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Yeast Ran-binding Protein Yrb1p Is Required for Efficient Proteolysis of Cell Cycle Regulatory Proteins Pds1p and Sic1p*

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Ubiquitin-dependent proteolysis of specific target proteins is required for several important steps during the cell cycle. Degradation of such proteins is strictly cell cycle-regulated and triggered by two large ubiquitin ligases, termed anaphase-promoting complex (APC) and Skp1/Cullin/F-box complex (SCF). Here we show that yeast Ran-binding protein 1 (Yrb1p), a predominantly cytoplasmic protein implicated in nucleocytoplasmic transport, is required for cell cycle regulated protein degradation. Depletion of Yrb1p results in the accumulation of unbudded G_1 cells and of cells arrested in mitosis implying a function of Yrb1p in the G_1/S transition and in the progression through mitosis. Temperature-sensitive *yrb1-51* mutants are defective in APC-mediated degradation of the anaphase inhibitor protein Pds1p and in degradation of the cyclin-dependent kinase inhibitor Sic1p, a target of SCF. Thus, Yrb1p is crucial for efficient APC- and SCF-mediated proteolysis of important cell cycle regulatory proteins. We have identified the *UBS1* gene as a multicopy suppressor of *yrb1-51* mutants. Ubs1p is a nuclear protein, and its deletion is synthetic lethal with a *yrb1-51* mutation. Interestingly, *UBS1* was previously identified as a multicopy suppressor of *cdc34-2* mutants, which are defective in SCF activity. We suggest that Ubs1p may represent a link between nucleocytoplasmic transport and ubiquitin ligase activity.

There are three major transitions in the cell cycle of the budding yeast *Saccharomyces cerevisiae*: the G_1/S transition when cells commit to replicate their genome, the metaphase to anaphase transition, when sister chromatids are separated, and the exit from mitosis, when the mitotic spindle is depolymerized and cytokinesis occurs. For efficient proliferation, cells must ensure the correct order of these events. Therefore, execution of these processes is tightly controlled.

One important cell cycle regulator is the cyclin-dependent kinase Cdk1p, also called Cdc28p (1). Cdk1p is active only in association with a member of either the B-type cyclins Clb1-6p or the G_1 cyclins Cln1-3p. Another important mechanism of cell cycle control is regulated protein degradation (2, 3). Such proteins are targeted for proteolytic destruction by the formation of covalently linked chains of ubiquitin molecules. The final step of this fusion process, the ligation of activated ubiquitin to the substrate, is catalyzed by ubiquitin ligases. Two of these ubiquitin-protein isopeptide ligase enzymes are essential for cell cycle progression: the anaphase-promoting complex (APC),[1] also called cyclosome, and the Skp1/Cullin/F-box (SCF) complex.

Components of the SCF complex are Skp1p, the cullin protein Cdc53p, the ring finger protein Hrt1p, the ubiquitin-conjugating enzyme Cdc34p, and one of several F-box proteins such as Cdc4p (4, 5). SCF is constantly active throughout the cell cycle, but ubiquitination of specific SCF substrates is regulated by substrate phosphorylation. An essential role of SCF is the destruction of Sic1p, which is an inhibitor specific for Cdk1p associated with B-type cyclins (6). At the G_1/S transition, G_1 cyclins Cln1p and Cln2p accumulate and activate Cdk1p to phosphorylate Sic1p (6). Phosphorylated Sic1p is then rapidly degraded by the SCF complex (5, 7). Thereby, Cdk1p associated with S phase cyclins Clb5p and Clb6p becomes active and triggers DNA replication.

APC is a large protein complex that consists of at least 12 subunits in *S. cerevisiae* and is highly conserved in eukaryotes (8, 9). At the metaphase to anaphase transition, APC triggers sister chromatid separation by targeting the anaphase inhibitor Pds1p for destruction (10). APC is also required for the mitotic exit. It triggers degradation of B-type cyclins leading to the inactivation of cyclin-dependent kinases (9, 11).

Several yeast strains that are mutated in essential components of the nucleocytoplasmic transport machinery do not stop cell cycle progression randomly but show distinct cell cycle arrest phenotypes, predominantly in the G_2/M phase. Such strains are *cse1* (12), *srp1* (13), *yrb1* (14), and *sac3* (15) mutants. These observations indicate an important role of nucleocytoplasmic transport for cell cycle progression. One of these mutants, *srp1-31*, was demonstrated to be defective in degradation of the mitotic cyclin Clb2p (13).

A crucial component for nuclear transport in all eukaryotes is the small Ras-like GTPase Ran, which exists in a GTP- and a GDP-bound form (16–18). Ran is present both in the nucleus and in the cytoplasm, but the two Ran pools differ with respect to their GTP/GDP binding state; because of a cytosolic Ran GTPase activating protein (Ran-GAP), cytosolic Ran is predominantly in its GDP-bound form. In the nucleus, Ran is kept in its GTP-bound form by the action of a nuclear Ran guanine-nucleotide exchange factor. Apparently, this asymmetric dis-

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[1] The abbreviations used are: APC, anaphase-promoting complex; CDK, cyclin-dependent kinase; RanBP, Ran-binding protein; SCF, Skp1/Cullin/F-box; HA, hemagglutinin; GFP, green fluorescent protein.

tribution of the two forms of Ran is the driving force for nucleocytoplasmic transport.

Most of the nuclear protein transport is mediated by a large superfamily of transport receptors, which are classified either as importins or as exportins, depending on the transport direction (19, 20). Importins and exportins differ with respect to the affinity for their substrates in the presence of RanGTP. Importins bind their substrate in the cytoplasm, where Ran-GTP is largely absent, and facilitate transport through the nuclear pore complex. In the nucleus, RanGTP binds to importin and thereby triggers the release of the substrate from the importin (21, 22), which is then transported back into the cytoplasm.

Exportins bind their substrates cooperatively with RanGTP to form a trimeric complex (23), which passages through the nuclear pore complex. Cytoplasmic dissociation of both RanGTP/importin and of RanGTP/exportin/cargo complexes requires the cytosolic Ran-binding protein 1 (RanBP1) (24). Binding of RanBP1 to RanGTP appears to trigger the transient formation of a RanGTP/RanBP1 dimer. In this dimer, RanGTP is accessible to RanGAP, which mediates GTP hydrolysis and renders complex disassembly irreversible (25, 26). Thus, RanBP1 appears to be a crucial factor for the recycling of transport factors and, as a consequence, for nuclear protein transport mediated by some of these receptors.

The yeast homologue of the RanBP1 is encoded by the essential *YRB1* (yeast RanBP1) gene (27–29). Temperature-sensitive mutants of *YRB1* show defects in nuclear import and mRNA export (30).[2] Like other mutants implicated in nucleocytoplasmic transport, temperature-sensitive *yrb1* mutants were shown to be impaired in cell cycle progression (14).

In this study, we show that cells depleted of Yrb1p arrest either in G_1 or in mitosis. These cell cycle defects prompted us to analyze whether Yrb1p is required for ubiquitin-mediated protein degradation during the cell cycle. We demonstrate that *yrb1-51* cells are impaired in proteolysis of crucial cell cycle regulatory proteins. The anaphase inhibitor Pds1p, a target of the APC and the CDK inhibitor Sic1p, whose degradation is normally triggered by the SCF, are both stabilized in these mutants. In a screen for high copy suppressors of *yrb1-51*, we have identified *UBS1*, which was previously identified as a putative positive regulator of Cdc34p, an ubiquitin-conjugating enzyme associated with SCF. Our data imply that Ran-binding protein Yrb1 is important for cell cycle-regulated proteolysis mediated by both the APC and SCF ubiquitin ligases and that Ubs1p may influence both the Ran pathway and ubiquitin ligases.

EXPERIMENTAL PROCEDURES

Yeast Strains—All yeast strains used in this study are derivatives of the *S. cerevisiae* W303 strain (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3, 12 his3-11, 15 ura3 GAL psi+*). The *yrb1-51* mutant was previously isolated as suppressor of the mating defect of a *fus1*Δ mutant.[2] To make the mutant strain congenic with the W303 wild-type strain, *yrb1-51* was backcrossed four times with this strain. The *YRB1* gene was disrupted by transformation of a diploid wild-type strain with a plasmid in which an internal *Bgl*II fragment of *YRB1* was replaced by a *Bam*HI *HIS3* fragment. To generate a haploid *yrb1::HIS3 GAL-YRB1* strain, a heterozygous *yrb1::HIS3*/*YRB1* strain was transformed with plasmid pMK114, containing the *GAL-YRB1* fusion gene. Haploid strains were obtained by tetrad dissection. The *ubs1::HIS3* disruption strain was derived from wild-type strains by disrupting the *UBS1* locus via transformation with a *Spe*I-*Sal*I fragment containing the *ubs1::HIS3* construct excised from plasmid pMK147i. Strains containing HA-tagged versions of Pds1p (10) and of Sic1p (6) were previously described.

Plasmid Construction—Plasmid pMK114 containing the *YRB1* gene under the control of the *GAL1* promoter (*GAL-YRB1*) was generated by a fusion of the *Eco*RI-*Bam*HI *GAL1* promoter fragment to the *YRB1* open reading frame (as a *Bam*HI-*Xba*I fragment) on plasmid pRS316. The *UBS1* gene was isolated as 1.4-kilobase *Hin*dIII-*Xba*I- fragment from the complementing plasmid pMK136 and subcloned into the 2μ plasmid YEp352, resulting in plasmid pMK145. pMK136 contains a genomic *Bgl*II-*Stu*I *UBS1* fragment in the *Bam*HI-*Eco*RV sites of pBluescriptKS(+). This fragment contains, in addition to the complete *UBS1* open reading frame (834 base pairs), 387-base pair upstream and 122-base pair downstream regions. The *ubs1::HIS3* disruption plasmid pMK147i was constructed by inserting a genomic *Bam*HI *HIS3* fragment into a single *Bam*HI site within the coding region of *UBS1* in pBluescriptKS(+)-*UBS1* (pMK136) in the opposite transcriptional orientation relative to *UBS1*. For construction of YEplac112-*NOP1p-GFP-UBS1* expressing a GFP fusion of *UBS1* under control of the *NOP1* promoter, a polymerase chain reaction-generated (using OUBS1–1 5′-GGGGCCATGGCTTACTCTTTAACAAGGAAATTGC-3′ and OUBS1–2 5′-GGGGAGATCTTTAGATTTTTTTCCTCTTTGTGTA-3′ as primers and YEp352-*UBS1* (pMK145) as template) *Nco*I/*Bgl*II fragment comprising the entire ORF of *UBS1* was inserted first into pNOPPATA1L (31) and subsequently recloned as a *Pst*I fragment into YEplac112-NOP1p-GFP (32).

Growth Conditions and Cell Cycle Arrests—Yeast cells were grown in YEP medium (2% bactopeptone, 1% yeast extract, 0.005% adeninsulfate) supplemented either with 2% glucose (YEPD) or 2% raffinose (YEP+Raf) or appropriate minimal mediums (33). Prior to cell cycle arrests, cultures were pregrown to log phase (A_{600} = 0.3–0.6) at 25 °C. When a gene was expressed from the inducible *GAL1* promoter, cells were pregrown in medium containing raffinose as the sole carbon source. The *GAL1* promoter was induced by the addition of galactose (final concentration, 2%). To turn off the *GAL1* promoter, cells were filtered and resuspended in medium containing 2% glucose (final concentration).

To arrest cells with the microtubule depolymerizing drug nocodazole, cells were incubated for 2.5–3 h in the presence of 15 μg/ml nocodazole (added from a 1.5 mg/ml stock solution in dimethyl sulfoxide). Cells were arrested in G_1 phase with α-factor by adding 0.5 or 5 μg/ml mating pheromone to *bar1* or *BAR1* strains, respectively, for 2.5 h.

Isolation of High Copy Suppressors of the yrb1-51 Mutation—To identify high copy suppressors of the *yrb1-51* mutation, *yrb1-51* strain JTY2026[2] was transformed with a genomic library on the high copy vector YEp352. This library contained 3-kilobase *Sau*3AI fragments of yeast genomic DNA ligated into the *Bam*HI site of the *URA3*-based plasmid YEp352 (34). Transformants were tested for their ability to grow at 32 °C on plates containing minimal medium without uracil (−URA). Plasmid dependence of the suppressing phenotype was tested by streaking the transformants on medium containing 5-fluoroorotic acid. Transformants whose survival at 32 °C was plasmid-dependent were tested for growth at 37 °C. Surviving transformants were presumed to contain the *YRB1* gene and not further analyzed. From the remaining clones, plasmids were isolated and transformed into *Escherichia coli*. After propagation and reisolation, plasmids were transformed back into the *yrb1-51* strain. Transformants were retested for plasmid dependent growth on −URA plates at 32 °C. Suppressing plasmids were isolated and analyzed by restriction digestion, Southern hybridization, and sequencing.

Immunoblot Analysis—Preparation of whole cell extracts and protein immunoblot analyses were performed as described (35). After separation on SDS gels, proteins were transferred to nitrocellulose membranes. The enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech) was used to detect specific proteins. Antibodies were used in 1:1000 (Clb2), 1:2000 (Cdc28), 1:1000 (Swi6), 1:5000 (Yrb1), and 1:100 (HA, 12CA5) dilutions, respectively.

Other Methods—RNA isolation and Northern hybridization was performed as described (36). Polymerase chain reaction-generated fragments of the respective genes were radiolabeled by random primer labeling (Stratagene) and used as hybridization probes. For immunfluorescence microscopy, cells were fixed in 3.7% formaldehyde, and spheroplasts were prepared as described by Pringle *et al.* (37). 4′,6-diamidino-2-phenylindole staining and anti-tubulin antibodies were used for visualization of nuclei and spindles, respectively. Fluorescence microscopy of living yeast cells expressing GFP fusion proteins was performed according to Hellmuth *et al.* (31).

RESULTS

Depletion of Yrb1p Results in the Accumulation of Unbudded and Large Budded Yeast Cells—The Ran-binding protein 1 is a predominantly cytoplasmic protein encoded by the essential

[2] M. Künzler, J. Trueheart, C. Sette, E. Hurt, and J. Thorner, submitted for publication.

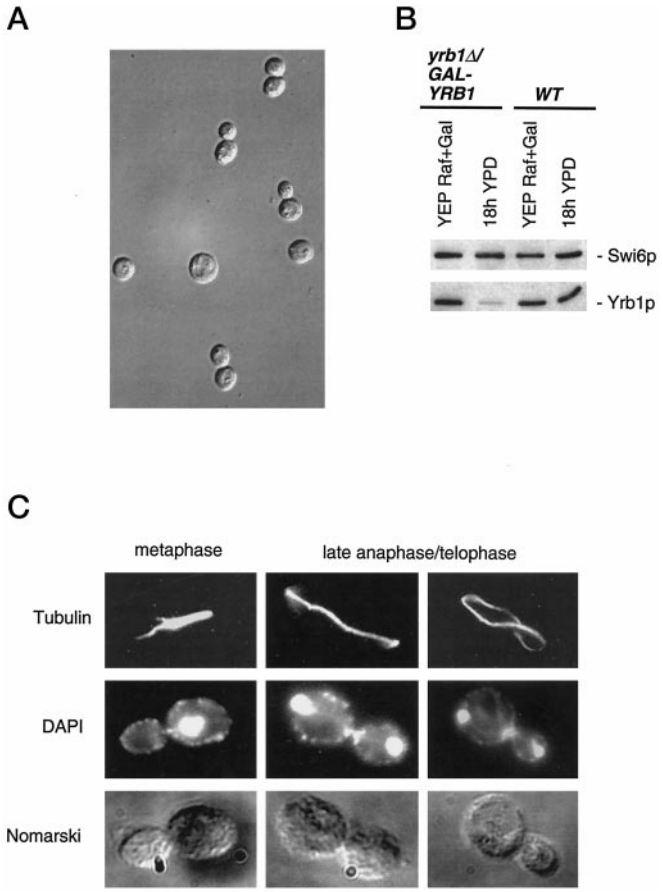

FIG. 1. **Depletion of Yrb1p is lethal and leads to the accumulation of unbudded and large budded yeast cells.** *yrb1*Δ/*GAL-YRB1* and wild-type cells were pregrown in YEP+Raf+Gal medium, filtered and then transferred to YEP+Glucose (YPD) medium. Samples were taken before (YEP+Raf+Gal) or 18 h after the glucose induced promoter shut off (18 h YPD). *A*, samples were fixed with formaldehyde and photographed by conventional light microscopy using Nomarski optics. Cells of the *yrb1*Δ/*GAL-YRB1* strain 18 h after transfer to glucose medium are shown. *B*, immunoblotting of *yrb1*Δ/*GAL-YRB1* and wild-type strains before and 18 h after the transfer to glucose medium. Yrb1p levels were monitored using an anti-Yrb1 antibody, and Swi6p was used as a loading control. *C*, immunofluorescence microscopy of *yrb1*Δ/*GAL-YRB1* cells. 4′,6-diamidino-2-phenylindole staining and anti-tubulin antibodies were used to visualize nuclei or mitotic spindles, respectively. *yrb1*Δ/*GAL-YRB1* cells 18 h after transfer to glucose medium are shown. Examples characteristic for the population of cells arrested with short or elongated spindles (metaphase or late anaphase/telophase cells, respectively) are shown.

YRB1 gene in yeast. Earlier studies showed that different temperature-sensitive *yrb1* mutant strains exhibit a cell cycle arrest phenotype at their restrictive temperature (14, 38). To further elucidate how Yrb1p might influence cell cycle progression, we first characterized the phenotype of a strain in which the *YRB1* gene was deleted. Because *YRB1* is an essential gene, *yrb1*Δ cells were kept alive by a *YRB1* gene under the control of the *GAL1*-promoter. These cells were dead on glucose medium but viable on galactose-containing plates.

To find out whether Δ*yrb1*/*GAL-YRB1* cells show a distinct cell cycle arrest phenotype upon a shift to glucose medium, cells were pregrown in galactose medium and then transferred to glucose medium. 18 h after the promoter shut-off Δ*yrb1*/*GAL-YRB1* cells mostly arrested either as unbudded or as large budded cells (Fig. 1*A*). The ratio of unbudded to large budded cells was approximately 1:1. The size of these cells increased considerably, reminiscent of a cell cycle arrest phenotype observed in other cell cycle mutants. Western analysis confirmed that Yrb1p levels were drastically reduced at this time point (Fig. 1*B*).

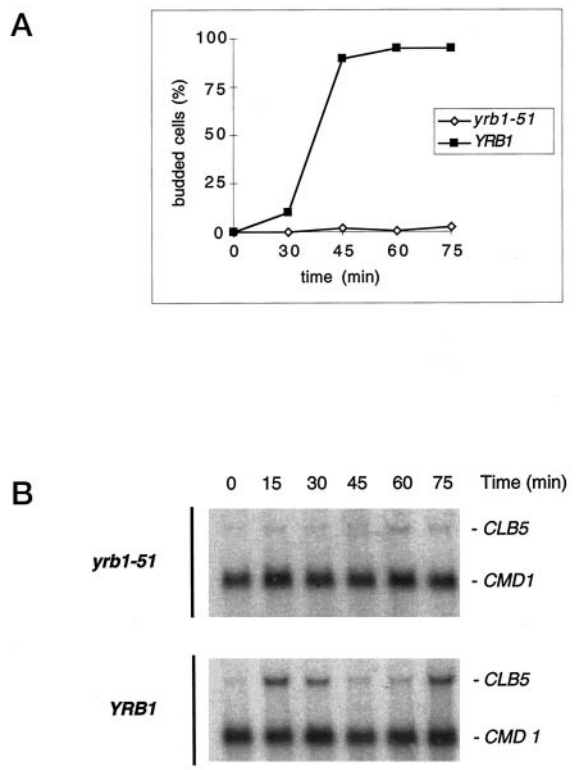

FIG. 2. **Temperature-sensitive *yrb1-51* are defective in the G_1/S transition.** Wild type (*YRB1*) and *yrb1-51* mutants were arrested at the permissive temperature (25 °C) with α-factor for 2.5 h. Cultures were then shifted to 37 °C for 1 h, then filtered, and transferred to fresh medium without α-factor. Cultures were further incubated at the restrictive temperature. *A*, the release from the G_1 arrest was monitored by counting the number of budded cells at the indicated time points. *B*, transcription of the *CLB5* gene, encoding an S phase cyclin, was monitored by Northern hybridization. *CMD1* mRNA (calmodulin mRNA) was used as a loading control.

Immunofluorescence microscopy revealed that about 35% of the large budded cells contained two separated nuclei and a distinctly elongated spindle, indicating a cell cycle arrest in late anaphase/telophase (Fig. 1*C*). These spindles had frequently an unusual twisted appearance. The remaining 65% of large budded cells had a short or moderately elongated spindle and mostly one nucleus. Thus, cells with reduced Yrb1p levels arrest either in G_1 or during mitosis. These findings confirm that Yrb1p is essential for cell cycle progression.

yrb1-51 Mutants Are Blocked at the G_1/S Transition and in the Passage through Mitosis—To further characterize the role of Yrb1p during the cell cycle, we used a temperature-sensitive *yrb1-51* mutant strain that represents a conditional null allele defective in nuclear import and mRNA export.[2]

To test whether a functional Yrb1p is required for the G_1/S transition, *yrb1-51* cells and isogenic wild-type cells were arrested in G_1 phase with α-factor at the permissive temperature, shifted to 37 °C, and then transferred to medium without α-factor. Whereas wild-type cells started to bud upon the release from the G_1 arrest, *yrb1-51* mutants failed to initiate budding (Fig. 2*A*). Furthermore, the expression of the S phase cyclin gene *CLB5*, which was rapidly induced in wild-type cells, was impaired in mutant cells (Fig. 2*B*). Thus, *yrb1-51* cells are defective in budding and the initiation of DNA replication, suggesting that Yrb1p is needed for the passage through START.

We next analyzed the role of Yrb1p in the progression through mitosis. For this purpose we used cells that were prearrested in metaphase with the microtubule-depolymerizing drug nocodazole. The temperature was then shifted to

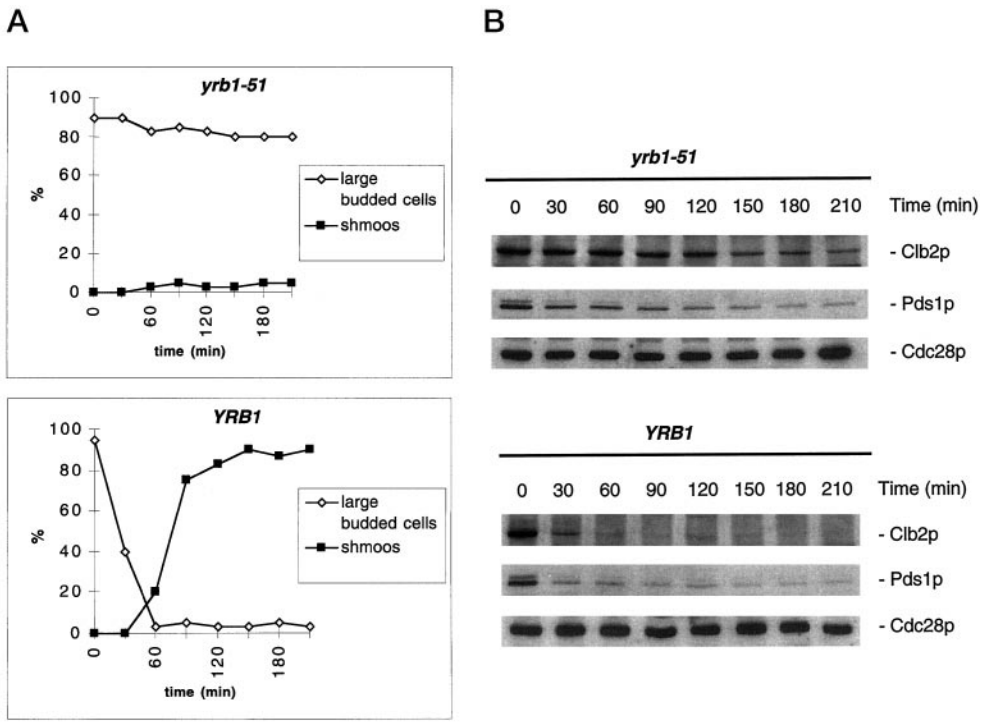

FIG. 3. ***yrb1-51* mutants are impaired in progression through mitosis and in degradation of APC substrates.** Wild type (*YRB1*) and *yrb1-51* mutants were arrested at the permissive temperature (25 °C) with nocodazole for 2.5 h. Cultures were shifted to 37 °C for 1 h, filtered, and transferred to fresh medium without nocodazole. Cultures were further incubated at the restrictive temperature. To monitor cells that were capable of exit mitosis, α-factor was added to block such cells in G_1 phase. *A*, the release from the metaphase arrest was monitored by counting the number of large budded (mitotic) and unbudded shmooing (G_1) cells. *B*, the abundance of the HA-tagged anaphase inhibitor protein Pds1p and the mitotic cyclin Clb2p was analyzed by immunoblotting using either the HA antibody to detect Pds1-HA protein or Clb2p antibodies, respectively. Cdc28p was used as a loading control.

37 °C, and cells were transferred to medium lacking nocodazole and released into medium containing α-factor. Thereby, cells capable to exit mitosis arrested in G_1 phase and started to form shmoos. Most wild-type cells completed mitosis 1 h after the removal of nocodazole and arrested in G_1 phase (Fig. 3*A*). In contrast, 80% of *yrb1* cells remained arrested as large budded cells for at least 3.5 h. Degradation of both the mitotic cyclin Clb2p and the anaphase inhibitor Pds1p, two substrates of the APC ubiquitin ligase, was severely delayed in these mutants (Fig. 3*B*).

These findings show that *yrb1-51* mutants display at least partial defects in APC-mediated proteolysis. The failure of these cells to exit mitosis suggests that Yrb1p is needed for the M/G_1 transition.

Proteolysis of the Anaphase Inhibitor Pds1p Is Impaired in the yrb1-51 Mutant Strain—Because *yrb1* mutants were delayed in Pds1p degradation when released from a metaphase arrest, we asked whether *yrb1-51* mutants are indeed impaired in degradation of Pds1p. To test this, the stability of Pds1p was determined in G_1 arrested cells. In this period of the cell cycle APC is normally fully active and, as a consequence, Pds1p is highly unstable in G_1 wild-type cells (10).

Wild-type and *yrb1-51* cells containing a *GAL-PDS1-HA* gene fusion were arrested in G_1 with α-factor, and Pds1p was transiently expressed. In wild-type cells, only low levels of Pds1p accumulated, and this protein was rapidly degraded after the promoter shut-off (Fig. 4*A*). In contrast, Pds1p was stabilized in *yrb1-51* mutant cells and was still detectable after 60 min. This experiment showed that proteolytic degradation of Pds1p is impaired in *yrb1-51* mutant. This phenotype therefore resembles the defect of mutants in the anaphase-promoting complex, such as *cdc16* or *cdc23* mutants (10).

A further characteristic of such mutants defective in APC function is their increased sensitivity toward elevated levels of the mitotic cyclin Clb2p (39). To test whether *yrb1-51* mutants display a similar phenotype, Clb2p was expressed in these cells to high levels from the *GAL1* promoter. Indeed, *yrb1-51 GAL-CLB2* cells were inviable on plates containing galactose at 25 °C, a temperature that is normally permissive for this mutant, similar to *cdc16-123 GAL-CLB2* cells, which were used as a control (Fig. 4*B*). These findings indicate that Yrb1p is necessary for proper degradation of targets of the APC.

yrb1-51 Mutants Are Defective in SCF-mediated Proteolysis of the CDK Inhibitor Sic1p—The defect of *yrb1-51* in APC-mediated degradation prompted us to test whether the SCF ubiquitin ligase is also affected. SCF is essential for entry into S phase, because it needs to trigger proteolysis of the CDK inhibitor Sic1p. To analyze whether Sic1p proteolysis is defective in *yrb1-51* mutants, a promoter shut-off experiment was performed with wild-type and *yrb1-51* cells containing a HA-tagged *SIC1* gene expressed from the *GAL1* promoter. In an unperturbed cell cycle, Sic1p is unstable from late G_1 to late anaphase (6, 40). To test Sic1p stability, strains were first arrested in a metaphase-like state with nocodazole, where Sic1p is normally unstable. Expression of the *SIC1-HA4* gene was transiently induced by galactose, and then the *GAL1* promoter was turned off by glucose addition. Immunoblot analysis showed that Sic1p is stabilized in *yrb1-51* cells at the restrictive temperature, compared with metaphase arrested wild-type cells (Fig. 5). This experiment shows that Yrb1p is required for proteolytic degradation of Sic1p.

Yrb1p Protein Levels Remain Constant during the Cell Cycle—Because Yrb1p is needed for crucial cell cycle transitions, the abundance of this protein might itself be regulated during the cell cycle. Indeed, the level of RanBP1 is cell cycle-regulated in mammalian cells (41). To test a possible fluctuation of Yrb1p, Yrb1p levels were analyzed in a culture of synchronized wild-type cells. Cells were arrested in G_1 phase by α-factor

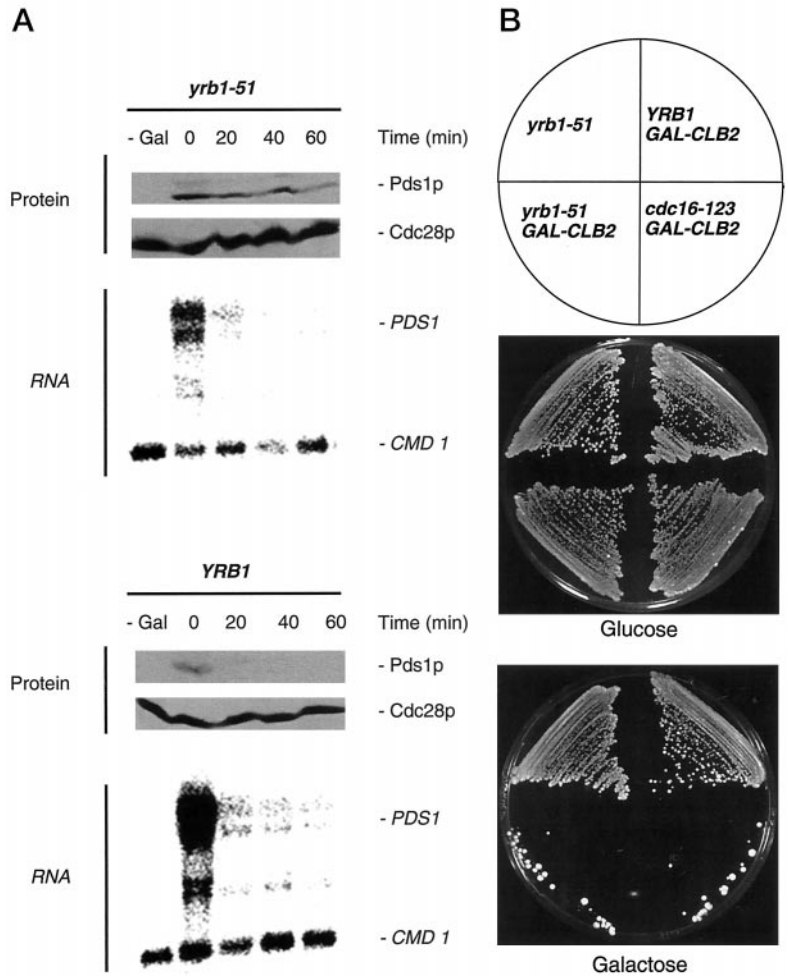

FIG. 4. ***yrb1-51* mutants are impaired in APC-mediated proteolysis.** *A*, Pds1p is stabilized in *yrb1-51* mutants. A wild-type (*YRB1*) and a *yrb1-51* strain, both containing an integrated *GAL-PDS1-HA* construct, were pregrown in YEP+Raf medium at 25 °C and subsequently treated with α-factor for 3 h to arrest cells in G_1. 2% galactose was added to express the *GAL-PDS1-HA* construct. After 30 min, the cultures were shifted to 35 °C to inactivate the Yrb1p, and incubation was continued for 30 min. Then, cells were filtered, transferred into medium containing glucose (YEPD), and further incubated in the presence of α-factor at 35 °C. Glucose repressed the *GAL1* promoter. Samples were collected at the indicated time points after the shift to YEPD medium (0 min time point). Levels of HA-tagged Pds1p were determined by immunoblotting using 12CA5 (HA) antibodies. Cdc28p was used as a loading control. To confirm that *PDS1* expression was indeed turned off in glucose medium, *PDS1* mRNA levels were analyzed by Northern hybridization. *CMD1* mRNA was used as a loading control. More than 95% of the cells had a shmoo-like appearance throughout the course of the experiment. *B*, high levels of Clb2p are toxic for *yrb1-51* mutants at permissive temperatures. A *yrb1-51*, a *cdc16-123*, and a wild-type strain, all containing an integrated *GAL-CLB2* fusion, as well as a *yrb1-51* strain without this construct, were streaked on a YEPD-plate and on a YEP+Gal plate and photographed after an incubation for 3 days at 25 °C.

pheromone and subsequently released to allow them to progress through the cell cycle. Immunoblotting showed that levels of Yrb1p remained constant during the cell cycle, whereas the abundance of a cell cycle-regulated control protein, the mitotic cyclin Clb2p, showed a distinct fluctuation during the course of the experiment (Fig. 6). A mRNA expression analysis of all yeast genes during the cell cycle previously identified all cell cycle regulated genes and revealed that *YRB1* mRNA is not distinctly fluctuating (42). These findings indicate that the abundance of Yrb1p is not regulated during the cell cycle and remains at constant levels. Similarly we found that steady-state cytoplasmic localization of a Yrb1-GFP fusion protein did not change significantly during the cell cycle (data not shown).

UBS1 Is a High Copy Suppressor of the yrb1-51 Mutant—A widely used approach to find out more about the cellular function of a specific protein is the screening for genetic suppressors. To identify genes whose overexpressions suppress the lethality of *yrb1-51* mutants, we performed a screen for high copy suppressors of a *yrb1-51* mutant strain at its restrictive temperature. Of 100,000 transformants, 38 clones were finally selected and restriction analysis and sequencing showed that suppressing plasmids could be grouped into four different complementation groups. 16 of them contained the *YRB1* gene. The crucial open reading frame on four plasmids was incomplete and encoded the N-terminal GTP-binding domain of Tef2, which is the yeast homologue of EF1α (43). The five plasmids of the third family contained the *SMY2* gene, which was previously identified as a suppressor for a defective myosin protein (44). The suppressing activity of the forth family comprising 13 plasmids was caused by the *UBS1* gene. This gene has previously been identified in a screen for high copy suppressors of a *cdc34* mutant (45), which is defective in the ubiquitin-conjugating enzyme Cdc34 required for SCF-mediated ubiquitination.

This interesting connection to the ubiquitination machinery prompted us to analyze the *UBS1* gene in more detail. The *UBS1* open reading frame was subcloned into the multi-copy vector YEp352 and tested for its suppressing activity of the *cdc34-2* and *yrb1-51* mutants. Indeed, this plasmid restored viability to both *cdc34-2* and *yrb1-51* mutants at their otherwise restrictive temperatures of 35 or 32 °C, respectively (Fig. 7, *A* and *B*). In contrast, multiple copies of *UBS1* failed to suppress the lethality of a *yrb1* deletion and did not influence mutants in the APC ubiquitin ligase (data not shown). In contrast to *UBS1*, overexpression of *YRB1* did not suppress *cdc34-2* mutants (Fig. 7*A*). Taken together, these results show that high levels of Ubs1p suppress mutations in both a Ran-binding protein and in the ubiquitin-conjugating enzyme of SCF.

The *yrb1-51* allele encodes a protein that is distinctly destabilized compared with the wild-type protein (Fig. 7*C*). To find out whether high amounts of Ubs1p simply rescue the *yrb1-51* mutant by stabilizing the mutant protein, *yrb1-51* cells expressing *UBS1* on a high copy plasmid were shifted to 37 °C, and levels of the mutant protein were monitored by immunoblotting. The mutant Yrb1p was unstable in the presence of Ubs1p, similar to mutants containing an empty vector plasmid (Fig. 7*C*). Thus, suppression of the *yrb1-51* mutants by high levels of *UBS1* is not caused by stabilization of the Yrb1p mutant protein. Multiple copies of *UBS1* rather restore viability to cells containing abnormally low levels of unstable Yrb1p.

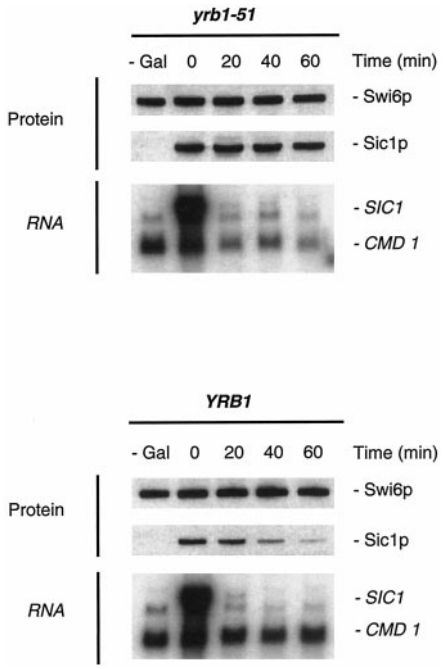

FIG. 5. ***yrb1-51* mutants are impaired in proteolytic degradation of the CDK inhibitor Sic1.** A wild-type (*YRB1*) and a *yrb1-51* strain, both containing an integrated *GAL-SIC1-HA* construct, were pregrown in YEP+Raf medium at 25 °C and subsequently treated with nocodazole for 2 h to arrest cells in mitosis. Then 2% galactose was added to express the *SIC1-HA* gene. After 30 min, the cultures were shifted to 35 °C to inactivate the Yrb1p, and incubation was continued for another 30 min. Then cells were filtered, transferred into medium containing glucose (YEPD) to turn off the *GAL1* promoter, and further incubated in the presence of nocodazole at 35 °C. Samples were collected at the indicated time points after the shift to YEPD medium (0 min time point). Levels of HA-tagged Sic1p were determined by immunoblotting using 12CA5 (HA) antibodies. Swi6p was used as a loading control. To confirm that *SIC1* expression was indeed turned off in glucose medium, *SIC1* mRNA levels were analyzed by Northern hybridization. *CMD1* mRNA was used as a loading control. More than 95% of the cells were large budded throughout the course of the experiment, and no spindle was visible when stained with tubulin antibodies.

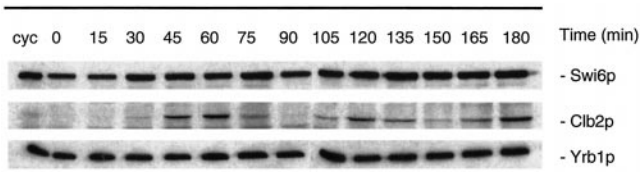

FIG. 6. **Yrb1 protein levels are constant during the cell cycle.** A wild-type culture was pregrown in YEPD medium at 25 °C to midlog phase and subsequently treated with α-factor for 3 h to arrest cells in G_1. Cells were then filtered, washed, and synchronously released into fresh YEPD medium without α-factor. Yrb1p was analyzed with Yrb1 antibodies. Swi6p was used as loading control and Clb2p was used as a control for a protein whose abundance fluctuates during the cell cycle. Synchrony of the culture was also verified by counting the number of budded cells (not shown).

Disruption of UBS1 Is Lethal for yrb1-51 Mutants—Because overexpression of *UBS1* suppressed *yrb1-51* mutant cells, we analyzed whether a disruption of *UBS1* is deleterious to this mutant. In a wild-type background, a disruption of *UBS1* displays no effect on cell viability or morphology (45). To analyze the situation in a *yrb1-51* background, the *yrb1-51* mutant strain was crossed with a *ubs1::HIS3* disruption strain, and the resulting segregants containing both mutations were incubated at semi-permissive temperatures. Whereas a *yrb1-51* single mutant is viable and forms colonies at 28 °C (Fig. 7*D*) and 30 °C (not shown), double mutants are inviable under these conditions. Therefore, *yrb1-51* and the *ubs1* disruption display a synthetic lethal phenotype, and we conclude that Ubs1p is required for viability of cells containing a partially destabilized Yrb1p.

Ubs1p Is a Nuclear Protein—Because Yrb1p is localized to the cytoplasm, we asked whether Ubs1p is also localized to a specific cellular compartment. To determine the localization of Ubs1p, the *UBS1* gene was fused at its N terminus to green fluorescent protein and expressed under the control of the *NOP1* promoter (31). The wild-type *UBS1* gene was replaced by this *NOP1-GFP-UBS1* fusion gene. To confirm that the *GFP-UBS1* gene product is functional, it was placed on a multi-copy plasmid and transformed in *yrb1-51 ubs1::HIS3* double mutants. Indeed, the conditional synthetic lethal phenotype was complemented by the fusion gene to a similar degree as by the *UBS1* gene, suggesting that the fusion protein is functional (data not shown). Fluorescence microscopy showed that GFP-Ubs1p is localized in the nucleus (Fig. 7*E*). We conclude that the multi-copy suppressor of *yrb1-51* mutants, Ubs1p, is a predominantly nuclear protein.

DISCUSSION

In this paper, we have analyzed the role of the Ran-binding protein 1 (Yrb1) in cell cycle progression in budding yeast. Yrb1p is a predominantly cytoplasmic protein implicated in nuclear import and export. A crucial role of Yrb1p in nucleocytoplasmic transport is thought to be the release of nuclear transport receptors from RanGTP in the cytoplasm. Thereby, Yrb1p is needed for the terminal step of nuclear export and for the recycling of import receptors (46).

A Role for Yrb1p in Cell Cycle-regulated Proteolysis—We found that depletion of Yrb1p results in a cell cycle arrest either in G_1 phase or in mitosis, and temperature-sensitive *yrb1-51* mutants fail to release from G_1 or M phase arrests at their restrictive temperature. These data confirm and extend previous findings showing that mutants in Yrb1p display cell cycle arrest phenotypes (14). Because ubiquitin-dependent proteolysis of specific target proteins is essential for cell cycle progression, we have particularly examined the role of Yrb1p in degradation of key regulatory proteins. We have shown here that *yrb1-51* mutants fail to degrade the anaphase inhibitor protein Pds1p, a substrate of the APC. Furthermore, mutant cells are sensitive against high levels of the mitotic cyclin Clb2p and thereby resemble mutants in the APC. We found that *yrb1-51* mutants are also impaired in degradation of an inhibitor of cyclin-dependent kinases, Sic1p, a target of the SCF complex (Fig. 5), and another substrate of SCF, the transcription factor Gcn4p, is also stabilized in these mutants.[3] We conclude from these results that Yrb1p plays an important role in cell cycle-regulated proteolysis mediated by both the APC and SCF ubiquitin ligases.

It was previously demonstrated that mutants in *SRP1*, encoding the yeast homologue of importin α, were unable to properly degrade Clb2p, suggesting that the nuclear import pathway is required for cyclin proteolysis (13). In contrast to *yrb1-51*, *srp1-31* arrest predominantly in G_2/M phase upon shift to their restrictive temperature, which may indicate that this importin is particularly important for proteolysis during mitosis. Our data imply that disruption of the Ran cycle stabilizes not only APC substrates but also SCF targets. Furthermore, cells lacking functional Yrb1p are impaired in the G_1/S transition and in progression through mitosis.

What could be the role of cytoplasmic Yrb1p in ubiquitin-dependent protein degradation? The findings that *srp1* and *yrb1* mutants are both impaired in this process implies that the obvious reason for this phenotype is their defect in nucleocyto-

[3] R. Pries, personal communication.

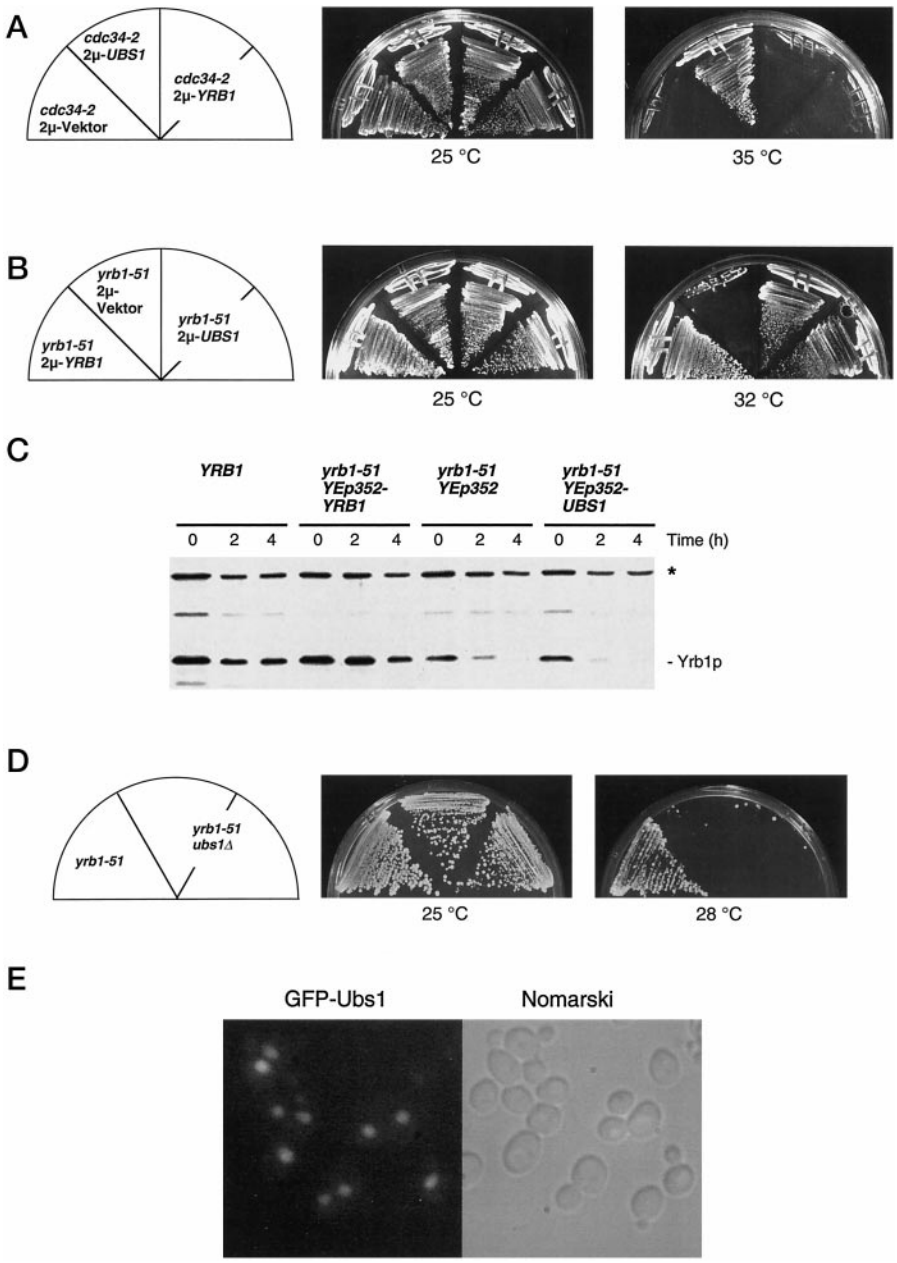

FIG. 7. **UBS1 is a high copy suppressor of both *cdc34-2* and *yrb1-51* mutants.** *A* and *B*, a 2μ plasmid containing the *UBS1* gene suppresses the lethality of *cdc34-2* and *yrb1-51* mutants. *cdc34-2* (*A*) and *yrb1-51* (*B*) mutant strains were transformed either with a high copy plasmid containing *UBS1* or *YRB1* or with the empty vector plasmid. Transformants were streaked on selective minimal medium and photographed after incubation for 3 days at the indicated temperatures. *C*, the *UBS1* high copy plasmid does not affect stability of the mutated Yrb1p in *yrb1-51* mutants. *yrb1-51* mutants carrying high copy plasmids containing either the *YRB1* gene, the *UBS1* gene, or no insert (YEp352) were pregrown at 25 °C and then shifted to 37 °C (0 min time point). Yrb1p was visualized by Yrb1 antibodies. The *asterisk* marks an unspecific band recognized by this antibody. *D*, synthetic lethality of *yrb1-51* and *ubs1* mutations. A *yrb1-51* mutant strain and two *yrb1-51* mutants containing *ubs1::HIS3* gene disruptions were streaked on YEPD plates and photographed after 2 days of incubation at the indicated temperature. *E*, subcellular localization of Ubs1p. Localization of GFP-Ubs1p in a *ubs1* disruption strain. Cells expressing a GFP-Ubs1 fusion protein under the control of the *NOP1* promoter (32) were cultivated in selective medium at 23 °C and viewed directly by fluorescence microscopy.

plasmic transport. Impaired nuclear import or export may cause defects in ubiquitin ligase activity, for example by mislocalization of crucial components or regulators of the ubiquitination machinery. Alternatively, target proteins of ubiquitin ligases may be stabilized because they are not transported to locations where the ubiquitination machinery is active. At least in the case of the APC, which triggers the separation of sister chromatids, it is obvious that the important function of this ubiquitin ligase resides in the nucleus (9). However, it is yet unknown whether the activity of ubiquitin ligases is restricted to a specific cellular compartment.

Another reason for the defect in proteolysis may be an impaired function of the proteasome. Indeed, recent findings showed that biogenesis of the 20 S proteasome depends on a functional nuclear protein import pathway.[4] As a consequence, cell cycle regulatory proteins targeted for destruction by ubiquitin ligases may get abnormally stabilized in *yrb1-51* mutants, because the proteasome is not properly active.

It is also tempting to speculate that Yrb1p may have other important functions than in Ran-mediated transport. Independently of their role in nuclear transport, Ran and RanBP1 were found to be involved in the formation of the mitotic spindle in mammalian cells. RanGTPase apparently regulates the assembly of the mitotic spindle in a transport-independent manner (47–49). It was also observed that RanBP1 levels oscillate during the cell cycle in mammalian cells and increased levels of RanBP1 disrupted various cell cycle events, such as the assembly of the mitotic spindle (41).

It is unknown whether yeast Yrb1p has additional functions besides nucleocytoplasmic transport. Overexpression of *YRB1* and of *GSP1* (the yeast RanGTPase) leads to an increased sensitivity toward the microtuble depolymerizing drug benomyl and to increased chromosome nondisjunction (28). We found that cells arrested in mitosis because of the absence of Yrb1p displayed an abnormal mitotic spindle (Fig. 1*C*). Thus, abnormal levels of Yrb1p, either increased or decreased, affect mitotic events, and these observed phenotypes might indicate that RanBP1 is important for spindle function also in lower eukaryotes.

[4] C. Enenkel, personal communication.

Although Yrb1p is localized predominantly in the cytoplasm and nuclear envelope breakdown during mitosis does not occur in this organism during mitosis, recent findings suggested that this Ran-binding protein may have a function in the nucleus. It was shown that Yrb1p shuttles between cytoplasm and nucleus (50, 51). However, it remains to be shown whether its transient localization in the nucleus is important for Yrb1p function.

Ubs1p, a Link of Yrb1p to Ubiquitin Ligase Activity?—In a screen for multi-copy suppressors of the *yrb1-51* mutation, we have identified the *UBS1* gene that may represent a link between the Yrb1p and ubiquitin ligases. Interestingly, *UBS1* was initially identified as a multi-copy suppressor of *cdc34* mutants (45). Cdc34p is an ubiquitin-conjugating enzyme associated with SCF, and mutants in this essential gene are defective in SCF function causing a drastic stabilization of SCF target proteins such as Sic1p. Ubs1p has no homology to any other gene, and its function is unknown. Because high levels of Ubs1p suppressed the lethality of *cdc34* mutants, it was suggested that Ubs1p represents a putative positive regulator of Cdc34 (45). Interestingly, high copy *UBS1* plasmids suppressed only some *cdc34* mutations but failed to suppress certain *cdc34* mutations in a specific region on the surface of the proteins. Because this surface sequences resembled a region in Ubs1p itself, it was suggested that Ubs1p might directly bind to Cdc34p and thereby regulate its activity by interaction or by modification. However, such an interaction has not yet been demonstrated.

A deletion of *UBS1*, which has no obvious effect on wild-type cells, is deleterious to both *cdc34* (45) and *yrb1-51* mutants at semipermissive temperatures. Thus, cells in which either Cdc34p or Yrb1p function is partially impaired are entirely dependent on Ubs1p. All these observations imply that Ubs1p promotes the function of both Yrb1p and Cdc34p.

A GFP-Ubs1 fusion protein is localized to the nucleus (Fig. 7*E*), and this nuclear localization is at least partially impaired in *yrb1-51* mutants (data not shown). As suggested by Prendergast *et al.* (45), Ubs1p might be an activator of Cdc34p, and its nuclear import triggered by the RanGTP cycle may be important for proper SCF function in the nucleus. However, this simple explanation would not explain why high levels of Ubs1p also suppress *yrb1-51* mutant cells. An alternative model, in which Ubs1p influences both nuclear transport and ubiquitin ligase activity, may be more likely. For example, Ubs1p may have a role in promoting nucleocytoplasmic transport and thereby accelerate nuclear localization of proteins, which induce SCF activity. The presence of high levels of this activatory protein then may cause suppression of a partially inactive Yrb1p. Accelerated transport by high levels of Ubs1p may then cause the nuclear accumulation of proteins that promote Cdc34/SCF activity leading to suppression of the *cdc34* mutant. Such a model would explain why Ubs1p affects both *yrb1* and *cdc34* mutants.

Nucleocytoplasmic Transport and the Regulation of Cell Cycle Events—Although it is possible that Yrb1p has essential roles other than nuclear import and export, cell cycle defect of mutants in several factors involved in nucleocytoplasmic transport, such as *srp1*, *sac3*, and *cse1* mutants, suggests that functional transport is crucial for cell cycle progression. Mutants in the importin-α gene, *SRP1*, display defects in mitosis and cyclin proteolysis (13). Sac3p, a protein localized to the nuclear pore, is implicated in the progression through mitosis (15). Cold-sensitive *cse1-1* mutants, defective in the nuclear export receptor gene of Srp1p, arrest as large budded cells (12), and its mammalian homologue, CAS, was also implicated in mitosis (52).

It is tempting to speculate that proteolysis of cell cycle regulatory proteins may be controlled by subcellular localization either of components of the destruction machinery or of the target proteins themselves. An example for regulated nuclear localization of a cell cycle regulatory protein is the transcription factor Swi5p. Swi5p is cytoplasmic until late anaphase, when it suddenly enters the nucleus and induces the transcription of late mitotic genes (53, 54). Phosphorylation of Swi5p by cyclin-dependent kinases prevents nuclear entry, and only the appearance of an antagonizing phosphatase, Cdc14p, allows nuclear import of Swi5p (55). Cdc14p is itself a well characterized example for regulated localization during the cell cycle. This phosphatase required for the mitotic exit is localized to the nucleolus for most of the cell cycle, and thereby it is kept inactive (56, 57). The release during anaphase allows Cdc14p to reach its targets, Swi5p, Sic1p, and Cdh1p, and the dephosphorylation of each of these proteins contributes to inactivation of cyclin-dependent kinases and the mitotic exit (11).

In analogy to these proteins, the regulated localization of components of the destruction machinery may be a possible mechanism to ensure a precisely controlled degradation of target proteins, which is crucial for faithful cell cycle progression. To identify such regulatory proteins whose activity is controlled by nucleocytoplasmic transport is a task for future experiment.

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