A Defect of Kap104 Alleviates the Requirement of Mitotic Exit Network
Gene Functions in Saccharomyces cerevisiae

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ABSTRACT

A subgroup of the karyopherin β (also called importin β) protein that includes budding yeast Kap104 and human transportin/karyopherin β2 is reported to function as a receptor for the transport of mRNA-binding proteins into the nucleus. We identified KAP104 as a responsible gene for a suppressor mutation of cdc15-2. We found that the kap104E604K mutation suppressed the temperature-sensitive growth of cdc15-2 cells by promoting the exit from mitosis and suppressed the temperature sensitivity of various mitotic-exit mutations. The cytoplasmic localization of these mitotic-exit mutants was not suppressed by kap104E604K. Furthermore, the kap104E604K mutation delays entry into DNA synthesis even at a permissive temperature. In cdc15-2 kap104E604K cells, SIR5 and SIC1, but not CDH1, became essential at a high temperature, suggesting that the kap104E604K mutation promotes mitotic exit via the Cdc15–Sic1 pathway. Interestingly, SPO12, which is involved in the release of Cdc14 from the nucleolus during early anaphase, also became essential in cdc15-2 kap104E604K cells at a high temperature. The kap104E604K mutation caused a partial delocalization of Cdc14 from the nucleolus during interphase. This delocalization of Cdc14 was suppressed by the deletion of SPO12. These results suggest that a mutation in Kap104 stimulates exit from mitosis through the activation of Cdc14 and implies a novel role for Kap104 in cell-cycle progression in budding yeast.

KARYOPHERINS (also known as importins/exportins/transportins), a family of soluble and structurally related proteins, serve as receptors in nucleocytoplasmic transport. Karyopherins bind their cargoes and transport them into and out of the nucleus. Ran GTPase regulates the interaction between karyopherins and their cargoes. Ran, in its GTP-bound form, which is enriched in the nucleus, promotes the assembly of karyopherin/cargo complexes in the export processes or the disassembly of karyopherin/cargo complexes in the import processes (Sazer and Dasso 2000; Macara 2001).

In the budding yeast Saccharomyces cerevisiae, some mutations in karyopherin genes affect cell-cycle progression. Srp1, the sole importin α protein in this organism, is required for the G1/M transition and for the degradation of the mitotic cyclin Clb2 in G1 (Loen et al. 1995; Hood and Silver 1998). Cse1/Kap109, a karyopherin β protein, is required for the progression through mitosis and for faithful chromosome segregation (Xiao et al. 1993; Schroeder et al. 1999). Yrb1, a budding yeast homolog of Ran-binding protein 1 (RanBP1; Coutavas et al. 1993), is required for cell-cycle progression of G2 phase and mitosis (Ouspenski 1998; Baumer et al. 2000). Involvement of karyopherins in exit from mitosis is suggested from the observation that the mutation either in SRP1 or in MTR10, encoding a karyopherin β protein, bypasses the requirement of Cdc15 kinase for exit from mitosis (Shou and Deshaies 2002).

A signaling system called the mitotic exit network (MEN), which includes Cdc15 and Cdc14, eventually inactivates mitotic cyclin-dependent kinases (CDKs) at the end of mitosis by promoting the expression of the CDK inhibitor Sic1 and the activation of the anaphase-promoting complex (APC)/cyclosome, which brings about the degradation of mitotic cyclins (Bardin and Amor 2001). Cdc15 functions as an effector of Tem1 GTPase, whose activation triggers the signal for mitotic exit, and activates the Mob1-Dbf2 complex by phosphorylation (Asakawa et al. 2001; Lee et al. 2001; Mah et al. 2001). The protein phosphatase Cdc14 is localized to the nucleolus (Shou et al. 1999; Visintin et al. 1999) and the spindle-pole body (SPB; Yoshida et al. 2002). The localization of Cdc14, which is regulated by the cdc14 early anaphase release (FEAR) network, which includes Cdc5, Esp1, Slk19, and Spo12 during early anaphase, and by MEN during anaphase/telophase, is dynamically changed upon its release from the nucleolus at the onset of anaphase (Stegmeier et al. 2002). Once activated during anaphase/telophase, Cdc14 promotes accumulation of Swi5, a major transcriptional activator for SIC1, in the nucleus by dephosphorylation of Swi5 (Nasmyth et al. 1990; Moll et al. 1991; Knapp et al. 1996; Toyn et al. 1997; Visintin et al. 1998). Cdc14 also activates APCcdh1 through dephosphorylation of Cdh1, which thereby promotes the ubiquitination of...

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mitotic cyclins (Zachariae et al. 1998; Jaspersen et al. 1999).

A subgroup of the karyopherin β (also called importin β) protein, which includes transportin/karyopherin β2 (hereafter referred to as transportin), is reported to function as a receptor for the transport of mRNA-binding proteins into the nucleus in mammalian cells (Bont-facit et al. 1997; Stomi et al. 1997). Kap104/transportin from budding yeast also imports mRNA-binding proteins, Nab2 and Hrp1/Nab4, into the nucleus (Artschon et al. 1996). From our search for the factors that genetically interact with CDC15, we identified KAP104. The kap104-E604K mutation suppressed the temperature-sensitive growth of cdc15-2 cells by promoting the exit from mitosis. Furthermore, the present study suggests that Kap104/transportin-related protein is required for the maintenance of the mitotic state. We also found that the kap104-E604K mutation caused a partial delocalization of Cdc14 from the nucleolus during interphase, suggesting that a mutation in Kap104/transportin-related protein stimulates exit from mitosis through the activation of Cdc14.

MATERIALS AND METHODS

Microbial manipulation: The principal yeast strains used in this study are listed in Table 1. Strains derived from them were also used as described in the text. Yeast cells were grown either in rich medium (YPD) consisting of yeast extract (DIFCO, Detroit), polyepetone (Nihon Seiyaku, Tokyo), and glucose or in synthetic glucose medium (SC), which is SD containing appropriate supplements (Sherman et al. 1986). Yeast transformations were performed by the method of Ito et al. (1983), and other standard yeast genetic manipulations were performed as described by Sherman et al. (1986). The Escherichia coli strain used is DH5α[pSU46ΔlacI169 (§600alZ3M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1].

Plasmid and strain construction: The SalI–45°–SalI–313° genomic fragment containing the KAP104 gene was cloned into the SalI- and the SmaI-digested pRS315 (Sikorski and Hieter 1989), generating pKZ006 (for the sequence coordinate, see below). The gap-repair plasmid for the kap104-E604K gene was constructed as follows. The ClaI–20°–ClaI–27° fragment of pKZ006 was deleted by ClaI digestion and religation, generating pKZ011. kap104-E604K cells (YKZ0239) were transformed with the ClaI-digested pKZ011 and the plasmid that contains the kap104-E604K gene was rescued from the resulting Ura+ transformants and designated pKZ012. The site of the kap104-E604K mutation was determined by DNA sequencing (Sanger et al. 1977) using a Thermo Sequenase dye terminator cycle sequencing premix kit (Amersham Life Science, Cleveland) and the ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Number +1 indicates the position of the adenine residue of the start codon. To generate the ΔuT5 strain, the DNA fragment of the SW15 locus was amplified by PCR using the yeast genome of the wu5::URA3 strain (K1999, the NclI–49° HindIII–20°, was replaced by the URA3 gene) and a pair of primers, SW15 1as/Bm (5′-CGGGATCCATGGATACATCGA ACTCT-3′) and SW15 2127as/Bm (5′-CGGGATCCCGCTTTTGA TTATTTTCCATTG-3′). Wild-type cells (YKZ0517) were transformed with the amplified PCR products. The Ura+ transformant was isolated and the strains with the URA3 gene integrated at the SW15 locus were selected (YKZ0504).

Cell-cycle synchronization by mating pheromone or hydroxyurea treatment: α-Factor (Sigma, St. Louis) was used to arrest cell growth at late G1 phase. BARI cells or bari1 cells growing asynchronously (OD600 = ~0.3) in 5–10 ml medium at 25°C were treated with 10 μg/ml or 1 μg/ml of α-factor for 2.5–5 hr at 25°C, respectively. After the treatment, α-factor was removed by washing cells three times (for BARI cells) or four times (for bari1 cells) with 5 ml of prewarmed medium. Then cells were released into fresh medium prewarmed at an indicated temperature. Hydroxyurea (Sigma) was used to arrest cell growth at S phase. Cells growing asynchronously (OD600 = ~0.3) in 5–10 ml medium at 25°C were treated with 0.2 m hydroxyurea for 2.5–3 hr at 25°C. After the treatment, hydroxyurea was removed by washing cells three times with 5 ml of prewarmed releasing medium. Then cells were released into fresh medium at the indicated temperature. Each washing step of these experiments took 8–9 min. The time point 0 min indicates the time when the cells were released from arrest.

Flow cytometry: Yeast cells were prepared for flow cytometry essentially as described by Hutter and Eipel (1979). Cells (430 μl of the culture at OD600 = ~0.5) were collected, fixed with 70% of ethanol, and washed with 0.2 M Tris- HCl (pH 7.5) solution. The fixed cells were sonicated thoroughly and treated with 1 mg/ml RNase A at 37°C overnight. Before analysis, the cells were stained with 100 μg/ml propidium iodide for 30 min at room temperature and then analyzed on an FACSCan/CellFIT DNA system (Becton Dickinson). Each histogram showing distribution of DNA contents was based on the accumulation of 20,000 nuclei.

Preparation of samples for Western blotting: Protein extraction for Western blotting analysis was performed as described by Kushnirov (2000). Yeast cells growing at log phase (OD600 = 0.3) were immediately placed on ice and harvested by centrifugation. These cells were resuspended in 100 μl of ice-cold distilled water, to which ice-cold 0.2 M NaOH (100 μl) was added, and incubated on ice for 10 min. After the incubation, the cells were pelleted by centrifugation and boiled in 50 μl of SDS-PAGE sample buffer (Laemmli 1970).

Microscopic analysis: For the indirect immunofluorescence method, cells (~300 μl of cell culture at OD600 = 0.5–0.6) were fixed by adding 37% formaldehyde to the culture (the final concentration was 3.7% formaldehyde) and incubated further for 20 min at the incubation temperature. The medium containing formalin was replaced with KPO4 buffer containing formalin (0.1 m KPO4, pH 6.4), 3.7% formalin] and the cells were incubated at room temperature for 1 hr to overnight. For spheroplasting, cells were incubated with 200 μl of SP [1.2 m sorbitol, 0.1 m KPO4 (pH 7.5)] containing zymolyase 100T (30 μg/ml; Seikagaku, Tokyo) and 0.2% of 2-mercaptoethanol (Wako, Osaka, Japan) for 1 hr at 30°C. Mouse monoclonal anti-α-tubulin antibody (1/50 dilution; clone DM 1A, Sigma) or mouse monoclonal anti-myc antibody (1/50 dilution; 9E10, Calbiochem, Cambridge, MA) was used as primary antibody. Goat anti-mouse IgG antibody conjugated with fluorescein (1/800 dilution; ICN Pharmaceuticals, Aurora, OH) was used as secondary antibody. Microscopic analyses were done using an Olympus IX70 epifluorescence microscope (Olympus, Tokyo) with a UPlanApo100x lens (Olympus) and a CCD camera (SenSys, Photometrics, Tucson, AZ).

RESULTS

A mutation in KAP104 suppressed the temperature sensitivity of cdc15-2 cells: To isolate factors functioning
genes (rcf KAP104 found to be recessive. Complementation analysis showed cdc15-2 sensitivity of cdc15-2 with a cdc15-2 rcf216 and incubated at a restrictive temperature of 34°C. H11034 of cdc15-2/H11034 downstream of Cdc15 in mitotic exit, we screened for extragenic suppressor mutations of the temperature sensitivity of cdc15-2-cells (the rcf mutation, for revertant of cdc15-2). Haploid cdc15-2-cells were streaked on plates and incubated at a restrictive temperature of 34°C. Of 216 spontaneous revertant strains isolated (cdc15-2 rcf1-cdc15-2 rcf216), 8 reproducibly generated two temperature-sensitive (ts) + and two ts- progenies when crossed with a cdc15-2 strain with the opposite mating type and subjected to tetrad analyses. These 8 suppressors were found to be recessive. Complementation analysis showed that the 8 rcf mutations were located in four different genes (RCF5, RCF70, RCF114, and RCF137). We observed that haploid progenies that were temperature sensitive at 34°C due to the cdc15-2 mutation were frequently obtained from crosses between the wild-type and any of the cdc15-2 rcf5, cdc15-2 rcf70, cdc15-2 rcf114, or cdc15-2 rcf137 strains; this indicated that none of the four rcf mutations occurred in CDC15. Each of the four rcf mutations suppressed the temperature sensitivity of cdc15-2 at 34°C but not at the higher temperature of 37°C (Figure 1A).

The rcf137 mutation alone caused temperature-sensitive growth (see below), which was suppressed by the introduction of a low-copy-number plasmid carrying the KAP104 gene (we isolated such a plasmid from our gene library), and the mutant locus showed a strong genetic linkage with the KAP104 locus on chromosome II (data not shown). Kap104 is a member of transportin-related...
proteins and Kap104 and human transportin/karyo- pherin B2 sequences are schematically shown in Figure 1B. DNA sequencing analysis of the open reading frame of Kap104 retrieved from the rcf137 strain revealed that E604 in the HEAT repeat was changed into K in the 12th HEAT repeat of Kap104 (Figure 1C). The L7 of Kap-B2 is required for the interaction with Ran (Cho et al. 1999). Glutamate 604 of Kap104 was changed to lysine by the kcap104-E604K mutation. Alignment of the 12th HEAT repeat of transportin-related proteins from various species was shown. Shaded letters are conserved amino acid residues. The amino acid residues that correspond to the kcap104-E604K mutation site are highlighted by a solid background. The numbers show amino acid positions where the methionine residue encoded by the start codon AUG is 1. NP_179287 and NP_496987 are accession numbers for putative transportin-related proteins registered at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Sc, S. cerevisiae; Hs, H. sapiens; Dm, Drosophila melanogaster; Xe, Xenopus laevis; At, Arabidopsis thaliana; Ce, Caenorhabditis elegans; Sp, Schizosaccharomyces pombe.

The kcap104-E604K mutation promoted mitotic exit, but not cytokinesis, in cdc15-2 cells: To understand precisely the feature of the kcap104-E604K-dependent suppression of the temperature sensitivity of cdc15-2 cells, we investigated the cell-cycle progression of cdc15-2 kcap104-E604K cells at 34°C. cdc15-2 cells and cdc15-2 kcap104-E604K cells were released in fresh medium (34°C) from the α-factor (G1 phase) arrest. After budding, α-factor was added back to prevent cells from entering the next cell cycle. cdc15-2 cells arrested at telophase with the elongated spindle and a high level of mitotic cyclin Clb2 (Figure 2A). On the contrary, cdc15-2 kcap104-E604K cells depolymerized the spindle and Clb2 was degraded as the cells proceeded through mitosis (Figure 2A), indicating that the kcap104-E604K mutation promoted mitotic exit in cdc15-2 cells. In the case in which pheromone was not added back, cdc15-2 kcap104-E604K cells continued the mitotic cycle and became multinucleated (Figure 2B, e and f) while cdc15-2 cells remained arrested at telophase with an extraordinarily elongated spindle (Figure 2B, b and c). Even though the mitotic cycle proceeded, ~90% of cdc15-2 kcap104-E604K cells displayed a defect in cytokinesis and became multi-budded (Figure 2B, d). Considering that the kcap104-E604K mutation alone does not cause a significant cytokinetic defect (see below), these results show that the cdc15 defect in cytokinesis is not suppressed by the kcap104-E604K mutation. We concluded that the kcap104-E604K mutation suppresses the temperature sensitivity of cdc15-2 cells by promoting the exit from mitosis, but does not suppress the cdc15 defect in cytokinesis.

Figure 1.—rcf137 is a mutation in KAP104. (A) Isolation of extragenic suppressors of cdc15-2 cdc15-2 cells (YKZ0200) were streaked on YPAD (yeast extract, peptone, adenine, dextrose) plates and incubated at 34°C for 3–4 days. Spontaneous revertant strains (216 colonies) were picked up. Each rcf mutation that occurred in a single gene was analyzed. Late log-phase cells (OD600 = 1) with the indicated genotypes (from the top, YKZ0517, YKZ0286, YKZ0287, YKZ0285, YKZ0341, and YKZ0200) were serially diluted by 10-fold and spotted onto YPAD plates. Plates were incubated at 25°C or 37°C for 3 days or at 34°C for 2 days. (B) A schematic diagram of transportins from S. cerevisiae (Kap104) and Homo sapiens (Kap-B2). The mutation site of the kcap104-E604K gene is indicated by an arrowhead. Stippled boxes and solid boxes indicate the twelfth HEAT repeat and L7, respectively. The L7 of Kap-B2 is required for the interaction with Ran (Cho et al. 1999). (C) Glutamate 604 of Kap104 was changed to lysine by the kcap104-E604K mutation. Alignment of the twelfth HEAT repeat of transportin-related proteins from various species was shown. Shaded letters are conserved amino acid residues. The amino acid residues that correspond to the kcap104-E604K mutation site are highlighted by a solid background. The numbers show amino acid positions where the methionine residue encoded by the start codon AUG is 1. NP_179287 and NP_496987 are accession numbers for putative transportin-related proteins registered at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Sc, S. cerevisiae; Hs, H. sapiens; Dm, Drosophila melanogaster; Xe, Xenopus laevis; At, Arabidopsis thaliana; Ce, Caenorhabditis elegans; Sp, Schizosaccharomyces pombe.
Cell Cycle in a Transportin Mutant

Figure 2.—The kap104-E604K mutation promoted mitotic exit in cdc15-2 cells at 34°C. (A) cdc15-2 kap104-E604K cells exited mitosis at 34°C. MATa cdc15-2Δbar1 cells (YKZ0635) and MATa cdc15-2 kap104-E604KΔbar1 cells (YKZ0640) were arrested at G1 phase with mating pheromone α-factor (1 μg/ml, treated for 3 hr at 25°C) and released in fresh medium at 34°C. Pheromone was added after bud formation to arrest cells in the next G1 phase (at 60 min to cdc15-2 cells and at 75 min to cdc15-2 kap104-E604K cells). Samples were taken at indicated time points for microscopic analysis and for Western blotting analysis (see materials and methods). Budding index (open circle) and the population of the cells with short spindle (<3 μm, triangle) and elongated spindle (>3 μm, solid circle) are shown. Spindles were detected by the indirect immunofluorescence method using antitubulin antibody. Amounts of the mitotic cyclins Clb2 and Cdc28 (control) were determined by Western blotting using anti-Clb2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-PSTAIRE antibody (Santa Cruz Biotechnology), respectively. The sample taken from the asynchronous culture at 25°C is shown as “cyc.” (B) cdc15-2 kap104-E604K cells continued the mitotic cycle but displayed a cytokinetic defect at 34°C. The α-factor arrest/release experiment was performed as described in A except that pheromone was not added after budding. Cells taken at 140 min after the release from the α-factor arrest are shown. Spindles and DNA were detected by the indirect immunofluorescence method using antitubulin antibody (b and c) and DAPI staining (c and f), respectively. A multi-budded cdc15-2 kap104-E604K cell containing both a short and an elongated spindle within a single cell (i.e., undergoing the second mitosis in the absence of cytokinesis) is shown (d, e, and f). cdc15-2 cells maintained the elongated spindle after nuclear division but continued the budding cycle by producing the bud only from the previous daughter cell (a, b, and c). Arrowheads (a and d) indicate the original mother cells that had been treated with mating pheromone. We confirmed that the multi-budded morphology of the cdc15-2 kap104-E604K cells remained after sonication. Bar, 5 μm.

Suppression spectrum of the kap104-E604K mutation: To test whether KAP104 interacts genetically with other MEN genes, double-mutant strains were constructed by crossing the kap104-E604K strain with the temperaturesensitive MEN mutant strains other than the cdc15-2 strains. We found that temperature-sensitive phenotypes of these MEN mutant strains were suppressed by the kap104-E604K mutation at a low restrictive temperature although the suppression of msd2-1 (allelic to CDC5) was less efficient than that of the other MEN mutations (Figure 3). The cdc14-1 mutation was suppressed by the kap104-E604K mutation at a lower restrictive temperature. Additionally, all of these double-mutant strains showed a multi-budded phenotype at a low restrictive temperature, as cdc15-2 kap104-E604K cells did (data not shown). These results indicate that the kap104-E604K mutation suppresses the defect in mitotic exit, but not in cytokinesis, of MEN in general.

The kap104-E604K mutation affects cell-cycle progression: The results described above established that Kap104 is involved in cell-cycle progression at least in cells defective in mitotic exit. To address the possibility that Kap104 is involved in cell-cycle progression, we examined the phenotypes of kap104-E604K cells at the restrictive temperature. During a 9-hr incubation at 37°C, kap104-E604K cells showed slow but continued growth until ~6 hr and then gradually stopped growing without a decline of viability (Figure 4A and data not shown). We found, however, that the population of the cells with the elongated spindle
(i.e., anaphase/telophase cells) was substantially decreased in the *kap104-E604K* culture as compared with that in the wild-type culture, regardless of the incubation temperatures (Figure 4B, type IV; 47–64% reduction). The population of the cells with the short spindle, although less prominent, was also decreased by the *kap104-E604K* mutation (Figure 4B, type III; 35–44% reduction). Interestingly, cells with two buds and a large nucleus, which were totally absent in the wild-type culture, had accumulated in a certain population (6%) of *kap104-E604K* cells after a 9-hr incubation at 37° (Figure 4B, type VI, and Figure 4C), suggesting that the *kap104-E604K* mutation leads to a weak defect in nuclear division.

Because *kap104-E604K* cells show a similar or slightly slower growth rate than the wild-type cells do at 25° or 34° (Figure 3), the reduced population of the cells with the mitotic spindle in the asynchronous *kap104-E604K* culture raises the possibility that the *kap104-E604K* cells undergo shortened mitosis. To confirm that the *kap104-E604K* mutation shortens mitosis, an α-factor arrest/release experiment was performed. Although the start of spindle elongation delayed by ~15 min, *kap104-E604K* cells completed the depolymerization of the spindle at almost the same time as wild-type cells did (Figure 4D), showing that *kap104-E604K* cells undergo a shorter mitosis than wild-type cells do. Fluorescence-activated cell sorter (FACS) analysis revealed that the 15-min delay occurred before initiation of DNA replication (Figure 4E), suggesting that *kap104-E604K* cells have a defect in G1/S transition.

Swi5, Sic1, and Spo12, but not APC<sup>Cdc11</sup>, are essential for the viability of *cdc15-2 kap104-E604K* cells at 34°: To address how Kap104 is involved in mitosis, we searched for the factor(s) required for the *kap104-E604K*-dependent promotion of mitotic exit. We found that the deletion of *SIC1* (encoding an inhibitor for mitotic CDKs) abolished the growth of *cdc15-2 kap104-E604K* cells at 34° while that of *CDH1* (a specificity factor for APC<sup>Cdc11</sup>) did not (Figure 5A). In addition, the deletion of *SWI5* (encoding another transcriptional activator for *SIC1*) and *ACE2* (encoding a CDK-inhibitory kinase) affected the growth of *cdc15-2 kap104-E604K* cells at 34°, suggesting that the Swi5-Sic1 pathway plays a major role in the *kap104-E604K*-dependent mitotic exit in *cdc15-2* cells. Deletion of neither *ACE2* (encoding another transcriptional activator for *SIC1*) nor *SWE1* (encoding a CDK-inhibitory kinase) affected the growth of *cdc15-2 kap104-E604K* cells at 34°, showing that *ACE2* and *SWE1* are dispensable for the suppression of *cdc15-2* by the *kap104-E604K* mutation (data not shown). Interestingly, *SPO12*, which is required for meiosis and for the release of Cdc14 from the nucleolus...
Figure 4.—Cell-cycle analysis of the kap104-E604K mutant. (A) Temperature-sensitive growth of kap104-E604K cells. Log-phase culture of KAP104 cells (YKZ0517) and kap104-E604K cells (YKZ0240) at 25°C was shifted up to 37°C (at the time point 0 hr) and the cell number (per milliliter) at each of the indicated time points was plotted. (B) Population of kap104-E604K cells at various cell-cycle stages at 37°C. Samples taken at indicated times in A were subjected to double staining of tubulin (by the indirect immunofluorescence method using antitubulin antibody) and DNA (DAPI staining). Cells at various cell-cycle stages were categorized into six groups (I–VI). I, unbudded cells; II, budded cells with the single SPB; III, cells with the short mitotic spindle; IV, cells with the elongated mitotic spindle; V, binucleate cells without the elongated mitotic spindle; VI, cells with a single nucleus and two buds. Nucleus and microtubule structure are indicated by shaded circles and solid bars, respectively. The result shown is representative of two independent experiments. (C) Typical image of kap104-E604K cells of type VI in B. The cells of type VI had two buds (top, DIC) and the nucleus was rather large. DNA was stained with DAPI (bottom, DAPI). Bar, 5 μm. (D and E) Cell-cycle progression of kap104-E604K cells at 34°C. MATa KAP104Δαα1 cells (YKZ0497) and MATa kap104-E604KΔαα1 cells (YKZ0498) were arrested in G1 phase with mating pheromone α-factor (1 μg/ml, treated for 3 hr at 25°C) and were synchronously released in fresh medium at 34°C. At 50 min after release, α-factor was added back to prevent cells from entering the next cell cycle. Spindles were detected by the indirect immunofluorescence method using antitubulin antibody. Spindle index indicating the proportion of cells with the elongated mitotic spindle (>3 μm) is shown. The result is representative of three independent experiments. DNA contents of the cells were investigated by FACS analysis (E). kap104-E604K cells delayed entering S phase by ~15 min.

The kap104-E604K mutation promoted the nuclear accumulation of Swi5 in a Cdc14-dependent manner: To verify that the Swi5-Sic1 pathway is activated by the kap104-E604K mutation in the first place, we tested whether the kap104-E604K mutation results in the nuclear accumulation of
Swi5, which promotes the expression of SIC1. As previously reported (Nasmyth et al. 1990; Moll et al. 1991), the nuclear accumulation of Swi5 was hardly observed in cdc15-2 cells at 37° (Figure 6A). On the contrary, the nuclear accumulation of Swi5 was observed in cdc15-2 kap104-E604K cells at telophase (Figure 6, A and B; 20% of binucleate cells, at the time point 120 min). Because the nuclear accumulation of Swi5 was detectable in 20–23% of binucleate cells in the wild-type culture (data not shown), we conclude that the kap104-E604K mutation promotes the nuclear accumulation of Swi5.

Next we investigated the amount of Sic1 in cdc15-2 kap104-E604K cells at 37°. Even at the elevated temperature at which the spindle depolymerization and the degradation of Cb2 were inefficient, the expression of Sic1 did occur in cdc15-2 kap104-E604K cells (Figure 6, C and D), showing that the kap104-E604K mutation primarily leads to the expression of Sic1 rather than to the degradation of Cb2. The stabilization of Cb2 occurred in cdc15-2 kap104-E604K cells as soon as the temperature shifted to 37°, which was less remarkable in the shift to 34° (Figure 2A). Additionally, in cdc15-2 kap104-E604K cells, a degradation of Sic1 after release from the α-factor arrest delayed 20–40 min longer than in cdc15-2 cells (Figure 6C). This is consistent with the delay in initiation of DNA replication observed in kap104-E604K cells (Figure 4E).

In these assays, we chose a higher restrictive temperature for cdc15-2 cells (37°), because our tagging construct (SWI5- myc or HA-SIC1) alone partially suppressed the temperature sensitivity of cdc15-2 cells at 34° (data not shown).

Cdc14-dependent dephosphorylation of Swi5 is crucial for the nuclear accumulation of Swi5 (Visintin et al. 1998). In either cdc14-1 or cdc14-1 kap104-E604K cells, the nuclear accumulation of Swi5 was not at all observed at 37° irrespective of the cell cycle (Figure 6E and data not shown). These results show that the kap104-E604K mutation promotes the nuclear accumulation of Swi5 in a manner dependent on Cdc14 and suggest that the kap104-E604K mutation promotes mitotic exit through Cdc14 function. This observation seems controversial because the kap104-E604K mutation suppresses the temperature sensitivity of the cdc14-1 mutation at a low restrictive temperature (Figure 3; for interpretation, see discussion). Unexpectedly, most (~70%) cdc14-1 kap104-E604K cells remained arrested with the short spindle (<3 μm) at 37°, suggesting that Kap104 is required for the onset of anaphase when the Cdc14 function is compromised.

Kap104 is required for the tight sequestration of Cdc14 to the nucleus during interphase: The result mentioned above raises the possibility that the kap104-E604K mutation leads to the activation of Cdc14. We therefore investigated the localization of Cdc14 in kap104-E604K cells at the permissive temperature of 34° using chromosomally integrated 18 myc-tagged CDC14. In kap104-E604K cells, Cdc14 was released from the nucleus when the nuclear division occurred, as observed in wild-type cells (Figure 7A). However, a faint but distinct Cdc14 stain was detectable around a discrete nucleolar staining region of DAPI (Figure 7B), showing that the kap104-E604K mutation results in a partial delocalization of Cdc14 from the nucleolus during interphase. We performed the same assay, except that the cells were incubated at 37° for 3 hr, and observed a similar pattern of the Cdc14 stain in kap104-E604K cells (data not shown).
Partial delocalization of Cdc14 from the nucleolus caused by the \textit{kap104-E604K} mutation is a \textit{Spo12}-dependent phenomenon: Because \textit{Spo12} is essential for the \textit{kap104-E604K}-dependent suppression of \textit{cdc15-2}, we tested whether \textit{Spo12} was responsible for the partial delocalization of Cdc14 from the nucleolus. The deletion of \textit{Spo12} from \textit{kap104-E604K} cells almost completely diminished the Cdc14 stain in the DAPI-staining region of the interphase nucleus, showing that the partial delocalization of Cdc14 from the nucleolus in \textit{kap104-E604K} cells occurred in a \textit{Spo12}-dependent manner (Figure 7A). We noted that the population of the cells with a dividing nucleus or with divided nuclei (i.e., mitotic cells) was almost at the same level in either
the Δspo12 or the Δspo12 kap104-E604K culture, suggesting that the shortened mitosis caused by the kap104-E604K mutation is also a Spo12-dependent phenomenon.

**DISCUSSION**

We identified KAP104 as a responsible gene for one of the suppressor mutations (the rcf mutation) of the temperature-sensitive cdc15-2 mutation in a search for the downstream elements of Cdc15 (Figure 1A). Further genetic analyses revealed that the kap104-E604K mutation generally suppresses the MEN defect and that the Swi5-Sic1 pathway is essential for the kap104-E604K-dependent suppression of cdc15-2 (Figures 3 and 6). Indeed, the kap104-E604K mutation promoted the nuclear accumulation of Swi5 and the expression of Sic1 in cdc15-2 cells (Figure 6). The fact that Cdc14 is essential for the kap104-E604K-dependent nuclear accumulation of Swi5 at telophase suggests that the kap104-E604K mutation suppresses the MEN defect through the activation of Cdc14 (Figure 6). To our surprise, the kap104-E604K mutation suppressed the cdc14-1 mutation at 31°C (Figure 3). However, the kap104-E604K mutation was not able to bypass the requirement of Cdc14. This controversial phenomenon will be explained later. Since one of the critical outputs of the MEN signaling is believed to be the regulation of Cdc14, these observations may place the Kap104 function at or near the downstream of MEN, but not immediately after the Cdc15 function.

The kap104-E604K mutation causes cell-cycle phenotypes not only in the MEN-defective cells but also in otherwise wild-type cells, which suggests a novel role for Kap104 in cell-cycle progression; the duration of mitosis in kap104-E604K cells is shorter than that of wild-type cells (Figure 4, B and D), and the kap104-E604K mutation delays initiation of DNA replication (Figure 4C). The kap104-null mutation leads to the elevated rate of chromosome loss (Entian et al. 1999) and the kap104-E604K mutation results in the emergence of the cells with multiple buds and a large nucleus at the restrictive temperature, although at a low frequency (Figure 4, B and C). These phenotypes may be the result of the commitment of the next cell cycle after the short and unfaithful mitosis. On the basis of these observations, we speculate that Kap104 is required for the temporal control of mitosis. Interestingly, we found that the

![Figure 7](chart.png)
The kap104-E604K mutation severely enhanced the defect in the initiation of spindle elongation in cdc14-1 cells at 37°C (Figure 6E). This is the third cell-cycle phenotype caused by the kap104-E604K mutation, which implies that Kap104 has a role for the anaphase onset at least when the function of Cdc14 is compromised.

The fact that the kap104-E604K mutation caused a partial delocalization of Cdc14 from the nucleolus during interphase further suggests that the kap104-E604K mutation promotes the exit from mitosis through the activation of Cdc14 (Figure 7). Because the release of a small amount of Cdc14 from the nucleolus is believed to be sufficient for execution of Cdc14 function (Shou et al. 1999), the shortened mitosis in kap104-E604K cells can be explained by their partial delocalization of Cdc14 from the nucleolus before anaphase. Moreover, the delay in the G1/S transition in kap104-E604K cells is also explained by the phosphatase activity derived from delocalized Cdc14 during G1 phase, which is supposed to stabilize Sic1 to delay DNA replication initiation (Figures 4C and 6C).

One possible mechanism for the kap104-E604K-dependent delocalization of Cdc14 from the nucleolus is the activation by the kap104-E604K mutation of the Spo12 pathway, leading to the precocious release of Cdc14 from the nucleolus during interphase. It will be interesting to determine the localization of FEAR factors such as Spo12, Esp1, Cdc5, and Slk19 (Stegmeier et al. 2002) in kap104-E604K cells to learn whether Kap104 is involved in the transport of these factors. So far, Spo12 seems not to be the target of Kap104 in the nucleocytoplasmic transport as it was reported that the localization of Spo12 was not affected by the deletion of KAP104 (Chaves and Blobel 2001). It was previously reported that some karyopherins (importin β and importin α) served as inhibitors for mitotic spindle assembly in Xenopus egg extract or in mammalian cells, in which the nuclear envelope does not exist during mitosis (Gruss et al. 2001; Nachury et al. 2001; Wiese et al. 2001). Given that an analogous feature of importin β is shared with transportin-related proteins, it is also a fascinating model that Kap104 serves as an inhibitor for a FEAR factor and that the kap104-E604K mutation attenuates this inhibitory effect to cause the precocious delocalization of Cdc14 from the nucleolus. Alternatively, it is possible that Kap104 is required for the return of Cdc14 (resequestration) to the nucleolus after mitotic exit and cell division because a defect in this process should extend the released state of Cdc14 and may cause the delocalization of Cdc14 during interphase.

As described above, a feature of the kap104-E604K mutation is that it causes partial constitutive delocalization of Cdc14. This phenomenon may explain our observation that kap104-E604K suppressed cdc14-1 at 31°C. According to the report by Jaspersen and Morgan (2000), the cdc14-1 mutation delays but does not prevent the release of green fluorescent protein (GFP)-tagged Cdc14-1 protein from the nucleolus at 34°C. Assuming that the release of Cdc14-1 from the nucleolus at 31°C is similar to that at 34°C and that Cdc14-1 shows a weak phosphatase activity at 31°C, a slightly higher level of delocalized Cdc14-1 in cdc14-1 kap104-E604K cells at 31°C due to the kap104-E604K mutation could fulfill a critical level of Cdc14 phosphatase activity needed for exit from mitosis.

Another model is that the defective transport of known cargoes of Kap104 (Nab2 and Hrp1/Nab4), or the defective nuclear architecture as a subsidiary consequence of the defective nucleocytoplasmic transport, causes the delocalization of Cdc14 during interphase because the deletion of KAP104 results in an abnormal nuclear morphology (Arthchison et al. 1996). However, we failed to detect an aberration of nuclear morphology in kap104-E604K cells except that a larger nucleus was observed in a small population (6%) of kap104-E604K cells 9 hr after shift up to 37°C (Figure 4, B and C). We also failed to notice any alteration of the nucleolar morphology through the microscopic observation of the Nop1-GFP signal in kap104-E604K cells (Figure 7B). Nonetheless, we cannot exclude the possibility that abnormal nucleolar architecture that is undetectable by microscopic analysis could cause the precocious delocalization of Cdc14.

This study provides the first evidence that transportin-related protein is involved in cell-cycle progression. It will be interesting to examine whether the defect in transportin-related protein causes similar cell-cycle phenotypes in eukaryotes other than budding yeast. Our results suggest that the kap104-E604K mutation activates Cdc14 in a Spo12-dependent fashion and that this leads primarily to the activation of the Swi5-Sic1 pathway rather than to that of APC^Cdh1. Identification of the binding partner(s) of Kap104 involved in the cell-cycle progression will deepen our understanding of the role of transportin-related proteins in the cell-cycle progression and the molecular mechanism of exit from mitosis.

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