J. Biochem.* **122**, 1133-1138
45. Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A.B., and Nasmyth, K. (1991) The role of *CDC28* and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell* **65**, 145-161
46. Cross, F., Hartwell, L.H., Jackson, C., and Konopka, J. B. (1988) Conjugation in *Saccharomyces cerevisiae*. *Annu. Rev. Cell Biol.* **4**, 429-457
47. Yeh, E., Skibbens, R.V., Cheng, J.W., Salmon, E.D., and Bloom, K. (1995) Spindle dynamics and cell cycle regulation of dynein in the budding yeast, *Saccharomyces cerevisiae*. *J. Cell Biol.* **130**, 687-700
48. Hoyt, M.A., Totis, L., and Roberts, B.T. (1991) *S. cerevisiae* genes required for cell-cycle arrest in response to loss of microtubule function. *Cell* **66**, 507-517
49. Li, R. and Murray, A.W. (1991) Feedback control of mitosis in budding yeast. *Cell* **66**, 519-531
50. Kitada, K., Johnson, A.L., Johnston, L.H., and Sugino, A. (1993) A multicopy suppressor gene of the *Saccharomyces cerevisiae* G1 cell cycle mutant gene *dbf4* encodes a protein kinase and is identified as *CDC5*. *Mol. Cell Biol.* **13**, 4445-4457
51. Gietz, R.D. and Sugino, A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base restriction sites. *Gene* **74**, 527-534
52. Broach, J.R., Strathern, J.N., and Hicks, J.B. (1979) Transformation in yeast: development of a hybrid cloning vector and isolation of the CAN1 gene. *Gene* **8**, 121-133
53. Sikorski, R.S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19-27
54. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I.W. (1997) XPO1/CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-1060
55. Taura, T., Krebber, H., and Silver, P.A. (1998) A member of the Ran-binding protein family, Yrb2p, is involved in nuclear protein export. *Proc. Natl. Acad. Sci. USA* **95**, 7427-7432
56. Kellogg, D.R., Kikuchi, A., Fujii-Nakata, T., Turck, C.W., and Murray, A.W. (1995) Members of NAP/SET family of proteins interact specifically with B-type cyclins. *J. Cell Biol.* **130**, 661-673
57. Kellogg, D.R. and Murray, A.W. (1995) NAP1 acts with Clb2 to perform mitotic functions and to suppress polar bud growth in budding yeast. *J. Cell Biol.* **130**, 675-685
58. Sikder, H., Funakoshi, M., Nishimoto, T., and Kobayashi, H. (1997) An altered nuclear migration into the daughter bud is induced by the cyclin A1-mediated Cdc28 kinase through an aberrant spindle movement in *Saccharomyces cerevisiae*. *Cell Struct. Funct.* **22**, 465-476
59. Hagting, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J. (1998) MPF localization is controlled by nuclear export. *EMBO J.* **17**, 4127-4138
60. Loeb, J.D.J., Schlenstedt, G., Pellman, D., Komitzer, D., Silver, P.A., and Fink, G.R. (1995) The yeast nuclear import receptor is required for mitosis. *Proc. Natl. Acad. Sci. USA* **92**, 7647-7651
61. Kutay, U., Izaurralde, E., Bischoff, F.R., Mattaj, I.W., and Görlich, D. (1997) Dominant-negative mutants of importin-β block multiple pathways of import and export through the nuclear pore complex. *EMBO J.* **16**, 1153-1163
62. Irniger, S., Piatti, S., Michaelis, C., and Nasmyth, K. (1995) Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell* **81**, 269-277
63. Matsuzaki, H., Nakajima, R., Nishiyama, J., Araki, H., and Oshima, Y. (1990) Chromosome engineering in *Saccharomyces cerevisiae* by using a site-specific recombination system of yeast plasmid. *J. Bacteriol.* **172**, 610-618
64. Tanaka, K., Matsumoto, K., and Toh-e, A. (1988) Dual regulation of the polyubiquitin gene by cyclic AMP and heat shock in yeast. *EMBO J.* **7**, 495-502
65. Oki, M., Noguchi, E., Hayashi, N., and Nishimoto, T. (1998) Nuclear protein import, but not mRNA export, is defective in all *Saccharomyces cerevisiae* mutants that produce temperature-sensitive forms of the Ran GTPase homologue Gsp1p. *Mol. Gen. Genet.* **257**, 624-634