DOI: 10.1534/genetics.109.105130

Mutations in the *Saccharomyces cerevisiae* Kinase Cbk1p Lead to a Fertility Defect That Can Be Suppressed by the Absence of Brr1p or Mpt5p (Puf5p), Proteins Involved in RNA Metabolism

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Manuscript received May 15, 2009
Accepted for publication June 8, 2009

ABSTRACT

In *Saccharomyces cerevisiae* the protein kinase Cbk1p is a member of the regulation of Ace2p and cellular morphogenesis (RAM) network that is involved in cell separation after cytokinesis, cell integrity, and cell polarity. In cell separation, the RAM network promotes the daughter cell-specific localization of the transcription factor Ace2p, resulting in the asymmetric transcription of genes whose products are necessary to digest the septum joining the mother and the daughter cell. RAM and *SSD1* play a role in the maintenance of cell integrity. In the presence of a wild-type *SSD1* gene, deletion of any RAM component causes cell lysis. We show here that some mutations of *CBK1* also lead to a reduced fertility and a reduced expression of some of the mating type-specific genes. As polarized growth is an integral part of the mating process, we have isolated suppressors of the fertility defect. Among these, mutations in *BRR1* or *MPT5* lead to a restoration of fertility and a more-or-less pronounced restoration of polarity; they also show genetic interactions with *SSD1*. Our experiments reveal a multilayered system controlling aspects of cell separation, cell integrity, mating, and polarized growth.

CELLULAR polarity is important in both multicellular and unicellular organisms and allows certain functions to be restricted to particular parts of the cell. In the yeast *Saccharomyces cerevisiae* this can be seen at different levels such as the positioning of the bud site and the ellipsoidal shape of the cells, which is due to a phase of polarized growth during G_2 of the cell cycle. Polarized growth is also particularly visible during filamentous growth and mating. The mating pheromones, secreted by haploid cells (*MAT***a** and *MAT*α), block cells of the opposite mating type in G_1 and provoke polarized growth leading to the formation of a long projection, or shmoo. The shmoos of opposite mating types fuse to form a diploid zygote. Setting up and maintaining cellular polarity involve the actin cytoskeleton, the septins, and the polarizome and are controlled by mitogen-activated protein kinase (MAPK) signal transduction pathways, such as the filamentation (f)-MAPK pathway and the mating (m)-MAPK pathway (for reviews see Pruyne *et al.* 2004; Park and Bi 2007).

In *S. cerevisiae* there is a network of six proteins that is necessary for a wild-type cellular polarity, the regulation of Ace2p and cellular morphogenesis (RAM) network: Cbk1p, Hym1p, Kic1p, Mob2p, Sog2p, and Tao3p (Nelson *et al.* 2003). The absence of any RAM component results in the formation of large aggregates of cells and a loss of polarity, leading to round rather than ellipsoidal cells (Dorland *et al.* 2000; Racki *et al.* 2000; Bidlingmaier *et al.* 2001; Colman-Lerner *et al.* 2001; Du and Novick 2002; Weiss *et al.* 2002; Nelson *et al.* 2003). This network is a two-kinase regulatory module that appears to be conserved among all eukaryotes and is involved in morphogenesis and cell cycle progression. Other well-defined examples are the mitotic exit network (MEN) in *S. cerevisiae* and the septation initiation network (SIN) in *Schizosaccharomyces pombe* (see Tamaskovic *et al.* 2003 and Hergovich *et al.* 2006 for reviews). The focal point of these networks is the regulation of a protein kinase of the nuclear Dbf2p related (NDR) family, Cbk1p in the case of the RAM network.

The NDR kinases are a subgroup of the protein kinase A (PKA)/PKG/PKC-like (AGC) serine threonine protein kinases (Hergovich *et al.* 2006). These kinases are regulated by the phosphorylation of at least two conserved sites (Millward *et al.* 1999). In Cbk1p, these

We dedicate this article to the memory of Piotr Slonimski, who died in Paris on April 25th, 2009 at the age of 86.

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.109.105130/DC1.

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correspond to serine 570, which undergoes an activating autophosphorylation, and threonine 743, which plays an essential but undefined role in the function of the protein (Jansen *et al.* 2006).

The role of the RAM network and the protein kinase Cbk1p in cell separation is reasonably well worked out and involves the transcription factor Ace2p (Racki *et al.* 2000). Ace2p controls the transcription of *CTS1* (which encodes chitinase) and other genes whose products are needed to degrade the chitin-rich septum that holds mother and daughter cells together after cytokinesis (Dohrmann *et al.* 1992; Doolin *et al.* 2001; Voth *et al.* 2007). The localization of Ace2p is controlled throughout the cell cycle, and for most of the cycle the protein is excluded from the nucleus because of phosphorylation by Cdc28p (O'Conallain *et al.* 1999); in late M, the protein accumulates specifically in the nucleus of the daughter cell, the Ace2p targets are transcribed, and cell separation occurs. Cbk1p is also localized transiently to the nucleus of the daughter cells and this localization is concomitant with and dependent upon that of Ace2p (Colman-Lerner *et al.* 2001; Weiss *et al.* 2002; Nelson *et al.* 2003; Jansen *et al.* 2006; Bourens *et al.* 2008). A combination of genetic and biochemical studies have shown that Cbk1p phosphorylates the Ace2p nuclear export sequence (NES), disrupting its interaction with the nuclear exportin Crm1p and resulting in the accumulation of Ace2p in the nucleus (Bourens *et al.* 2008; Mazanka *et al.* 2008; Sbia *et al.* 2008; reviewed in Parnell and Stillman 2008).

In certain genetic backgrounds the RAM network is also necessary for cell integrity. Laboratory yeast strains show a polymorphism for the *SSD1* gene: some, such as CEN.PK2, carry a full-length wild-type version of the gene (*SSD1* or *SSD1-v*); others, such as W303, carry a truncated version (*ssd1-d*), which is thought to be inactive (Uesono *et al.* 1997). In the presence of the *ssd1-d* allele the deletion of any of the *RAM* genes is viable, but in the presence of the wild-type *SSD1* gene Δ*ram* alleles are lethal and lead to cell lysis (Du and Novick 2002; Jorgensen *et al.* 2002; Kurischko *et al.* 2005). Ssd1p is thought to play a role in cell wall integrity (Kaeberlein and Guarente 2002; Wheeler *et al.* 2003) and has been implicated in a large variety of other cellular functions, either via genetic interactions or as a high/low-copy suppressor. The protein has been shown to bind RNA (Uesono *et al.* 1997) and to interact directly with Cbk1p (Racki *et al.* 2000; Ho *et al.* 2002). However, despite a large amount of published data, the exact role of *SSD1* is unclear.

In recent years, much progress has been made in understanding the role of the RAM network in cell separation and cell integrity, but its role in cell polarity remains unknown. Studies of the polarity phenotype involving the Δ*ram* strains are hampered by the aggregation phenotype. We have isolated an allele of *CBK1*, *cbk1::3HA* (identical to the allele isolated by Bidlingmaier *et al.* 2001), which has a loss of polarity with no significant accumulation of cell aggregates. Here we show that strains carrying the *cbk1::3HA* allele have a reduced fertility. As polarized growth is an integral part of the mating process, we reasoned that the fertility defect could be linked to the polarity defect. We have isolated a series of extragenic suppressors of the fertility defect of the *cbk1::3HA* allele, some of which show a concomitant restoration of cell polarity. Two of these suppressors have been cloned and are loss-of-function alleles of *BRR1* and *MPT5* (*PUF5*). We have determined the effect of the deletions of these genes on the other Δ*ram* phenotypes and the genetic interactions between *CBK1*, *BRR1*, *MPT5*, and *SSD1*.

MATERIALS AND METHODS

Strains and media: For standard DNA propagation the *Escherichia coli* strain XL2-Blue (Stratagene, La Jolla, CA) was used. All the *S. cerevisiae* strains used are derived from W303 (Thomas and Rothstein 1989) and CEN.PK2 (a gift from K. Entian) and are listed in supporting information, Table S1. All media for bacteria were prepared as described in Sambrook *et al.* (1989). Yeast media were prepared according to Dujardin *et al.* (1980).

Basic genetic techniques and nucleic acid manipulation: The genetic techniques were used as described in Adams *et al.* (1997). Gene deletions were made using the PCR procedure of Wach *et al.* (1994) and Petracek and Longtine (2002); GFP fusions were constructed as described by Wach *et al.* (1997), using the plasmid pFA6-5GA super bright (Knop *et al.* 1999). The *CBK1* gene was mutagenized using the transposon mutagenesis system of Ross-MacDonald *et al.* (1997), which creates an in-phase tandem insertion of three copies of the HA epitope. One of these mutant alleles (*cbk1::3HA*), an insertion of the 3HA epitope at residue 567, is identical to *Cbk1-mTn3F1* isolated by Bidlingmaier *et al.* (2001). The suppressors were cloned using a genomic bank of the strain FL100 in the plasmid *URA3* multicopy plasmid pFL44L (Bonneaud *et al.* 1991), which was provided by François Lacroute and Nathalie Bonnefoy. The strain SupM1 rapidly accumulated fast growing suppressors (the *supM1* allele carries a nonsense mutation), and to circumvent this problem the corresponding gene was cloned by transforming several independent freshly dissected spores.

Transformation of *E. coli*, plasmid isolation, small-scale genomic DNA isolation, and gel electrophoresis were performed as described by Sambrook *et al.* (1989). Yeast were transformed as described by Gietz *et al.* (1992).

Isolation of extragenic suppressors of the mating deficiency of the *cbk1::3HA* mutant: Ten independent subclones of WR580-13b (*MAT*α *cbk1::3HA ade2 LEU2*) were grown to confluence on rich glucose plates for 2 days at 28°, and they were then stored in the dark at 4° for 1 week. Small patches of these cells were plated to obtain a unicellular lawn of cells and UV mutagenized to give 1–5% survival. After 2 days' growth in the dark, the plates were washed and the cell suspensions corresponding to each mutagenesis were spread on rich glucose medium to give ~200 colonies per plate. These were replica mated to a lawn of WR580-4d (*Mat a cbk1::3HA ADE2 leu2*) on a medium selecting for diploids (minus adenine, minus leucine) and were examined regularly over a 48-hr period to identify colonies that were able to cross more efficiently. A strain carrying the *cbk1::3HA* allele was used as

the tester strain because the mating deficiency is more pronounced in *cbk1::3HA* × *cbk1::3HA* crosses and this facilitated the identification of the suppressors. In total, 20,000 colonies were tested and after verification and retesting 13 suppressors with an improved mating efficiency were selected.

Mating assays: Quantitative mating assays were performed using a protocol adapted from Sprague (1991). The experimental and tester strains carry auxotrophic markers that allow the selection of diploids. Strains were grown in rich glucose medium to an OD_{600} between 1 and 2, the experimental strain was mixed with a fivefold excess of the tester strain, and 300 μl were plated on a 3-cm petri dish of rich glucose medium and incubated at 28° for 4 hr. After this the cells were resuspended, diluted, and plated on a series of selective media that allowed us to count the haploid parents and the resulting diploids. The parental cultures were also plated to check for reversion of the auxotrophic markers. The mating efficiency was calculated as the number of diploids divided by the sum of the diploids and the experimental haploid.

The mating time course was determined according to Gammie and Rose (2002). Equal numbers of mutant cells of both mating types grown in rich glucose medium were mixed and incubated on 2.5-cm filter discs placed on rich glucose medium at 28° for 2 or 4 hr. The cells were then washed off and fixed, and the different mating stages were counted; the results presented are the mean and standard deviation from three independent experiments ($n > 100$).

Microscopy: Live cells were grown to early logarithmic phase in rich glucose medium and observed with a Zeiss Axioplan 2 microscope linked to a Cool Snap camera (Princeton Instruments).

Quantitative PCR: Total RNA was extracted with hot acidic phenol (Racki *et al.* 2000). cDNA was generated using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), using p(dN)6 random hexamers and anchored oligo(dT)23 primers (Sigma, St. Louis). Individual cDNAs were quantified by real-time PCR, using the LightCycler instrument and the FastStart DNA Master SYBR Green I reaction mixture (Roche, Indianapolis). *ACT1* was used as a reference gene. Data were analyzed with the LightCycler data analysis software. All the oligonucleotides used in this study are listed in Table S2.

Transcriptome analysis: Total RNA of haploid strains was isolated by extraction with hot acidic phenol (Racki *et al.* 2000) and labeled using the LILRAK PLUS kit; internal standards came from the Two-Color RNA Spike-in kit (Agilent Technologies). The labeled targets were purified using the RNeasy Mini kit (QIAGEN, Valencia, CA). All microarrays are phosphoramidite arrays manufactured by Agilent Technologies (ref G4140B) and correspond to whole-genome coverage long oligonucleotides (60 mers). The labeling efficiency and product integrity were checked according to criteria defined by Graudens *et al.* (2006). Identical amounts (0.25 μg) of Cy3- and Cy5-labeled targets were mixed according to the manufacturer's instructions and incubated on the microarray slides for 17 hr at 65°, in a rotating oven at 5–6 rpm, using an Agilent hybridization system. The slides were washed as described by the manufacturer and any traces of water were removed by centrifugation at 800 rpm for 1 min. For each comparison, four microarrays were hybridized, including two biological dye swaps.

The slides were scanned with an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA) equipped with 532- and 635-nm excitation lasers for Cy3 and Cy5. Each slide was scanned at 100% laser power at 5-μm resolution. Photomultiplier tube voltages were automatically adjusted to balance the distributions of the red and green intensities and to optimize the dynamics of image quantification. The level of saturated pixels was limited to 0.05%. The resulting 16-bit images were analyzed using the GenePix Pro 6.0 software, the segmentation being computed with the "adaptive circle" method.

Data were processed with the MAnGO software (Marisa *et al.* 2007). Backgrounds were estimated with the "morphological closing followed by opening" method and were subtracted. Not found, saturated, and bad spots were discarded from the subsequent analysis. Raw data were normalized using the print-tip loess method (Smyth and Speed 2003), which is a local regression normalization within artificial print-tip blocks. Once the biases were corrected, the differential expression of genes between two compared conditions was assessed from the biological replications. A moderated *t*-test with adjustment of *P*-values (Benjamini and Hochberg 1995) was computed to measure the significance associated with each expression difference. Genes were selected as significantly differentially expressed when their adjusted *P*-value was <5%, their mean fold change (average of the fold changes of the four repeats for each comparison) was >1.5 in absolute value, and their mean intensity level $[\log_2(\text{Cy5} \times \text{Cy3})/2]$ was >7.

RESULTS

Mutations in Cbk1p lead to a fertility defect and a reduction in the expression of the mating type-specific genes: The *cbk1::3HA* allele contains an insertion of three copies of the HA epitope at residue 567 and, as noted by Bidlingmaier *et al.* (2001), it presents a loss of polarity without significant aggregate formation (see Figure S1). We also noted that in plate crosses strains carrying the *cbk1::3HA* allele showed a reduced mating efficiency that was more pronounced in *cbk1::3HA* × *cbk1::3HA* crosses; in quantitative tests they yielded 40% diploids when crossed to the wild type, compared to 70% in wild-type (wt) × wt crosses (Figure 1). Like other protein kinases in the NDR family, Cbk1p is regulated by phosphorylation (Stegert *et al.* 2005; Hergovich *et al.* 2006), the autophosphorylation of serine 570 in the activation loop, and phosphorylation of threonine 743, in a hydrophobic C-terminal motif (Jansen *et al.* 2006). In Cbk1::3HAp, the three copies of the HA epitope are inserted close to the serine that is autophosphorylated. We reasoned that the phenotype of this mutation might be due in part to a perturbation of the autophosphorylation, so we created derivatives of Cbk1p that cannot be phosphorylated, Cbk1S570Ap and Cbk1T743Ap. Cbk1S570Ap shows a mating defect similar to that of Cbk1::3HAp (Figure 1). Like Jansen *et al.* (2006), we found that Cbk1S570Ap has a polarity defect (see the axial ratios in Figure 5) with no accumulation of aggregates, whereas Cbk1T743Ap has a phenotype close to the deleted strain with large aggregates of round cells and is therefore not suitable for quantitative mating assays.

To refine our understanding of the fertility defect in strains carrying the *cbk1::3HA* allele, we determined a time course of the mating process as described in materials and methods. Mating mixtures of wild-type

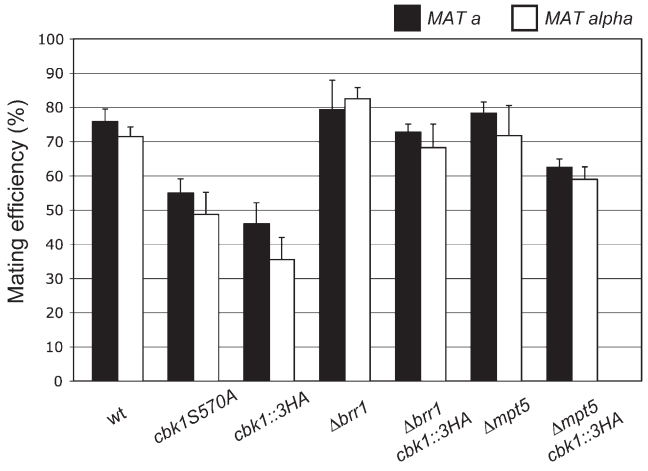

FIGURE 1.—Mating efficiency of strains carrying different *cbk1*, *brr1*, and *mpt5* mutations. The experimental strain was mated with a fivefold excess of the wild-type strain. The mating efficiency was calculated as diploids/(diploids + experimental haploid) × 100. The results show the mean and standard deviation for three independent experiments.

or *cbk1::3HA* strains were examined after 2 and 4 hr, and the percentages of the different intermediates [unaffected cells, cells with a shmoo, cells that have fused (dumbbell), and zygotes producing the first diploid cell (cloverleaf)] were calculated. The results in Figure 2 show that at a given time point the level of the different mating intermediates is considerably reduced in *cbk1::3HA* × *cbk1::3HA* crosses compared to the wild type, suggesting that in the presence of the *cbk1::3HA* allele the mating process is slowed down, beginning with shmoo formation.

To identify the genes or networks that are regulated by *CBK1* in the mating process and the establishment of cellular polarity we decided to undertake a microarray study of the different *cbk1* mutants. The most striking result was a reduction in the level of the expression of many of the mating type-specific genes (Table 1). Thus the different *cbk1* mutations affect the expression of the mating type-specific genes and this effect is more pronounced in *MAT*α strains.

To validate these results, we decided to perform quantitative qPCR experiments and selected three genes for each mating type. For the *MAT***a** strains we chose *MFA2*, *STE2*, and *AGA2*, but no significant and reproducible effects were seen on the expression of these genes (data not shown). For the *MAT*α strains we chose *MF*α*1*, *STE3*, and *SAG1*. The results in Figure 3A show that in the *cbk1* mutations, all three genes have a similar profile of expression, with *SAG1* being the most strongly affected. In general, the *cbk1* mutations cause a significant reduction in the expression of all three genes tested in a way that essentially parallels the severity of the *cbk1* mutation. Because Cbk1p is known to regulate the activity of the transcription factor Ace2p via its localization, we also determined the effect of deleting *ACE2* on the transcription of the mating type-specific genes. Our results show that deletion of *ACE2* did not reduce the transcript level of our test genes.

Taken together, our results show that certain *cbk1* mutant alleles lead to a reduction in fertility and a slowing down of the mating process. There is also a clear reduction in the transcription of many of the *MAT*α mating type-specific genes in a way that is independent of the transcription factor Ace2p.

Isolation of extragenic suppressors of the fertility defect of the *cbk1::3HA* mutation: As polarized growth is an integral part of the mating process, we reasoned that extragenic mutations that increased the mating efficiency of *cbk1::3HA* might show a concomitant restoration of cellular polarity and thus help us understand the role of Cbk1p in cellular polarity. To this end we isolated a series of 13 suppressors that showed an increased mating efficiency; of these, some showed no obvious effect on polarity, while others showed a partial restoration of polarity or even a hyperpolarized growth phenotype. Here we present the analysis of 2 suppressors that increase the mating efficiency and show a concomitant restoration of polarized growth: SupM1 and a complementation group of three alleles, SupM2, SupM3, and SupM4.

In plate crosses the mating efficiency phenotype is weak and not suitable as the basis of a cloning strategy; therefore we looked for other more tractable phenotypes associated with the suppressors. In a wild-type *CBK1* background, *supM1* conferred a recessive slow growth phenotype (Figure 4A). After transformation of a *supM1 CBK1* strain by a wild-type library and the selection of transformants with a normal growth phenotype, we showed that *supM1* is an allele of *BRR1* (bad response to refrigeration) (NOBLE and GUTHRIE 1996a), with a nonsense mutation in codon 121 (Brr1p contains 341 aa). We have deleted *BRR1* and found that this mutation confers a cryosensitivity on complete medium as reported by NOBLE and GUTHRIE (1996a,b); however, we also detected an additional growth defect at 28° in two different genetic backgrounds, W303 (*ssd1-d*) and CEN.PK2 (*SSD1*) (Figures 4A and 7A).

Strains carrying suppressors of the complementation group SupM2 all show a recessive thermosensitivity (Figure 4B). After transformation of a strain carrying the *supM2* allele by a wild-type multicopy library we recovered 12 plasmids that allowed growth at 36° (File S1 and Table S3). The sequencing of candidate genes showed that the SupM2 group of suppressors are alleles of *MPT5*. The protein Mpt5p/Puf5p (859 aa) is a member of the RNA-binding Pumilio FBF (PUF) family (WHARTON and AGGARWAL 2006) and contains a PUF domain of eight repetitions between residues 223 and 530 (BARKER *et al.* 1992; MACDONALD *et al.* 1992). The different suppressor alleles correspond to (1) a nonsense mutation in codon 222, (2) a frameshift in codon

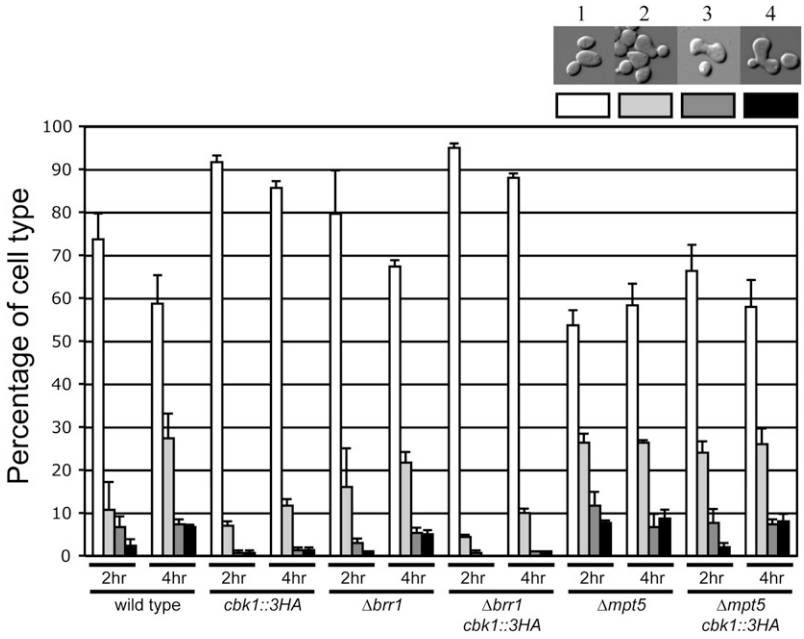

FIGURE 2.—Time course of the mating process. Equal numbers of mutant cells of both mating types were grown in rich glucose medium, mixed, and incubated on 2.5-cm filter discs placed on a rich glucose medium plate at 28° for 2 or 4 hr. The cells were then washed off and fixed and the different mating stages were counted; Open bars, unaffected cells; bars with light shading, cells with shmoos; bars with dark shading, fused cells (dumbbells); and solid bars, zygotes with the first diploid cell emerging (cloverleaf). The results are the mean and standard deviation from three independent experiments ($n > 100$).

407 that leads to a premature stop, and (3) a missense mutation Y520H (Figure 4C). These mutations lead to the loss of all or part of the PUF domain or the modification of one repetition. We have constructed the Δ*mpt5* allele in a W303 background and, as previously reported, this allele, like our suppressors, is thermosensitive (KIKUCHI *et al.* 1994; KAEBERLEIN and GUARENTE 2002). Our genomic clone gave only a partial complementation of the thermosensitive phenotype of the *mpt5* mutants; initially we assumed that this was because overexpression of Mpt5p is toxic (CHEN and KURJAN 1997; TADAUCHI *et al.* 2001). In fact the *MPT5* clone isolated from our library carries a frameshift due to the insertion of an adenine in a series of eight adenine nucleotides. This results in the formation of a truncated protein of 236 aa missing almost all the PUF domain. It is unlikely that the overexpression of this protein is able to complement the *mpt5* mutations; more probably, there is some ribosome slippage, allowing the production of some full-length protein. Subsequently, we have shown that this mutation is present in the genome of the strain used to make our library (FL100) (LACROUTE 1968). Interestingly, this mutation has also been described in another "wild-type" strain (KENNEDY *et al.* 1997). Thus it appears that *MPT5* presents a polymorphic variation in some laboratory strains.

Deletion of *BRR1* and *MPT5* also suppresses the fertility defect of *cbk1::3HA*: The nature of the mutations isolated in the *BRR1* and *MPT5* genes suggested that the suppression of the fertility defect of the *cbk1::3HA* allele was due to a loss of function. To determine if deletion of these genes also suppressed the fertility defect we measured the mating efficiency of strains carrying the deleted alleles in the presence of either the wild-type *CBK1* gene or *cbk1::3HA* and in both mating types. In the presence of *cbk1::3HA*, the deletion of either *BRR1* or *MPT5* significantly increased the mating efficiency, with Δ*brr1* restoring an essentially wild-type mating efficiency (Figure 1). Deletion of the genes in a wild-type *CBK1* background had little or no effect. Thus Δ*brr1* and Δ*mpt5* alleles also act as suppressors of the fertility defect of *cbk1::3HA*, and these were used in preference to the original suppressors in our subsequent experiments.

When we examined the effect of the deletion of *BRR1* and *MPT5* on the time course of the mating process, we found that the two genes had different effects: the deletion of *BRR1* leads to a very slight slowing of the mating process, while deletion of *MPT5* accelerates the mating process. In terms of the suppressors, Δ*brr1* has no discernible effect as *cbk1::3HA* Δ*brr1* strains are indistinguishable from *cbk1::3HA* strains. The deletion of *MPT5* has a dramatic effect, as *cbk1::3HA* Δ*mpt5* strains show an accelerated mating process compared to the wild type, albeit less than the Δ*mpt5* strains (Figure 2).

As our *cbk1* mutants have a defect in the expression of some of the mating type-specific genes, we examined the effect of Δ*brr1* and Δ*mpt5* on the expression of these genes. In *MAT*α strains deleting *BRR1* or *MPT5* in a *CBK1* background does not significantly affect the transcription of *MF*α*1*, *STE3*, or *SAG1*. In the *cbk1* mutant backgrounds, Δ*brr1* seems to have only a slight effect, essentially on *MF*α*1*, but Δ*mpt5* produces a clear increase in the transcript levels of all three genes (Figure 3B).

From these results it is clear that the deletion of *BRR1* or *MPT5* suppresses the fertility defect of *cbk1::3HA* in both mating types, and this suppression could be due in part to a restoration of the transcription of the mating type-specific genes. Also we have shown that the deletion of *MPT5* is able to overcome the slowing down of the mating process caused by the *cbk1::3HA* allele.

TABLE 1

Mutations in the *CBK1* gene affect the expression of some of the mating type-specific genes

Genes	Function	Δ*cbk1*	*cbk1S570A*	*cbk1::3HA*	*cbk1T743A*
		*MAT*α strains			
MF(α*1*)	α-Factor	−2.0 ± 1.0[a]	−2.2 ± 0.8[a]	−2.6 ± 0.4[a]	−5.5 ± 3.9[a]
MF(α*2*)	α-Factor	−6.5 ± 3.0[a]	−4.6 ± 2.6[a]	−5.8 ± 1.4[a]	−4.5 ± 1.2[a]
SAG1	α-Agglutinin	−12.0 ± 5.4[a]	−4.0 ± 1.8[a]	−5.1 ± 1.2[a]	−15.6 ± 7.4[a]
STE3	a-Factor receptor	−2.4 ± 0.4[a]	−1.6 ± 0.2[a]	−1.9 ± 0.4[a]	−10.2 ± 7.9[a]
YLR040c	Unknown function	−1.5 ± 0.4	−1.6 ± 0.8[a]	−1.9 ± 1.0[a]	−3.5 ± 1.8[a]
*MAT*α*1*	Locus MATα	−1.6 ± 0.2[a]	−1.3 ± 0.2	−1.6 ± 0.2[a]	−2.0 ± 0.8[a]
*MAT*α*2*	Locus MATα	−1.6 ± 1.0	−1.2 ± 0.4	−1.3 ± 0.2	−2.6 ± 1.0[a]
*HML*α*1*	Locus MATα	−1.6 ± 0.4	−1.4 ± 0.2	−1.6 ± 0.2	−2.0 ± 0.8[a]
*HML*α*2*	Locus MATα	−1.5 ± 1.0	−1.2 ± 0.6	−1.3 ± 0.4	−2.6 ± 0.9[a]

Genes	Function	*cbk1S570A*	*cbk1T743A*
		*MAT***a** strains	
AGA2	α-Agglutinin	1.1 ± 0.2	−1.4 ± 0.4
BAR1	α-Factor protease	−1.1 ± 0.4	−2.3 ± 1.1[a]
MFA1	**a**-Factor	−1.4 ± 0.4	−2.2 ± 0.8
MFA2	**a**-Factor	−1.4 ± 0.4	−2.3 ± 0.8[a]
STE2	α-Factor receptor	−1.2 ± 0.6	−3.4 ± 1.9[a]
STE6	a-Factor transporter	−1.1 ± 0.2	−2.5 ± 1.2[a]
HMRA1	Locus MAT**a**	−1.9 ± 1.6	−2.3 ± 1.0[a]
HMRA2	Locus MAT**a**	−1.1 ± 0.6	−1.1 ± 0.8

The results are the variation intervals of the fold changes of the four biological repeats compared to the wild type (mean ± 2 standard deviations).

[a] Genes having a statistically significant difference in expression between the mutants and the wild type are indicated (see MATERIALS AND METHODS).

The *brr1* and *mpt5* suppressor alleles have a polarity phenotype: The suppressors of the fertility defect of *cbk1::3HA* were isolated in an attempt to clarify the role of Cbk1p and the RAM network in cellular polarity. Both the *BRR1* and the *MPT5* deletions affect cellular polarity. We measured the axial ratio of haploid strains carrying the deletion alleles in the presence of three forms of *CBK1*: wild type, *cbk1::3HA*, and Δ*cbk1*. It is clear from the images and the axial ratios that deletion of *MPT5* has a much stronger effect on polarity than the deletion of *BRR1* (Figure S2 and Figure 5). Deletion of *BRR1* leads to a modest hyperpolarization in a *CBK1* background (axial ratio 1.29 compared to 1.2 for the wild type) and a limited restoration of polarity in *cbk1::3HA* or Δ*cbk1*. In contrast, deletion of *MPT5* leads to a clear hyperpolarization (axial ratio 1.43 compared to 1.2 for the wild type, Figure 5; see also PRINZ *et al.* 2007) and restores cellular polarity in a *cbk1::3HA* or Δ*cbk1* background, leading to a slightly hyperpolarized axial ratio (1.3 and 1.29 compared to 1.2 for the wild-type strain).

Thus to different extents, the suppression of the *cbk1::3HA* fertility defect by both Δ*brr1* and Δ*mpt5* is accompanied by a restoration of cellular polarity during vegetative growth; and at least in the case of Δ*mpt5* the polarity effect is independent of *CBK1* as it is also seen in a Δ*cbk1* background.

Deletion of *BRR1* or *MPT5* leads to an aggregation phenotype: Our initial observations of the fertility defect suppressors of *cbk1::3HA* showed that they contained aggregates of cells, a phenotype that is associated with a loss of Cbk1p function; we decided to quantify this effect. In a wild-type *CBK1* background, Δ*brr1* and Δ*mpt5* lead to a slight reduction in the level of individual cells, but no significant increase in the level of large aggregates (containing >12 cells). In the presence of *cbk1::3HA*, the deletion of either gene leads to a clear shift toward larger aggregates (>29 cells: *cbk1::3HA*, 1.3%; *cbk1::3HA* Δ*brr1*, 11%; and *cbk1::3HA* Δ*mpt5*, 16.5%) (Figure 6A). Thus the deletion of *BRR1* or *MPT5* appears to accentuate the aggregation phenotype of the *cbk1::3HA* allele.

As the aggregation phenotype is known to be due to a mislocalization of Ace2p and that Cbk1p is necessary for the correct localization of Ace2p, we decided to determine if the accentuated aggregation phenotype of the suppressors was caused by a modification of the localization of Ace2p or Cbk1p. To do this we looked at the localization of GFP-tagged proteins in strains (*CBK1* and *cbk1::3HA*) deleted for *BRR1* or *MPT5*. Because the addition of a C-terminal GFP to Cbk1::3HAp aggravates the aggregation phenotype of this mutant (data not shown), we determined its localization using Mob2-GFP, a RAM component that both colocalizes with Cbk1p and is dependent on Cbk1p for its localization (NELSON *et al.* 2003). These results show no significant change in the localization of Ace2p, suggesting that the aggregation phenotype of the Δ*brr1* and Δ*mpt5* strains is indepen-

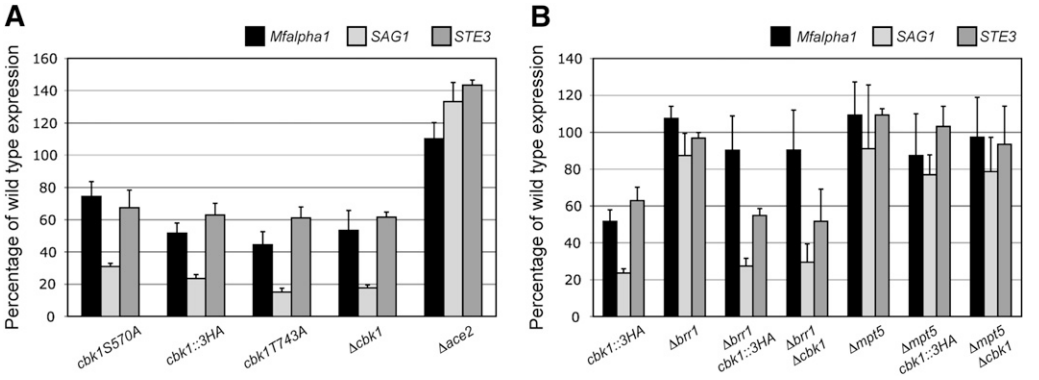

FIGURE 3.—(A and B) Effect of different mutations in *CBK1*, *BRR1*, and *MPT5* on the expression of the mating type-specific genes *MFα1*, *SAG1*, and *STE3* in a W303 *MAT*α background. Total RNA was extracted from haploid strains and analyzed by qPCR; *ACT1* was used as an internal control. The results are derived from at least three independent experiments and are expressed as a percentage of the wild-type level.

dent of the localization of Ace2p (Figure S2). The situation is less clear for Cbk1p/Mob2p, especially in the Δ*brr1 cbk1::3HA* context, as these proteins have weak nuclear signals that are difficult to see in cell aggregates or cells that have perturbed morphologies. Thus, it is not possible to rule out a subtle variation in the localization of these proteins.

To see if Ace2p function is affected by the deletion of *BRR1* or *MPT5*, we examined the level of transcription of two genes that are directly controlled by this factor, *CTS1* and *DSE2* (Dohrmann *et al.* 1992; Colman-Lerner *et al.* 2001). The results of the qPCR experiments presented in Figure 6B show essentially identical effects on the transcript levels of both *CTS1* and *DSE2*. In a wild-type context (*CBK1*), Δ*brr1* has no effect, while Δ*mpt5* results in a 40–50% reduction in the level of *CTS1* and *DSE2* transcripts. In the presence of *cbk1::3HA*, transcription of the genes is already reduced by ~60% and deletion of either *BRR1* or *MPT5* causes a further reduction, which is more severe in the case of *MPT5*. As one would expect, the severity of the effect of Δ*brr1* and Δ*mpt5* on the transcription of *CTS1* and *DSE2* follows the severity of the aggregation phenotypes (Figure 6, A and B). We have also shown that Δ*brr1* and Δ*mpt5* do not affect the transcription of *ACE2* (data not shown). These results show that Δ*brr1* and Δ*mpt5* reduce the levels of at least two Ace2p targets in a way that appears to be independent of both the transcription of the *ACE2* gene and the localization of the protein.

Genetic interactions between *CBK1*, *BRR1*, *MPT5*, and *SSD1*: In the presence of a wild-type *SSD1* gene, *CBK1* and all the *RAM* genes are essential (Du and Novick 2002; Jorgensen *et al.* 2002; Kurischko *et al.* 2005). Kikuchi *et al.* (1994) and Kaeberlein and Guarente (2002) have shown that Δ*mpt5* strains are thermosensitive at 36° in a Δ*ssd1* context and that this thermosensitivity is suppressed by *SSD1*; we have confirmed these results in our strains (data not shown). We have also deleted *BRR1* in CEN.PK2, which carries the wild-type *SSD1* gene. The deletion of *BRR1* leads to a significant slow growth phenotype at 28°, which is clearly aggravated by the deletion of *SSD1* (Figure 7A); this effect can also be seen at 18° and 36° (data not shown). Thus *SSD1* acts as a suppressor of the growth defects of both Δ*brr1* and Δ*mpt5* strains. This led us to ask if there is also an interaction between *BRR1* and *MPT5*. To address this we analyzed the segregation of a Δ*brr1/BRR1* Δ*mpt5/MPT5* diploid in a W303 (*ssd1-d*) and CEN.PK2 (*SSD1*) background. Figure 7B shows that in the absence of *SSD1* (W303 *ssd1-d*), the double deletion, Δ*brr1* Δ*mpt5*, is inviable at both 28° and 36°, whereas in the presence of *SSD1*, the double mutant is able to grow slowly at 36°. Thus Δ*brr1* and Δ*mpt5* show a synthetic lethal phenotype that is partially suppressed by *SSD1* at 36°.

Taken together, these results show that *BRR1*, *MPT5*, and *SSD1* are intricately related and we have previously shown that Cbk1p interacts physically with Ssd1p (Racki *et al.* 2000). To determine how *MPT5* affects the genetic interaction between *SSD1* and *CBK1*, we analyzed the segregation of a Δ*cbk1/CBK1* Δ*mpt5/MPT5* diploid in a CEN.PK2 (*SSD1*) background (Figure 7C). As expected, the deletion of *CBK1* led to a lethal phenotype, but this was partially suppressed when *MPT5* was also deleted. The double mutant, Δ*cbk1* Δ*mpt5*, is thermosensitive: the strain showed a uniform slow growth at 28° and formed aggregates similar to a Δ*cbk1* Δ*ssd1* strain; individual cells had a very heterogeneous shape and tended to lyse easily. Thus Δ*mpt5* is a weak suppressor of the lethality of Δ*cbk1* in a wild-type *SSD1* background.

These experiments have uncovered new interactions between *CBK1*, *BRR1*, *MPT5*, and *SSD1* and reveal a complex network that controls aspects of cell separation, cell integrity, mating, and polarized growth.

DISCUSSION

The protein kinase Cbk1p and the RAM network are critical for maintaining cell polarity and efficient cell separation. We have shown here that *cbk1::3HA* and *cbk1S570A* have a reduced mating efficiency in both *MAT***a** and *MAT*α strains and that all our *cbk1* mutants show a reduction in the level of the transcripts of some mating type-specific genes in *MAT*α strains. It is known

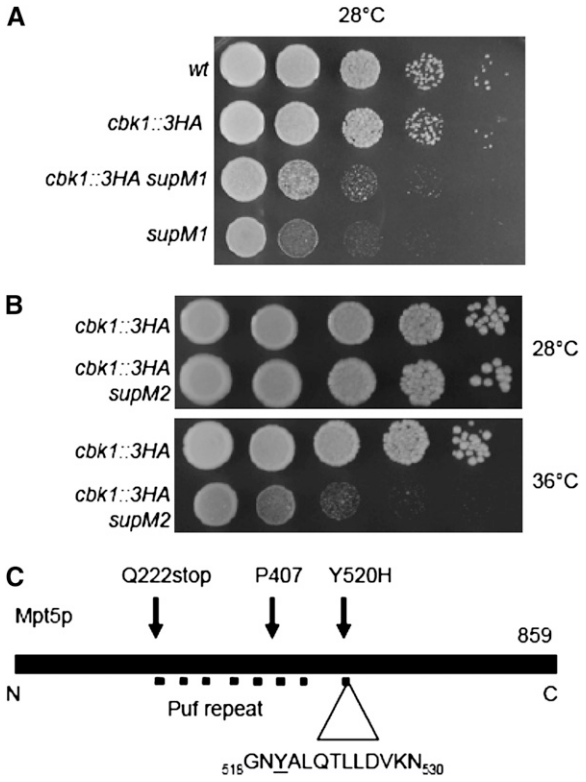

FIGURE 4.—Characterization of the suppressors of the mating defect of *cbk1::3HA*. Exponentially growing cultures were diluted and plated onto rich glucose medium and incubated as indicated. (A) A tetrad derived from the diploid *CBK1/cbk1::3HA SUP⁺/supM1* (*brr1*), incubated at 28° for 48 hr. (B) *cbk1::3HA* haploids with or without *supM2* (*mpt5*), incubated at 28° and 36° for 72 hr. (C) A schematic representation of Mpt5p showing the position of the eight repeats that make up the RNA-binding PUF domain and the mutations present in the three suppressor alleles of *mpt5*. P407 is a frameshift that leads to a premature stop.

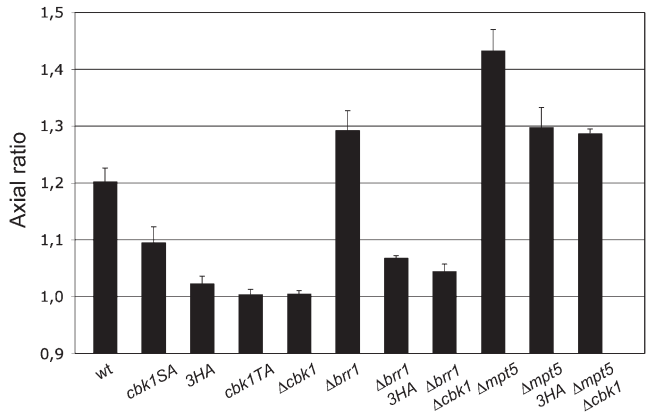

FIGURE 5.—Effect of different mutations in *CBK1*, *BRR1*, and *MPT5* on cell polarity. The axial ratio (length/width) was used to measure cellular polarity; unpolarized round cells will have an axial ratio of 1. Three independent experiments were performed with >200 cells measured in each experiment. The results show the mean and standard deviation between the three experiments. The strains are all haploids in a W303 background and were grown in rich glucose medium. *cbk1SA*, *cbk1S570A*; *3HA*, *cbk1::3HA*; and *cbk1TA*, *cbk1T743A*.

that Δ*ram* strains have difficulty forming robust shmoos when treated with α-factor (BIDLINGMAIER *et al.* 2001; NELSON *et al.* 2003) and we have observed that *cbk1::3HA* and *cbk1S570A* strains are slow to form shmoos and form small shmoos (data not shown). Furthermore, our analysis of the time course of the mating process shows that in *cbk1::3HA* strains, the onset of shmoo formation is delayed, and it appears that other critical steps, such as cell fusion (to form the dumbbells) are also delayed (see Figure 2). Taken together, these data suggest that the fertility defect of the *cbk1::3HA* is not simply the result of a reduction in the transcription of some of the mating type-specific genes.

We have identified Δ*brr1* and Δ*mpt5* as suppressors of the mating defect of *cbk1::3HA* strains, and subsequent experiments have shown that to a greater or a lesser extent they also affect the polarity, aggregation, and cell integrity phenotypes of these strains.

Brr1p is a nuclear protein (HUH *et al.* 2003) and this localization is not modified in Δ*cbk1* strains (data not shown). Originally *BRR1* was identified as being required for the stability of the snRNAs and shown to interact with the snRNPs (NOBLE and GUTHRIE 1996a,b). The deletion was cryosensitive, and we have shown that it has a severe growth defect at 28° in two different strain backgrounds. The cryosensitivity is partially suppressed by the overexpression of the spliceosomal protein SmD1. The *BRR1* deletion is also synthetic lethal with *ccr4* and *pop2* (which encode components of the Ccr4–Not complex that mediates 5′–3′ mRNA deadenylation) and with *snu114* (GTPase component of the U5 snRNP) (BRENNER and GUTHRIE 2005; PAN *et al.* 2006). It has been found in association with the spliceosomal proteins Smd1p, Smd2p, Smx2p, Smx3p, and Sto1p, which are part of the nuclear cap-binding complex (GAVIN *et al.* 2002, 2006; KROGAN *et al.* 2006), and recently Δ*brr1* was shown to have a synthetic phenotype with the deletion of *TGS1*, the trimethylguanosine synthase (HAUSMANN *et al.* 2008). Thus many interactions link Brr1p with RNA metabolism, particularly splicing and degradation, but the exact molecular role of the protein is not known.

Although it is clear that Δ*brr1* suppresses the fertility defect of *cbk1::3HA* in a way that is independent of mating type, the mechanism by which this happens is unclear. Δ*brr1* has very little effect on the expression of the mating type-specific genes and the mating time courses of *cbk1::3HA* and *cbk1::3HA* Δ*brr1* strains are essentially identical.

Mpt5p/Puf5p is a member of the PUF family of RNA-binding proteins and it recruits the Ccr4–Not complex to the 3′-UTR of some mRNAs, stimulating deadenylation and reducing translation (BARKER *et al.* 1992; MACDONALD *et al.* 1992; GOLDSTROHM *et al.* 2006,

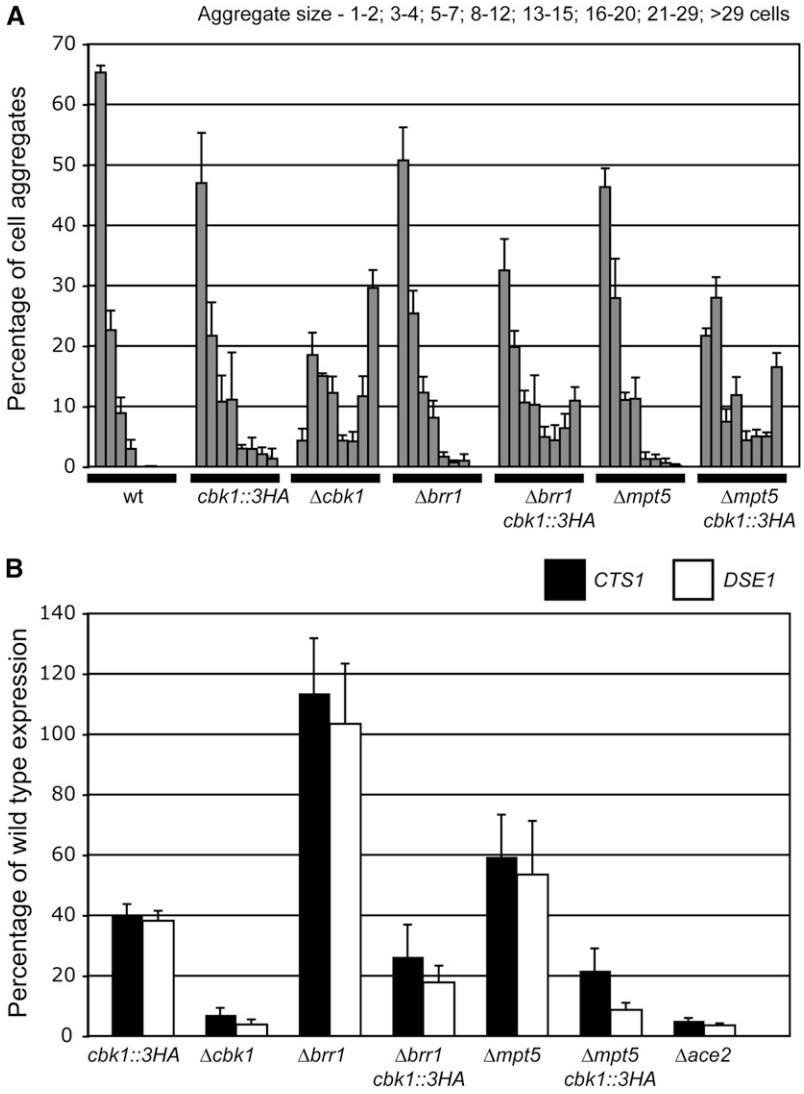

FIGURE 6.—(A) Aggregation phenotype of different *cbk1*, *brr1*, and *mpt5* mutations. The aggregation phenotype was quantified by counting the number of attached cells in a group. Three independent experiments were performed with >200 cell aggregates counted in each experiment. The aggregate categories are 1–2, 3–4, 5–7, 8–12, 13–15, 16–20, 21–29, and >29 cells. The mean and standard deviation between the three experiments are shown. The strains are all haploids in a W303 background and were grown in rich glucose medium. (B) Expression of *CTS1* and *DSE2* in the presence of different *cbk1*, *brr1*, and *mpt5* mutations. The level of expression of *CTS1* and *DSE2* was determined on total RNA extracted from haploid strains in a W303 background; *ACT1* was used as an internal control. The results are derived from at least three independent experiments and are expressed as a percentage of the wild-type level.

2007; WHARTON and AGGARWAL 2006). Systematic studies have suggested that Mpt5p can bind to >200 mRNAs, indicating that Mpt5p could regulate many cellular processes (GERBER *et al.* 2004; SEAY *et al.* 2006). Several Mpt5p mRNA targets are well characterized such as *HO*, involved in mating type switching, and *STE7* and *TEC1*, two components of the (f)-MAPK pathway. Mpt5p has also been implicated in the regulation of many other pathways, such as silencing and replicative life span, although the mechanisms involved remain unclear (KENNEDY *et al.* 1997; COCKELL *et al.* 1998; TADAUCHI *et al.* 2001; IRIE *et al.* 2002; OHKUNI *et al.* 2006; PRINZ *et al.* 2007). Mpt5p also regulates, at least in part, the cell wall integrity (CWI) pathway. The GTPase Rho1p, which is in turn activated by the sensors Mid2p and Slg1p, activates Pkc1p, the central regulatory kinase in the pathway; Lrg1p inhibits this pathway. Mpt5p binds to the *LRG1* mRNA, inhibiting its expression, thus activating the CWI pathway. The growth defect of Δ*mpt5 ssd1* strains is suppressed by the absence of Lrg1p or the overexpression of *MID2*, *SLG1*, and *PKC1* (TAKEUCHI *et al.* 1995; HATA *et al.* 1998; KAEBERLEIN and GUARENTE 2002; LEVIN 2005; OHKUNI *et al.* 2006; STEWART *et al.* 2007). We also isolated *SLG1* and *PKC1* as multicopy suppressors of the *mpt5* mutation during the cloning of the gene (see Table S3).

Mpt5p also shows a complex series of interactions with different factors involved in mating: it interacts physically with Sst2p, the GTPase activating protein for Gpa1p that regulates desensitization to the α-pheromone; the protein also interacts with Fus3p (m-MAPK) and Kss1p (m-MAPK and f-MAPK), and it regulates *STE7* mRNA, which is also part of the m-MAPK cascade. Finally, the deletion of *MPT5* suppresses the mating defect of a *fus3* mutant (CHEN and KURJAN 1997; BARDWELL 2005; PRINZ *et al.* 2007). In general Mpt5p seems to regulate pathways, or individual proteins, by binding to the 3′-UTR of mRNAs, leading to degradation or a reduction of translation.

It is clear that the mating defect of the *cbk1::3HA* strains results from a combination of causes. Recently KURISCHKO *et al.* (2008) have shown that Cbk1p and the

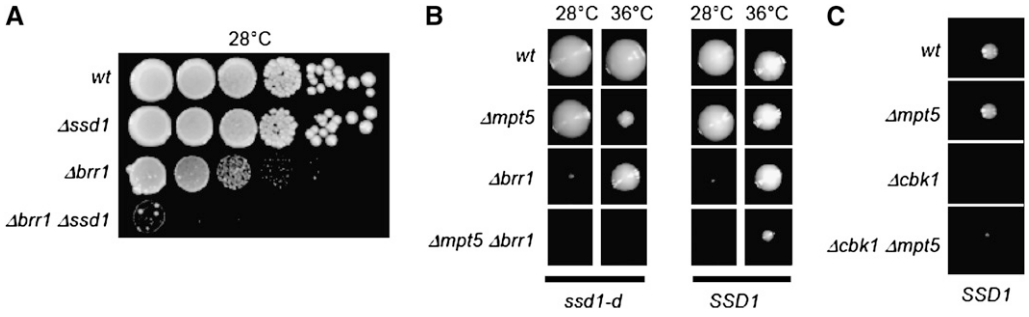

FIGURE 7.—Genetic interactions between *CBK1*, *BRR1*, *MPT5*, and *SSD1*. (A) Serial dilutions of a tetrad showing the segregation and phenotypes of Δ*brr1* and Δ*ssd1* in a CEN.PK2 background after 72 hr incubation at 28°. (B) Tetrads showing the segregation and phenotypes of Δ*brr1* and Δ*mpt5* in either an *ssd1-d* background (W303) or a wild-type *SSD1* background (CEN.PK2). The plates were incubated at 28° or 36° for 4 days. (C) A tetrad showing the segregation and interaction between Δ*cbk1* and Δ*mpt5* in a wild-type *SSD1* background (CEN.PK2), showing that Δ*mpt5* is a weak suppressor of the Δ*cbk1* lethality in this context. The plate was incubated at 28° for 2 days.

RAM network are involved in regulating growth, bud emergence, and secretion. In particular, Cbk1p can phosphorylate Sec2p and inhibition of Cbk1p leads to a delay in the polarized localization of the exocytosis regulators Sec4p and Sec2p. Exocytosis and remodeling of the cell wall and cell membrane are crucial elements of shmoo formation and cell fusion during mating. Thus a partial disruption of these processes, together with the reduced transcription of some of the mating type-specific genes, could explain the delay in the onset of mating in the *cbk1::3HA* strains.

In a wild-type background, deletion of *MPT5* leads to a hyperpolarization of the cells and an acceleration of the mating process. In *cbk1::3HA* cells, Δ*mpt5* stimulates the expression of some of the mating type-specific genes, restores polarity, and accelerates the mating process. PRINZ *et al.* (2007) have shown that the hyperpolarization of Δ*mpt5* strains requires the presence of *TEC1*. In conjunction with Ste12p, Tec1p activates the filamentation pathway; Mpt5p binds the *TEC1* mRNA and inhibits the expression of Tec1p. Thus in the absence of Mpt5p, Tec1p probably causes a low-level constitutive expression of the filamentation pathway, leading to a hyperpolarization of the cells. In a similar way, given the known interactions between Mpt5p and elements of the m-MAPK pathway, it is possible that deletion of *MPT5* leads to a low-level activation of this pathway. Lrg1p could also constitute an element of this system: the protein is required for efficient cell fusion (FITCH *et al.* 2004) and its expression is inhibited by Mpt5p; thus deletion of *MPT5* would lead to increased levels of Lrg1p and improved cell fusion.

Our results and those from several other laboratories have shown that a complex series of genetic interactions link *CBK1* (and the *RAM* network in general) with *SSD1*, *BRR1*, and *MPT5* and also that Ssd1p interacts physically with Cbk1p and Mpt5p (RACKI *et al.* 2000; TARASSOV *et al.* 2008). No clear unified picture is immediately evident from all these interactions, but there are certain parallels that draw these proteins together. Cbk1p, Ssd1p, and Mpt5p have all been linked to the maintenance of cell integrity; Ssd1p, Brr1p, and Mpt5p are all involved in RNA metabolism; and recently Tao3p, another RAM component, has been implicated in snoRNA biogenesis (QIU *et al.* 2008). Brr1p and Mpt5p are known to interact with the Ccr4–Not complex that mediates 5′–3′ mRNA deadenylation and Cbk1p is also linked to this complex as it is a two-hybrid partner of one of the subunits (Not3p) (RACKI *et al.* 2000). Cbk1p is also two-hybrid partner of Dsf2p, which is a deletion suppressor of the Δ*mpt5* thermosensitive phenotype (W. J. RACKI, unpublished results; OHKUNI *et al.* 2006). All these results are suggestive of a role for these proteins in regulating gene expression.

Recently MAZANKA *et al.* (2008) determined a consensus motif for phosphorylation by Cbk1p and noted an unusual requirement for a histidine residue at −5 to the phosphorylation site. Ssd1p has an exact match to this consensus and both Brr1p and Mpt5p have very close matches including the histidine at −5; thus it is possible that all three proteins are phosphorylated by Cbk1p. The observation that in each case the phenotype associated with the *CBK1* deficiency is overcome by deletion of one of the genes (lethality for Δ*ssd1* and reduced fertility for Δ*brr1* and Δ*mpt5*) suggests that phosphorylation by Cbk1p has an inhibitory role. In the case of Ssd1p and Mpt5p, we can speculate a little further: Cbk1p could negatively regulate Ssd1p by phosphorylation, with a constitutively active Ssd1p leading to cell lysis in the absence of RAM. There is no clear evidence to indicate if Brr1p and Ssd1p are positive or negative effectors, but in general Mpt5p seems to be a negative regulator. Thus no, or a reduced, Cbk1p inhibitory phosphorylation would lead to a constitutive repression that would be relieved by the deletion of the *MPT5* gene (see the discussion of Lrg1p above). These different interactions are summarized in the model in Figure 8. Given the links between Cbk1p and Mpt5p, and Mpt5p and the m-MAPK and f-MAPK pathways, it is interesting to note that the *Neurospora crassa* homolog of Cbk1p, COT1, has recently been shown to interact genetically with two MAP kinases, MAK1 and MAK2, which participate in the regulation of filamentous growth, hyphal fusion, and sexual development (MAERZ *et al.* 2008).

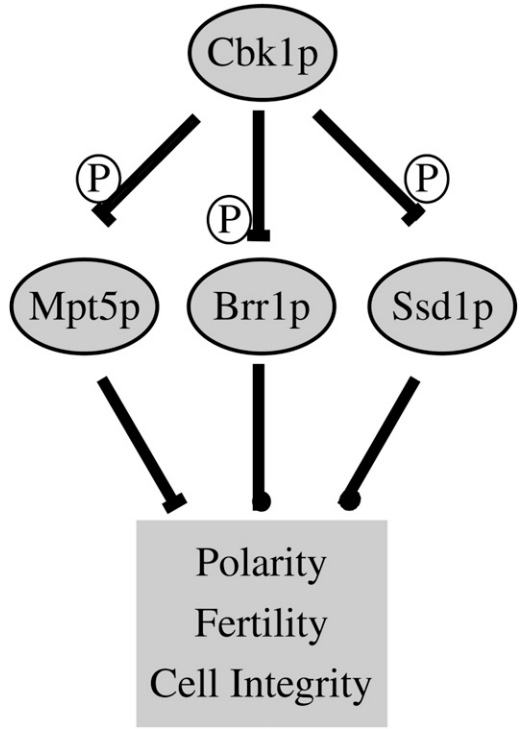

FIGURE 8.—A model for the role of Cbk1p in the regulation of cell polarity, fertility, and integrity. We propose that Cbk1p negatively regulates Mpt5p, Brr1p, and Ssd1p by phosphorylation and that these proteins have overlapping roles in the regulation of polarity, fertility, and cell integrity. Lines that terminate with a "dot" indicate that the role of the effectors could be positive or negative.

It is clear that the RAM network interacts with Ssd1p, Brr1p, and Mpt5p to regulate a series of parallel, and probably at least partially redundant, pathways controlling aspects of cell separation, cell integrity, mating, and polarized growth (Figure 8). Some of these functions are essential for viability but may be lethal when overexpressed in an uncoordinated way leading to cell lysis, and this could be why the cell evolved such a complicated multilayered system to control them.

We thank W. Racki for providing the original *cbk1::3HA* strain, A.-M. Bécam for invaluable technical assistance, G. Dujardin and N. Bonnefoy for critical reading of the manuscript, H. Delacroix for helpful discussions concerning the microarray experiments, and L. Kuras for help with the qPCR experiments. This work was financed by the Centre National de la Recherche Scientifique (CNRS), by a "Subvention Fixe" from the Association pour la Recherche sur le Cancer, and by an Action Concertée Incitative Biologie Cellulaire, Moléculaire et Structurale grant from the French Ministry of Research. M.B. thanks the Institut de Chimie des Substances Naturelles du CNRS, Gif-sur-Yvette, France, for financial support.

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Communicating editor: M. D. Rose

GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.109.105130/DC1

Mutations in the *Saccharomyces cerevisiae* Kinase Cbk1p Lead to a Fertility Defect That Can Be Suppressed by the Absence of Brr1p or Mpt5p (Puf5p), Proteins Involved in RNA Metabolism

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DOI: 10.1534/genetics.109.105130

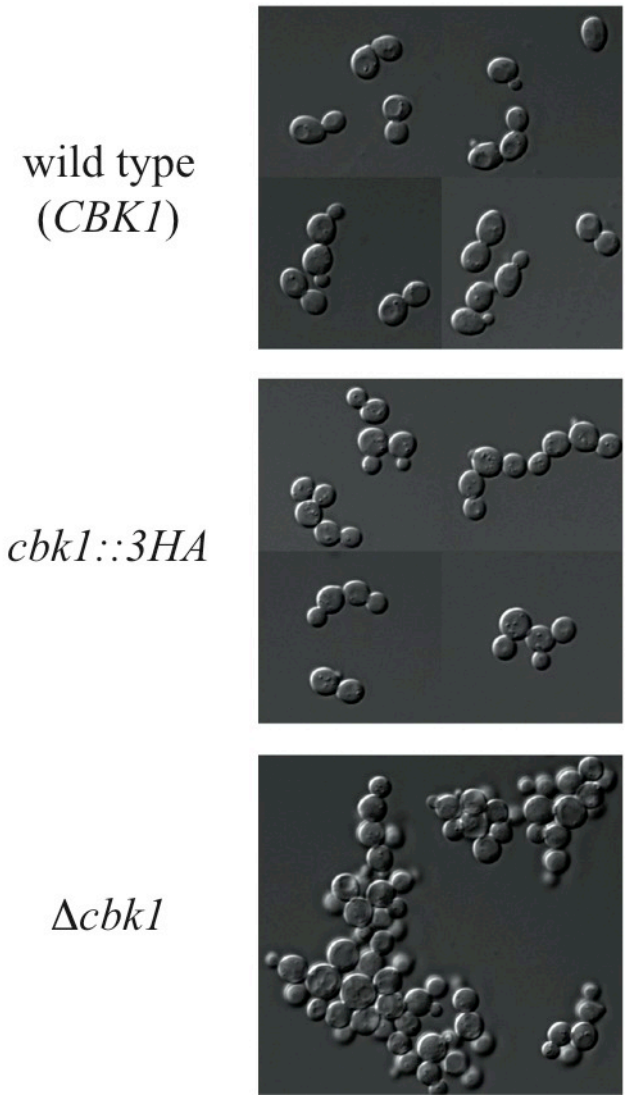

FIGURE S1.—Cell morphology of strains carrying different *CBK1* alleles; wild type, *cbk1::3HA* and Δ*cbk1*.

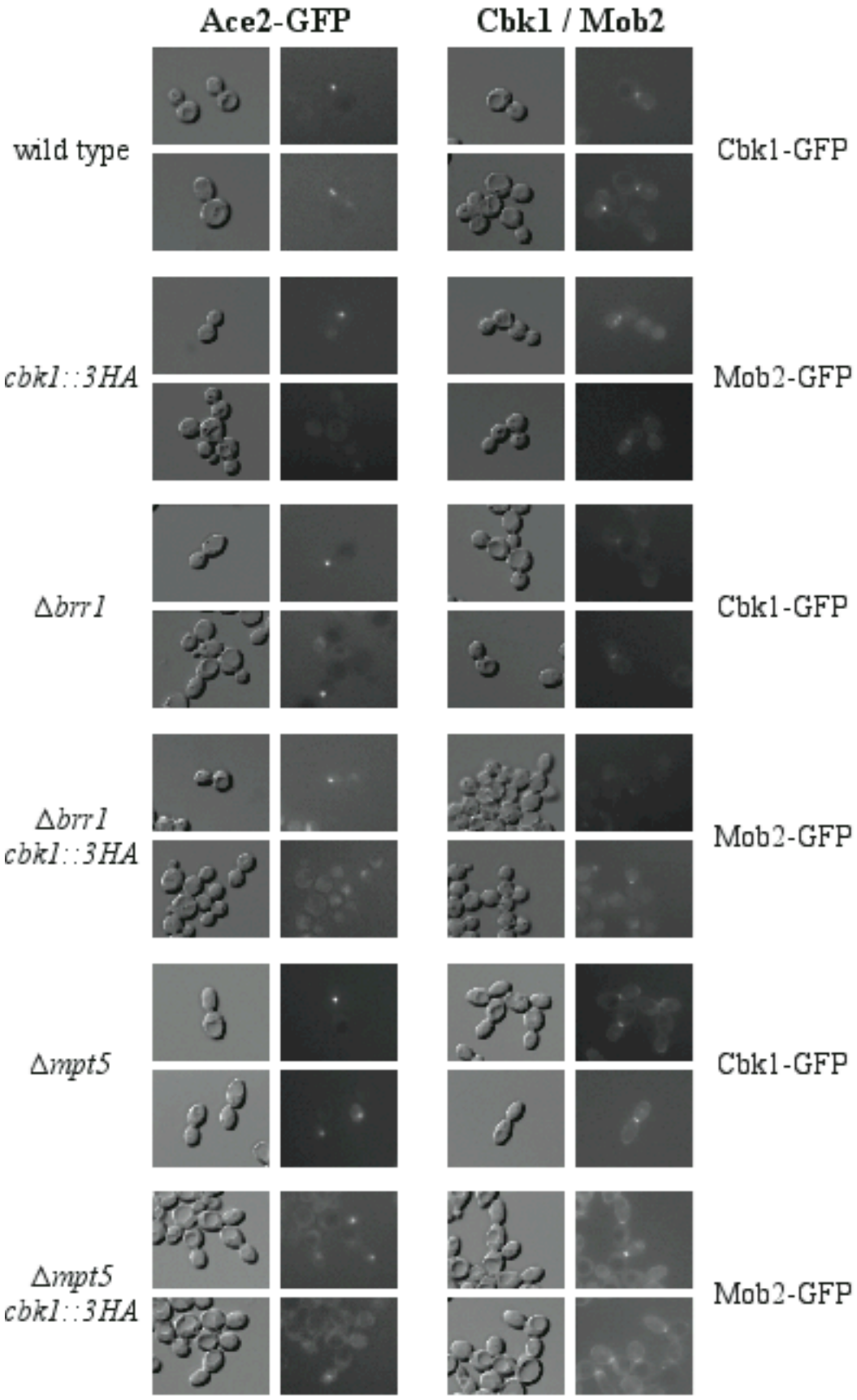

FIGURE S2.—Effect of the suppressor mutations on the localization of Ace2-GFP and Cbk1-GFP, or Mob2-GFP. The DIC and GFP images show the localization of Ace2p and Cbk1p, or Mob2p in the different suppressor contexts. Because the addition of a GFP tag to Cbk1::3HAp leads to a significant aggravation of the associated phenotype, Mob2-GFP, which co-localizes with, and is dependent on Cbk1p for its localization, was used to indicated the position of Cbk1::3Hap.

TABLE S1

***Saccharomyces cerevisiae* strains used in this study**

Strains	Genotype
WR762-3c	*MATa ADE2*
WR762-5c	*MATalpha LEU2*
WR761-14a	*MATa Δbrr1::HPH LEU2*
WR811-4c	*MATa Δmpt5::G418 LEU2*
WR763-2b	*MATa cbk1::3HA LEU2*
WR580-4d	*MAT a cbk1::3HA ADE2*
WR761-8a	*MATa cbk1::3HA Δbrr1::HPH LEU2*
WR821-5b	*MAT a cbk1::3HA Δmpt5::G418 LEU2*
WR768-2b	*MATa cbk1S570A LEU2*
WR580-7c	*MATalpha LEU2*
WR774-6b	*MATalpha ADE2*
WR767-10c	*MATalpha Δbrr1::HPH LEU2*
WR811-10b	*MATalpha α Δmpt5::G418 LEU2*
WR580-13b	*MATalpha cbk1::3HA LEU2*
WR761-12c	*MATalpha cbk1::3HA Δbrr1::HPH LEU2*
WR821-13d	*MATalpha cbk1::3HA Δmpt5::G418 LEU2*
WR768-2a	*MATalpha cbk1S570A LEU2*
SupM1	*MATalpha cbk1::3HA supM1 LEU2*
CHY022	*MATa/ MATalpha ade2/ADE2 leu2/LEU2 CBK1/cbk1::3HA SUP+/supM1*
SupM2	*MATalpha cbk1::3HA supM2 LEU2*
SupM3	*MATalpha cbk1::3HA supM3 LEU2*
SupM4	*MATalpha cbk1::3HA supM4 LEU2*
WR766-2c	*MATa Δcbk1::HIS3 LEU2*
WR766-3a	*MATalpha Δcbk1::HIS3 LEU2*
WR769-1c	*MATa cbk1T743A LEU2*
WR769-4b	*MATalpha cbk1T743A LEU2*
WR765-3a	*MATa Δace2::HPH LEU2*
WR765-2a	*MATalpha Δace2::HPH LEU2*
WR903	*MATa/MATα Δbrr1::HPH/BRR1 Δmpt5::G418/MPT5*
WR767-7d	*MATa Δcbk1::HIS3 Δbrr1::HPH LEU2*
WR767-7c	*MATalpha Δcbk1::HIS3 Δbrr1::HPH LEU2*
WR811-6a	*MATa Δcbk1::HIS3 Δmpt5::G418 LEU2*
WR811-7d	*MATalpha Δcbk1::HIS3 Δmpt5::G418 LEU2*
WR155-1a	*MATalpha ACE2-GFP::G418*
WR162-1d	*MATa Δcbk1::HIS3 ACE2-GFP::G418*
WR539-2b	*MATa cbk1::3HA ACE2-GFP::G418*
WR910-9a	*MATa Δbrr1::HPH ACE2-GFP::G418*
WR818-2d	*MATa Δmpt5::G418 ACE2-GFP::G418*
WR911-3b	*MATa Δbrr1::HPH ACE2-GFP::G418 cbk1::3HA*

WR834-18b	*MATa Δmpt5::G418 ACE2-GFP::G418 cbk1::3HA*
WR152-1a	*MATa ade3 CBK1-GFP::G418*
CHY063-9c	*MATa Δcbk1::HIS3 MOB2-GFP::G418*
WR835-1b	*MATa cbk1::3HA MOB2-GFP::G418 LEU2*
WR859-8b	*MATa Δbrr1::HPH MOB2-GFP::G418*
WR817-4b	*MATa Δmpt5::G418 CBK1-GFP::G418 ADE2*
WR912-4b	*MATa Δbrr1::HPH cbk1::3HA MOB2-GFP::G418*
WR835-2b	*MATa Δmpt5::G418 cbk1::3HA MOB2-GFP::G418*
WR762-5c	*MATa LEU2*
WR763-2b	*MATa LEU2 cbk1::3HA*
WR763-4c	*MATalpha ADE2 cbk1::3HA*
WR821-20d	*MATa Δmpt5::G418 cbk1::3HA*
WR821-14c	*MATalpha Δmpt5::G418 cbk1::3HA*
WR809-15b	*MATa Δmpt5::G418*
WR809-15c	*MATalpha Δmpt5::G418*
WR911-18b	*MATa Δbrr1::HPH cbk1::3HA*
WR911-18a	*MATalpha Δbrr1::HPH cbk1::3HA*
WR685-15a	*MATa Δbrr1::HPH*
WR685-15b	*MATalpha Δbrr1::HPH*
WR850 (CENPK2)	*MATa/ MATalpha Δbrr1::HPH/BRR1 SSD1/Δssd1::G418*
WR829 (CENPK2)	*MATa/ MATalpha Δcbk1::HPH/CBK1 Δmpt5::G418/MPT5*
WR906 (CENPK2)	*MATa/ MATalpha Δbrr1::HPH/BRR1 Δmpt5::G418/MPT5*

Except when indicated all strains were derived from the homozygous diploid W303 (*MAT a/MAT alpha ade2-1 his3-11,15 leu2-3,12 trp1-1 ura3-1 can1-100 ssd1-d*), for simplicity, only differences from the W303 genotype are shown. CEN.PK2 is also a homozygous diploid (*MAT a/MAT alpha ura3-52 trp1-289 leu2-3,112 his3Δ1 SSD1*), again , only differences from the parental genotype are shown.

TABLE S2

Oligonucleotide primers used in this study

Name	Oligonucleotide Sequence
MFA2-5'	GCAACCGATCACCACTGCTTCC
MFA2-3'	GCGATAACACAGGCGGGATCCC
AGA2-5'	CGGGAAGGCAATGCAAGGAG
AGA2-3'	CTGTGTGTTTATGGGGCTGCC
STE2-5'	CAGTTGCCCACACCTACGAGTTC
STE2-3'	CTGGCTTCCTCATCAGCTGCCG
MFALPHA1-5'	CGCTGAAGCTTGGCATTGGCTGC
MFALPHA1-3'	GGTTGGCCGGGTTTTAACTGC
SAG1-5'	CAGCAGTCTCATCTCAGGGAACG
SAG1-3'	CTGTTGGATACCGGACAATTGG
STE3-5'	CAGATGATATTCTTGATGAAATAGACC
STE3-3'	CGAGAGTAACTTTTGAGAATCCTCC
MATalpha1-5'	GCAGCACGGAATATGGGACTACTTCG
MATalpha1-3'	CTTCCCAATATCCGTCACCACG
HMRA1-5'	CAATATCACCCCAAGCACGGGC
HMRA1-3'	CTCTTACTTGAAGTGGAGTAATGCCAC
CTS1-5'	GCTCTGCTGATGGGAAGTTCG
CTS1-3'	GGGTATAGACTGAGTCGCCGG
ACT1-5'	CCATGTTCCCAGGTATTGCC
HERB44	CACCAATCCAGACGGAGTAC
CBK1-5'	CGGTGTTGATTGGAATACAATCAGAC
CBK1-3'	GTGCCATAGCTGGGGAATCTG
DSE2-5'	GCTCGGATGGCACTTGTTACG
DSE2-3'	GTGGTGGCGGCATCAACG
ACE2-5' (1325)	GAAGAGCAAGAAGAGGTGGCG
ACE2-3' (9473)	GGAGGAGGAAATTGATATCC
BRR1-5'	CATATTCTCCACCGCGGCTGG
BRR1-3'	GGAAGGCATTATCGTCGGCTG
MPT5-5' (MPT5-9)	CCGTAATTCAATGTACTAGAGC
MPT5-3' (MPT5-5)	GCAGTTGCTTCTGATGACGTG

TABLE S3

Multicopy suppressors isolated during the cloning of *MPT5*

Chromosome Coordinates	Genes present in the insert	Candidates
VII 166492-171468	*MPT5, YGL177W…*	*MPT5* Multicopy suppressor of Pop Two
XV 580561-584874	*IDH2*, *SIA1*	*SIA1* suppressor of eIF5A
XV 581123-585019	*SIA1*, *RUP1*	
XV 339523-343078	*SLG1*, *YOR0008C-A*	*SLG1* Synthetic lethal with gap1
XIII 810329-816398	*ZDS1*, *RCE1*, *ARS1330*	*ZDS1* Zillion different screens 1
XIII 810566-818366	*ZDS1*, *RCE1*, *ARS1330*, *BUL1*	
XIII 46400-54755	*BUL2*, *COQ5*, *ZDS2*	*ZDS2* Zillion different screens 2
XIII 340004-346363	*IMP2*, *MIH1*, *MSN2*	*MIH1* Mitotic Inducer Homolog
XIV 268902-273162	*YNL195C*, *SLZ1*, beginning of *WHI3*	*?*
II 13201-19134	*PKC1*	*PKC1* Protein Kinase C
IV 174014-179861	*YDR159C-B*, *YDL157C*, *YDL156W*, *CLB3*	*CLB3* Cyclin B 3
IV 174014-179087	*YDL156W*, *CLB3*	

Multicopy suppressors isolated during the identification of the *mpt5* suppressor alleles. In the experiments to clone the wild type gene corresponding to the suppressor *SupM3* (*mpt5Y520H*) several other plasmids were isolated that were able to restore growth at 36°C and therefore contain multicopy suppressors of the *mpt5* thermosensitive phenotype.

FILE S1

Discussion of Table S3

Two of the plasmids carry genes that have previously been identified as Δ*mpt5* suppressors: *PKC1* and *SLG1* which both belong to the cell wall integrity pathway (CWI) and lead to the over-expression of this pathway (TAKEUCHI *et al.* 1995; HATA *et al.* 1998; KABERLEIN and GUARENTE, 2002; OHKUI *et al.* 2006). *MPT5* activates the CWI in a wild type strain via the repression of *LRG1* (LEVIN *et al.* 2005; STEWART *et al.* 2007). Thus these two suppressors probably compensate the repression of this pathway caused by the absence of Mpt5p. Other plasmids carrying *SIA1*, *ZDS1* or its homologue *ZDS2* probably also act through the CWI pathway, but their exact role is unclear. *SIA1* has been isolated as a suppressor of the thermosensitive phenotype of a *HYP2* mutant, as have *PKC1*, *WSC2*, *WSC3*, *SLG1* and *PAB1*, which are all members of the CWI (VALENTINI *et al*. 2002). *ZDS1* is necessary for *PKC1* to suppress the *hyp2* growth and polarity defect (ZANELLI and VALENTINI, 2005). We also isolated *MIH1*, which encodes a phosphatase that dephosphorylates tyrosine 19 of Cdc28p (YANG *et al.* 2000). It is know that Δ*swi1* is also a suppressor of Δ*mpt5*. Swe1p is a protein kinase implicated in the G2/M transition and inhibits the Cdc28p kinase activity by phosphorylation of tyrosine 19 (SIA *et al.* 1998). It is interesting to note that these two suppressors both lead to the dephosphorylation of tyrosine 19 activating Cdc28p. Mpt5p has been shown to interact with Cdc28p (CHEN and KURJAN, 2001), so it is possible that a variation in the activity of Cdc28p can compensate for the absence of Mpt5p. An insert containing *CLB3*, a G2/M-phase specific cyclin that regulates cyclin-dependent protein kinase activity (MENDENHALL and HODGE, 1998), also suppresses Δ*mpt5*, perhaps by regulating Cdc28p. These results may suggest that Mpt5p regulates the cell cycle *via* Cdc28p, or in parallel with Cdc28p. It is significant that all the multicopy suppressors we obtained lead to a loss of the hyper-polarization of the Δ*mpt5* cells (data not shown), indicating that the activity of Mpt5p in cell polarity is at least partially under the control of these suppressors and therefore the CWI pathway.

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