# *Saccharomyces cerevisiae MPT5* and *SSD1* Function in Parallel Pathways to Promote Cell Wall Integrity

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ABSTRACT

Yeast *MPT5* (*UTH4*) is a limiting component for longevity. We show here that *MPT5* also functions to promote cell wall integrity. Loss of Mpt5p results in phenotypes associated with a weakened cell wall, including sorbitol-remedial temperature sensitivity and sensitivities to calcofluor white and sodium dodecyl sulfate. Additionally, we find that mutation of *MPT5*, in the absence of *SSD1-V*, is lethal in combination with loss of either Ccr4p or Swi4p. These synthetic lethal interactions are suppressed by the *SSD1-V* allele. Furthermore, we have provided evidence that the short life span caused by loss of Mpt5p is due to a weakened cell wall. This cell wall defect may be the result of abnormal chitin biosynthesis or accumulation. These analyses have defined three genetic pathways that function in parallel to promote cell integrity: an Mpt5p-containing pathway, an Ssd1p-containing pathway, and a Pkc1p-dependent pathway. This work also provides evidence that post-transcriptional regulation is likely to be important both for maintaining cell integrity and for promoting longevity.

CELL integrity in the budding yeast *Saccharomyces cerevisiae* is governed by the regulated expression of many genes. The cell wall must be remodeled in response to environmental cues, as well as cell-cycle-specific changes such as budding and mating (reviewed in Cid *et al.* 1995). This highly coordinated process requires precise transcriptional regulation, much of which is accomplished via signaling through a mitogen-activated protein kinase (MAPK) cascade regulated by the Pkc1 protein kinase (Heinisch *et al.* 1999).

Pkc1p is a homolog of the mammalian protein kinase C. It is activated by the Rho-like GTPase, Rho1p, which binds to Pkc1p in a GTP-dependent manner (Nonaka *et al.* 1995; Kamada *et al.* 1996). On the basis of genetic and *in vitro* data, activated Pkc1p is thought to phosphorylate the MAPKKK Bck1p (Levin *et al.* 1994), which in turn is thought to phosphorylate the redundant MAPKKs, Mkk1p and Mkk2p (Irie *et al.* 1993). These proteins then activate the MAPK Slt2p, which phosphorylates downstream targets, including the transcription factors Swi4p and Swi6p (Madden *et al.* 1997). Mutations that perturb signaling through this pathway can result in sensitivity to changes in external osmolarity, defective budding, and cell lysis (Levin and Bartlett-Heubusch 1992).

Many of the genes important for promoting proper cell wall structure and cell integrity have been identified by their genetic interactions with components of the *PKC1* signaling pathway. The polymorphic locus *SSD1* is an example of one such gene. Two types of *SSD1* alleles have been described from different laboratory strains: *SSD1-V* and *ssd1-d*. *SSD1-V* alleles are defined by the ability to confer viability on a *sit4* mutant, while *ssd1-d* alleles are synthetically lethal in combination with a deletion of *SIT4* (Sutton *et al.* 1991). *SSD1* has been shown to interact genetically with genes downstream of *PKC1*. A single copy of *SSD1-V* expressed from an ARS-CEN plasmid is sufficient to restore growth at 37° to cells lacking Bck1p (Costigan *et al.* 1992). Mutation of *SSD1* also reduces the permissive temperature of a strain mutant for *SLT2* from 37° to 30° (Lee *et al.* 1993) and increases its sensitivity to caffeine (Martin *et al.* 1996). These observations suggest that *SSD1-V* could act in parallel to *PKC1* to promote cell integrity.

In addition to suppressing mutations in the *PKC1* pathway, *SSD1-V* alleles have been shown to affect several other cellular processes. For example, *SSD1-V* has been isolated as a suppressor of mutations in genes involved in the response to cAMP (Sutton *et al.* 1991; Uesono *et al.* 1994), genes coding for subunits of RNA polymerase III (Stettler *et al.* 1993), and genes coding for splicing factors (Luukkonen and Seraphin 1999). Ssd1p has homology to several ribonucleases and preferentially binds poly(A) mRNA (Uesono *et al.* 1997). It has been speculated that Ssd1p acts as a post-transcriptional regulator.

The *MPT5* (*UTH4*) gene shares some striking similarities to *SSD1*. *MPT5* interacts genetically with *PKC1* (Hata *et al.* 1998), affects diverse cellular processes, and can function as a post-transcriptional regulator (Tadauchi *et al.* 2001). Mpt5p is required for growth

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at high temperature (Kikuchi *et al.* 1994), affects telomeric silencing and Sir2/3/4p localization within the cell (Gotta *et al.* 1997; Cockell *et al.* 1998), and is involved in the haploid response to pheromone (Chen and Kurjan 1997). *MPT5* has also been identified as a gene required for resistance to starvation and for wild-type life span (Kennedy *et al.* 1995). Overexpression of Mpt5p extends life span, demonstrating that Mpt5p is a limiting determinant of longevity in wild-type cells (Kennedy *et al.* 1997).

*MPT5* was independently identified as a multicopy suppressor of *POP2* and *CCR4* (Hata *et al.* 1998). Pop2p is a member of the Ccr4p transcriptional complex (Draper *et al.* 1995; Liu *et al.* 1997). This complex is required for transcription of several genes involved in nonfermentative growth (Denis 1984) and includes Ccr4p, Pop2p (Malvar *et al.* 1992), Dbf2p (Liu *et al.* 1997), and Not1-4p (Liu *et al.* 1998). In addition to its role in the transcriptional response to glucose, Ccr4p also functions as one component of a distinct RNA polymerase II complex along with Paf1p, Cdc73p, and Hpr1p (Chang *et al.* 1999). The primary role of this complex appears to be the *PKC1*-dependent transcriptional regulation of genes required for proper cell wall structure. Loss of Ccr4p causes decreased transcription of several genes involved in cell wall biosynthesis, including *KRE6*, *VAN2*, and *MNN1*.

A second *PKC1*-dependent transcriptional activator is Swi4p. Swi4p interacts with Swi6p to form the heterodimeric transcriptional complex SBF (Andrews and Herskowitz 1989), which is primarily involved in the cell-cycle-regulated transcription of the G1 cyclins *CLN1* and *CLN2* (Nasmyth and Dirick 1991). In addition to its role in cell-cycle progression, *SWI4* is also required for the cell-cycle-regulated transcription of the cell wall biosynthetic genes *FKS1*, *GAS1*, *KRE6*, *MNN1*, *VAN2*, and *CSD2* (Igual *et al.* 1996). Mutation of either *SWI4* or *SWI6* results in sensitivity to both sodium dodecyl sulfate (SDS) and calcofluor white (CFW), indicating a defect in cell wall integrity. Genetic analysis suggests that the role of Swi4p in regulating cell wall biosynthesis consists of both a *PKC1*-dependent and *PKC1*-independent component (Madden *et al.* 1997). Like *MPT5*, the slow growth and temperature sensitivity caused by loss of Swi4p can be suppressed by *SSD1-V* (Cvrckova and Nasmyth 1993; Chen and Rosamond 1998).

On the basis of the genetic relationships described above, we hypothesized that the post-transcriptional regulators *MPT5* and *SSD1* may share overlapping functions with the *CCR4* complex and SBF, both of which are important for transcriptional regulation. We therefore examined the effect of mutations in either *CCR4* or SBF in combination with *MPT5* deletion. We describe here the resulting synthetic lethal interactions. Furthermore, we show that these synthetic effects are dependent on the *SSD1* gene and define at least three genetic pathways regulating cell wall integrity.

## MATERIALS AND METHODS

**Strains and genetic techniques:** The strains used in this study are listed in Table 1. All strains were derived from W303R (described in Mills *et al.* 1999). Genetic crosses, sporulation, and tetrad analysis were carried out as described (Sherman and Hicks 1991). The genotype of inviable spore clones was inferred when possible on the basis of marker segregation in viable spore clones from the same tetrad. Unless otherwise noted, cells were cultured in YPD or synthetic media prepared using conventional methods (Guthrie and Fink 1991). YPDS is YPD supplemented with 1 M sorbitol. YPDCFW is YPD supplemented with 0.05 mg/ml calcofluor white (Fluorescent Brightener 28; Sigma, St. Louis). YPDSDS is YPD supplemented with 0.02% SDS. YPDN is YPD supplemented with 5 mg/ml D-glucosamine.

Yeast transformation was accomplished by the lithium acetate method (Gietz *et al.* 1992). The *ccr4::HIS3, swi6::TRP1*, *mpt5::LEU2*, and *mpt5::HIS3* disruptions were constructed using the described plasmids (Table 2). All other gene deletions were generated by transforming cells with PCR-amplified disruption cassettes as described (Kaeberlein *et al.* 1999). In each case, the entire open reading frame (ORF) was removed except for the *swi4::HIS3* disruption, which replaces the first 2 kb of the *SWI4* ORF with *HIS3*. All disruptions were verified phenotypically or by PCR.

**Serial dilutions:** Serial dilution assays were performed by growing cells overnight in YPD at 30°. Cells were then diluted back 50-fold in fresh YPD and cultured for 3–4 hr. For each strain, a series of 10-fold dilutions was prepared in fresh YEP over a range of concentrations from $10^{-1}$ to $10^{-5}$, relative to the initial culture. A total of 5 μl of the original culture and each dilution was spotted sequentially onto the appropriate media. The cells were then grown at 30° for 2 days prior to visualization.

**Synthetic lethality:** To verify the synthetic lethality between deletions of *CCR4* and *MPT5*, *mpt5 ccr4 ssd1-d2* pYK690 cells were grown overnight in YPD. Fifty-microliter aliquots were then plated onto either SC-URA or synthetic media supplemented with 1.2 mg/ml 5-fluoroorotic acid (5-FOA) and incubated at 30° for 3 days. Only cells that have lost the *URA3* plasmid are able to form colonies in the presence of 5-FOA. To verify the synthetic lethality between deletions of *SWI4* and *MPT5*, the same procedure was used with *mpt5 swi4 ssd1-d2* pSWI4 cells.

**Generation of *SSD1-V* strains:** W303R has been previously shown to carry the *ssd1-d2* allele (Sutton *et al.* 1991). *SSD1-V* integrating plasmids containing either *TRP1* or *URA3* were constructed by PCR amplifying the *SSD1-V* allele from pFK1CU (Kikuchi *et al.* 1994) and cloning into the *Not*I and *Bam*HI sites of pRS404 or pRS406 (Sikorski and Hieter 1989). Unless otherwise indicated, all *SSD1-V* strains contain *SSD1-V* integrated at the marker locus and still carry the *ssd1-d2* allele at the *SSD1* locus. Deletion of *ssd1-d2* does not affect life span, growth, or sensitivity to CFW (not shown), suggesting that *ssd1-d2* is a null allele. Integration of *SSD1-V* at the *SSD1* locus has the same effects as integration at the marker locus for all phenotypes tested.

**Life-span analysis:** Life spans were performed as described (Kaeberlein *et al.* 1999) with the following modifications. Cells were grown overnight at 30° on fresh YPD for two consecutive days. Cells were then patched onto fresh YPD and incubated at 30° for 3 hr. Using a micromanipulator, individual cells were isolated and allowed to undergo at least one cell cycle. Virgin daughter cells were then isolated and life span was measured. Life span is defined as the number of daughter cells produced by an individual cell prior to senescence. Virgin

**TABLE 1**

**Yeast strains used in this study**

| Strain | Relevant genotype |
|---|---|
| W303R | *MAT***a** *ADE2::rDN1 his3 leu2 trp1 ura3 ssd1-d2 RAD5* |
| MKY1324 | W303R *mpt5::LEU2* |
| MKY1320 | W303R *ccr4::HIS3* |
| MKD145 | W303R/W303R *ccr4::HIS3/CCR4 mpt5::LEU2/MPT5* |
| MKD146 | W303R/W303R *ccr4::HIS3/CCR4 mpt5::LEU2/MPT5 SSD1-V/URA3* |
| MKY1510 | W303R *SSD1-V/URA3* |
| MKY1332 | W303R *mpt5::LEU2 SSD1-V/URA3* |
| MKY1331 | W303R *ccr4::HIS3 SSD1-V/URA3* |
| MKY1345 | W303R *ccr4::HIS3 mpt5::LEU2 SSD1-V/URA3* |
| MKY1535 | W303R *ccr4::HIS3 mpt5::LEU2 pYK690* |
| MKY673 | W303R *mpt5::TRP1* |
| MKY645 | W303R *mpt5::HIS3* |
| MKD167 | W303R/W303R *mpt5::TRP1/MPT5 ccr4::HIS3/CCR4* |
| MKD171 | MKd167 YEpPKC1 |
| MKD172 | MKd167 YEpWSC1 |
| MKD168 | MKd167 pRS425 |
| MKY1527 | W303R YEpPKC1 |
| MKY652 | W303R *mpt5::TRP1* YEpPKC1 |
| MKY647 | W303R *mpt5::TRP1* pRS425 |
| MKY1529 | W303R *ccr4::HIS3 mpt5::TRP1* YEpPKC1 |
| MKY1514 | W303R *ccr4::HIS3 mpt5::TRP1* pWSC1 |
| MKD138 | W303R/W303R *swi4::HIS3/SWI4 mpt5::LEU2/MPT5 SSD1-V/URA3* |
| MKY639 | W303R *swi4::HIS3* |
| MKY1385 | W303R *swi4::HIS3 SSD1-V/URA3* |
| MKY1387 | W303R *mpt5::LEU2 swi4::HIS3 SSD1-V/URA3* |
| MKD195 | W303R/W303R *swi4::HIS3/SWI4 mpt5::TRP1/MPT5* pRS425 |
| MKD196 | W303R/W303R *swi4::HIS3/SWI4 mpt5::TRP1/MPT5* YEpPKC1 |
| MKD197 | W303R/W303R *swi4::HIS3/SWI4 mpt5::TRP1/MPT5* YEpWSC1 |
| MKY1551 | W303R *swi4::HIS3 mpt5::TRP1* YEpPKC1 |
| MKY1552 | W303R *swi4::HIS3 mpt5::TRP1* pWSC1 |
| MKY664 | W303R *swi4::HIS3 mpt5::LEU2* pFK1CU |
| MKY1542 | W303R *swi4::HIS3 mpt5::LEU2* pSWI4 |
| MKY1447 | W303R *mbp1::URA3* |
| MKY1452 | W303R *mbp1::URA3 mpt5::LEU2* |
| MKY1495 | W303R *swi6::URA3* |
| MKY1497 | W303R *swi6::URA3 mpt5::LEU2 SSD1-V/TRP1* |
| MKY1496 | W303R *swi6::URA3 SSD1-V/TRP1* |

All strains are isogenic to W303R. MKY strains numbered less than MKY1000 are haploid and derived by direct transformation. MKY strains numbered greater than MKY1000 are haploid and derived by crossing. MKD strains are diploid.

cells that fail to produce at least one daughter were not included in the viability curves. Terminal morphology is defined as the morphology of a cell upon senescence and prior to lysis. Cells were tallied as having a chain-forming morphology if a mother cell could not be detached from a daughter cell by micromanipulation and if that daughter cell had undergone at least one cell division. For the purposes of calculating life span, daughters of chain-forming mothers were included only if those cells subsequently produced at least one daughter. During the course of each life-span experiment, cells were incubated at 30° during the day and 10° overnight. Statistical significance was determined by a Wilcoxon rank-sum test. Average life span is stated to be different for $P < 0.05$. Each figure represents data derived from a single experiment.

The described life-span procedure was modified as follows for the experiment shown in Figure 5D. To allow for plasmid selection, cells were grown overnight at 30° in 10 ml of SC-LEU. Ten-microliter aliquots were then spread onto YPD agar and allowed to dry into the plates. After incubation at 30° for 1 hr, individual cells were isolated and life span was measured directly.

**Microscopy:** Differential interference contrast (DIC) microscopy was performed on a Nikon E600 microscope with a Plan Apo ×100 objective lens. Logarithmically growing cells in YPD or YPDN were placed on a slide with a coverslip and visualized directly. For CFW staining, cells were grown overnight in YPD. Overnight cultures were diluted 1:50 into fresh YPD and incubated at 30° for 3–4 hr. Cells were then resuspended in fresh YPD supplemented with 0.01% CFW at a density of $10^7$ cells/ml. Cells were incubated at 30° for 5 min and then washed with fresh YPD prior to visualization. Digital images were obtained using a CCD camera controlled by OpenLab image acquisition software.

**TABLE 2**

**Plasmids used in this study**

| Plasmid | Description | Source |
|---|---|---|
| YEpMPT5 | 2μ *LEU2 MPT5* | Hata *et al.* (1998) |
| YEpPKC1 | 2μ *LEU2 PKC1* | Hata *et al.* (1998) |
| pWSC1 | 2μ *LEU2 WSC1* | Verna and Ballester (1999) |
| pΔUTH4 | *mpt5::LEU2* deletion plasmid | Kennedy *et al.* (1997) |
| pΔCCR4 | *ccr4::HIS3* deletion plasmid | Hata *et al.* (1998) |
| pΔMPT5 | *mpt5::HIS3* deletion plasmid | Hata *et al.* (1998) |
| pΔSWI6 | *swi6::TRP1* deletion plasmid | Nasmyth and Dirick (1991) |
| pYK690 | ARS-CEN *URA3 MPT5* | Kikuchi *et al.* (1994) |
| pFK1CU | ARS-CEN *URA3 SSD1-V* | Kikuchi *et al.* (1994) |
| pSWI4 | ARS-CEN *URA3 SWI4* | Nasmyth and Dirick (1991) |
| p406SSD1 | Integrating *URA3 SSD1-V* | This study |
| p404SSD1 | Integrating *TRP1 SSD1-V* | This study |

## RESULTS

**Mutation of both *CCR4* and *MPT5* results in *SSD1*-dependent spore inviability:** Mpt5p and Ccr4p both act as multicopy suppressors of *pop2* (Hata *et al.* 1998). Therefore, we began our analysis of Mpt5p function by examining the possibility that a *ccr4 mpt5* double mutant might be inviable. Our wild-type strain, W303R, carries the *ssd1-d2* allele (Sutton *et al.* 1991). It is worth noting that this allele behaves as a null allele for all phenotypes we have tested (see materials and methods). A *MAT***a** *ccr4::HIS3 ssd1-d2* haploid was constructed and crossed with a *MAT*α *mpt5::LEU2 ssd1-d2* haploid. The resulting diploid was sporulated and tetrads were dissected. Out of 23 expected *ccr4 mpt5 ssd1-d2* double-mutant spores, none were able to form colonies (Figure 1A and Table 3). Microscopic examination of these spores revealed that most formed microcolonies with 1–10 enlarged cells prior to cell lysis. A few *ccr4 mpt5 ssd1-d2* spore clones formed mini-colonies with >50 cells, but when streaked to fresh YPD no viable colonies were recovered.

*SSD1-V* suppresses the temperature-sensitive cell lysis phenotype of the *mpt5 ssd1-d2* haploid (Figure 1B; Kikuchi *et al.* 1994). We were, therefore, interested in determining whether *SSD1-V* is able to suppress the inviability of the *ccr4 mpt5 ssd1-d2* spore clones. A single copy of the *SSD1-V* allele was integrated at the *URA3* locus in a *ccr4::HIS3/CCR4 mpt5::LEU2/MPT5 ssd1-d2/ssd1-d2* diploid. The resulting strain was sporulated and tetrads were dissected. *SSD1-V* was able to fully suppress the inability of the *ccr4 mpt5 ssd1-d2* spores to form

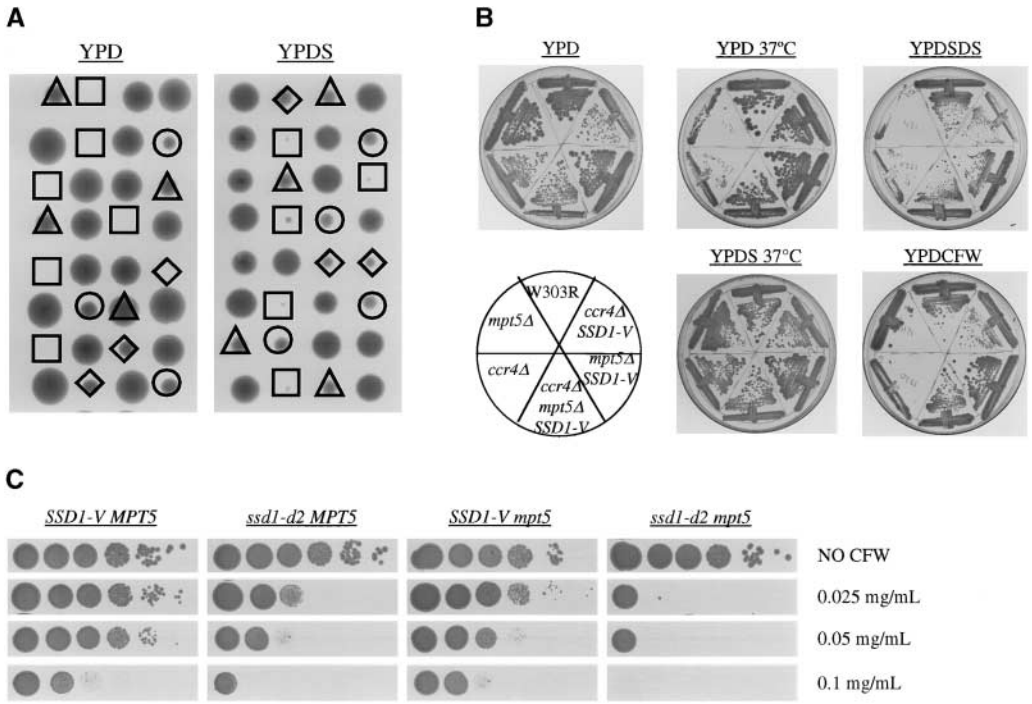


Figure 1.—Growth of *mpt5*, *ccr4*, and *SSD1-V* strains. (A) Strain MKd145 was sporulated and tetrads were dissected onto either YPD or YPDS. Growth after 4 days at 30° is shown. Genotypes are shown for *ccr4 SSD1-V* (△), *ccr4 ssd1-d2* (○), *ccr4 mpt5 SSD1-V* (◇), and *ccr4 mpt5 ssd1-d2* (□) spore clones. (B) Strains were incubated overnight on YPD at 30° and then streaked onto the designated media. The pictures show cells after growth for 3 days. (C) Five-microliter aliquots of 10-fold serial dilutions were plated onto the designated media. Cells were incubated at 30° for 2 days.

**TABLE 3**

**Spore clone viability**

| Spore clone genotype | Spore clone % viability |
|---|---|
| *ssd1-d2* | 98 (172) |
| *SSD1-V* | 100 (65) |
| *mpt5 ssd1-d2* | 94 (162) |
| *mpt5 SSD1-V* | 100 (81) |
| *ccr4 ssd1-d2* | 100 (23) |
| *ccr4 SSD1-V* | 100 (8) |
| *swi4 ssd1-d2* | 16 (97) |
| *swi4 SSD1-V* | 88 (65) |
| *swi6 ssd1-d2* | 65 (91) |
| *swi6 SSD1-V* | 100 (23) |
| *mbp1 ssd1-d2* | 100 (11) |
| *mbp1 SSD1-V* | 100 (8) |
| *mpt5 ccr4 ssd1-d2* | 0 (23) |
| *mpt5 ccr4 SSD1-V* | 83 (6) |
| *mpt5 swi4 ssd1-d2* | 0 (74) |
| *mpt5 swi4 SSD1-V* | 84 (79) |
| *mpt5 swi6 ssd1-d2* | 0 (65) |
| *mpt5 swi6 SSD1-V* | 100 (10) |
| *mpt5 mbp1 ssd1-d2* | 100 (13) |
| *mpt5 mbp1 SSD1-V* | 100 (9) |

Tetrads were dissected onto YPD and incubated at 30°. Spore clones that failed to form visible colonies after 4 days were examined microscopically to verify inviability. Numbers in parentheses give the total number of representative spores analyzed.

colonies (Table 3). Furthermore, *SSD1-V* was also able to suppress the growth defect of the *ccr4 ssd1-d2* haploid at 37° (Figure 1B). This is the first reported genetic interaction between *SSD1* and *CCR4*.

We verified that the inviability of *mpt5 ccr4 ssd1-d2* spores represented a true synthetic lethality by generating *ccr4 mpt5 ssd1-d2* cells carrying the *MPT5* gene on an *URA3* ARS-CEN plasmid. These cells were unable to grow in the presence of 5-FOA, indicating that the plasmid is required for viability (see MATERIALS AND METHODS). The same effect was observed when a *URA3* plasmid carrying *SSD1-V* was used instead. Therefore, this can be considered a case of three-gene synthetic lethality. Simultaneous mutation of *MPT5*, *CCR4*, and *SSD1* results in inviability, but the presence of a functional copy of any one of these genes allows growth.

**Mutation of *MPT5* results in a loss of cell integrity during mitotic growth:** The ability of *SSD1-V* to suppress the cell lysis phenotype of the *ccr4 mpt5* spores suggests that the double mutant suffers from a defect in the cell wall. To test this hypothesis, tetrads were dissected onto YPD supplemented with 1 M sorbitol (YPDS), which provides osmotic stabilization and prevents lysis caused by a weakened cell wall. Under these conditions it was possible to obtain very-slow-growing *ccr4 mpt5 ssd1-d2* spore clones (Figure 1A). When maintained on YPDS, the *ccr4 mpt5 ssd1-d2* haploids grew mitotically; however, when restreaked onto YPD the cells rapidly lysed and were unable to form colonies (not shown). The *ccr4 mpt5 ssd1-d2* cells grown on YPD underwent lysis as primarily (>80%) large-budded cells (not shown). This phenotype is indistinguishable from *mpt5 ssd1-d2* cells grown on YPD at 37° (KIKUCHI *et al.* 1994; M. KAEBERLEIN and L. GUARENTE, unpublished results) and suggests that the inviability of *mpt5 ccr4 ssd1-d2* cells is due to the same defect that causes temperature sensitivity in *mpt5 ssd1-d2* cells.

In addition to osmotic stabilization, we observed that overexpression of either *PKC1* or *WSC1* is sufficient to suppress the inviability of the *ccr4 mpt5 ssd1-d2* spores (Table 5). Pkc1p is a central regulator of cell integrity that acts to promote transcription of cell wall biosynthetic genes, and Wsc1p is a transmembrane sensor that functions as an upstream activator of *PKC1*. Doubly heterozygous diploids with plasmids containing either *PKC1* or *WSC1* were able to form viable *ccr4 mpt5 ssd1-d2* spore clones only when the spores received the plasmid. Diploid cells transformed with a vector control were unable to produce viable double-mutant spores (not shown).

It has been demonstrated that mutation of *CCR4* results in altered transcription of some cell wall biosynthetic genes and in sensitivity to the cell wall perturbing agents SDS and CFW (IGUAL *et al.* 1996; CHANG *et al.* 1999). We therefore tested the *mpt5 ssd1-d2* mutant for similar phenotypes. Relative to wild-type cells, *mpt5 ssd1-d2* and *ccr4 ssd1-d2* haploid cells are sensitive to both SDS and CFW (Figure 1B and Table 4). These defects are completely suppressed by the presence of *SSD1-V*.

To further characterize this phenotype, we quantitatively examined the sensitivity to CFW caused by loss of Mpt5p. Mutation of both *MPT5* and *SSD1* results in increased sensitivity at 0.025 mg/ml, 0.05 mg/ml, and 0.1 mg/ml CFW, relative to cells that carry mutations in either one of these genes (Figure 1C). Sensitivity appears to be increased between 10- and 100-fold. Interestingly, *MPT5 SSD1-V* cells are more resistant to CFW than either *mpt5 SSD1-V* or *MPT5 ssd1-d2* cells. Thus, *MPT5* and *SSD1* act synergistically with respect to CFW sensitivity, as predicted for genes in parallel pathways.

Taken together, the synthetic lethality, suppression by osmotic stabilization and increased Pkc1p activity, and sensitivities to SDS and CFW all suggest that *MPT5* and *CCR4* function in parallel pathways to ensure cell integrity by promoting stabilization of the cell wall. *SSD1* defines a third pathway in this process.

***MPT5* is required for viability in the absence of SBF and *SSD1-V*:** In addition to the Ccr4p complex, the transcriptional activator SBF has also been shown to function downstream of Pkc1p to regulate cell wall biosynthesis (MADDEN *et al.* 1997). SBF is a heterodimeric complex of Swi4p and Swi6p. *SWI4* is required for the cell-cycle-regulated transcription of several cell wall biosynthetic genes and mutation of *SWI4* results in slow

**TABLE 4**

**Summary of cell integrity phenotypes tested in this study**

| Genotype | YPD 30° | YPD 37° | YPDS 30° | YPDS 37° | YPDN 37° | SDS | CFW |
|---|---|---|---|---|---|---|---|
| *ssd1-d2* | ++ | ++ | ++ | ++ | ++ | R | R |
| *SSD1-V* | ++ | ++ | ++ | ++ | ++ | R | R |
| *mpt5 ssd1-d2* | ++ | − | ++ | ++ | + | S | S |
| *ccr4 ssd1-d2* | + | +/− | ++ | + | − | S | S |
| *swi4 ssd1-d2* | + | +/− | ++ | + | + | S | S |
| *swi6 ssd1-d2* | + | +/− | ++ | + | ++ | S | S |
| *mbp1 ssd1-d2* | ++ | ++ | ++ | ++ | ND | R | R |
| *mpt5 SSD1-V* | ++ | ++ | ++ | ++ | ND | R | R |
| *ccr4 SSD1-V* | ++ | ++ | ++ | ++ | ND | R | R |
| *swi4 SSD1-V* | ++ | ++ | ++ | ++ | ND | I | R |
| *swi6 SSD1-V* | ++ | ++ | ++ | ++ | ND | I | R |
| *mbp1 SSD1-V* | ++ | ++ | ++ | ++ | ND | R | R |
| *mpt5 ccr4 ssd1-d2* | − | ND | +/− | − | ND | ND | ND |
| *mpt5 swi4 ssd1-d2* | − | ND | − | ND | ND | ND | ND |
| *mpt5 swi6 ssd1-d2* | − | ND | − | ND | ND | ND | ND |
| *mpt5 mbp1 ssd1-d2* | ++ | − | ++ | ++ | ND | S | S |
| *mpt5 ccr4 SSD1-V* | ++ | ++ | ++ | ++ | ND | R | R |
| *mpt5 swi4 SSD1-V* | ++ | ++ | ++ | ++ | ND | I | R |
| *mpt5 swi6 SSD1-V* | ++ | ++ | ++ | ++ | ND | I | R |
| *mpt5 mbp1 SSD1-V* | ++ | ++ | ++ | ++ | ND | R | R |

Growth was measured by visual observation of cells incubated under the conditions indicated for 3 or 4 days. ++, wild-type levels of growth; +, slow growth; +/−, very slow growth; −, no observable growth; ND, the experiment was not performed. For SDS and CFW sensitivities R indicates the given strain is resistant and grew well and S indicates the cells are sensitive and grew poorly or not at all in the presence of 0.05 mg/ml CFW or 0.02% SDS. I indicates that cell growth was intermediate between wild-type and sensitive strains.

growth and sensitivity to SDS and CFW (Igual *et al.* 1996). We therefore wished to place the *SWI4* gene relative to the genetic pathways described above.

Nasmyth and Dirick (1991) reported that *swi4* mutants are inviable in the W3031-A background; however, we were able to obtain *swi4* haploids in our W303R strain both by tetrad dissection and by direct transformation of haploid cells (see materials and methods). The *swi4 ssd1-d2* mutant is slow growing, exhibits lowered spore viability (Table 3), and has an altered cell morphology. To determine whether any synthetic effects are between *SWI4* and *MPT5*, a *mpt5/MPT5 swi4/SWI4 ssd1-d2/ssd1-d2* diploid was constructed and a single copy of *SSD1-V* was integrated at the *URA3* locus. This diploid was sporulated and tetrads were dissected. As was the case for *mpt5 ccr4 ssd1-d2* spores, *mpt5 swi4 ssd1-d2* spore clones were inviable. None of the 74 spores with this inferred genotype were able to form colonies (Table 3 and Figure 2A). When examined microscopically, these spores invariably formed mini-colonies containing one to six lysed cells. Haploid *mpt5 swi4 ssd1-d2* cells were able to grow in the presence an ARS-CEN plasmid carrying *URA3* and *SWI4*, but were unable to lose this plasmid as evidenced by their inability to grow on 5-FOA (not shown; see materials and methods).

Similar to the *ccr4 mpt5 ssd1-d2* strain, the inviability of the *mpt5 swi4 ssd1-d2* triple mutant is suppressed by the presence of *SSD1-V* (Tables 3 and 5). *mpt5 swi4 SSD1-V* haploid cells showed mitotic growth comparable to wild type, and *SSD1-V* was able to suppress the sensitivity of a *swi4 ssd1-d2* strain to CFW (Figure 2B). In contrast to the *ccr4 mpt5 ssd1-d2* strain, we were unable to obtain viable *swi4 mpt5 ssd1-d2* spore clones by dissecting tetrads on YPD supplemented with 1 m sorbitol (Figure 2A and Table 4). This may indicate that the defect in the *swi4 mpt5 ssd1-d2* strain is more severe than in the *ccr4 mpt5 ssd1-d2* strain. Overexpression of either *PKC1* or *WSC1*, however, was sufficient to confer viability on the *swi4 mpt5 ssd1-d2* double-mutant cells (Table 5).

The synthetic lethality between *MPT5* and *SWI4* suggests that SBF and *MPT5* operate in parallel pathways to promote cell integrity. Consistent with this hypothesis, we observed that *mpt5 swi6 ssd1-d2* spore clones are also unable to form colonies (Table 3). As was the case with *SWI4*, a single copy of *SSD1-V* suppresses the synthetic lethality between deletions of *MPT5* and *SWI6*. Likewise, the *swi6 ssd1-d2* mutant demonstrates sensitivities to CFW and SDS that are suppressed by *SSD1-V* (Table 4).

In addition to SBF, Swi6p also forms a second heterodimeric complex with Mbp1p, known as MBF (Koch *et al.* 1993). To determine whether loss of MBF activity is

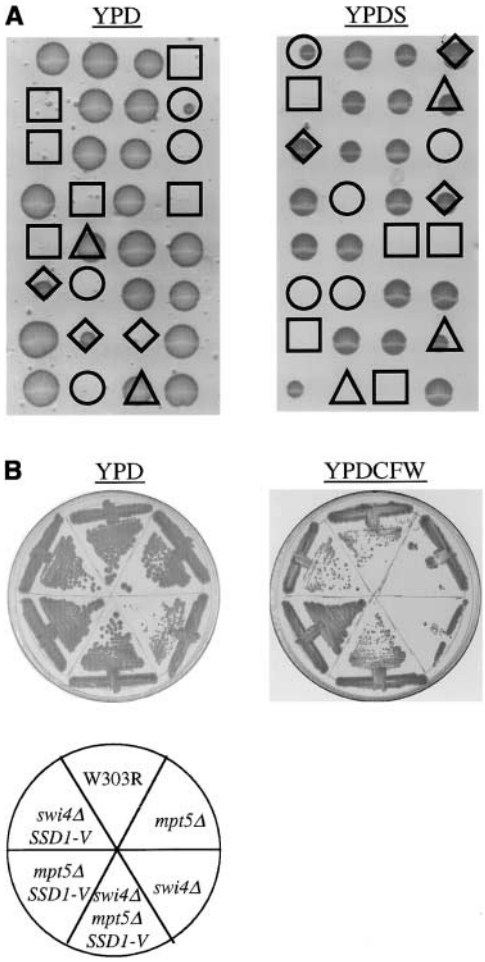


FIGURE 2.—Growth of *mpt5*, *swi4*, and *SSD1-V* strains. (A) Strain MKd138 was sporulated and tetrads were dissected onto either YPD or YPDS. Growth after 4 days at 30° is shown. Genotypes are shown for *swi4 SSD1-V* (△), *swi4 ssd1-d2* (○), *swi4 mpt5 SSD1-V* (◇), and *swi4 mpt5 ssd1-d2* (□) spore clones. (B) Strains were incubated overnight on YPD at 30° and then streaked onto the designated media. The pictures show cells after growth for 3 days.

also lethal in combination with mutation of *MPT5*, we generated an *mpt5/MPT5 mbp1/MBP1 ssd1-d2/ssd1-d2* diploid and examined spore viability. In contrast to *SWI4* and *SWI6*, we observed no synthetic interactions between *MPT5* and *MBP1* (Table 3). As previously reported (IGUAL *et al.* 1996), mutation of *MBP1* did not result in sensitivity to CFW or SDS (Table 4). Thus, SBF, but not MBF, is required for viability in the absence of functional Mpt5p and Ssd1p.

***SSD1-V* restores wild-type life span to the *mpt5* mutant but not the *ccr4* or *swi4* mutants:** *MPT5* is a regulator of yeast life span. We therefore wished to determine whether the role of Mpt5p in cell wall stability is related to its role in promoting longevity. Life spans were measured for *mpt5 ssd1-d2*, *mpt5 SSD1-V*, *ccr4 ssd1-d2*, *ccr4 SSD1-V*, and *mpt5 ccr4 SSD1-V* haploid strains. As expected, mutation of *MPT5* in an *ssd1-d2* background results in a life span that is ~50% shorter than wild type (Figure 3A). Mutation of *CCR4* leads to an even more dramatic 80% reduction in life span (Figure 3B). A single copy of the *SSD1-V* allele is able to suppress the short life span caused by loss of Mpt5p (Figure 3A). In contrast, *SSD1-V* is unable to suppress the extremely short life span of the *ccr4 ssd1-d2* or *mpt5 ccr4 ssd1-d2* strains (Figure 3B), even though it does suppress the cell wall defects associated with loss of Ccr4p.

Mutation of *SWI4* in an *ssd1-d2* background results in a more severe growth defect at 30° than mutation of either *MPT5* or *CCR4*. Not surprisingly, the *swi4 ssd1-d2* strain also has an extremely short life span (Figure 3C). This life-span defect is partially suppressed by the presence of the *SSD1-V* allele, but the mean life span of the *swi4 SSD1-V* strain is still ~80% shorter than the wild-type life span. The life span of the *mpt5 swi4 SSD1-V* strain is comparable to the *swi4 SSD1-V* strain. Thus, the short life spans caused by mutation of *CCR4* or *SWI4* are apparently due to something other than the cell integrity defect.

***SSD1-V* alters the morphology of a *swi4* mutant:** In addition to a shortened life span, the *swi4 ssd1-d2* strain also displayed abnormal cellular senescence. Nearly all wild-type virgin cells produced one or more daughters. In contrast, ~35% of *swi4 ssd1-d2* virgin cells failed to produce even a single daughter (not shown). Instead, these cells became grossly enlarged and arrested in the unbudded state. This likely reflects an inability of these cells to accumulate G1 cyclins and proceed into S phase (NASMYTH and DIRICK 1991). After a few hours, the enlarged unbudded cells lysed, perhaps due to the cell

**TABLE 5**

**Suppressors of the synthetic lethal interactions described in this study**

| Synthetic lethal interaction | YEpPKC1 | 2μ WSC1 | SSD1-V | 1 M sorbitol |
|---|---|---|---|---|
| *mpt5 ccr4* | YES | YES | YES | YES |
| *mpt5 swi4* | YES | YES | YES | NO |
| *mpt5 swi4* | YES | YES | YES | NO |

Viability of the indicated strains was determined by the growth of spore clones following tetrad dissection. YES indicates that we were able to obtain viable double-mutant spores in the presence of the indicated plasmid or intervention. NO indicates that we were unable to obtain double-mutant spores.

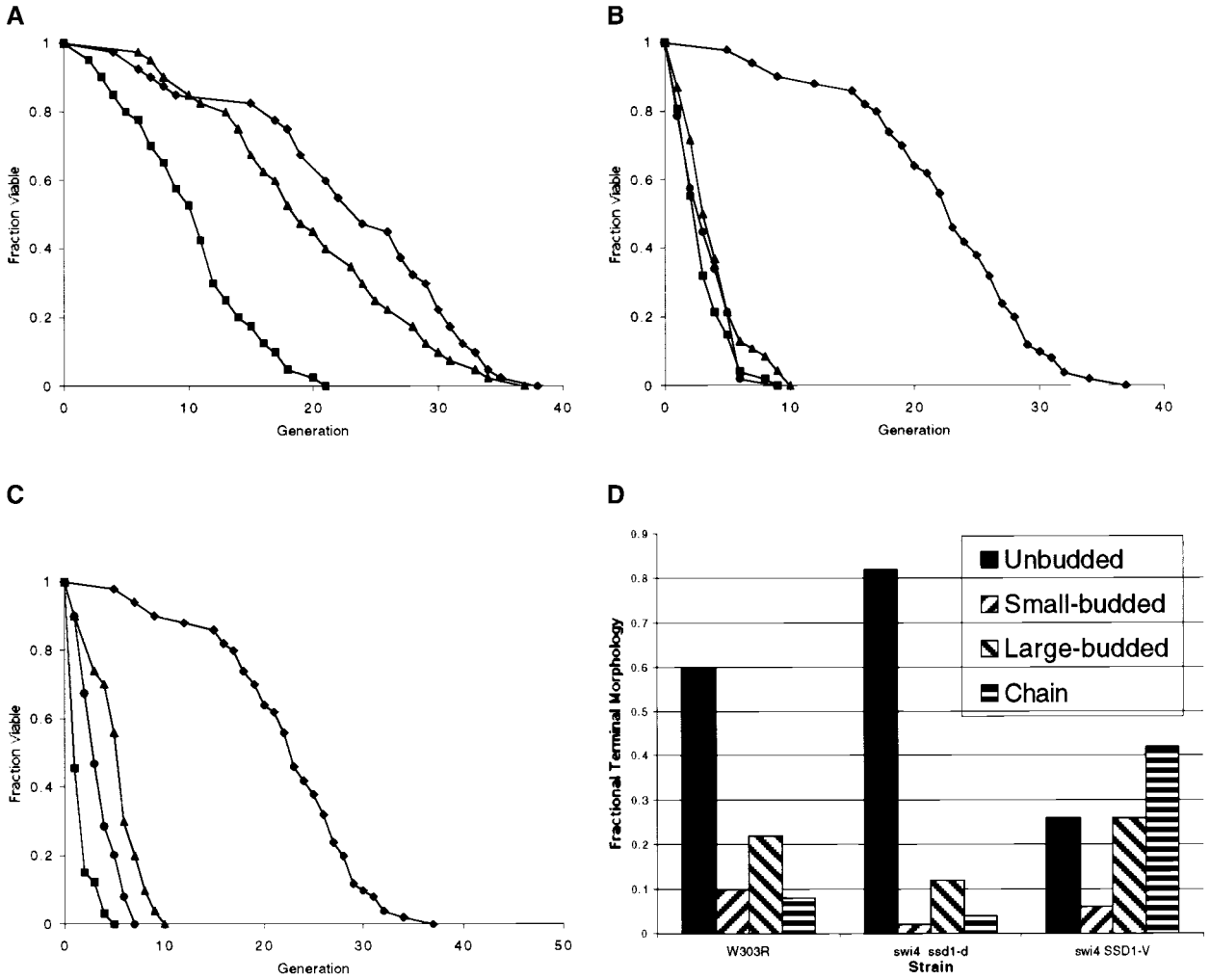


FIGURE 3.—Life-span analysis of *mpt5*, *ccr4*, *swi4*, and *SSD1-V* strains. (A) Life spans were determined for W303R (◆), *mpt5 ssd1-d2* (■), and *mpt5 SSD1-V* (▲) strains. Mean life spans and number of cells analyzed were W303R 22.7 ($n$ = 40), *mpt5 ssd1-d2* 10.2 ($n$ = 40), and *mpt5 SSD1-V* 19.5 ($n$ = 40). (B) Life spans were determined for W303R (◆), *ccr4 ssd1-d2* (■), *ccr4 SSD1-V* (▲), and *mpt5 ccr4 SSD1-V* (●) strains. Mean life spans and number of cells analyzed were W303R 22.4 ($n$ = 50), *ccr4 ssd1-d2* 3.2 ($n$ = 47), *ccr4 SSD1-V* 4.0 ($n$ = 46), and *ccr4 mpt5 SSD1-V* 3.5 ($n$ = 46). (C) Life spans were determined for W303R (◆), *swi4 ssd1-d2* (■), *swi4 SSD1-V* (▲), and *mpt5 swi4 SSD1-V* (●) strains. Mean life spans and number of cells analyzed were W303R 22.4 ($n$ = 50), *swi4 ssd1-d2* 1.8 ($n$ = 33), *swi4 SSD1-V* 5.3 ($n$ = 50), and *swi4 mpt5 SSD1-V* 3.6 ($n$ = 49). (D) Terminal morphology of W303R, *swi4 ssd1-d2*, and *swi4 SSD1-V* mother cells. A cell was defined as senescent and terminal morphology was recorded if it failed to divide after 12 hr incubation at 30° or upon lysis.

integrity defect. The presence of the *SSD1-V* allele fully suppresses this phenotype. While *swi4 SSD1-V* virgin cells still showed delayed exit from G1 and became enlarged, the cells failed to lyse and usually completed at least one cell cycle prior to senescence. Virgin cells that failed to produce at least one daughter are excluded from the data used to generate the life-span curves (see MATERIALS AND METHODS).

Terminal morphology of mother cells has recently been shown to provide information regarding the mechanisms of senescence in different mutant backgrounds (MCVEY *et al.* 2001). We therefore examined the terminal morphology of *swi4 ssd1-d2* and *swi4 SSD1-V* cells that produced at least one daughter (Figure 3D). Approximately two-thirds of wild-type cells arrest in the unbudded state. An even greater fraction of *swi4 ssd1-d2* cells senesce as unbudded cells prior to lysis, again likely reflecting a defect in G1 cyclin accumulation. (Note that these data do not include those virgin cells that fail to produce at least one daughter. If these cells were included, the fraction of unbudded *swi4 ssd1-d2* cells would be even greater.) In contrast, *swi4 SSD1-V* cells are likely to senesce with a large-budded or chain-forming morphology. Cells were defined as having a chain-forming morphology if mother cells senesced while attached to at least one daughter cell that divided at least one time. No difference in terminal morphology was observed between *SWI4 ssd1-d2* and *SWI4 SSD1-V* cells (not shown).

***swi4 SSD1-V* mutants appear to have a defect in cytokinesis:** Due to the extremely short life span of the *swi4 SSD1-V* strain, we hypothesized that the abnormal chain-

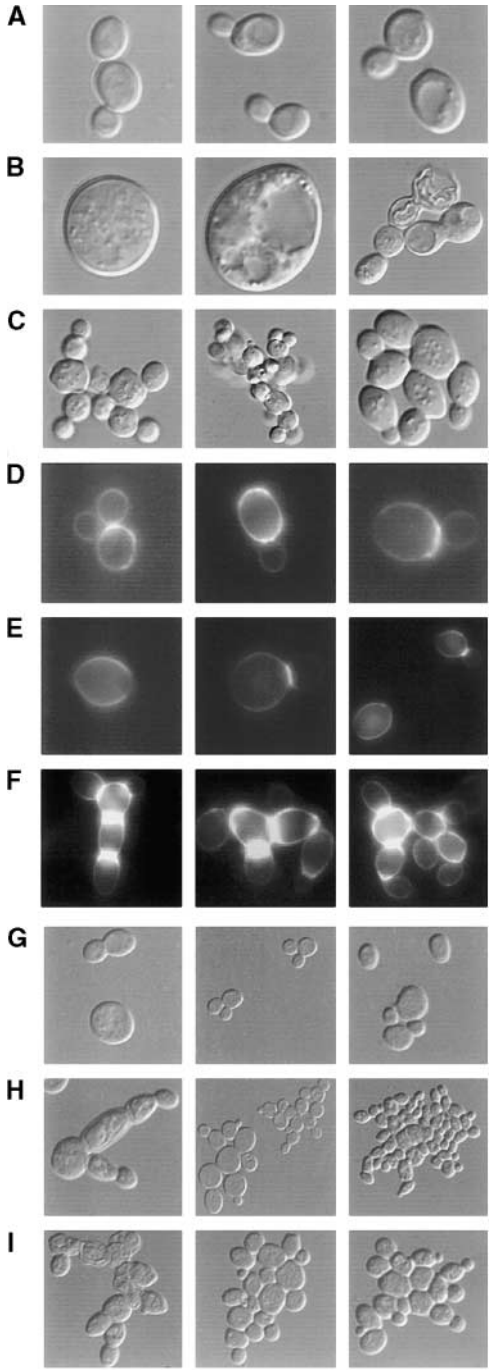


FIGURE 4.—Microscopic analysis of W303R (A, D, G), *swi4 ssd1-d2* (B, E, H), and *swi4 SSD1-V* (C, F, I) cells. For A–C, cells were grown in YPD and visualized using DIC microscopy. For D–F, cells were grown in YPD, stained with CFW, and visualized with the 4′6-diamidino-2-phenylindole channel. For G–I, cells were grown in YPDN and visualized using DIC microscopy.

forming morphology would be apparent in a logarithmically growing culture. Indeed, we observed that a large proportion of *swi4 SSD1-V* cells were found in clumps or chains (Figure 4C). These chains were observed only infrequently in *swi4 ssd1-d2* cultures (Figure 4B) and almost never in wild-type cultures (Figure 4A). In contrast to wild type, *swi4 ssd1-d2* cells often showed enlarged vacuoles or a clearly altered cellular morphology, perhaps just preceding lysis. These phenotypes were largely suppressed by the presence of *SSD1-V*.

The chain-forming morphology of *swi4 SSD1-V* cells suggested a defect in cytokinesis. To further examine this possibility, we microscopically analyzed cells stained with CFW, a fluorescent molecule that preferentially binds chitin in the cell wall. Wild-type and *swi4 ssd1-d2* cells showed relatively normal CFW staining (Figure 4, D and E). In contrast, *swi4 SSD1-V* cells often had brightly staining chitin rings between mother and daughter cells present in the chains (Figure 4F). Often a single central mother was present with three or more daughters still attached. We propose that mutation of *swi4* results in several different defects: delayed accumulation of G1 cyclins, a defect in cell wall stability during G1, and incomplete cytokinesis. *SSD1-V* is apparently able to suppress the cell wall defect and prevent lysis, but is unable to suppress the other phenotypes.

**Glucosamine alters phenotypes caused by mutation of *SWI4* and *MPT5*:** The *swi4 SSD1-V* strain displays a defect in cytokinesis and chitin distribution during logarithmic growth in YPD. A similar defect in cytokinesis was observed in a *swi4 agm1* double mutant (IGUAL *et al.* 1997). This defect was suppressed by addition of glucosamine to the media. *AGM1* codes for an enzyme involved in chitin biosynthesis, and glucosamine is a precursor of chitin. We therefore wished to determine whether glucosamine could also suppress the cytokinesis defect of the *swi4 SSD1-V* strain.

Wild-type cells grown in YPDN (YPD supplemented with 5 mg/ml glucosamine) grew normally and showed no obvious changes in morphology relative to untreated cells (Figure 4G). Surprisingly, growth in YPDN caused *swi4 ssd1-d2* cells to adopt a chain-forming morphology similar to that of the *swi4 SSD1-V* cells in YPD (Figure 4H), suggesting that glucosamine may act like *SSD1-V* to prevent lysis prior to budding. Addition of glucosamine failed to suppress the cytokinesis defect of the *swi4 SSD1-V* cells (Figure 4I).

We also examined whether glucosamine could suppress any of the phenotypes associated with mutation of *MPT5*. Glucosamine had no effect on the growth of *mpt5 ssd1-d2* or *mpt5 SSD1-V* cells at 30°; however, *mpt5 ssd1-d2* cells were able to grow on YPDN at 37° (Table 4). *mpt5 ssd1-d2* cells were unable to grow on YPD supplemented with either 5 mg/ml glucose or 5 mg/ml sorbitol at 37°, demonstrating that this effect is specific to glucosamine. The ability of glucosamine to suppress the temperature sensitivity caused by loss of Mpt5p may suggest that *mpt5 ssd1-d2* cells suffer from a defect in chitin biosynthesis or chitin distribution.

**The short life span caused by loss of Mpt5p is likely to be caused by a defective cell wall:** Is the role of *MPT5* in life-span regulation related to its role in cell integrity? Overexpression of Mpt5p extends life span in W303R by ~20% (Figure 5A) and deletion shortens life span by 50% (Figure 1, A, C, and D). Having identified several

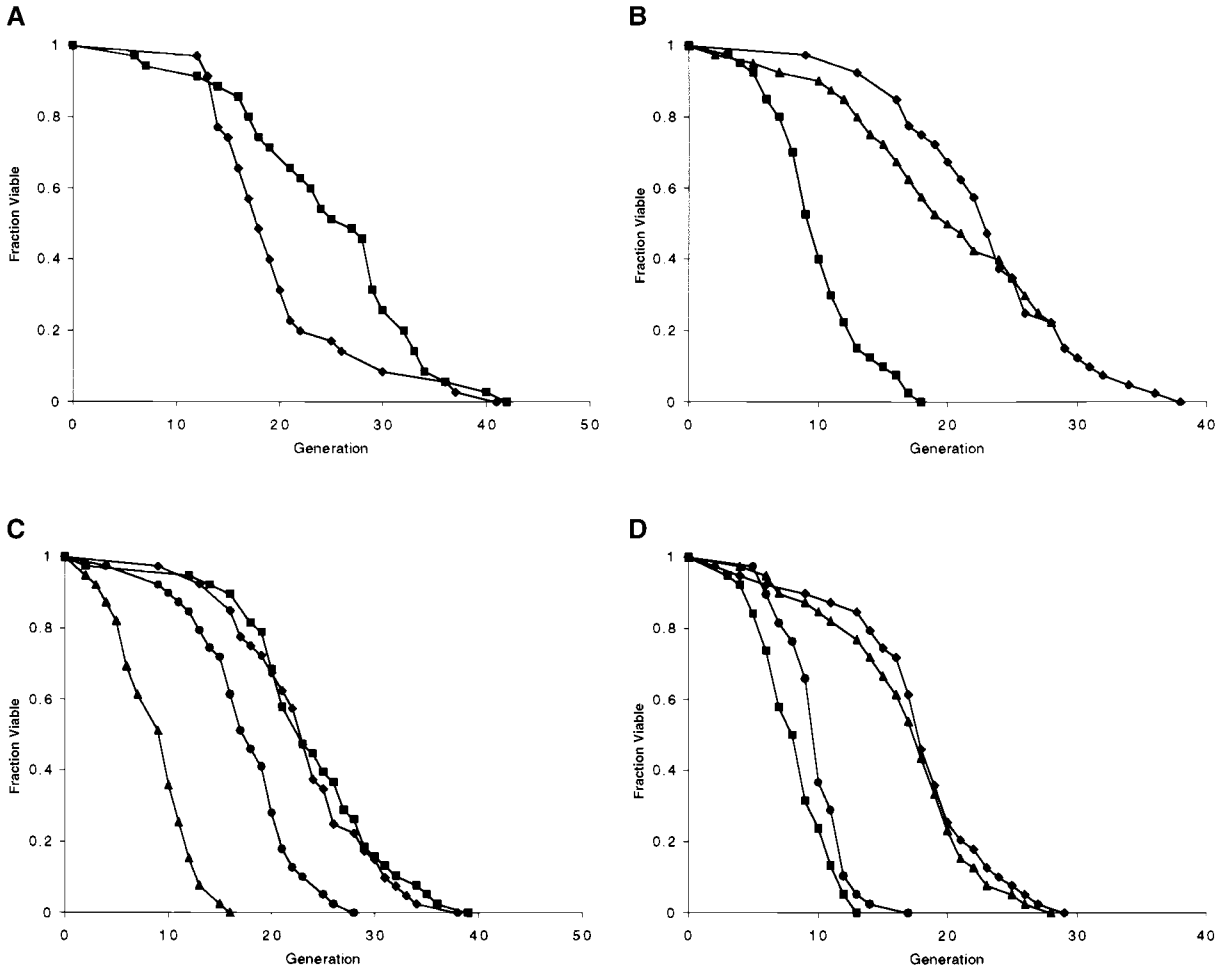


FIGURE 5.—Cell wall integrity and life span. (A) Life spans were determined for (◆) W303R and (■) W303R overexpressing Mpt5p (W303R *ADH_MPT5*). Mean life spans and number of cells analyzed were W303R 20.1 ($n = 40$) and W303R *ADH_MPT5* 25.1 ($n = 40$). (B) Life spans were determined for W303R grown on YPD (◆), *mpt5 ssd1-d2* grown on YPD (■), and *mpt5 ssd1-d2* grown on YPD supplemented with 1 M sorbitol (YPDS) (▲). Mean life spans and number of cells analyzed were W303R YPD 23.0 ($n = 40$), *mpt5 ssd1-d2* YPD 10.7 ($n = 40$), and *mpt5 ssd1-d2* YPDS 20.6 ($n = 40$). (C) Life spans were determined for W303R grown on YPD (◆), W303R grown on YPD supplemented with 0.5 mg/ml D-glucosamine (YPDN) (■), *mpt5 ssd1-d2* grown on YPD (▲), and *mpt5 ssd1-d2* grown on YPDN (●). Mean life spans and number of cells analyzed were W303R YPD 23.2 ($n = 40$), W303R YPDN 23.8 ($n = 40$), *mpt5 ssd1-d2* YPD 9.0 ($n = 40$), and *mpt5 ssd1-d2* YPDN 17.6 ($n = 40$). (D) Life spans were determined for W303R pRS425 (◆), *mpt5 ssd1-d2* pRS425 (■), W303R YEpPKC1 (▲), and *mpt5 ssd1-d2* YEpPKC1 (●) strains. Mean life spans and number of cells analyzed were W303R pRS425 17.7 ($n = 40$), *mpt5 ssd1-d2* pRS425 8.3 ($n = 40$), W303R YEpPKC1 16.9 ($n = 40$), and *mpt5 ssd1-d2* YEpPKC1 10.0 ($n = 40$).

suppressors of the temperature sensitivity caused by mutation of *MPT5*, we wished to determine whether life span was also affected. *SSD1-V* suppressed the short life caused by loss of Mpt5p to the level of wild type (Figure 3A). Likewise, growth in the presence of 1 M sorbitol (Figure 5B) or 5 mg/ml glucosamine (Figure 5C) largely suppressed the short life span of the *mpt5 ssd1-d2* strain. Overexpression of *PKC1* only slightly extended the life span of the *mpt5* mutant, and neither glucosamine nor *PKC1* overexpression was capable of extending wild-type life span (Figure 5, C and D).

## DISCUSSION

**Three pathways for cell integrity:** *MPT5* regulates cell integrity, response to pheromone, distribution of silencing factors within the nucleus, life span, and resistance to environmental stress. We show here that genetic analysis places *MPT5* parallel to *SSD1* in one pathway promoting cell integrity and also parallel to *PKC1*, *CCR4*, and *SBF* in a second pathway (Figure 6). Strains carrying mutations in all three pathways are inviable, whereas the cell is able to survive loss of function in any two pathways. We also provide evidence that the short life span caused by loss of Mpt5p is due to a defect in cell wall stability, perhaps related to chitin biosynthesis.

Our three-pathway model is based on the discovery of synthetic lethal interactions between *MPT5*, *CCR4*, and *SSD1* and between *MPT5*, SBF, and *SSD1*. One concern with using synthetic lethality to construct parallel genetic pathways is the possibility that the mutations contributing to the lethality affect unrelated processes

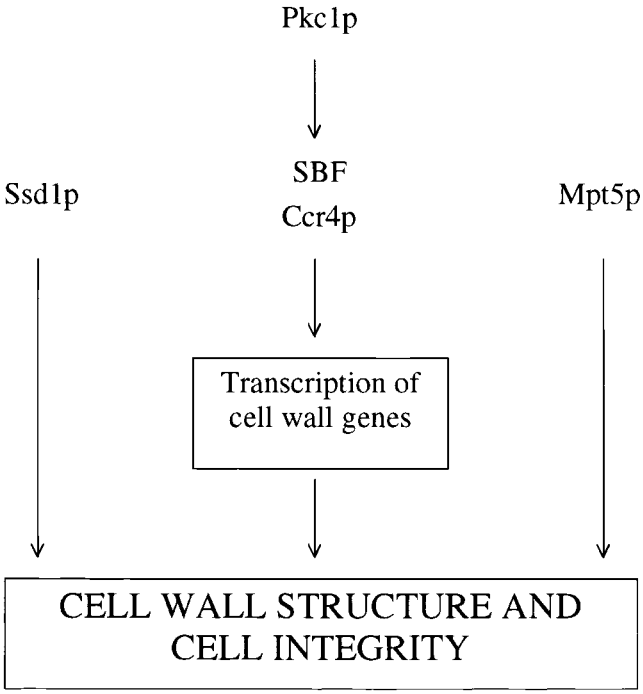


FIGURE 6.—Genetic model describing the function of Mpt5p and Ssd1p in cell wall integrity. Mpt5p, Ssd1p, and Pkc1p define three parallel pathways that regulate cell integrity. SBF and Ccr4p function as transcriptional activators downstream of Pkc1p. Mpt5p and Ssd1p could act either post-transcriptionally to regulate genes involved in cell wall biosynthesis or as upstream regulators that promote cell integrity by an indirect mechanism.

that happen to result in inviability when simultaneously disrupted. We do not believe this is the case here for several reasons. First, mutants for *MPT5*, *CCR4*, or SBF all display phenotypes associated with a weakened cell wall, such as sorbitol-remedial temperature sensitivity and sensitivity to CFW and SDS. Second, these phenotypes are suppressed either by *SSD1-V* or by overexpression of *PKC1*. Third, *SSD1-V* promotes increased resistance to CFW even in the presence of functional Mpt5p, Ccr4p, and SBF. Finally, both *CCR4* and SBF are known to act downstream of *PKC1* to regulate cell wall biosynthesis. Taken together, we conclude that it is likely that all of these proteins have a role in promoting cell wall stability. Thus, we feel that it is appropriate to use synthetic lethality to order these genes into three parallel pathways.

***MPT5*, *CCR4*, and *SWI4* are required for wild-type life span:** The fact that *MPT5* functions to promote both longevity and cell integrity might suggest that the cell wall can become a limiting factor in old cells. Overexpression of Mpt5p could extend life span by increasing cell integrity and preventing lysis. This hypothesis makes two predictions: first, overexpression of Mpt5p should result in increased cell wall stability, and second, treatments that suppress the temperature sensitivity of the *mpt5 ssd1-d2* mutant should also extend life span. We were unable to observe increased resistance to either SDS or CFW in the Mpt5p-overexpressing strain (not shown). It is possible, however, that in Mpt5p-overexpressing cells, the cell wall structure could be stabilized, yet not result in enhanced resistance to these chemicals.

Several treatments that suppress the temperature sensitivity caused by loss of Mpt5p also suppress the life-span defect. *SSD1-V*, osmotic stabilization, and growth in the presence of glucosamine all restored wild-type longevity to the *mpt5 ssd1-d2* mutant (Figure 3A and Figure 5, B and C). Overexpression of *PKC1* failed to fully suppress the life-span defect; however, hyperactivation of the *PKC1* pathway is known to be toxic (WATANABE *et al.* 1995), which could account for the slow growth and short life span of these cells. We, therefore, favor the interpretation that the short life span of the *mpt5 ssd1-d2* strain is likely to be caused by a defect in the cell wall. It remains to be determined whether the life-span extension observed in cells overexpressing *MPT5* is due to increased cell wall stability or to an additional function of Mpt5p, such as the regulation of Sir2p localization.

The short life span caused by loss of either Ccr4p or Swi4p is likely due to a defect other than a weakened cell wall. This is evidenced by the fact that *SSD1-V* is able to suppress the sensitivity of these strains to CFW or SDS, but fails to restore wild-type life span. We do not propose that mutation of either *ccr4* or *swi4* results in premature aging *per se*. Rather, it seems likely that these mutations result in defects that shorten replicative capacity by a mechanism unrelated to the normal aging process.

**Function of *SSD1-V* and *MPT5* in promoting cell integrity:** How do *MPT5* and *SSD1-V* act to promote cell integrity? The ability of glucosamine to suppress the temperature sensitivity and short life span caused by mutation of *MPT5* suggests a role for Mpt5p in chitin biosynthesis or accumulation. Interestingly, mutation of the chitin synthase *CHS3* also results in sorbitol-remedial temperature sensitivity in some strain backgrounds (BULAWA 1992). We speculate that, like the cases for *MPT5*, *CCR4*, and *SWI4*, the strain-specific phenotypes associated with loss of *CHS3* are due to the presence of different *SSD1* alleles.

Mpt5p shares homology with the Drosophila translational repressor pumilio (KENNEDY *et al.* 1997) and is required for post-transcriptional repression of *HO* (TADAUCHI *et al.* 2001). It is interesting to note that *SWI4* and *SWI6* were originally identified as regulators of *HO* expression, as well (BREEDEN and NASMYTH 1987). Like *MPT5*, *SSD1-V* also shares homology to RNA-binding proteins and it has been proposed that *SSD1-V* functions as a post-transcriptional regulator of mRNA stability. UESONO *et al.* (1997) have shown that Ssd1p preferentially binds poly(A) RNA, although no catalytic activity has been detected.

One intriguing model is that *CCR4*, *SWI4*, *MPT5*, and *SSD1* all function to regulate a common subset of genes important for proper cell wall structure. *CCR4* and *SWI4*

would act at the level of mRNA transcription to regulate basal and cell-cycle-specific expression of these genes. The mRNA-binding factors Mpt5p and Ssd1p would act post-transcriptionally to regulate translational efficiency and mRNA stability. Reports that both Mpt5p and Ssd1p are cytoplasmically localized are consistent with such a model (Uesono *et al.* 1997; K. Mills and L. Guarente, unpublished results).

An alternative possibility is that Mpt5p and/or Ssd1p act indirectly by regulating gene expression of upstream factors. These factors would then act to regulate expression of genes important for cell integrity. Consistent with an upstream regulatory role, Mpt5p has been shown to interact physically with the cyclin-dependent kinase Cdc28p (Chen and Kurjan 1997). Cdc28p, in turn, is known to activate both Pkc1p (Marini *et al.* 1996) and Swi4p (Amon *et al.* 1993; Siegmund and Nasmyth 1996). Likewise, there is ample evidence that *SSD1* affects a wide variety of cellular processes, suggesting a more global role for this gene.

Finally, it is also possible that Mpt5p and Ssd1p could act transcriptionally, or post-transcriptionally, to increase expression of Ccr4p, Swi4p, and Swi6p. We have used microarray analysis to examine the transcriptional profile of *SSD1-V* cells relative to *ssd1-d* cells. We find that *SSD1-V* does not significantly alter the abundance of *SWI6*, *SWI4*, or *CCR4* transcripts in logarithmically growing cells (M. Kaeberlein, A. Andalis, G. Fink and L. Guarente, unpublished data). Moreover, this model fails to explain why the presence of functional Mpt5p or Ssd1p is sufficient for viability in the absence of Ccr4p or SBF. If Mpt5p and Ssd1p promote cell integrity by acting as activators of *CCR4*, *SWI4*, and *SWI6*, then Mpt5p and Ssd1p should have no effect in strains mutant for these genes. In contrast, we find that either Ssd1p or Mpt5p is absolutely required for viability under these conditions. Therefore, we do not favor this model to explain the function of Mpt5p and Ssd1p.

Mpt5p and Ssd1p have many features in common. Both are likely to regulate gene expression post-transcriptionally by binding mRNA. *MPT5* and *SSD1* are both polymorphic loci, and mutation of either gene results in pleiotropic phenotypes affecting diverse cellular processes. We have shown here that both genes are involved in promoting cell integrity by functioning parallel to a pathway containing *CCR4*, *SWI4*, and *PKC1*. In addition, both genes act as positive regulators of longevity. Further research should be directed toward determining the precise transcripts that these proteins bind to and regulate.

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