

# A Role for the *Saccharomyces cerevisiae* Regulation of Ace2 and Polarized Morphogenesis Signaling Network in Cell Integrity

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## ABSTRACT

*Saccharomyces cerevisiae* RAM is a conserved signaling network that regulates maintenance of polarized growth and daughter-cell-specific transcription, the latter of which is critical for septum degradation. Consequently, cells defective in RAM function (designated *ramΔ*) are round in morphology, form feeble mating projections, and fail to separate following cytokinesis. It was recently demonstrated that RAM genes are essential in strains containing functional *SSD1* (*SSD1-v*), which encodes a protein of unknown function that binds the RAM Cbk1p kinase. Here we investigated the essential function of RAM in *SSD1-v* strains and identified two functional groups of dosage suppressors for *ramΔ* lethality. We establish that all *ramΔ* mutants exhibit cell integrity defects and cell lysis. All dosage suppressors rescue the lysis but not the cell polarity or cell separation defects of *ramΔ* cells. One class of dosage suppressors is composed of genes encoding cell wall proteins, indicating that alterations in cell wall structure can rescue the cell lysis in *ramΔ* cells. Another class of *ramΔ* dosage suppressors is composed of *ZRG8* and *SRL1*, which encode two unrelated proteins of unknown function. We establish that *ZRG8* and *SRL1* share similar genetic interactions and phenotypes. Significantly, Zrg8p coprecipitates with Ssd1p, localizes similarly to RAM proteins, and is dependent on RAM for localization. Collectively, these data indicate that RAM and Ssd1p function cooperatively to control cell integrity and suggest that Zrg8p and Srl1p function as nonessential inhibitors of Ssd1p.

THE conserved *Saccharomyces cerevisiae* regulation of Ace2p transcription factor and polarized morphogenesis (RAM) signaling network controls two genetically distinct cellular processes. One function of RAM is to regulate the daughter-cell-specific nuclear localization and activation of Ace2p transcription factor during mitotic exit (WEISS *et al.* 2002). In wild-type cells, Ace2p localizes specifically to the daughter nucleus during mitotic exit and induces the expression of several genes encoding septum degradation proteins (COLMAN-LERNER *et al.* 2001; WEISS *et al.* 2002). In addition to regulating cell separation, Ace2p also regulates a G<sub>1</sub> delay in daughter cells (LAABS *et al.* 2003). In RAM mutant cells (designated throughout as *ramΔ* cells), Ace2p localizes to both nuclei and is not active as a transcription factor. Thus, *ramΔ* cells accumulate as large clusters of unseparated cells, similar to *ace2Δ* cells (O'CONNOR *et al.* 1998; BIDLINGMAIER *et al.* 2001). RAM is also required to maintain the polarity of the actin cytoskeleton during polarized growth, which is important for bud site selection, cell morphogenesis, and mating projection formation (WEISS *et al.* 2002; NELSON *et al.* 2003). Consequently, *ramΔ* cells are spherical in morphology, exhibit random bud site selection, and form feeble mating projections. Notably, *ace2Δ*

cells do not display defects in polarized morphogenesis, indicating that the morphogenetic function of RAM is independent of Ace2p.

RAM is composed of two protein kinases, Cbk1p and Kic1p, and several associated proteins: Mob2p, Hym1p, Tao3p, and Sog2p (JØRGENSEN *et al.* 2002; NELSON *et al.* 2003). All RAM proteins are conserved from yeast to human and localize to the sites of polarized growth, which include the bud cortex during bud emergence and growth, the tips of mating projections, and the bud neck region during mitotic exit. In addition, Cbk1p and Mob2p colocalize with Ace2p in the daughter cell nucleus during mitotic exit (COLMAN-LERNER *et al.* 2001; WEISS *et al.* 2002). Cbk1p kinase belongs to the LATS/NDR protein kinase family, which includes the *S. cerevisiae* mitotic exit network (MEN) protein Dbf2p and the *Schizosaccharomyces pombe* proteins Orb6p and Sid2p (DORLAND *et al.* 2000; HOU *et al.* 2000, 2003; RACKI *et al.* 2000; BIDLINGMAIER *et al.* 2001; TAMASKOVIC *et al.* 2003). Mob2p is a Cbk1p-binding protein that is related to the *S. cerevisiae* MEN protein Mob1p (COLMAN-LERNER *et al.* 2001; LUCA *et al.* 2001; WEISS *et al.* 2002). Mob1p and Mob2p are MOB family proteins, which function as regulatory subunits of LATS/NDR family kinases (LUCA and WINEY 1998; HOU *et al.* 2000, 2003; WEISS *et al.* 2002). Kic1p is a GCK-II family kinase related to the MEN kinase Cdc15p (DAN *et al.* 2001). The molecular functions of Hym1p, Tao3p, and Sog2p are unknown. Hym1p interacts with Kic1p, and Cbk1p and

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is an ortholog of the *Aspergillus nidulans* hyphal growth protein *hymA* and the uncharacterized mouse protein MO25 (MIYAMOTO *et al.* 1993; KAROS and FISCHER 1999; DORLAND *et al.* 2000; BIDLINGMAIER *et al.* 2001). Tao3p (aka Pag1p) is a conserved 270-kDa protein that also interacts with both Cbk1p and Kic1p (DU and NOVICK 2002; NELSON *et al.* 2003). Sog2p is a leucine-rich-repeat-containing protein that binds Hym1p, Kic1p, and Cbk1p (NELSON *et al.* 2003). Homologs to Cbk1p, Mob2, Tao3, and Kic1p were identified in *S. pombe* and were shown to play a role in polarized growth (VERDE *et al.* 1998; HIRATA *et al.* 2002; HOU *et al.* 2003; HUANG *et al.* 2003).

Mob2p and Cbk1p appear to function late in RAM signaling (NELSON *et al.* 2003). Mob2p binds Cbk1p throughout the cell cycle and is required for Cbk1p kinase activity, which is critical for all known RAM functions (WEISS *et al.* 2002). Nevertheless, Mob2p binding is not sufficient for Cbk1p activation in *kic1Δ*, *hym1Δ*, *sog2Δ*, or *tao3Δ* cells (NELSON *et al.* 2003). The daughter-cell-specific nuclear localization of Mob2p, Cbk1p, and Ace2p is also dependent on all other RAM proteins (NELSON *et al.* 2003). These data suggest that Kic1p, Hym1p, Sog2p, and Tao3p function upstream of Mob2p-Cbk1p. By analogy to MEN, it is likely that Mob2p-Cbk1p is directly activated by Kic1p kinase, since Mob1p-Dbf2p is phosphorylated and activated by the Kic1p-related kinase Cdc15p (MAH *et al.* 2001). Thus far, substrates of Mob2p-Cbk1p are unknown.

Intriguingly, the RAM signaling network is essential for cell viability in strains that contain wild-type *SSDI* (designated *SSDI-v*), such as S288C-derived strains from the *S. cerevisiae* deletion consortium (DU and NOVICK 2002; JORGENSEN *et al.* 2002). In contrast, none of the RAM genes is essential for viability in strains that express a nonfunctional allele of *SSDI* (designated *ssdl-d*), such as W303 and our laboratory's S288C-derived strains (LUCA and WINEY 1998; DORLAND *et al.* 2000; RACKI *et al.* 2000; BIDLINGMAIER *et al.* 2001; DU and NOVICK 2002; JORGENSEN *et al.* 2002; NELSON *et al.* 2003). Thus, *ssdl-d* serves as an extragenic suppressor of *ramΔ* lethality. In contrast, *SSDI* does not exhibit the same genetic relationship with *ACE2*, since *ace2Δ* mutations are viable in *SSDI-v* and *ssdl-d* strains. These data suggest that RAM regulation of Ace2p is independent of *SSDI*.

Insight into the essential function of RAM may come from understanding *SSDI* function and regulation. Ssd1p is a conserved, nonessential protein of unknown function that localizes to punctate spots or patches in the cytoplasm (SUTTON *et al.* 1991; UESONO *et al.* 1997). It contains an RNB domain that binds poly(A) RNA *in vitro*. Cells lacking *SSDI* have higher concentrations of chitin and mannan in the cell wall and decreased concentrations of 1,3-β-glucan and 1,6-β-glucan, suggesting a role for *SSDI* in cell wall integrity (WHEELER *et al.* 2003). Genetic interactions suggest that *SSDI* participates in a cell integrity pathway that functions

in parallel to the protein kinase C (PKC) pathway (KAEBERLEIN and GUARENTE 2002). In agreement, *ssdl-d* is synthetic lethal with a variety of mutations in the PKC, cAMP/PKA, TOR signaling, and secretory pathways (SUTTON *et al.* 1991; COSTIGAN *et al.* 1992; UESONO *et al.* 1997; LI and WARNER 1998; KOSODO *et al.* 2001; ROSENWALD *et al.* 2002; VINCENT *et al.* 2003; REINKE *et al.* 2004). Significantly, Ssd1p interacts with Cbk1p in two-hybrid assays, suggesting that Ssd1p and RAM are functionally interconnected (RACKI *et al.* 2000). Moreover, recent data suggest that RAM functions in parallel to PKA signaling, as observed for *SSDI*, perhaps to control cell proliferation via regulation of Rho1p (BOGOMOLNAYA *et al.* 2004; SCHNEPER *et al.* 2004).

Several lines of evidence point to a role of Cbk1p, Tao3p, and Kic1p in maintenance of cell wall integrity. Two-hybrid assays revealed that Cbk1p interacts with Lre1p, a protein that affects cell wall chitinase and trehalose accumulation and functions antagonistically to protein kinase A (RACKI *et al.* 2000; VERSELE and THEVELEIN 2001). Moreover, the lethality of *cbk1Δ* and *tao3Δ* mutations in *SSDI-v* strains is suppressed by overexpression of the cell wall protein Sim1p (DU and NOVICK 2002). It was also demonstrated that, in some strains, *kic1* mutations lead to severe cell damages and eventually to cellular lysis (SULLIVAN *et al.* 1998; VINK *et al.* 2002). Furthermore, diminished Kic1p expression reduces 1,6-β-glucan levels in the cell wall (VINK *et al.* 2002). Despite these data, it was not known if the apparent cell integrity function of Cbk1p, Kic1p, and Tao3p was universal for all RAM proteins. Nor was it known if the essential function of RAM was genetically distinct from its function in polarized growth.

To investigate the essential function of RAM, we analyzed the phenotypes of *ramΔ SSDI-v* cells and employed a dosage suppressor screen. Here, we present evidence that RAM functions in an *SSDI*-dependent cell integrity pathway that is genetically distinct from its Ace2p regulation and polarized growth. Furthermore, we identified several components of the *SSDI*-dependent cell integrity pathway, including two cell wall proteins and a novel Ssd1p-binding protein, Zrg8p, which localizes similarly to RAM proteins. Our data suggest that Zrg8p and another dosage suppressor, Srl1p, function in a RAM- and Ssd1p-dependent cell integrity pathway.

## MATERIALS AND METHODS

**Yeast strains and cultures:** Yeast strains used in this study are listed in Table 1. Yeast were grown and manipulated using standard methods (GUTHRIE and FINK 1991). Yeast proteins were GFP-, HA-, and Myc-tagged by integration of PCR-based cassettes, as described (LONGTINE *et al.* 1998).

**Dosage suppressor screen:** Strain Y25654 (*mob2Δ::KANMX/MOB2 SSDI-v/SSDI-v*) was obtained from Open Biosystems and transformed with pRS316-MOB2, which contains *URA3* as a selectable marker. Upon sporulation and tetrad dissection, a

**TABLE 1**  
**Yeast strains**

Name	Genotype	Source
FLY849	<i>MATa ACE2-GFP::KANMX mob2Δ::HIS3 ssd1-d</i>	WEISS <i>et al.</i> (2002)
FLY858	<i>MATa mob2Δ SSD1-v</i> [pRS316-MOB2]	This work
FLY1279	<i>MATa ZRG8-GFP::HIS3</i>	This work
BY4742	<i>MATα SSD1-v</i>	Deletion Consortium
FLY1306	<i>MATα ccw12Δ SSD1-v</i>	Deletion Consortium
FLY1307	<i>MATα sim1Δ SSD1-v</i>	Deletion Consortium
FLY1308	<i>MATα srl1Δ SSD1-v</i>	Deletion Consortium
FLY1309	<i>MATα zrg8Δ SSD1-v</i>	Deletion Consortium
FLY1310	<i>MATα zap1Δ SSD1-v</i>	Deletion Consortium
FLY1313	<i>MATα cbp3Δ SSD1-v</i>	Deletion Consortium
FLY1327	<i>MATa ZRG8-GFP::HIS3 cbk1Δ::KANMX ssd1-d</i>	This work
FLY1632	<i>MATα ace2Δ SSD1-v</i>	Deletion Consortium
FLY1662	<i>MATα cbk1Δ SSD1-v</i> [pRS316-CBK1]	This work
FLY1663	<i>MATa ACE2-GFP::KANMX mob2Δ SSD1-v</i> [YEp13-CBP3]	This work
FLY1665	<i>MATa ACE2-GFP::KANMX mob2Δ SSD1-v</i> [YEp13-ZRG8]	This work
FLY1667	<i>MATa ACE2-GFP::KANMX mob2Δ SSD1-v</i> [YEp13-SRL1]	This work
FLY1669	<i>MATa ACE2-GFP::KANMX mob2Δ SSD1-v</i> [YEp13-SIM1]	This work
FLY1671	<i>MATa ACE2-GFP::KANMX mob2Δ SSD1-v</i> [YEp13-CCW12]	This work
FLY1680	<i>MATα ZRG8-GFP::HIS3 ace2Δ::KANMX ssd1-d</i>	This work
FLY1687	<i>MATa hym1Δ SSD1-v</i> [pRS316-HYM1]	This work
FLY1692	<i>MATa sog2Δ SSD1-v</i> [pYES-SOG2]	This work
FLY1718	<i>MATa ZRG8-myc::KANMX SSD1-HA::HIS3 SSD1-v</i>	This work
FLY1641	<i>MATa zrg8Δ::KANMX ccw12Δ::KANMX SSD1-v</i>	This work
FLY1722	<i>MATa srl1Δ::KANMX ccw12Δ::KANMX SSD1-v</i>	This work
FLY1741	<i>MATa mob2Δ::KANMX srl1Δ::KANMX SSD1-v</i> [pRS316-MOB2]	This work
FLY1745	<i>MATa mob2Δ::KANMX zrg8Δ::KANMX SSD1-v</i> [pRS316-MOB2]	This work
FLY1739	<i>MATα srl1Δ::KANMX zrg8Δ::KANMX SSD1-v</i>	This work
FLY1735	<i>MATα ZRG8-Myc::KANMX SSD1-v</i>	This work
FLY1737	<i>MATα SSD1-HA::HIS3 SSD1-v</i>	This work

*KANMX URA3* segregant (FLY858) was obtained and transformed with a YEp13-based genomic *S. cerevisiae* library (DEMARINI *et al.* 1997). Transformants were replica plated on 5-FOA to select for cells that lost the *URA3*-containing pRS316-MOB2 plasmid. Plasmids were rescued and retransformed into FLY858 to confirm the *mob2Δ* suppressor activity. Suppressor plasmids containing *MOB2* were identified by PCR and discarded. We isolated 23 dosage suppressor plasmids from ~100,000 transformants. The genomic DNA was identified in each plasmid by DNA sequencing. Eleven plasmids contained *SIM1*, which was previously described as a multicopy suppressor of *cbk1Δ* and *tao3Δ* (DU and NOVICK 2002). The *SIM1* plasmid used in this analysis, YEp13-SIM1, contains a genomic fragment of chromosome IX (125,951–133,378). Of the 23 dosage suppressor plasmids, 5 contained *SRL1*, 2 contained *CCW12*, 3 contained *ZAP1* and YJL055w, 1 contained *ZRG8*, 1 contained *CBP3*, and 1 contained *FSP2-HXT9*. *CCW12*, *CPB3*, *SRL1*, and *ZRG8* were subcloned as described below.

**Plasmid construction:** A *SacI-ClaI* fragment (2.6 kb) of YEp13-CCW12 containing ORFs *AHP1*, *CCW12*, and *YLR111* was subcloned into pRS423. From this plasmid, the *EcoRI-KpnI* fragment containing *CCW12* was subcloned into pRS424 to yield pRS424-CCW12. A *DraI* fragment (2.7 kb) of YEp13-CBP3 containing *CBP3* was subcloned into *SmaI* of pRS425 to yield pRS425-CBP3. YEp13-SRL1 contains the fragment of chromosome XV with *APC5* and *SRL1*. An *EcoRI-ApaI* fragment (2.2 kb) of YEp13-SRL1 was subcloned into pRS423 to yield pRS423-SRL1. The *PstI-HindIII* fragment (4.1 kb) of YEp13-ZRG8 was subcloned into pRS425 to produce pRS425-ZRG8.

**Microscopy and image analysis:** Differential interference contrast (DIC) and fluorescence microscopy was conducted as described (LUCA *et al.* 2001; WEISS *et al.* 2002).

**Calcofluor white sensitivity assays:** Calcofluor white sensitivity assays were performed as described using medium supplemented with 10, 50, and 100 μg/ml of Calcofluor white (RAM *et al.* 1994). Cells were grown at 22°, unless otherwise stated.

**Immunoprecipitation and immunoblotting:** Co-immunoprecipitation and immunoblot analyses were conducted using monoclonal anti-HA antibody (12CA5, Covance) and monoclonal anti-Myc antibody (9E10, Covance), as described (WEISS *et al.* 2002).

## RESULTS

**RAM signaling is required for maintaining cell integrity:** To determine the essential function of RAM, we analyzed the phenotypes of RAM deletion mutations in *SSD1-v* cells. We sporulated diploid cells that were heterozygous for RAM gene deletions and homozygous for *SSD1-v* and assayed the phenotypes of the meiotic products. We confirmed that *cbk1Δ*, *sog2Δ*, and *hym1Δ* cells were inviable in the presence of *SSD1-v* (data not shown). Most of the *cbk1Δ SSD1-v* spores germinated and lysed after several cell divisions. Some cells could form colonies of <100 cells before completely dying out (data

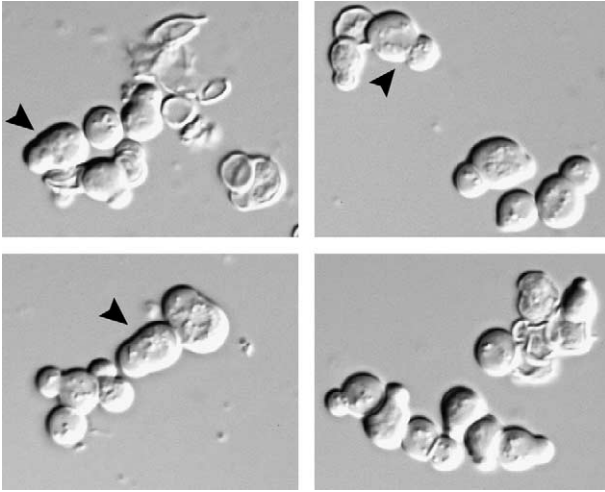


FIGURE 1.—*mob2Δ SSD1-v* cells exhibit severe morphology and lysis defects. DIC images of *mob2Δ SSD1-v* cells (segregants of strain Y25654, Open Biosystems) show cell separation, morphology, and cell lysis defects. Arrowheads point to abnormally wide bud necks.

not shown). In rare instances, some *cbk1Δ SSD1-v* segregants formed microcolonies after 5 days (data not shown). The viable *cbk1Δ SSD1-v* cells exhibited severe cell lysis phenotypes, which diminished in severity when cells were continually grown on rich media. Some *kic1Δ* cells also progressively improve over time (VINK *et al.* 2002), suggesting that both *kic1Δ* and *cbk1Δ* cells can readily obtain suppressor mutations. In contrast to *cbk1Δ SSD1-v* cells, all *mob2Δ SSD1-v* segregants formed slowly growing microcolonies on YPD plates. Many *mob2Δ SSD1-v* cells contained broken cell walls and exhibited varying degrees of cellular lysis (Figure 1). The remaining cells appeared very sick and contained wide bud necks. Similar phenotypes were described for conditional *kic1* mutants (SULLIVAN *et al.* 1998). The cellular lysis and lethality of *ramΔ SSD1-d* strains were not suppressed by supplementing the growth media with 1 M sorbitol (data not shown). Collectively, these data indicate that RAM is required for maintaining cell integrity and suggest a role for RAM in cell wall maintenance.

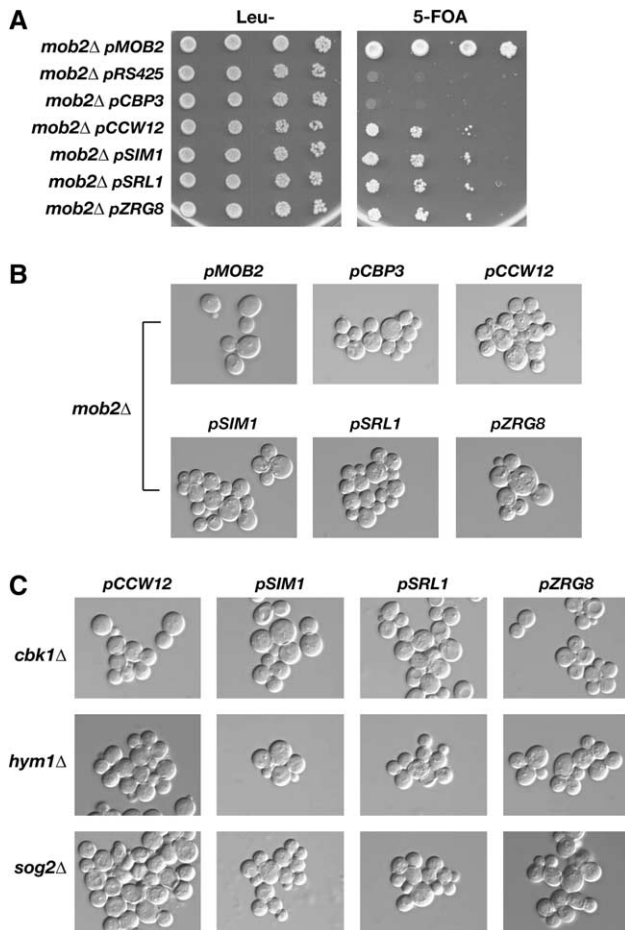
**High-copy suppressors of the *SSD1*-dependent function of RAM:** To elucidate the role of RAM in maintaining cell integrity and possibly identify important regulators or targets of RAM or Ssd1p, we conducted a dosage suppressor screen in *mob2Δ SSD1-v* cells (see MATERIALS AND METHODS). We introduced a yeast genomic DNA library of high-copy plasmids into *mob2Δ SSD1-v* cells that also contained a counterselectable *MOB2* plasmid. Upon counterselection for the *MOB2* plasmid on 5-FOA plates (see MATERIALS AND METHODS), we selected for robustly growing colonies and recovered the dosage suppressor plasmids. We identified the corresponding dosage suppressor genes by DNA sequencing and confirmed their suppressor activities by subcloning and retransforming them into *mob2Δ SSD1-v* cells.

We identified several dosage suppressors of *mob2Δ SSD1-v* cells, including *SIMI*, *CCW12*, *SRL1*, *ZRG8*, *CBP3*, and a DNA fragment encoding truncated *ZAP1* and *YJL055w*. Intriguingly, none of these genes is essential for viability (<http://www.yeastgenome.org/>). High-copy plasmids of *SIMI*, *CCW12*, *SRL1*, and *ZRG8* allowed *mob2Δ SSD1-v* cells to grow robustly on plates, although not as well as *MOB2*-containing cells (Figure 2A; supplementary Figure S1 at <http://www.genetics.org/supplemental/>). In contrast, *CBP3* plasmids provided much more modest growth improvement for *mob2Δ* cells. The cell lysis phenotypes of *mob2Δ SSD1-v* cells were greatly diminished by the high-copy *SIMI*, *CCW12*, *SRL1*, *ZRG8*, and *CBP3* plasmids (Figure 2B). The plasmid-containing *mob2Δ SSD1-v* cells appeared healthy and were nearly indistinguishable in morphology from *mob2Δ ssd1-d* cells. The cells were spherical in morphology and persisted as clusters of connected cells. These data indicate that overexpression of *SIMI*, *CCW12*, *SRL1*, *CBP3*, and *ZRG8* suppresses the cellular lysis phenotypes, but not other phenotypes of *mob2Δ* cells.

Most of the *mob2Δ* dosage suppressor genes were not well characterized; however, previous data implicate *SIMI*, *CCW12*, and *SRL1* in cell wall biogenesis. *SIMI* encodes a protein that is noncovalently bound to the cell wall and is highly glycosylated (VELOURS *et al.* 2002). *SIMI* overexpression was demonstrated to suppress the lethality of *tao3Δ SSD1-v* and *cbk1Δ SSD1-v* cells (DU and NOVICK 2002). *CCW12* encodes a glycosylphosphatidylinositol (GPI)-anchored mannosylated protein and *SRL1* encodes a putative structural mannoprotein that localizes to the cortex of small buds (MRSA *et al.* 1999; TERASHIMA *et al.* 2002; SHEPARD *et al.* 2003; HAGEN *et al.* 2004; TEPARIC *et al.* 2004). Recent work suggests that deletion of GPI-anchored proteins, such as Ccw12p, activates an *SRL1*-dependent compensatory pathway for cell wall integrity (HAGEN *et al.* 2004). Thus, the dosage suppressors *SIMI*, *CCW12*, and *SRL1* implicate RAM signaling in cell wall maintenance and suggest a cell-wall-dependent mechanism for suppressing the lethality of *ramΔ* mutations.

Prior to this study *ZRG8*, *ZAP1*, and *CBP3* were not implicated in cell wall maintenance or function. Both *ZRG8* and *ZAP1* are zinc-regulated genes whose expression is increased during zinc deficiency 1.9- and 5.7-fold, respectively (YUAN 2000). *ZRG8* encodes an uncharacterized 1076-amino-acid protein that contains no obvious protein motifs. *ZAP1* encodes a transcription factor that regulates expression of proteins involved in zinc homeostasis (ZHAO *et al.* 1998) and *CBP3* encodes a putative mitochondrial chaperonin that is required for cytochrome-c reductase assembly (SHI *et al.* 2001). It is possible that *ZRG8*, *ZAP1*, and *CBP3* overexpression suppresses the cell wall defects of *mob2Δ* cells indirectly or via a cell-wall-independent mechanism.

To determine if the *mob2Δ* dosage suppressors could suppress the lethality of other *ramΔ* mutations, we introduced each *SIMI*, *CCW12*, *SRL1*, *ZRG8*, and *CBP3*



**FIGURE 2.**—Dosage suppressors of *mob2Δ* *SSD1-v* lethality suppress the cell lysis but not the cell separation defect of *ramΔ* *SSD1-v* cells. (A) *mob2Δ* *SSD1-v* cells (FLY858) containing pRS316-MOB2 were transformed with empty vector (pRS425) or with high-copy plasmids containing *MOB2* (pMOB2), *CBP3* (pCBP3), *CCW12* (pCCW12), *SIM1* (pSIM1), *SRL1* (pSRL1), or *ZRG8* (pZRG8). All of the high-copy plasmids contained *LEU2* and thus were selectable on leucine deficient (Leu<sup>-</sup>) medium. The cells were serially diluted (10-fold) and spotted onto Leu<sup>-</sup> and 5-FOA plates. 5-FOA selects for cells that do not contain pRS316-MOB2. Note that *CBP3* is a weak suppressor and that none of the high-copy suppressors rescue the lethality of *mob2Δ* as well as that of *MOB2*. (B) DIC images of the suppressed *mob2Δ* *SSD1-v* cells. High-copy *CBP3*, *CCW12*, *SIM1*, *SRL1*, and *ZRG8* plasmids suppress the cell lysis defects but not the cell separation defects of *mob2Δ* *SSD1-v* cells (FLY858). *mob2Δ* *SSD1-v* cells containing pMOB2 are indistinguishable from wild-type cells. (C) High-copy *CCW12*, *SIM1*, *SRL1*, and *ZRG8* plasmids suppress the lethality of *cbk1Δ* *SSD1-v* (FLY1662), *hym1Δ* *SSD1-v* (FLY1687), and *sog2Δ* *SSD1-v* (FLY1692) cells. The rescued cells display cell separation defects that are identical in phenotype to *mob2Δ* *ssd1-d* and *cbk1Δ* *ssd1-d* cells (FLY168 and FLY757), which were previously described in WEISS *et al.* (2002). *cbk1Δ* *SSD1-v*, *hym1Δ* *SSD1-v*, and *sog2Δ* *SSD1-v* cells containing cognate *CBK1*, *HYM1*, and *SOG2* plasmids are indistinguishable from wild-type cells (data not shown). All high-copy suppressor plasmids are derived from a YEp13-based genomic library (DEMARINI *et al.* 1997).

multicopy plasmids into *cbk1Δ*, *sog2Δ*, and *hym1Δ* cells and analyzed their viability. We found that high-copy *SIM1*, *CCW12*, *SRL1*, and *ZRG8* plasmids suppressed the lethality and slow-growth defects of *cbk1Δ*, *sog2Δ*, and *hym1Δ* cells (Figure 2C), indicating that the dosage suppression is not specific to *mob2Δ* *SSD1-v* cells. Thus, *SIM1*, *CCW12*, *SRL1*, and *ZRG8* overexpression can bypass the essential function of the RAM signaling network. Intriguingly, high-copy *CBP3* plasmids, which provided only modest growth improvement for *mob2Δ* cells, did not rescue the lethality of *cbk1Δ*, *sog2Δ*, or *hym1Δ*.

**Overexpression of *SIM1*, *CCW12*, *SRL1*, and *ZRG8* suppress the cell lysis defects but not the cell separation and polarized growth defects of *ramΔ* cells:** It was established that RAM signaling is important for (1) maintenance of polarized growth and (2) regulating the daughter-cell-specific localization and activity of Ace2p transcription factor, which controls the expression of cell separation proteins (WEISS *et al.* 2002). We therefore tested if the dosage suppressor plasmids could suppress the cell polarity and cell separation defects of *ramΔ* *SSD1-v* and *ramΔ* *ssd1-d* cells. As noted above, high-copy *SIM1*, *CCW12*, *SRL1*, and *ZRG8* plasmids suppressed the cell lysis phenotypes of *mob2Δ* *SSD1-v*, *cbk1Δ* *SSD1-v*, *hym1Δ* *SSD1-v*, and *sog2Δ* *SSD1-v* cells. When we analyzed the cell morphology, we found that the cells were round and persisted in clusters of unseparated cells (Figure 2, B and C). The round cell shape suggests that, despite the presence of the dosage suppressor plasmids, *ramΔ* *SSD1-v* cells fail to maintain apical growth after bud emergence. It was previously demonstrated that *ramΔ* cells also fail to maintain polarized growth during mating projection formation (NELSON *et al.* 2003). Thus, we assayed whether *SIM1*, *CCW12*, *SRL1*, *ZRG8*, and *CBP3* plasmids could restore proper mating projection formation in *mob2Δ* cells. As can be observed in Figure 3, none of the dosage suppressor plasmids allowed *mob2Δ* cells to form robust mating projections in response to mating pheromone.

The persistent cell separation defects in plasmid-containing *ramΔ* cells indicate that Ace2p function is not restored by *SIM1*, *CCW12*, *SRL1*, *ZRG8*, or *CBP3* overexpression. In agreement, none of the dosage suppressor plasmids rescued the cell separation defects of *ace2Δ* cells (Figure 4). Nevertheless, it was possible that one or more of the dosage suppressors could influence Ace2p localization, which is normally controlled by RAM. We therefore monitored the effects of high-copy *SIM1*, *CCW12*, *SRL1*, *ZRG8*, and *CBP3* plasmids on the localization of Ace2-GFP in both *mob2Δ* *ssd1-d* and *mob2Δ* *SSD1-v* cells. As previously observed, Ace2-GFP localizes to the daughter cell nucleus at the end of mitosis and mislocalizes to both mother and daughter cell nuclei in *ramΔ* *ssd1-d* cells. We found that Ace2p localizes to the daughter cell nucleus in *SSD1-v* cells (Figure 5). High-copy *SIM1*, *CCW12*, *SRL1*, *ZRG8*, and *CBP3* plasmids did not restore proper

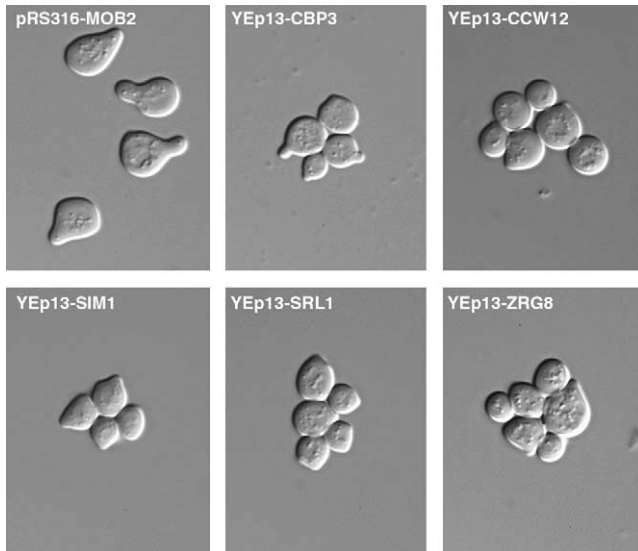


FIGURE 3.—*mob2Δ SSD1-v* cells containing high-copy *CBP3*, *CCW12*, *SIM1*, *SRL1*, and *ZRG8* plasmids do not form robust mating projections. *mob2Δ SSD1-v* cells containing high-copy *CBP3*, *CCW12*, *SIM1*, *SRL1*, and *ZRG8* plasmids were treated with  $\alpha$ -factor for 3 hr. All *mob2Δ SSD1-v* cells containing pRS316-MOB2 (top left) formed normal mating projections. In contrast, cells lacking pRS316-MOB2 formed feeble mating projections and remained connected.

daughter-specific localization of Ace2p in any *mob2Δ* cells (Figure 5). These data indicate that *SIM1*, *CCW12*, *SRL1*, *ZRG8*, and *CBP3* are not involved in Ace2p regulation. Furthermore, these results demonstrate that RAM performs genetically distinct functions in maintaining cell integrity (via *SSD1*) and Ace2p regulation.

***SIM1*, *CCW12*, *SRL1*, and *ZRG8* influence cell wall biogenesis:** It is possible that overexpression of *SIM1*, *CCW12*, *SRL1*, *ZRG8*, and *CBP3* suppresses the cellular lysis phenotypes of *ramΔ SSD1-v* cells by inducing a compensatory change in cell wall architecture. If this model is correct, then high-copy *SIM1*, *CCW12*, *SRL1*, *ZRG8*, and *CBP3* plasmids might induce detectable changes in cell wall architecture in wild-type cells (such as BY4742). Thus, we introduced the high-copy plasmids into *SSD1-v* cells and assayed for Calcofluor white sensitivity. We found that cells containing *SIM1*, *CCW12*, *SRL1*, or *ZRG8* plasmids were more sensitive to Calcofluor white than control cells that contained empty vector (Figure 6A). In contrast, *CBP3* overexpression caused no observable effect on Calcofluor sensitivity. These results are consistent with the model that overexpression of *SIM1*, *CCW12*, *SRL1*, or *ZRG8* suppresses *ramΔ SSD1-v* cells by inducing a compensatory change in cell wall architecture.

*CCW12* and *SIM1* were shown to encode cell wall proteins. Srl1p is a serine- and threonine-rich protein, which is tightly associated with the cell wall (TERASHIMA *et al.* 2002). If *ZRG8*, *ZAP1*, and *CBP3* are also important for cell wall biosynthesis, then deletion of those genes may induce cell wall defects that are detectable by

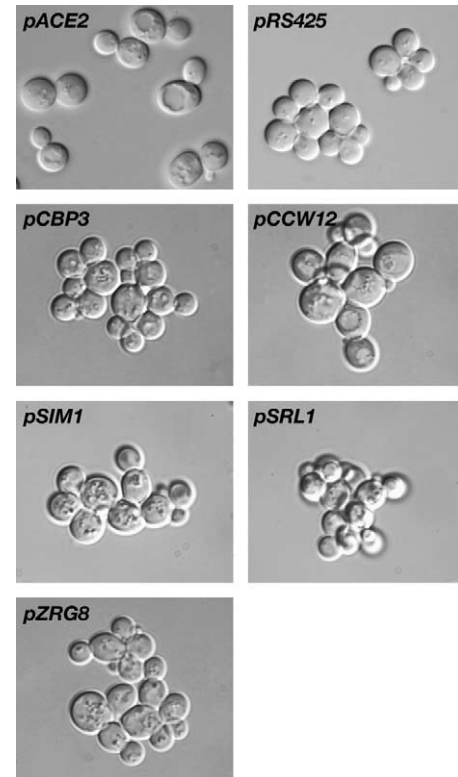


FIGURE 4.—High-copy *CBP3*, *CCW12*, *SIM1*, *SRL1*, and *ZRG8* plasmids do not suppress the cell separation defects of *ace2Δ* cells. High-copy *CBP3*, *CCW12*, *SIM1*, *SRL1*, and *ZRG8* (YEp13-based plasmids) and low-copy *ACE2* plasmids were introduced into *ace2Δ SSD1-v* cells (FLY1632). Cells were sonicated and analyzed for morphology. Only pACE2 rescued the cell separation defects of *ace2Δ* cells. Cells containing the empty high-copy vector pRS425 are shown as a negative control.

assaying Calcofluor sensitivity. Indeed, it was previously established that *ccw12Δ* cells are more sensitive to Calcofluor than control cells (MRSA *et al.* 1999). Thus, we assayed isogenic *sim1Δ*, *srl1Δ*, *zrg8Δ*, *zap1Δ*, and *cbp3Δ* strains for increased Calcofluor sensitivity. We found that *ccw12Δ*, *srl1Δ*, and *zap1Δ* cells were much more sensitive to Calcofluor than control *SSD1-v* cells (Figure 6B). At 22°, *zrg8Δ* cells were slightly more Calcofluor sensitive than wild-type cells, but *sim1Δ* cells were as Calcofluor sensitive as wild-type cells (Figure 6B). Calcofluor sensitivity of *ccw12Δ*, *srl1Δ*, and *zrg8Δ* cells is significantly enhanced at 37° (see Figure 8B). Intriguingly, *ssd1Δ* cells are also more sensitive to Calcofluor than *SSD1-v* cells and have elevated levels of chitin and mannan in their cell wall, indicating that *SSD1* is required for normal cell wall biosynthesis (WHEELER *et al.* 2003). Collectively, these data indicate that both overexpression and loss of function of *CCW12*, *SRL1*, and *ZRG8* alter cell wall biosynthesis. Thus, it is possible that *SIM1*, *CCW12*, *SRL1*, or *ZRG8* overexpression suppresses the lethality of *ramΔ* cells by altering cell wall integrity.

**Zrg8p localizes similarly to RAM proteins:** One or more of the *mob2Δ* dosage suppressors might function

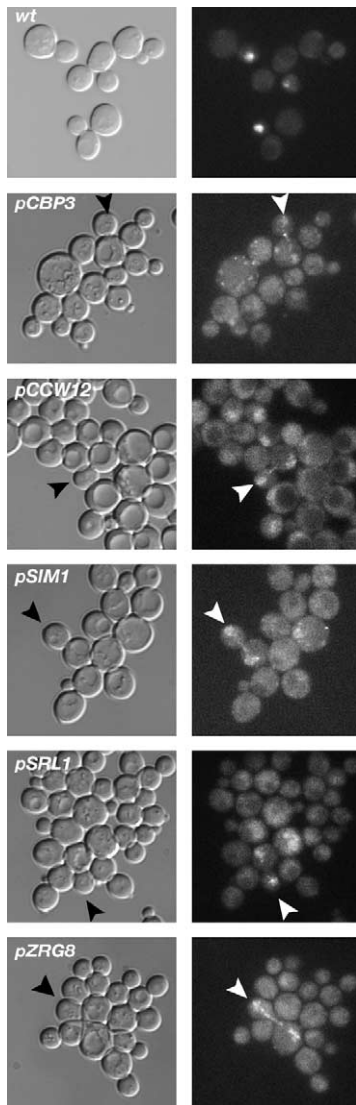


FIGURE 5.—High-copy *CBP3*, *CCW12*, *SIM1*, *SRL1*, and *ZRG8* plasmids do not restore the daughter-specific localization of Ace2p in *mob2Δ SSD1-v* cells. High-copy plasmids containing *CBP3*, *CCW12*, *SIM1*, *SRL1*, or *ZRG8* were introduced into *mob2Δ SSD1-v* cells expressing Ace2-GFP. The localization of Ace2p-GFP was analyzed by fluorescence microscopy. Wild-type cells (wt) are shown as a control to illustrate the daughter-specific nuclear localization of Ace2p in *SSD1-v* cells, as previously observed for *ssd1-d* cells (WEISS *et al.* 2002). Arrowheads point to late mitotic cells where Ace2p can be detected in both the mother and the daughter cell nucleus. The Ace2-GFP nuclear fluorescence is consistently weaker in *mob2Δ* cells than in wild-type cells. One hundred percent of the wild-type cells with detectable Ace2-GFP displayed the daughter-cell-specific localization in contrast to 0% of the *mob2Δ* cells ( $n = 50$ ).

as a RAM substrate or regulator. A RAM substrate or regulator is likely to localize similarly to RAM proteins at the bud cortex, bud neck, or daughter cell nucleus. Indeed, Srl1p was shown to localize to the cortex of small buds (SHEPARD *et al.* 2003). In contrast, Ccw12p, Sim1p, and Cbp3p may not directly interact with RAM proteins because they localize to the cell wall (Ccw12p, Sim1p) and mitochondria (Cbp3p), where RAM pro-

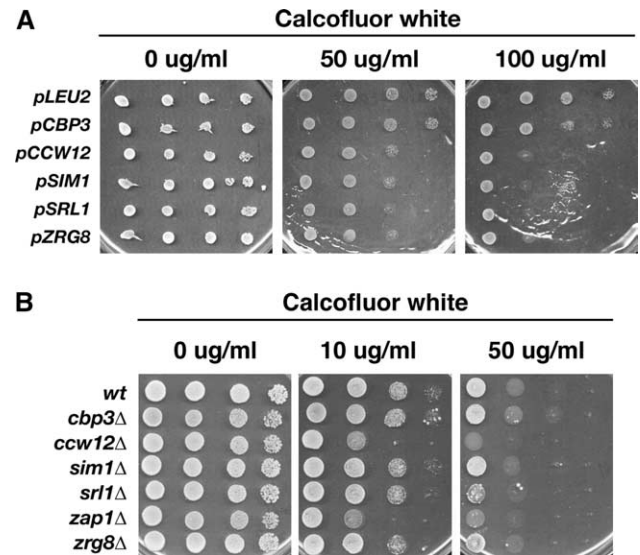
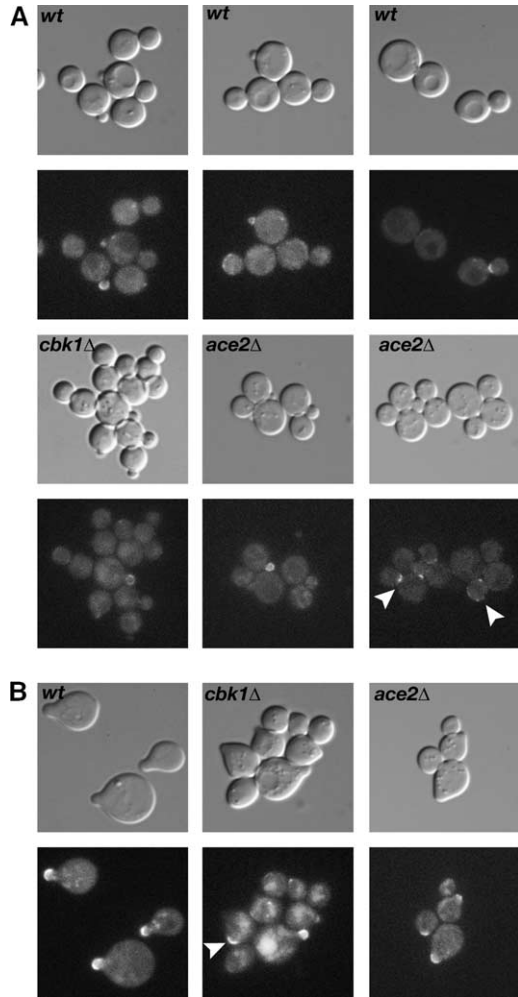


FIGURE 6.—Calcofluor white sensitivity assays for *mob2Δ* dosage suppressors. (A) Wild-type cells (BY4742) containing multicopy *CBP3*, *CCW12*, *SIM1*, *SRL1*, and *ZRG8* plasmids were assayed for Calcofluor white sensitivity on selective media. (B) Cells deleted for *CBP3* (FLY1313), *CCW12* (FLY1306), *SIM1* (FLY1307), *SRL1* (FLY1308), *ZAP1* (FLY1310), and *ZRG8* (FLY1309) were analyzed for their ability to grow on plates containing 10 or 50  $\mu\text{g/ml}$  Calcofluor white. Serial dilutions (10-fold) of cells were spotted onto each plate and grown at 22°.

teins are not known to localize (MRSA *et al.* 1999; SHI *et al.* 2001; VELOURS *et al.* 2002). To determine if Zrg8p localizes similarly to RAM proteins, we investigated the distribution of GFP-tagged Zrg8p in live cells. Zrg8-GFP localized to the cortex of small and large buds during bud growth and to the bud neck during mitotic exit (Figure 7A). Moreover, in pheromone-treated cells, Zrg8-GFP localized to the tips of mating projections (Figure 7B).

To test if Zrg8p localization is dependent on RAM, we monitored Zrg8-GFP in *cbk1Δ* and *ace2Δ* cells. We found that Zrg8p localization was greatly diminished or absent from the bud cortex and bud neck in *cbk1Δ* cells (Figure 7A). Zrg8p-GFP was detectable on the cortex in 61% ( $n = 65$ ) and 54% ( $n = 60$ ) of the small budded wild-type and *ace2Δ* cells, but was detectable only in 21% ( $n = 79$ ) of the small budded *cbk1Δ* cells. Zrg8p localization was also aberrant in *cbk1Δ* cells that were treated with mating pheromone (Figure 7B). Zrg8p was completely absent from mating projections and cell cortices in 64% ( $n = 133$ ) of the pheromone-treated *cbk1Δ* cells (Figure 7). In 22% of the pheromone-treated *cbk1Δ* cells, Zrg8p localized aberrantly to spots or patches on the sides of the mating projection or cell cortex. The remaining 14% of cells contained Zrg8p at the tips of mating projections. Moreover, some Zrg8-GFP appeared to localize to vacuoles in many of the mating-pheromone-treated *cbk1Δ* cells, suggesting that in the absence of Cbk1p, some Zrg8p is targeted for degradation. Zrg8p localization appeared normal in





**FIGURE 7.**—Localization of Zrg8p-GFP. (A) The localization of Zrg8p-GFP was analyzed in wild type (wt; FLY1279), *cbk1Δ* (FLY1327), and *ace2Δ* (FLY1680) cells by fluorescence microscopy. Zrg8p-GFP localizes to small and large buds and to the bud neck at the end of mitosis (top right) in wild-type cells. In *cbk1Δ* cells, Zrg8p-GFP is absent from the cortex and bud neck of most large budded cells and is greatly diminished on the cortex of most small buds. In contrast, Zrg8p localization appears normal in *ace2Δ* cells. Zrg8-GFP was detectable on the bud cortex in 63% ( $n = 60$ ), 21% ( $n = 79$ ), and 54% ( $n = 65$ ) of small budded wild-type, *cbk1Δ*, and *ace2Δ* cells, respectively. Arrowheads in *ace2Δ* indicate Zrg8p on the bud neck and the cortex of large budded cells. (B) Zrg8p-GFP was analyzed in cells that were treated with mating pheromone for 2 hr. In wild-type cells (wt) and *ace2Δ* cells, Zrg8p-GFP localizes to the tip of all mating projections. Zrg8p-GFP is undetectable at the cortex of most (64%,  $n = 133$ ) pheromone-treated *cbk1Δ* cells. In the remaining *cbk1Δ* cells, Zrg8p localizes to the tips of mating projections or to cortical spots or patches (arrowhead) that are not associated with mating projections.

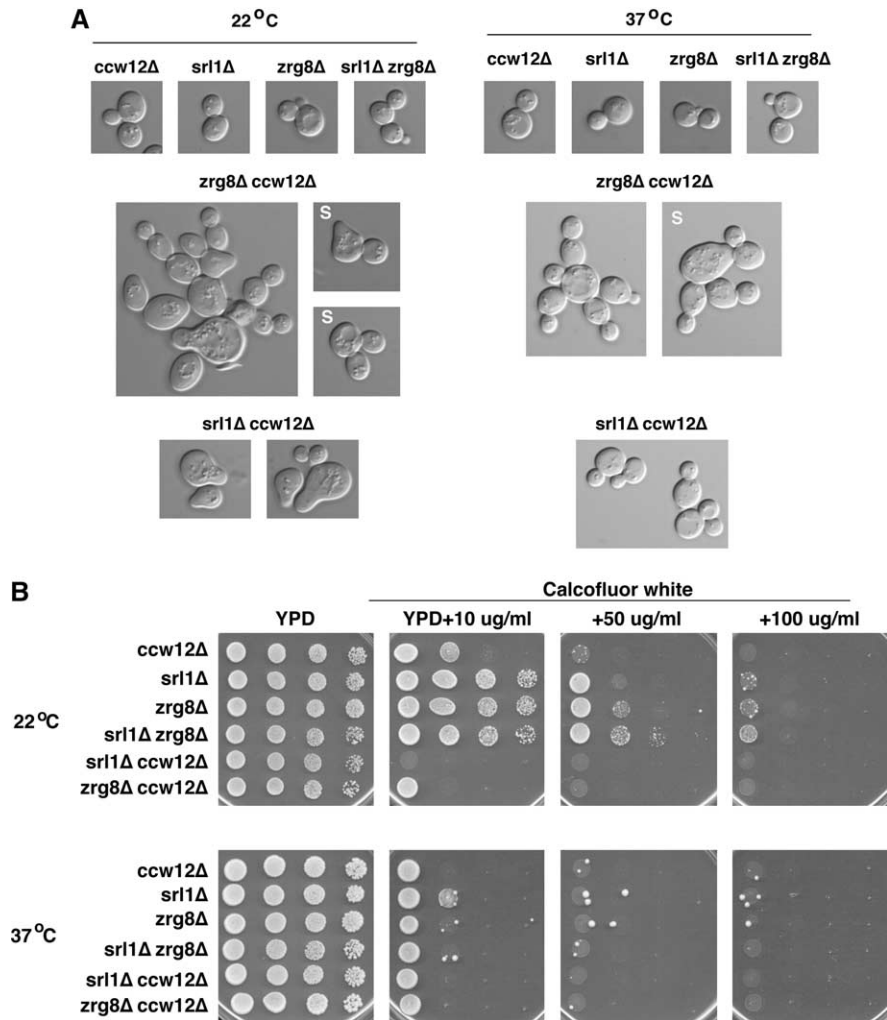
*ace2Δ* cells and in *srl1Δ* cells (Figure 7, A and B, and data not shown). These results indicate that Zrg8p localizes similarly to all RAM proteins throughout the cell cycle and is RAM dependent, but Ace2p independent, for localization. The RAM-like cortical localizations establish Zrg8p and Srl1p as viable candidates for RAM substrates or regulators.

***zrg8Δ* and *srl1Δ* mutations do not cause additive phenotypes:** The similar (albeit not identical) localization patterns of Zrg8p and Srl1p suggest that *ZRG8* and *SRL1* function in the same genetic pathway. If this model is correct, then the phenotypes of *zrg8Δ srl1Δ* cells should be similar to *zrg8Δ* and *srl1Δ* single-mutant cells. Alternatively, if Zrg8p and Srl1p function in different or parallel pathways, *zrg8Δ srl1Δ* cells may exhibit more severe phenotypes than the single-mutant cells. Thus, we crossed *zrg8Δ* and *srl1Δ* strains to construct a *zrg8Δ srl1Δ* double-mutant strain and assayed for synthetic phenotypes. The *zrg8Δ srl1Δ* cells did not exhibit any obvious growth or morphological differences from the *zrg8Δ* and *srl1Δ* single-mutant cells (Figure 8A). Likewise, *zrg8Δ srl1Δ* cells exhibited the same degree of Calcofluor sensitivity as *zrg8Δ* and *srl1Δ* single-mutant cells (Figure 8B). These data are consistent with the model that *ZRG8* and *SRL1* function in the same genetic pathway.

***SRL1* and *ZRG8* are not required for dosage suppression of *mob2Δ SSD1-v* cells:** If Zrg8p and Srl1p function together, then they may be dependent on each other to suppress the lethality of *mob2Δ SSD1-v* cells. Thus, we conducted a plasmid shuffle strategy, similar to that described for the dosage suppressor screen, to determine if dosage suppression of *mob2Δ* requires both *SRL1* and *ZRG8*. We introduced high-copy *ZRG8* or *SRL1* plasmids into *mob2Δ srl1Δ* and *mob2Δ zrg8Δ* double-mutant cells that also contained a counterselectable *MOB2* plasmid. We then selected for cells that lost the *MOB2* plasmid and assayed cell viability. We observed that high-copy *SRL1* and *ZRG8* plasmids could restore the viability of *mob2Δ srl1Δ* double-mutant cells (Figure 9). Similarly, *SRL1* and *ZRG8* plasmids could suppress the lethality of *mob2Δ zrg8Δ* double-mutant cells. We performed similar experiments to determine if *CBP3*, *CCW12*, or *SIM1* overexpression could suppress *mob2Δ* lethality in the absence of *ZRG8* or *SRL1*. We found that *CCW12* and *SIM1*, but not *CBP3*, plasmids suppressed the lethality of *mob2Δ zrg8Δ* and *mob2Δ srl1Δ* cells (Figure 9). Collectively, these data suggest that suppression of the cell integrity defects of *ramΔ* cells by *CCW12* and *SIM1* overexpression is independent of *ZRG8* and *SRL1*. These data also support the model that *ZRG8* and *SRL1* function independently. Alternatively, *ZRG8* and *SRL1* may function redundantly when overexpressed.

***ZRG8* and *SRL1* function in parallel to *CCW12*:** To elucidate the mechanism of *ramΔ* suppression, we investigated the relationship between *CCW12*, *ZRG8*, and *SRL1*. We assayed *zrg8Δ ccw12Δ* and *srl1Δ ccw12Δ* double-mutant cells for enhanced morphological and cell wall defects. In contrast to *zrg8Δ*, *srl1Δ*, and *ccw12Δ* single-mutant cells, which were wild type in morphology (Figure 8A), *srl1Δ ccw12Δ* and *zrg8Δ ccw12Δ* double-mutant cells exhibited aberrant cellular morphologies. At 22°, 79% ( $n = 311$ ) of the *srl1Δ ccw12Δ* cells displayed





**FIGURE 8.—***SRL1* and *ZRG8* exhibit synthetic genetic interactions with *CCW12*. (A) The morphologies of *srl1Δ*, *zrg8Δ*, *ccw12Δ* single- and double-mutant cells were monitored at 22° and 37°. *srl1Δ* (FLY1308), *zrg8Δ* (FLY1309), *ccw12Δ* (FLY1306) single-mutant cells and *srl1Δ zrg8Δ* (FLY1739) double-mutant cells appear normal in morphology at 22° and 37° (top). In contrast, many *zrg8Δ ccw12Δ* double-mutant cells (FLY1641) are aberrant in morphology and budding patterns at 22° and 37° (middle). The presence of cellular chains indicates that budding occurred in a polar fashion, as opposed to axial budding that is typical for haploid cells. In addition, *zrg8Δ ccw12Δ* cells fail to separate efficiently at 22° and 37°. The clusters of *zrg8Δ ccw12Δ* cells are resistant to disruption by sonication (S) at 37° or EDTA treatment (data not shown). *srl1Δ ccw12Δ* cells (FLY1722) exhibit severe morphology defects at 22°, but not at 37°. At 22°, *srl1Δ ccw12Δ* and some *zrg8Δ ccw12Δ* cells resemble mating-pheromone-treated wild-type cells. Seventy-nine percent of *srl1Δ ccw12Δ* and 68% of *zrg8Δ ccw12Δ* cells displayed aberrant morphologies at 22° ( $n > 260$ ). (B) *srl1Δ*, *zrg8Δ*, *ccw12Δ* single- and double-mutant cells were assayed for Calcofluor white sensitivity at 22° and 37°. At 22°, *srl1Δ ccw12Δ* and *zrg8Δ ccw12Δ* cells exhibit enhanced Calcofluor white sensitivity in comparison to the corresponding single-mutant cells. At 37°, all single- and double-mutant strains are hypersensitive to Calcofluor white.

aberrant cell shapes that were similar in morphology to pheromone-treated cells. Intriguingly, the aberrant morphology of *srl1Δ ccw12Δ* cells was completely abolished at 37°. Most (68%,  $n = 268$ ) *zrg8Δ ccw12Δ* cells also appeared swollen or misshapen. Moreover, they persisted in cell clusters at 22°, which could be partially separated by brief sonication. At 37°, the cell clusters were more resistant to sonication (Figure 8A). The cell clusters were not diminished by addition of 20 mM EDTA (data not shown). The haploid *srl1Δ ccw12Δ* and *zrg8Δ ccw12Δ* cells displayed apparent defects in bud site selection, since many buds emerged at opposite poles of cells, which is typical for diploid cells but not for haploid cells.

We also assayed *srl1Δ ccw12Δ* and *zrg8Δ ccw12Δ* cells for Calcofluor sensitivity. As shown in Figures 6B and 8B, *zrg8Δ*, *srl1Δ*, and *ccw12Δ* single-mutant cells are sensitive to low concentrations of Calcofluor, indicating cell wall defects. Intriguingly, all three single-mutant strains were more sensitive to Calcofluor at 37° than at 22° (Figure 8B). At 22°, both the *srl1Δ ccw12Δ* and *zrg8Δ ccw12Δ* double-mutant cells display enhanced Calcofluor sensitivity in comparison to the single mutants. Indeed, even

the most densely plated aliquot of *srl1Δ ccw12Δ* cells died in the presence of 10  $\mu$ g/ml Calcofluor. At 37°, *srl1Δ ccw12Δ* and *zrg8Δ ccw12Δ* cells displayed the same degree of Calcofluor sensitivity as the single mutant cells. Collectively, these data are consistent with the model that *ZRG8* and *SRL1* function in the same genetic pathway and that both *ZRG8* and *SRL1* function in parallel to *CCW12*. Recent results suggest that deletion of *CCW12* and other genes encoding GPI-anchored cell wall proteins activates a novel *SRL1*-dependent cell integrity pathway (HAGEN *et al.* 2004). Thus, our data may suggest that *ZRG8* and *SRL1* function together in this novel cell integrity pathway, which is probably *SSD1* dependent for function.

**Zrg8p coprecipitates with Ssd1p:** It was demonstrated that *SSD1* inactivation rescues the lethality of *ramΔ* cells (DU and NOVICK 2002; JØRGENSEN *et al.* 2002). Thus, it is possible that one or more of the dosage suppressors restore the viability of *ramΔ SSD1-v* cells by regulating Ssd1p function. For example, Zrg8p overexpression might suppress the lethality of *ramΔ SSD1-v* cells by inhibiting Ssd1p or preventing Ssd1p from interacting with other RAM-associated proteins. If this is

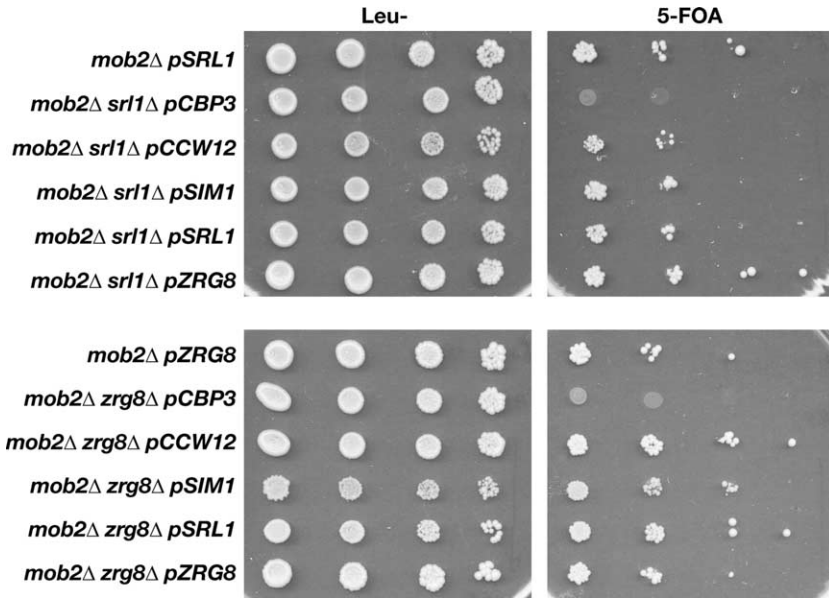


FIGURE 9.—Dosage suppression of *mob2Δ* lethality does not require *SRL1* or *ZRG8*. *mob2Δ srl1Δ SSD1-v* and *mob2Δ zrg8Δ SSD1-v* cells containing pRS316-MOB2 (FLY1741 and FLY1745, respectively) were transformed with YEp13-based plasmids containing *CBP3*, *CCW12*, *SIM1*, *SRL1*, or *ZRG8*. Cells were serially diluted (10-fold) and spotted onto leucine deficient (Leu<sup>-</sup>) and 5-FOA plates to counterselect for pRS316-MOB2. Each dosage suppressor plasmid, with the exception of pCBP3, rescued the lethality of *mob2Δ srl1Δ SSD1-v* and *mob2Δ zrg8Δ SSD1-v* cells.

true, then Zrg8p might physically associate with Ssd1p. Indeed, large-scale affinity precipitation methods suggest that Zrg8p associates in a complex with Ssd1p (KROGAN *et al.* 2004). Thus, to determine if Zrg8p interacts with Ssd1p, we conducted coprecipitation experiments in strains expressing Myc-tagged Zrg8p and HA-tagged Ssd1p. We established that Zrg8p-Myc and Ssd1p-HA are functional *in vivo* because Zrg8p-Myc does not cause morphology defects in *ccw12Δ* cells and Ssd1p-HA expression is lethal in *mob2Δ ssd1-d* cells (data not shown). We immunoprecipitated Ssd1p-HA from extracts of asynchronously growing cells and assayed for the presence of coprecipitated Zrg8p-Myc by immunoblot and vice versa. We found that a significant portion of Zrg8p coprecipitated Ssd1p (and vice versa) (Figure 10). These data indicate that Zrg8p and Ssd1p associate as a complex and are consistent with the model that Zrg8p functions as an inhibitor or cofactor of Ssd1p. We therefore hypothesize that Zrg8p dosage suppression of *ramΔ SSD1-v* cells occurs via inhibition of Ssd1p function.

## DISCUSSION

The *S. cerevisiae* RAM signaling network is critical for maintaining polarized growth and for regulating Ace2p transcription factor, which controls cell separation at the end of mitosis. In this article, we demonstrate that RAM has a third genetically separable function in maintaining cell integrity that involves *SSD1*. Deletion of RAM genes in *SSD1-v* cells causes severe cell lysis, which ultimately leads to cell death. The lethality of *ramΔ* cells is suppressed by *ssd1-d*, which encodes a nonfunctional form of *SSD1* (SUTTON *et al.* 1991; DU and NOVICK 2002; JORGENSEN *et al.* 2002; McDONALD *et al.* 2002). Although the cell lysis defects are suppressed by a loss of *SSD1* function, all *ramΔ ssd1-d* cells exhibit cell polarity and cell separation defects (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). Interestingly, deletion of *SSD1* does not cause severe defects in otherwise wild-type cells (WHEELER *et al.* 2003). Given the two-hybrid interactions between Cbk1p and Ssd1p (RACKI *et al.* 2000), it is likely that RAM and Ssd1p function in a common biochemical pathway to maintain cell integrity; however, it remains a mystery why cells lacking both RAM and *SSD1* exhibit no cellular lysis defects.

In an effort to elucidate the essential function of RAM, we identified several dosage suppressors of *ramΔ* lethality, including *SIM1*, *CCW12*, *SRL1*, and *ZRG8*.

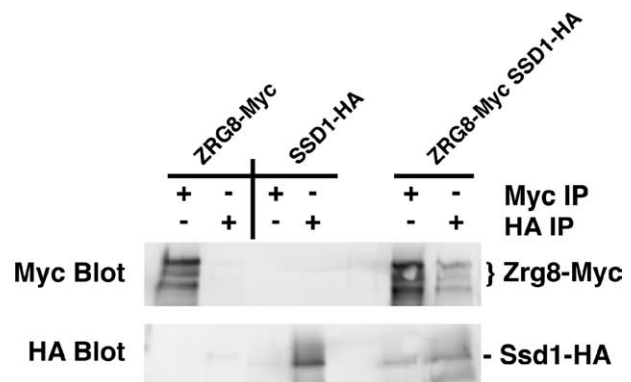


FIGURE 10.—Zrg8p and Ssd1p coprecipitate. Lysates of cells expressing Zrg8p-Myc (FLY1735), Ssd1p-HA (FLY1735), or both Zrg8p-Myc and Ssd1p-HA (FLY1718) were immunoprecipitated with anti-Myc (Myc IP) or anti-HA (HA IP) antibodies. Immunoprecipitated material was loaded onto a protein gel, immunoblotted, and probed with anti-Myc (Myc blot) or anti-HA (HA blot). Immune complexes of Zrg8p-Myc contain Ssd1p-HA and immune complexes of Ssd1p-HA contain Zrg8p-Myc (right lanes). Note that Zrg8p-Myc migrates on protein gels as three bands. This could be caused by post-translational modification to Zrg8p or by partial proteolysis.

Although each dosage suppressor rescued the severe cell wall defects of *ramΔ* cells, none suppressed the polarized growth or Ace2p regulatory defects. These data establish that the maintenance of cell integrity function of RAM is genetically distinct from its polarized growth and Ace2p regulatory functions. The dosage suppressors could rescue *ramΔ* lethality by several possible mechanisms. In one mechanism, overexpression of the suppressor gene may alter or strengthen the cell wall and thereby prevent cellular lysis of *ramΔ SSDI-v* cells. In another mechanism, overexpression of an Ssd1p-binding protein or regulator might functionally inhibit Ssd1p and thus restore cell viability to *ramΔ* cells. Specific inhibition of Ssd1p in *ramΔ SSDI-v* cells would yield cells that are phenotypically indistinguishable from *ramΔ ssd1-d* cells. Alternatively, *ramΔ SSDI-v* cells might be suppressed by overexpression of a downstream target of RAM or Ssd1p that bypasses the essential cell integrity function of RAM.

Genetic and cytological experiments provide insight into the mechanism of *ramΔ* suppression. We found that although none of the identified *ramΔ* suppressor genes is essential for viability, most of them are required for proper cell wall biosynthesis, as detected by increased Calcofluor sensitivity in deletion mutants. Furthermore, we established that the *ramΔ* dosage suppressors fall into at least two functional groups, with *CCW12* and *SIMI* constituting one group and *SRL1* and *ZRG8* constituting a second group. *CCW12* and *SIMI* encode cell wall proteins, which implies that the suppression of the lethality of *ramΔ* cells occurs via altering, and possibly strengthening, cell wall architecture. Ccw12p is a GPI-anchored cell wall protein and Sim1p is a member of the SUN protein family (MRSA *et al.* 1999; VELOURS *et al.* 2002). SUN family proteins share a common stretch of 258 aa at their C terminus, but appear to be involved in different cellular processes (VELOURS *et al.* 2002). Sim1p was recently shown to be a noncovalently linked cell wall protein (VELOURS *et al.* 2002) that was previously identified as a dosage suppressor of *tao3Δ* and *cbk1Δ*. It is possible that Sim1p and Ccw12p are downstream targets of RAM signaling; however, thus far, there is no evidence that RAM proteins directly interact with the cell wall. Thus, it may be more likely that *CCW12* and *SIMI* overexpression suppresses the lethality of *ramΔ* cells via indirect mechanisms that alter cell wall structure.

Several lines of evidence suggest that *SRL1* and *ZRG8*, which encode proteins of unknown function, belong to the same pathway. First, Srl1p and Zrg8p localize similarly to the cortex of small buds (Figure 7; SHEPARD *et al.* 2003). Moreover, the phenotypes of *srl1Δ* and *zrg8Δ* mutations are not additive. As shown in Figure 8, *srl1Δ zrg8Δ* double-mutant cells do not exhibit more severe phenotypes than the corresponding single-mutant cells, as would be expected if Srl1p and Zrg8p function in separate or parallel pathways. *SRL1* and *ZRG8* also

display similar genetic interactions with *CCW12*. Specifically, deletion of *SRL1* or *ZRG8* in *ccw12Δ* cells enhances Calcofluor sensitivity and morphological defects. These data suggest that *SRL1* and *ZRG8* function in parallel or cooperating pathways with *CCW12* to maintain cell integrity. Nevertheless, the morphological differences between *srl1Δ ccw12Δ* and *zrg8Δ ccw12Δ* double-mutant cells indicate that *SRL1* and *ZRG8* are not functionally redundant. Thus, collectively these data support the model that *SRL1* and *ZRG8* function in a RAM- and *SSDI*-dependent cell integrity pathway.

*ZRG8* may interact directly with RAM. In support of this, Zrg8p localizes similarly to RAM proteins throughout the cell cycle. *ZRG8* was first identified as a zinc-regulated gene that encodes a serine-rich protein of unknown function. Its expression is enhanced 1.9-fold when cells are grown in zinc-deficient media (YUAN 2000), although the significance of this phenomenon is unknown. We established that Zrg8p localizes to the cortex of small and large buds, to the bud neck region during mitotic exit, and to the tips of mating projections. Significantly, these localizations are dependent on the RAM kinase Cbk1p, but are not aberrant in zinc-limiting medium (data not shown) or in *ace2Δ* cells (Figure 7). These findings suggest that Zrg8p is involved in the cell polarity or integrity functions of RAM, but not in the cell separation functions of RAM. Evidence that Zrg8p coprecipitates with Ssd1p supports a role for Zrg8p in regulating cell integrity (Figure 10) and is consistent with the model that Zrg8p overexpression disrupts *SSDI* function and thereby suppresses the lethality of *ramΔ SSDI-v* cells. Similar coprecipitation approaches did not reveal an interaction between Zrg8p and Cbk1p in *ssd1-d* cells (data not shown). Nevertheless, since Ssd1p interacts with Cbk1p (RACKI *et al.* 2000), it is likely that Zrg8p associates with at least one of the RAM proteins at the cell cortex. Thus, Zrg8p may functionally link Ssd1p with RAM activity.

Although genetically related, *SRL1* is probably not functionally redundant to *ZRG8*. *SRL1* encodes a 210-amino-acid mannoprotein that may associate with membranes or cell walls (TERASHIMA *et al.* 2002; HAGEN *et al.* 2004). *SRL1* expression is regulated by Swi4p during G<sub>1</sub>, as was shown by direct binding of Swi4p to the promoter region of *SRL1* (BAETZ *et al.* 2001). Like Zrg8p, Srl1p localizes to the cortex of small buds (SHEPARD *et al.* 2003); however, unlike Zrg8p, Srl1p is undetectable in large buds or on the bud neck (our unpublished observations). These localization patterns suggest that Srl1p functions at the cortex from late G<sub>1</sub> to early S phase, whereas Zrg8p functions at the sites of polarized growth throughout the cell cycle. Recent data suggest that Srl1p is required for a cell integrity pathway that is triggered in the absence of multiple GPI-anchored cell wall proteins, such as Ccw12p (HAGEN *et al.* 2004). Moreover, *SRL1* expression is enhanced by cell-wall-damaging agents and in *ramΔ ssd1-d* strains

(JORGENSEN *et al.* 2002; GARCIA *et al.* 2004). These data suggest that cell wall and/or polarized growth defects (such as those caused by *ramΔ* mutations) enhance the activation of a cell integrity pathway via *SRL1* activation. Thus, RAM may regulate cell integrity by indirectly controlling *SRL1* expression and/or by regulating *Srl1p* function at the bud cortex. Alternatively, *Srl1p* may function as an inhibitor of *Ssd1p* function, as discussed below.

It is probable that *Zrg8p* and *Srl1p* contribute to cell integrity via *Ssd1p* and/or RAM. In support of this, *Zrg8p* coprecipitates with *Ssd1p* and both *Zrg8p* and *Srl1p* localize similarly to RAM proteins. Nevertheless, the bulk of *Zrg8p* does not appear to associate with *Ssd1p in vivo*. *Ssd1p* localizes to poorly defined punctate spots in the cytoplasm, but does not accumulate at the bud cortex or bud neck, as do *Zrg8p* and RAM proteins (UESONO *et al.* 1997). Thus, a fraction of *Ssd1p* is likely to function independently of RAM and *Zrg8p* and vice versa. In agreement, RAM functions independently of *Ssd1p* for its cell polarity and cell separation function. Curiously, *ZRG8* and *SRL1* are not essential for viability, indicating that they are not critical for *Ssd1p*- or RAM-dependent roles in maintenance of cell integrity (Figure 8). Moreover, deletion of *ZRG8* or *SRL1* does not rescue the lethality of *mob2Δ SSD1-v* cells. To resolve these observations, we propose that *Zrg8p* and *Srl1p* function as nonessential inhibitors of *Ssd1p*. In accordance with this model, *Ssd1p* would still be able to function with RAM to ensure cell integrity in the absence of these nonessential *Ssd1p* inhibitors.

Although the specific molecular role that *SSD1* plays in RAM signaling is unknown, insight has come from analyzing its genetic interactions. *Ssd1p* contains a RNase-II-like RNA-binding domain that can bind RNA *in vitro* (UESONO *et al.* 1997), although the physiological relevance of RNA binding has not been demonstrated *in vivo*. *SSD1* is linked genetically to several important cellular processes. Mutations in *SSD1* are synthetic lethal with mutations in genes involved in mRNA splicing, cell wall integrity, and vesicle transport (LUUKKONEN and SERAPHIN 1999; KAEBERLEIN and GUARENTE 2002; ROSENWALD *et al.* 2002). *SSD1* exhibits a different type of genetic relationship with RAM genes and *RVS167*. Mutations in *SSD1*, such as *ssd1-d*, suppress the lethality of *ramΔ* and *rvs167Δ* mutations (BRETON *et al.* 2001; DU and NOVICK 2002; JORGENSEN *et al.* 2002). Intriguingly, *RVS167* encodes a BAR adapter protein that interacts with actin and participates in regulation of endocytosis and vesicle transport (BRETON *et al.* 2001). The similarity of the genetic interactions suggests that RAM and *RVS167* are functionally linked. In agreement, both *RVS167* and the RAM gene *KIC1* share genetic interactions with *KRE6*, which encodes a putative  $\beta$ -1,6-glucan synthase whose activity is dependent on vesicle transport (BRETON *et al.* 2001; VINK *et al.* 2002). Furthermore, the *S. pombe* *Kic1p* homolog was recently

shown to associate with the *S. pombe* *Rvs167p* homolog (HUANG *et al.* 2005). It is therefore possible that *SSD1* and RAM function cooperatively to regulate vesicle transport and/or endocytosis, which is critical for polarized growth, cell wall biosynthesis and ultimately cell integrity. Further work is necessary to test this model for RAM and *SSD1* function and establish the specific molecular roles of *Zrg8p* and *Srl1p* in RAM signaling and *Ssd1p* regulation.

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