

# The Cdc34/SCF Ubiquitination Complex Mediates *Saccharomyces cerevisiae* Cell Wall Integrity

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## ABSTRACT

To identify novel functions for the Cdc34/SCF ubiquitination complex, we analyzed genomewide transcriptional profiles of *cdc53-1* and *cdc34-2* *Saccharomyces cerevisiae* mutants. This analysis revealed altered expression for several gene families, including genes involved in the regulation of cell wall organization and biosynthesis. This led us to uncover a role for the Cdc34/SCF complex in the regulation of cell wall integrity. In support of this, *cdc53-1* and *cdc34-2* mutants exhibit phenotypes characteristic of cell wall integrity mutants, such as SDS sensitivity and temperature-sensitive suppression by osmotic stabilizers. Examination of these mutants revealed defects in their induction of Slt2 phosphorylation, indicating defects in Pkc1-Slt2 MAPK signaling. Consistent with this, synthetic genetic interactions were observed between the genes encoding the Cdc34/SCF complex and key components of the Pkc1-Slt2 MAPK pathway. Further analysis revealed that Cdc34/SCF mutants have reduced levels of active Rho1, suggesting that these defects stem from the deregulated activity of the Rho1 GTPase. Altering the activity of Rho1 via manipulation of the Rho1-GAPs *LRG1* or *SAC7* affected Cdc34/SCF mutant growth. Strikingly, however, deletion of *LRG1* rescued the growth defects associated with Cdc34/SCF mutants, whereas deletion of *SAC7* enhanced these defects. Given the differential roles that these GAPs play in the regulation of Rho1, these observations indicate the importance of coordinating Cdc34/SCF activity with specific Rho1 functions.

SEVERAL signaling networks exist within eukaryotic cells to mediate the complex series of events regulating cellular proliferation. A major impetus in promoting cell proliferation is the coordinated expression of distinct families of genes encoding factors that promote the transition between distinct cell cycle phases. Many of these gene families are under the control of signaling pathways that respond to extracellular signals such as nutrient availability or stress. In this way, eukaryotic cells have developed an elegant method of responding to varying environmental conditions. One of the many means by which gene expression can be modulated is through ubiquitination of transcriptional regulatory proteins. This involves the covalent ligation of ubiquitin (Ub) onto protein substrates through an enzymatic cascade consisting of a Ub-activating enzyme, a Ub-conjugating enzyme, and a Ub ligase. Ub attachment or the assembly of poly(Ub) chains onto a protein may destine it for a variety of fates, commonly targeting that protein for degradation

by the 26S proteasome. The relationship between Ub and transcription can be observed at many different levels, such as in the regulation of signal transduction effectors and the function of transcription factors (MURATANI and TANSEY 2003).

The Cdc34 Ub-conjugating enzyme together with the Skp1/Cdc53/Rbx1/F-box (SCF) Ub-ligase functions as a key regulator of several transcriptional events. Studies in *Saccharomyces cerevisiae* have defined several essential roles for this complex. One of the best-studied roles for this complex in transcription is the regulation of the Gcn4 transcription factor, which functions to regulate genes responsible for the biosynthesis of amino acids, vitamins, and purines in response to amino acid starvation (BRAUS 1991; NATARAJAN *et al.* 2001). Under favorable growth conditions, the Cdc34/SCF complex ubiquitinates Gcn4, targeting it for proteasomal degradation. In this manner, Gcn4 protein levels are kept low, thereby sustaining low-level expression of its target genes. However, under conditions of amino acid starvation, Gcn4 protein levels are stabilized, allowing for activation of its target genes (HINNEBUSCH 1997; IRNIGER and BRAUS 2003). Interestingly, under these conditions, Cdc34/SCF-mediated ubiquitination of Gcn4 is also required for the transcriptional activation of these genes, indicating that Ub also plays a direct role in transcriptional activation (LIPFORD *et al.* 2005).

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Many other similar examples of Cdc34/SCF-mediated control of transcriptional regulation also exist, including the regulation of the transcriptional factors Met4 (KAISER *et al.* 2000; ROUILLOIN *et al.* 2000) and Tec1 (BAO *et al.* 2004), the transcriptional repressor Mth1 (SPIELEWOW *et al.* 2004), as well as several components of signaling pathways that affect gene expression, such as the mitogen-activated protein kinase (MAPK) Ste7 (WANG *et al.* 2003b). The widespread importance of the Cdc34/SCF complex in the regulation of transcriptional events prompted us to examine its transcriptional roles on a global nature. Using DNA microarrays to examine the transcriptional variance in *cdc53-1* and *cdc34-2* mutants, we identified a novel role for the Cdc34/SCF complex in the regulation of cell wall integrity.

In *S. cerevisiae*, cell wall integrity is maintained by an integrated network of signaling pathways that function to regulate cell wall metabolism and actin reorganization during the cell cycle and in response to stress. These pathways initially converge on, and then diverge from, the GTPase Rho1 (GUSTIN *et al.* 1998; LEVIN 2005). In response to cell wall stress, plasma membrane sensors stimulate a process leading to an increase in the activity of Rho1. Several distinct roles have been identified for Rho1, including (1) activating the protein kinase Pkc1 and subsequently the Slt2 MAPK pathway (NONAKA *et al.* 1995; MARTIN *et al.* 2000); (2) participating as a regulatory component of the 1,3- $\beta$ -glucan synthase enzyme, which is essential for cell wall biosynthesis (DRGONOVA *et al.* 1996; QADOTA *et al.* 1996); (3) mediating actin polymerization via the regulation of the formin proteins Bni1 and Bnr1 (IMAMURA *et al.* 1997); (4) activating the transcription factor Skn7 (ALBERTS *et al.* 1998); and (5) regulating vesicle transport (GUO *et al.* 2001). Several regulators of Rho1 activity exist to direct its differential roles, including guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The two major GEFs that positively regulate Rho1 function in response to cell wall signals are Rom1 and Rom2 (OZAKI *et al.* 1996; BICKLE *et al.* 1998). Negative regulation of Rho1 is achieved by several different GAPs, including Sac7 and Lrg1, each of which act to regulate distinct functions of Rho1. For example, Sac7 has been implicated as a regulator of actin polarization (DUNN and SHORTLE 1990; SCHMIDT *et al.* 1997) whereas Lrg1 has been implicated as a negative regulator of 1,3- $\beta$ -glucan synthase activity (WATANABE *et al.* 2001; FITCH *et al.* 2004).

In this study we show that yeast strains possessing mutations in the Cdc34/SCF complex exhibit cell wall integrity defects. Our analysis suggests that these defects stem from the misregulation of Rho1 activity, implicating a role for the Cdc34/SCF complex in its regulation. Consistent with this, we observe that signaling via the Slt2 MAPK pathway, which lies downstream of Rho1 activation, is compromised in Cdc34/SCF mutants. Furthermore, we observe strong genetic interactions be-

tween genes encoding components of the Cdc34/SCF complex and the Slt2 pathway. Together, these results suggest that the Cdc34/SCF complex plays a key role in regulating yeast cell integrity.

## MATERIALS AND METHODS

**Yeast strains:** All yeast strains used were isogenic to K699 (Table 1). Strains harboring *sic1::kan<sup>r</sup>*, *slt2::kan<sup>r</sup>*, *bck2::kan<sup>r</sup>*, *sac7::kan<sup>r</sup>*, or *lrg1::kan<sup>r</sup>* deletions were generated by homologous recombination using PCR products. Each PCR product was synthesized from genomic DNA derived from the appropriate *kan<sup>r</sup>* deletion strain (Open Biosystems) and included the *kan<sup>r</sup>* gene flanked by ~100–500 bp of DNA sequence from the upstream and downstream flanking regions of the target gene. Each strain was subsequently transformed with the appropriate PCR product and plated onto YPD medium containing 200  $\mu$ g/ml G418 to select for integrants. For *slt2::kan<sup>r</sup>* and *bck2::kan<sup>r</sup>* strains, selection was carried out on medium containing both G418 and 1 M sorbitol. The resulting knockout strains were then confirmed by PCR and functional analysis.

**Growth media:** Yeast strains were cultured in rich liquid medium (1% yeast extract, 2% bacto-peptone) containing 2% dextrose (YPD). Exceptions included the *slt2::kan<sup>r</sup>* and *bck2::kan<sup>r</sup>* strains, which were grown in YPD medium supplemented with 1 M sorbitol. Strains transformed with the YCp111.GAL (YCp), YCp111.GAL-Lrg1 (YCp-Lrg1), or YCp111.GAL-Sac7 (YCp-Sac7) plasmids were cultured in liquid SD medium (0.67% yeast nitrogen base lacking amino acids, supplemented with the required amino acids) containing 2% raffinose (SD-R). Solid YPD, SD-D (2% dextrose), and SD-G (2% galactose) media were prepared as above except that 2% bacto-agar was added to each. For the cell integrity plating experiments, YPD solid medium was supplemented with 1 M sorbitol, 0.5 M NaCl, or sodium dodecyl sulfate (SDS) to a final concentration of either 0.005% or 0.0075%.

**Microarray analysis:** Wild-type, *cdc53-1*, and *cdc34-2* cells were grown at 30° in YPD to an OD<sub>600</sub> of 0.8. Three separate cultures of each strain were generated for subsequent microarray analysis. Cells were collected by centrifugation and washed twice with ice-cold water and total RNA was immediately extracted from the cells by the hot-acid phenol method (KOHNER and DOMDEY 1991). Messenger RNA (mRNA) was then isolated using the Easy-mRNA kit (QIAGEN, Chatsworth, CA), and cDNA was synthesized from the mRNA by reverse transcription, and biotin-labeled cRNA was constructed and purified using the procedures described by Affymetrix. UV spectroscopy was used to quantitate the RNA.

Biotin-labeled cRNA was hybridized to Affymetrix yeast S98 whole-genome oligonucleotide microarray chips according to the manufacturer's procedures. The chips were processed using the Affymetrix Fluidics Station 400 and the arrays were imaged using the Affymetrix GeneArray Scanner (570 nm, 3- $\mu$ m pixel resolution). Each array was scanned twice and the images were averaged. The acquired images were then analyzed using the default parameters in Affymetrix's MicroArray Suite 5.0 (MAS 5.0). Subsequently the data was analyzed using Micro DB, Data Mining Tool 3.0 (DMT 3.0) and GeneSpring 6.2.

Statistical analysis (MAS 5.0) revealed that 84 and 85% of the genome in the *cdc53-1* and *cdc34-2* cells, respectively, was significantly present. Approximately 14% of the genes were absent in all the samples analyzed. The present genes from the triplicate *cdc53-1* and *cdc34-2* samples were compared in every various combination to those from the triplicate wild-type

**TABLE 1**  
**Yeast strains used in this study**

Yeast strains	Genotype	Source/reference
K699 (W303)	<i>MATa ade2-101 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100</i>	WILLEMS (1996)
MTY740	W303 <i>MATa cdc53-1</i>	WILLEMS (1996)
MTY670	W303 <i>MATa cdc34-2</i>	WILLEMS (1996)
BVY036	W303 <i>MATa Irg1::kan'</i>	This study
BVY037	W303 <i>MATa cdc34-2 Irg 1::kan'</i>	This study
BVY038	W303 <i>MATa cdc53-1 Irg 1::kan'</i>	This study
BVY039	W303 <i>MATa slt2::kan'</i>	This study
BVY040	W303 <i>MATa cdc34-2 slt2::kan'</i>	This study
BVY041	W303 <i>MATa cdc53-1 slt2::kan'</i>	This study
BVY086	W303 <i>MATa sic1::kan'</i>	This study
BVY087	W303 <i>MATa cdc34-2 sic1::kan'</i>	This study
BVY088	W303 <i>MATa cdc53-1 sic1::kan'</i>	This study
BVY121	W303 <i>MATa sac7::kan'</i>	This study
BVY122	W303 <i>MATa cdc34-2 sac7::kan'</i>	This study
BVY123	W303 <i>MATa cdc53-1 sac7::kan'</i>	This study
BVY130	W303 <i>MATa bck1::kan'</i>	This study
BVY131	W303 <i>MATa cdc34-2 bck1::kan'</i>	This study
BVY132	W303 <i>MATa cdc53-1 bck1::kan'</i>	This study

samples (for example, nine different fold changes were calculated for the *cdc53-1* strain by comparing the three *cdc53-1* samples to the three different wild-type samples), and from this an average fold gene expression was generated.

**Plating experiments:** For all plating experiments, a single colony of each strain examined was used to inoculate YPD liquid medium. Each culture was incubated at 30° and grown to midlog phase (OD<sub>600</sub> = ~0.5–1.0) prior to plating. A dilution series of each culture was subsequently prepared, and 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, and 10<sup>2</sup> cells were spotted onto four separate plates of the appropriate solid culture medium. The plates were then incubated at 30°, 33°, 35°, and 37° for 3 days prior to documentation. All plating experiments were carried out in duplicate using two separate colonies and were confirmed by at least one repetition of the experiment.

For *LRG1* overexpression the wild-type, *cdc34-2*, and *cdc53-1* strains were transformed either with an empty control plasmid (YCp111.GAL/YCp) or with the same plasmid carrying the *LRG1* coding sequence under control of the *GALI* promoter (YCp111.GAL-Lrg1/YCpLrg1). Plating experiments using each transformed strain followed the same procedure as described above except that single colonies were initially used to inoculate SD-R liquid medium. A dilution series of each culture was subsequently prepared and the appropriate volume of each spotted onto SD-D or SD-G solid medium lacking leucine. Plates were then incubated at 30° for 3 days prior to documentation.

YCp111.GAL was constructed using a fragment of the pESC(Trp) plasmid (Invitrogen, San Diego) that contains a multiple cloning sequence, a transcriptional terminator, and the *GALI* and *GAL10* promoters. This fragment was generated by PCR using oligonucleotides that introduced a 5' *Pst*I and a 3' *Mfe*I restriction site. These sites were used to ligate this fragment into the *Pst*I and *Eco*RI restriction sites of the YCplac111 plasmid. The *LRG1* coding region was PCR amplified from genomic DNA and inserted into the *Eco*RI and *Sst*I sites of the YCp111.GAL multiple cloning sequence to generate YCp111.GAL-Lrg1.

**Flow cytometry:** To assess the cell cycle position of various cultures, the DNA content of the cells was determined. An aliquot of each culture was taken, and the cells were collected

by centrifugation. Cells were then fixed in 70% ethanol followed by overnight incubation at 4°. Fixed cells were subsequently stained with propidium iodide and analyzed by flow cytometry using a FACScan instrument (Becton Dickinson, San Jose, CA), as described previously (EPSTEIN and CROSS 1992).

**Slt2 phosphorylation assays:** Cultures were grown in YPD liquid medium at 30° to an OD<sub>600</sub> of ~0.5 after which they were shifted to 37° or into YPD liquid medium supplemented with 2 mM caffeine. Aliquots of each culture were taken prior to the shift as well as at 15-, 30-, and 60-min time points after the shift for immunoblot analysis. The level of Slt2 or phosphorylated Slt2 present at each time point was determined by immunoblot analysis. To this end, a 1.5-ml aliquot was taken at each time point, the cells were collected by centrifugation, and the cell pellet was immediately frozen in liquid nitrogen. Cells were subsequently lysed by resuspending the pellet in SDS load buffer (500 mM Tris-HCl, pH 6.8, 20% glycerol, 10% SDS, 0.1% bromophenol blue, 100 mM DTT) followed by boiling for 5 min. The volume of SDS load buffer used for each aliquot was normalized on the basis of the OD<sub>600</sub> of each culture at each time point. Cells lysates were clarified by centrifugation and then separated by electrophoresis through a 10% SDS-polyacrylamide gel followed by transfer to a polyvinylidene difluoride membrane. To detect dually phosphorylated Slt2, the membranes were probed with an antiphospho-p44/42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) primary antibody (Cell Signaling Technology and New England Biolabs, Beverly, MA) diluted 1:1000 in TBS-T (50mM Tris-HCl, pH 7, 150 mM NaCl, 0.1% Tween-20) with 2% w/v skim milk powder overnight at 4°. Membranes were then washed with TBS-T and probed with a HRP-conjugated anti-rabbit (Cell Signaling Technology and New England Biolabs) secondary antibody at 1:1000 dilution in TBS-T for 2 hr at room temperature. Total Slt2 was detected by stripping and reprobing the membranes with an anti-Mpk1 primary antibody (Santa Cruz Biotechnology) at 1:100 dilution in TBS-T followed by a HRP-conjugated anti-mouse antibody (Santa Cruz Biotechnology) at a dilution of 1:1000 in TBS-T using the same conditions as described for detection of phosphorylated Slt2.

**Rho1 GTP assays:** Wild-type, *cdc34-2*, and *cdc53-1* strains were transformed either with an empty control plasmid (YCp111.GAL) or with the same plasmid carrying the *RHO1* coding sequence followed by a HA epitope tag under control of the *GALI* promoter (YCp-Rho1-HA). These cells were grown to midlog phase at 30° in SD-R and then galactose was added to a final concentration of 0.2% and the cells were grown for an additional 2 hr to induce a moderate level of Rho1-HA expression. Cells were then lysed in GPLB buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5% NP40, 5 mM glycerophosphate, 5 mM NaF, 1 mM DTT, 1 mM PMSF) and the extracts were incubated either with GST-rhotekin-binding domain bound to glutathione agarose beads (GST-RBD) (Cytoskeleton) or with GST bound to glutathione agarose beads for 1 hr at 4°. The beads were then washed five times with GPLB buffer, and SDS load buffer was added. The samples were then analyzed by immunoblotting with a mouse anti-HA antibody (12CA5, Roche).

**Microscopy:** To examine cell morphology, cells from an exponentially growing culture were initially fixed by the addition of 4% formaldehyde and subsequently incubated at room temperature for 10 min. Cells were then collected by centrifugation, resuspended in phosphate buffered saline (PBS) containing 4% formaldehyde, and incubated at room temperature for 1 hr. The samples were washed twice using PBS, briefly sonicated at the lowest setting to disperse cell clumps, and then spotted onto a microscope slide for analysis. Cells were visualized by DIC microscopy using a Zeiss Axioskop 2 microscope and documented using a Spot digital camera and Spot software 3.0.4 (Diagnostic Instruments).

## RESULTS

**The transcriptional profiles of *cdc53-1* and *cdc34-2* mutants reflect Cdc34/SCF function:** In an attempt to uncover novel functions for the Cdc34/SCF ubiquitination complex, we analyzed the transcriptional profiles of the *cdc53-1* and *cdc34-2* temperature-sensitive mutants using DNA microarray analysis. To mitigate the potentially confounding effects of heat-stress-related transcriptional changes, we performed our analysis at the permissive temperature of 30°. We reasoned that at this temperature the mutants would grow efficiently with minimal defects, yet may still exhibit sufficient transcriptional variation to delineate novel roles. Transcription profiles were generated from three independent, asynchronous, midlog phase cultures of *cdc53-1* and *cdc34-2* mutants and compared to that of wild-type cells (see supplemental Table 1 at <http://www.genetics.org/supplemental/> for the complete data set).

Relative to wild-type cells, the *cdc53-1* and *cdc34-2* mutants exhibited increased gene expression (twofold or higher) for 175 and 146 genes, respectively, and a decreased expression (twofold or more) for 19 and 17 genes, respectively. Comparison of the transcription profiles for the two mutants showed a high correlation ( $r^2 = 0.534$ ) with an overlap of 92 genes showing an induction and 9 genes showing a reduction of twofold or more. Overall, 229 different genes were induced and 27 genes were repressed for expression when the two gene sets were combined.

The similar effects that the *cdc53-1* and *cdc34-2* mutations had on global transcription indicated that Cdc53 and Cdc34 affect the expression of a common set of genes, an observation consistent with the known roles for the Cdc34/SCF complex in transcriptional regulation. Analysis of the microarray data revealed enrichment for several groups of genes, many of which are regulated by Cdc34/SCF targets, including (1) the transcriptional activator Gcn4, which regulates the expression of genes involved in the metabolism and biosynthesis of small molecules including amino acids, vitamins, cofactors, and purine bases; (2) the transcriptional activator Met4, which regulates the expression of genes involved in sulfur metabolism and biosynthesis of the sulfur containing the amino acids methionine and cysteine; (3) the transcription factors Tec1 and Ste12 and the signaling effectors Ste7 and Ste3, which regulate various aspects of the mating signaling pathway; and (4) Mth1, which downregulates the expression of genes involved in glucose metabolism and transport.

Taken together, the microarray analysis confirms that the data set is of high confidence as known functions for the Cdc34/SCF complex were identified. Therefore, we demonstrate that examining Cdc34/SCF function via the analysis of the *cdc53-1* and *cdc34-2* transcriptional profiles at a temperature permissive for growth is a valid approach.

**Novel functions for the *cdc53-1* and *cdc34-2* mutants are revealed by the microarray analysis, including an induced expression of cell-wall-related genes:** In addition to genes known to be regulated by the Cdc34/SCF complex, our microarray analysis identified changes in expression of several gene clusters that had not been previously linked to Cdc34/SCF function. Most notably, these included genes involved in sporulation, heavy metal ion homeostasis, and cell wall organization and biosynthesis (Tables 2 and 3).

Considering that the majority of the genes (at least 70%) for which expression changes by twofold or more in *cdc53-1* and *cdc34-2* mutants can be explained by the misregulation of well-characterized Cdc34/SCF targets, there exists a clear enrichment for genes involved in cell wall organization and biogenesis among those left currently unexplained. At least nine genes with gene ontology (GO) annotations related to cell wall synthesis were upregulated by twofold or greater in our microarray analysis (Table 2). Furthermore, a comparison of our data set with studies of cells that have undergone cell wall damage show a significant overlap in clusters of induced genes, which tend to be enriched for regulators of cell wall biosynthesis (GARCIA *et al.* 2004; LESAGE *et al.* 2004; supplemental Figure 1 at <http://www.genetics.org/supplemental/>). Of the genes induced by twofold or more, the transcription of at least three (*FKS2/GSK2*, *PIR3*, and *KTR2*) are critical under conditions that compromise the cell wall (BOORSMA *et al.* 2004; GARCIA *et al.* 2004). In particular, *FKS2*, the gene encoding the

**TABLE 2**  
**Selected groups of genes induced in the *cdc53-1* and *cdc34-2* mutants**

Biological process (based on gene ontology)	<i>P</i> -value	Genes	Genes in input cluster/genes in category	Fold enrichment <sup>a</sup>	Cdc34/SCF target(s) regulating this process
Amino acid metabolism (GO:0006520)	1.011e-10	<i>GDH3, HIS4, LYS20, ARO3, MET32, ARO10, SER3, STR3, ARG4, ARO9, LYS1, ARG3, MET3, CPA2, MET14, MET1, ECM40, MET2, LYS9, ARG1, HIS3, CPA1, ICL2, MET16</i>	24/140	4.8	Gcn4, Met4
Sulfur utilization (GO:0006791)	5.288e-08	<i>MET10, MET3, ECM17, MET14, MET1, MET16</i>	6/8	20.9	Met4
Vitamin metabolism (GO:0006766)	2.857e-05	<i>TKL2, RIB5, BNA1, BNA2, TH111, BNA5, SNO1, SNZ1, SNZ2, THI21</i>	10/57	4.9	Gcn4
Sodium transport (GO:0006814)	0.003759	<i>ENA5, ENA2</i>	2/3	18.6	Gcn4
Cell–cell adhesion (GO:0016337)	0.00734	<i>AGA2, MUC1</i>	2/4	13.9	Gcn4
Sporulation (GO:0030435)	0.007634	<i>UBC5, SPO71, SPS1, PRB1, SHC1, SGA1, SPS19, SPO21</i>	8/80	2.8	?
Purine base metabolism (GO:0006144)	0.01	<i>ADE1, MTD1, ADE2</i>	3/13	6.4	Gcn4
Cell wall organization and biogenesis (GO:0007047)	0.03079	<i>ECM13, YDL222C, TIR1, GSC2, ECM17, PIR3, KTR2, ECM40, PKH2</i>	9/122	2.1	?
Heavy metal ion homeostasis (GO:0030006)	0.03227	<i>PCA1, SIT1, ISU2, ISU1</i>	4/34	3.3	?
Signal transduction of mating signal (GO:0007330)	0.03769	<i>AFR1, MFA1, STE3</i>	3/21	4.0	Tec1, Ste7

Table was generated by analyzing the combined list of genes induced in the *cdc53-1* and *cdc34-2* data sets using Funspec (ROBINSON *et al.* 2002).

<sup>a</sup>Fold enrichment is the percentage of category genes induced in the mutant/percentage of category genes found in the genome.

catalytic subunit of the 1,3- $\beta$ -glucan synthase enzyme, was upregulated. This enzyme is a key player in the generation of cell wall material and genes encoding this complex are known to be induced following cell wall insult and in mutants with chronic cell wall injuries. In addition to these genes, other genes that likely affect the yeast cell wall were also upregulated. In particular, there was a large enrichment for genes encoding known glycosyl–phosphatidylinositol-anchored cell wall proteins (21.6-fold enrichment). Apart from providing structural support, these mannoproteins are incorporated into the yeast cell wall to accommodate changes to environmental conditions as well as to mediate cell wall reorganization during the cell cycle (MOUZYNA *et al.* 2000). More generally, the induction of several of these cell-wall-specific genes parallels that observed in response to cellular stress, such as osmotic and temperature stress (BOORSMA *et al.* 2004), as well as cell wall damage that may occur as a result of reduced 1,3- $\beta$ -glucan incorporated into the cell wall (TERASHIMA *et al.*

2000). Since our analysis was performed at 30° under rich growth conditions, the alteration of expression of these cell-wall-specific genes suggests a role for the Cdc34/SCF complex in maintaining cell wall integrity distinct from that of its previously characterized functions.

**The *cdc53-1* and *cdc34-2* mutants exhibit cell wall integrity defects:** The increased transcription of cell-wall-related genes within the *cdc53-1* and *cdc34-2* mutants may be a consequence of cell wall defects associated with these mutants. If so, we anticipated that these mutants would manifest phenotypes representative of cell wall integrity defects, such as (1) temperature sensitivity; (2) suppression of the temperature sensitivity by the presence of high concentrations of extracellular solutes, such as sorbitol or NaCl; and (3) sensitivity to low levels of the anionic detergent SDS (LEVIN and BARTLETT-HEUBUSCH 1992; PARAVICINI *et al.* 1992; MARTIN *et al.* 1996). We found that the temperature sensitivity of both *cdc53-1* and *cdc34-2* mutants was markedly

**TABLE 3**  
**Selected groups of genes repressed in the *cdc53-1* and *cdc34-2* mutants**

Biological process (based on gene ontology)	P-value	Genes	Genes in input cluster/genes genes in category	Fold enrichment <sup>a</sup>	Cdc34/SCF target(s) regulating this process
Ammonium transporter (GO:0015696)	6.341e-08	<i>MEP1, MEP2, MEP3</i>	3/3	232	?
Hexose transport (GO:0008645)	0.003633	<i>HTX1, HTX4</i>	2/22	21	Mth1

Table was generated by analyzing the combined list of genes induced in the *cdc53-1* and *cdc34-2* data sets using Funspec (ROBINSON *et al.* 2002).

<sup>a</sup>Fold enrichment is the percentage of category genes induced in the mutant/percentage of category genes found in the genome.

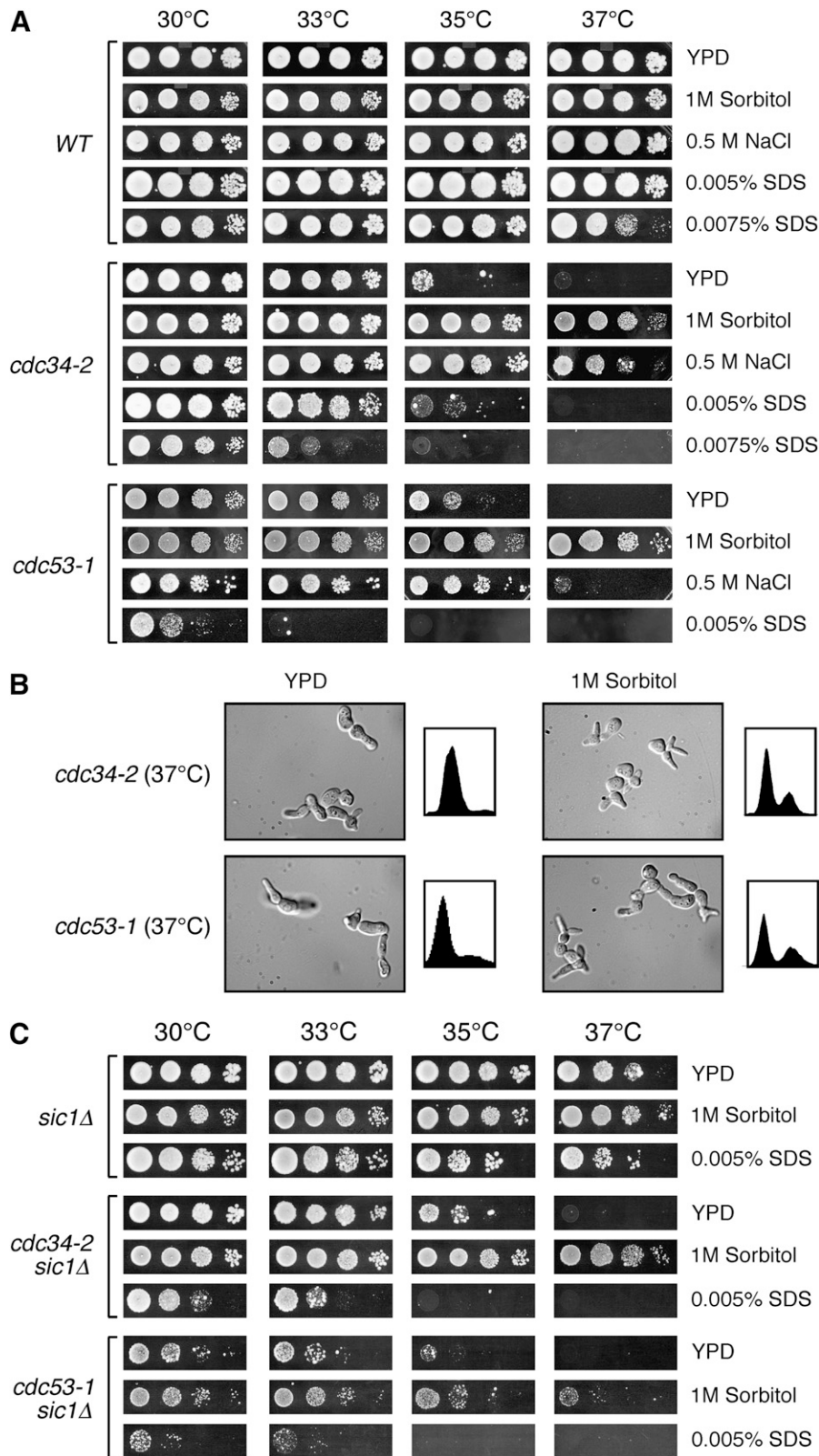
suppressed by the presence of 1 M sorbitol or 0.5 M NaCl in the growth media (Figure 1A). While these mutants grew at elevated temperature under these growth conditions, they still displayed an elongated multi-budded phenotype and an accumulation of cells in the G<sub>1</sub> phase of the cell cycle, indicating that their cell cycle defects were not fully suppressed (Figure 1B). This suggested that the primary reason for suppression of the temperature-sensitive growth defects in these mutants resulted from cell wall stabilization rather than from stabilization of the Cdc34 or Cdc53 heat-labile proteins in the presence of osmotic stabilizers. Further evidence for cell wall defects was observed when the mutants were grown on medium containing low levels of SDS. When grown on medium containing 0.005% SDS, the nonpermissive temperature of the *cdc53-1* mutant was drastically reduced (Figure 1A). The *cdc34-2* strain also showed SDS sensitivity; however, a slightly higher SDS concentration of 0.0075% was required to observe a strong phenotype. On the basis of these observations, the *cdc53-1* and *cdc34-2* mutants exhibit phenotypes consistent with cell wall integrity defects suggestive of a role for the Cdc34/SCF complex in the maintenance of the yeast cell wall.

A possible cause for the *cdc34-2* and *cdc53-1* cell integrity phenotypes might stem from Sic1 stabilization. Failure to degrade Sic1 in Cdc34/SCF mutants causes hyperpolarized growth that leads to an elongated, multi-budded phenotype (SCHWOB *et al.* 1994; VERMA *et al.* 1997). It is possible that this aberrant morphology perturbs the cell wall sufficiently to cause the observed cell integrity phenotypes. If true, it would be expected that deletion of *SIC1* in the *cdc34-2* and *cdc53-1* backgrounds, which is known to suppress their morphological defects, would also suppress the cell integrity defects associated with these strains. However, rather than suppressing the *cdc53-1* and *cdc34-2* cell integrity defects, the deletion of *SIC1* either had no effect or exacerbated the SDS sensitivity of these mutants, respectively (Figure 1C). In fact, deletion of *SIC1* in the *cdc53-1* mutant showed very strong synthetic growth defects under all conditions tested. Moreover, when the double

mutants were grown in the presence of 1 M sorbitol, a partial suppression of the growth defects was observed (Figure 1C). Therefore, these results indicate that the cell wall integrity defects associated with the *cdc53-1* and *cdc34-2* mutants occur independently of hyperpolarized growth caused by Sic1 accumulation, and rather reveal the importance Sic1 in mitigating these defects.

**The *cdc53-1* and *cdc34-2* mutants are defective in the induction of Slt2 phosphorylation:** Our data are consistent with the notion that the *cdc53-1* and *cdc34-2* mutations cause cell wall defects, likely leading to the increased transcription of cell-wall-related genes. These cell wall defects do not appear to result simply from the aberrant morphology associated with these mutants, and therefore it is possible that the Cdc34/SCF complex plays a direct role in cell wall maintenance that is compromised in the *cdc53-1* and *cdc34-2* mutants. To uncover such a function, we considered the effect that these mutations might have on signaling through cellular signaling pathways and in particular on the cell-integrity-regulating Slt2 MAPK pathway.

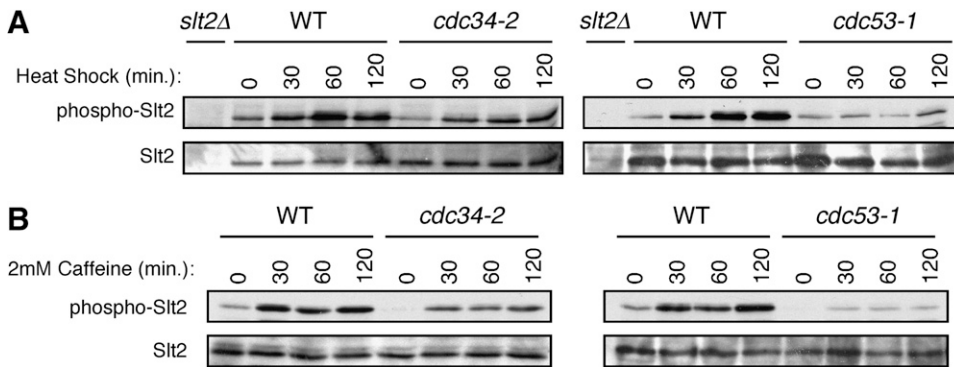
The Slt2 signaling pathway is central to the maintenance of yeast cell wall integrity and consists of a cascade of phosphorylation events that leads to the phosphorylation of the Slt2 kinase in response to cell-wall-related stress (LEVIN 2005). Activation of Slt2 ultimately leads to the expression of genes required for cell wall biosynthesis and cell cycle progression (IGUAL *et al.* 1996; MADDEN *et al.* 1997; JUNG *et al.* 2002). Misregulation of this pathway often leads to cell integrity phenotypes such as those observed in the *cdc53-1* and *cdc34-2* mutants (LEVIN and BARTLETT-HEUBUSCH 1992; PARAVICINI *et al.* 1992; MARTIN *et al.* 1996). We therefore monitored the induction of Slt2 phosphorylation in the wild-type, *cdc53-1*, and *cdc34-2* cells following different conditions that are known to induce the phosphorylation of Slt2, such as heat shock or treatment with caffeine (MARTIN *et al.* 2000). Wild-type cells showed a strong induction of Slt2 phosphorylation in response to heat shock (Figure 2A) or caffeine treatment (Figure 2B) as previously described (MARTIN *et al.* 2000). The *cdc34-2* strain



**FIGURE 1.**—The *cdc53-1* and *cdc34-2* mutants display cell integrity defects. (A) The *cdc53-1* and *cdc34-2* mutants display phenotypes consistent with cell integrity defects. Wild-type (WT), *cdc53-1*, and *cdc34-2* cells were grown on YPD or YPD containing 1 M sorbitol, 0.5 M NaCl, 0.0050% SDS, or 0.0075% SDS. The growth of the cells incubated at 30°, 33°, 35°, and 37° is shown. (B) *cdc53-1* and *cdc34-2* mutants grown in the presence of 1 M sorbitol display morphological and cell cycle defects. Cultures of *cdc53-1* and *cdc34-2* cells were grown in YPD media or YPD media containing 1 M sorbitol at 30° until midlog phase. These cells were shifted to 37° and samples were taken from each temperature and examined for morphology by microscopy and for DNA content as shown. (C) Deletion of *SIC1* does not suppress the *cdc34-2* and *cdc53-1* cell integrity defects. *sic1Δ*, *cdc34-2 sic1Δ*, and *cdc53-1 sic1Δ* cells were grown on YPD and YPD containing 1 M sorbitol or 0.0050% SDS. The growth of the cells incubated at 30°, 33°, 35°, and 37° is shown.

also showed an induction of Slt2 phosphorylation, but to a slightly lesser extent than that seen for the wild-type cells (Figure 2, A and B). By comparison, almost no induction of Slt2 phosphorylation was observed in the

*cdc53-1* mutant after any of the conditions tested (Figure 2, A and B). Therefore, these observations indicate that the *cdc53-1* mutation, and to a lesser extent the *cdc34-2* mutation, leads to a defect in the induction of Slt2



**FIGURE 2.**—The *cdc34-2* and *cdc53-1* mutants display defects in Slt2 phosphorylation. (A) Wild-type (WT), *cdc34-2*, and *cdc53-1* cells were grown in YPD medium to midlog phase at 30° and then shifted to 37°. Samples were taken before (0 min) or at the indicated time points (15, 30, and 60 min) following heat shock. As a negative control, a sample from the *slt2Δ* strain after 60 min of growth at 37° was analyzed. (B) WT, *cdc34-2*, and *cdc53-1*

cells were grown in YPD medium to midlog phase at 30° and then shifted in YPD containing 2 mM caffeine at 30°. Samples were taken before (0 min) or at the indicated time points (15, 30, and 60 min) following caffeine treatment. The samples were analyzed for phosphorylated Slt2 and total Slt2 protein levels by immunoblotting.

phosphorylation. Furthermore, the degree to which the *cdc53-1* and *cdc34-2* mutations exhibit SDS sensitivity correlates with the extent to which Slt2 phosphorylation is compromised in each mutant. Together, these observations indicate that the cell integrity phenotype and the strength of this phenotype relate to the relative degree to which Slt2 signaling is defective in each mutant.

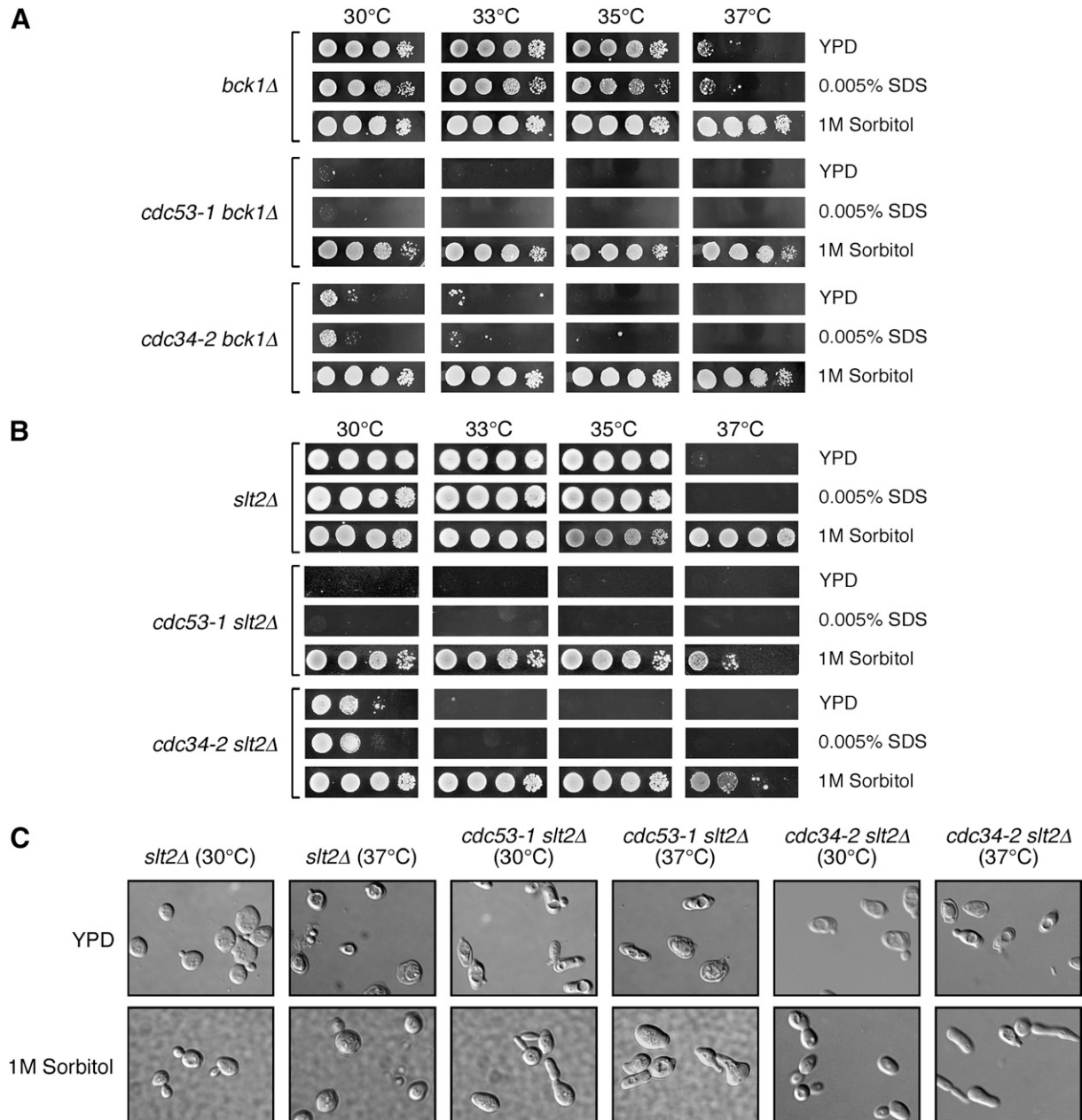
**The Slt2 MAPK pathway is required for maintaining cell wall integrity in *cdc53-1* and *cdc34-2* mutants:** We reasoned that, if Slt2 misregulation contributes directly to the cell integrity defects observed in the *cdc53-1* and *cdc34-2* mutants, then eliminating Slt2 signaling in these mutants should enhance their growth defects. Deletion of *SLT2* or its upstream activating kinase *BCK1* is known to block signaling via this MAPK pathway, resulting in temperature-sensitive growth defects that are suppressed by growth on media containing 1 M sorbitol (Figure 3, A and B; LEE *et al.* 1993). Microscopic inspection of the *slt2Δ* cells revealed that these cells undergo cell lysis at 37°, and this was suppressed by growth in 1 M sorbitol as previously reported (Figure 3C; MADDEN *et al.* 1997). Severe synthetic growth defects were observed at all temperatures tested when either *SLT2* or *BCK1* were deleted in the *cdc34-2* or the *cdc53-1* mutants, and these defects were also suppressed by growth on 1 M sorbitol media (Figure 3, A and B). Like *slt2Δ* cells, microscopic inspection of *cdc34-2 slt2Δ* and *cdc53-1 slt2Δ* cells revealed a cell lysis phenotype that could be suppressed by the presence of 1 M sorbitol (Figure 3C). Interestingly, the *SLT2* or *BCK1* deletion strains did not display any significantly increased sensitivity to SDS as compared to growth on YPD media (Figures 3, A and B). The observed synthetic phenotype indicates that an intact cell integrity pathway is required to maintain *cdc53-1* and *cdc34-2* mutant viability and further indicates that at least some of the defects that occur in these mutants are independent of this signaling pathway. In particular, since the *slt2Δ* and *bck1Δ* cells do not display SDS sensitivity, it appears that this phenotype in the *cdc53-1* and *cdc34-2* mutants occurs as a result of other defects.

Taken together, these observations suggest that the Cdc34/SCF complex mediates some aspect of cell wall integrity that is independent of, but affects, Slt2 signaling.

**Cdc34/SCF mutants possess defects in distinct Rho1 function(s):** One potential link between cell wall integrity and the phenotypes observed in the *cdc53-1* and *cdc34-2* mutants is the Rho1 GTP-binding protein. Rho1 is a central mediator of many aspects of cell wall integrity, and its misregulation severely impairs cell growth (LEVIN 2005). Interestingly, low levels of SDS treatment have been shown to deregulate Rho1 activity (BICKLE *et al.* 1998), which has the potential to affect the phosphorylation of Slt2. This suggested the possibility that the cell wall integrity defects in the *cdc53-1* and *cdc34-2* mutants might result from alterations in Rho1 activity. To test this, we examined the levels of GTP-bound Rho1 in these mutants. We transformed a *RHO1-HA*-expressing plasmid into wild-type, *cdc53-1*, and *cdc34-2* cells and measured the ability of Rho1-HA in these cells to bind to the rotoxin-binding domain (RBD), a domain that tightly interacts with GTP-bound Rho1 (REN *et al.* 1999; GARCIA *et al.* 2006). Using GST-RBD coupled to beads, we precipitated GTP-bound Rho1 from wild-type, *cdc53-1*, and *cdc34-2* cell extracts and found that the levels of GTP-bound Rho1 in *cdc53-1* and *cdc34-2* mutants were considerably less than that in wild-type cells (Figure 4). This interaction was specific since GST beads alone did not precipitate any Rho1-HA and was not due to variation in Rho1-HA protein levels as the total Rho1-HA expressed in these cells was similar. This indicates that the active state of Rho1 is reduced in these Cdc34/SCF mutants. Interestingly, the amounts of GTP-bound Rho1 in each mutant correlated with their defects in phospho-Slt2 induction and the extent of their cell integrity phenotypes, suggesting that the cell integrity defects observed in these cells are a result of deregulated Rho1 activity.

To try to gain further insight into how Rho1's function might be affected, we tested whether we could alter the phenotypes of *cdc53-1* and *cdc34-2* mutants by





**FIGURE 3.**—Abrogation of the Slt2 signaling pathway in *cdc53-1* and *cdc34-2* mutants results in severe synthetic lysis defects. (A) Deletion of *BCK2* in *cdc34-2* and *cdc53-1* mutants results in synthetic growth defects. *bck2Δ*, *cdc34-2 bck2Δ*, and *cdc53-1 bck2Δ* cells were grown on YPD and YPD containing 1 M sorbitol or 0.005% SDS at the indicated temperatures. (B) Deletion of *SLT2* in *cdc34-2* and *cdc53-1* mutants results in synthetic growth defects. *slt2Δ*, *cdc34-2 slt2Δ*, and *cdc53-1 slt2Δ* cells were grown on YPD plates and YPD plates containing 1 M sorbitol or 0.005% SDS at the indicated temperatures. (C) Deletion of *SLT2* results in cell lysis defects. *slt2Δ*, *cdc34-2 slt2Δ*, and *cdc53-1 slt2Δ* cells were grown in YPD containing 1 M sorbitol at 30° to midlog phase and then shifted to 37°, or transferred into YPD medium without sorbitol and grown at 30° or 37° for 6 hr. Samples from each were analyzed by microscopy.

modulating the activity of Rho1. We initially pursued this by overexpressing *RHO1* in these mutants, but found that overexpression of *RHO1* alone had no phenotypic effects on their growth (data not shown). We also tested the expression of a hyperactive allele of Rho1 (Q58H) in wild-type, *cdc53-1*, and *cdc34-2* mutants, but found that the expression of this gene was extremely toxic to the mutants (data not shown) as well as to the

wild-type cells as previously reported (MADAULE *et al.* 1987). Therefore, we chose to affect Rho1 function indirectly by manipulating the expression of Rho1-specific regulators. In particular, we placed the genes encoding the Rho1-specific GAPs Lrg1 and Sac7 under control of a galactose (GAL)-inducible promoter. These GAPs induce Rho1's GTPase activity and so function to reduce GTP-bound Rho1 levels, effectively acting as

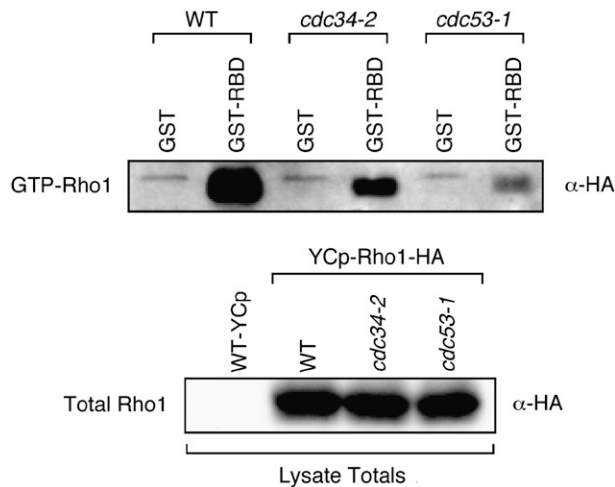


FIGURE 4.—*cdc34-2* and *cdc53-1* mutants have reduced levels of GTP-bound Rho1. WT, *cdc34-2*, and *cdc53-1* cells were transformed with empty YCP.GAL or YCP.GAL-Rho1-HA and then grown in the presence of galactose to induce the expression of HA-Rho1. Extracts were prepared from these cells, which expressed approximately equal amounts of HA-tagged Rho1 (bottom), and incubated with either GST-RBD glutathione beads to pull down GTP-bound Rho1 or GST glutathione beads as a control. The beads were washed and then analyzed for their retention of GTP-Rho1-HA by immunoblotting with an anti-HA antibody (top).

negative regulators. Galactose induction of *LRG1* or *SAC7* expression had no effect on the growth of wild-type cells when compared to growth on glucose media (GAL-repressing conditions) at 30° (Figure 5A). In contrast, the galactose induction of either *LRG1* or *SAC7* expression in the *cdc53-1* and *cdc34-2* mutants was toxic (Figure 5A), particularly in the case of *LRG1* expression. Given the role of these GAPs, their overexpression likely further reduces the GTP-bound Rho1 levels in these mutants, indicating that proper regulation of Rho1 activity is essential in Cdc34/SCF mutants.

To investigate the consequences of increasing Rho1 activity, and to try to delineate which function(s) of Rho1 might be compromised in the Cdc34/SCF mutants, we examined the effects of deleting *SAC7* or *LRG1* in *cdc53-1* and *cdc34-2* mutants. Both of these GAPs operate to reduce the levels of active GTP-bound Rho1; however, each of these proteins has been shown to regulate distinct aspects of Rho1 function. Thus, deletion of *LRG1* or *SAC7* would lead to a stimulation of Rho1 activity, but could also bias Rho1 activity toward a certain functional pathway. Both GAPs may affect signaling leading to Slt2 phosphorylation; however, deletion of *LRG1* would negatively affect Rho1 function pertaining to its 1,3- $\beta$ -glucan synthase activity (WATANABE *et al.* 2001). In contrast, deletion of *SAC7* would affect Rho1 function pertaining to its actin cytoskeleton remodeling activity (DUNN and SHORTLE 1990; SCHMIDT *et al.* 2002). Therefore, deletion of the genes encoding these GAPs in the context of Cdc34/SCF mutants could

reveal interactions between the function(s) of the Cdc34/SCF complex and Rho1-specific roles.

We observed that the deletion of *SAC7* in wild-type cells resulted in SDS sensitivity at high temperatures, but had no obvious growth defects on YPD (Figure 5B). Deletion of *SAC7* in the *cdc53-1* or *cdc34-2* mutants had a slight effect on cell growth on YPD media and also resulted in extreme sensitivity to SDS at all temperatures tested. Interestingly, the defects associated with the *cdc34-2 sac7 $\Delta$*  mutant could be efficiently suppressed by the presence of 1 M sorbitol, whereas this was not the case with the *cdc53-1 sac7 $\Delta$*  mutant. Together, these results establish a genetic relationship between *Sac7* and the Cdc34/SCF complex and suggest that a particular function of Rho1 that is regulated by *Sac7* may be defective in the *cdc53-1* and *cdc34-2* mutants.

Deletion of *LRG1* had a much different effect. No growth differences were observed between *lrg1 $\Delta$*  and wild-type cells under the conditions that we tested, but when *LRG1* was deleted in the *cdc53-1* and *cdc34-2* mutants, we observed a suppression of their growth defects on YPD in both the presence and the absence of SDS (Figure 5B). The cell cycle defects associated with these mutants were not fully suppressed since these cells still displayed an elongated multi-budded morphology and had an accumulation of 1 N DNA content (Figure 5C). Therefore, deletion of *LRG1* likely does not affect the function of the Cdc34/SCF complex in Sic1 degradation, but rather must suppress other defects within the *cdc53-1* and *cdc34-2* mutants that could alleviate the defects in these cells sufficiently to allow for growth. Deletion of *LRG1* leads to increased activity via the Pkc1-Slt2 pathway (LORBERG *et al.* 2001), even when combined with the *cdc53-1* and *cdc34-2* mutants (data not shown). This increased pathway activity may contribute to the effects observed; however, deletion of *SAC7* also leads to similar increases in Pkc1-Slt2 pathway activity (MARTIN *et al.* 2000; SCHMIDT *et al.* 2002; data not shown). Therefore, activation of this pathway alone is not capable of explaining the suppressive effects observed from the deletion of *LRG1*. A likely defect suppressed by deletion of *LRG1* may relate to its role in 1,3- $\beta$ -glucan synthesis, as *Lrg1* functions primarily as a negative regulator of this Rho1 function. Although a definitive cause for the suppression by *LRG1* deletion has not been delineated, these results support the notion that the Cdc34/SCF complex functions to mediate cell integrity by regulating the activity of Rho1.

## DISCUSSION

Our global expression analysis of *cdc53-1* and *cdc34-2* mutants revealed that the Cdc34/SCF complex regulates multiple aspects of cellular growth. In some cases the observed effect directly correlated with a known role for the Cdc34/SCF complex in transcriptional regulation. These included pathways that regulate methionine

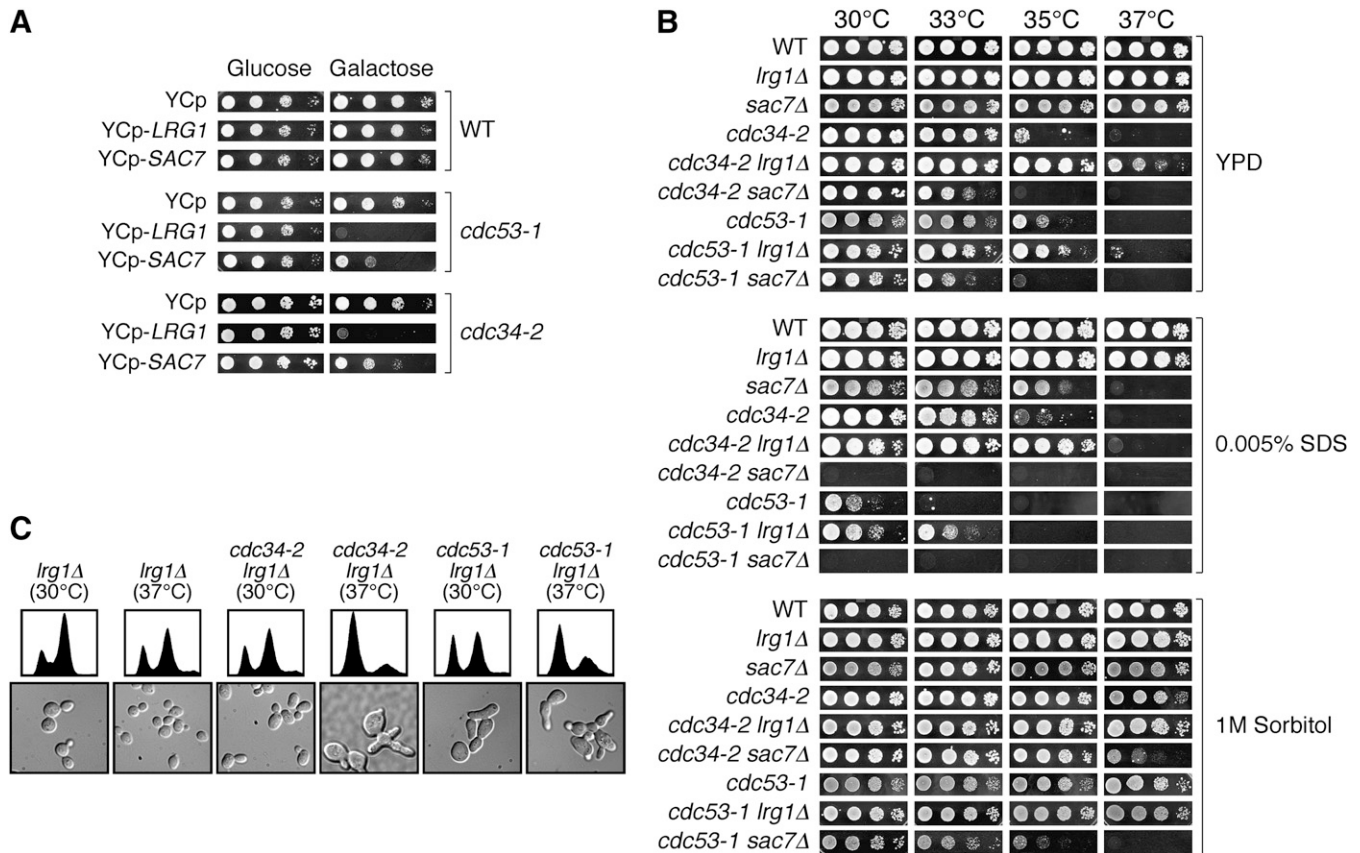


FIGURE 5.—*CDC34* and *CDC53* display genetic interactions with the Rho1-GAPs *LRG1* and *SAC7*. (A) Overexpression of *LRG1* or *SAC7* is toxic to *cdc53-1* and *cdc34-2* mutants. Wild-type (WT), *cdc34-2*, and *cdc53-1* cells carrying an empty control plasmid (YCP) or with plasmids expressing *LRG1* or *SAC7* from the *GAL1* promoter (YCP-*LRG1* and YCP-*SAC7*, respectively) were grown on medium containing glucose (left) or galactose (right) at 30° for 3 days. (B) Deletion of *LRG1* suppresses the growth defects associated with *cdc53-1* and *cdc34-2* mutants, whereas the deletion of *SAC7* enhances these defects. *cdc53-1* and *cdc34-2* mutants having either *LRG1* or *SAC7* deleted were grown on YPD, YPD containing 1 M sorbitol, or YPD containing 0.0050% SDS at the indicated temperatures. (C) *cdc53-1 lrg1Δ* and *cdc34-2 lrg1Δ* mutants display cell cycle and morphological defects. *cdc53-1* and *cdc34-2* cells having *LRG1* deleted were grown in YPD at 30° to midlog phase and shifted to 37°. Samples from both temperatures were examined for morphology by microscopy and for DNA content as shown.

and general amino acid biosynthesis. In other cases, however, subsets of genes not previously known to be regulated by the Cdc34/SCF complex were found to be upregulated within *cdc53-1* and *cdc34-2* mutants, including groups that regulate sporulation, heavy metal ion homeostasis, and cell wall organization and biosynthesis.

The observed induction of sporulation-specific genes was interesting since our analysis was done in haploid cells proliferating in rich medium, conditions that normally preclude the induction of these genes. With the exception of *PRB1*, all of the sporulation-specific genes identified are normally induced in the middle and late phases of meiotic differentiation (CHU *et al.* 1998). Consistent with this finding, most of these genes possess middle sporulation elements within their promoters and are induced by the meiosis-specific transcription factor Ndt80 (CHU *et al.* 1998). These observations implicate the Cdc34/SCF complex in the repression of these sporulation-specific genes in haploid cells. The induction of genes regulating heavy metal ion homeo-

stasis were also interesting, given recent studies suggesting a role for Cdc34 in the resistance of yeast to methylmercury (FURUCHI *et al.* 2002).

The link between the Cdc34/SCF complex and cell wall integrity was further substantiated by our observations that (1) these mutants display growth phenotypes consistent with cell integrity defects; (2) genetic interactions occur between *CDC34* or *CDC53* and genes composing the Slt2 MAPK pathway, a critical regulator of cell wall integrity; (3) *cdc53-1* and *cdc34-2* mutants display defects in the regulation of Slt2 phosphorylation, a key event in coordinating cell wall biosynthesis and cell cycle progression; (4) genetic interactions occur between *CDC34* or *CDC53* and genes encoding regulators of Rho1, a major player in cell wall synthesis and remodeling; and (5) active GTP-bound Rho1 levels are reduced in *cdc53-1* and *cdc34-2* mutants.

The connection between Cdc34/SCF function and cell integrity was generated in large part from observations that *cdc34-2* or *cdc53-1* mutants exhibit sensitivity

to low concentrations of SDS. This treatment imposes stress on the cell wall, thereby lowering the permissive temperature at which these mutants proliferate. These data, combined with the observation that high concentrations of the osmotic stabilizers sorbitol or NaCl raise the nonpermissive temperature for their proliferation, suggest that mutations in the Cdc34/SCF complex result in cell wall defects. A trivial explanation for these defects may be the inability of Cdc34/SCF mutants to degrade the Cdk inhibitor Sic1 at elevated temperatures. Increased Sic1 levels lead to a prolonged G<sub>1</sub> phase of the cell cycle that in turn causes the formation of single or multiple elongated buds, which could cause sufficient stress to produce the observed cell integrity defects. However, we believe this possibility is unlikely, given that (1) *cdc34-2 sic1Δ* and *cdc53-1 sic1Δ* cells, which do not display elongated multiple buds and proceed through the G<sub>1</sub>-S transition of the cell cycle, remain SDS sensitive and their growth is partially suppressed by the presence of 1 M sorbitol; (2) suppression of growth by high concentrations of sorbitol does not alleviate the elongated multi-budded phenotype associated with *cdc34* and *cdc53* mutants, indicating that sorbitol functions to osmotically stabilize cell wall defects rather than to stabilize the mutant proteins within the cell; (3) SDS treatment reduces the restrictive temperature of these mutants to temperatures at which Sic1 is efficiently degraded; and (4) there is an increased expression of cell-wall-related genes even at a temperature at which Sic1 is efficiently degraded. Of particular interest is the increased expression of *FKS2*, which comprises a component of the cell wall synthesis machinery and whose expression is normally increased in response to cell wall stress. Together, these data indicate a novel function for the Cdc34/SCF complex in the regulation of cell wall integrity that is distinct from its cell cycle function related to Sic1 degradation. Nevertheless, the importance of Sic1 regulation in maintaining cell growth does become apparent when *SIC1* is deleted, as *cdc34-2 sic1Δ* and *cdc53-1 sic1Δ* cells have greater defects, emphasizing the importance of a proper coordination between cell cycle progression and cell wall synthesis.

It is logical that a relationship exists between factors that control cell integrity and cell division, given the extensive cell wall remodeling and synthesis that occurs throughout the cell cycle. This increased level of cell wall metabolism coincides with the formation of a nascent bud and subsequent polarized bud growth. Under these conditions, the synthesis of proteins engaged in the assembly of cell wall components is induced and these proteins, along with required materials, are directed toward the emerging bud in a controlled fashion (CABIB *et al.* 1998). This involves a restructuring of the actin cytoskeleton and includes the concerted action of many pathways. Several cell cycle regulators (*e.g.*, Sic1, Cln2, and Cdc6) as well as effectors of actin cytoskeleton remodeling (*e.g.*, Gic2) have been identified as Cdc34/

SCF substrates. Therefore, a strategy linking these processes by a common regulator, such as the Cdc34/SCF complex, likely exists.

Several results suggest that cell integrity defects seen in the *cdc53-1* and *cdc34-2* mutants arise from the misregulation of Rho1 function. This conclusion is supported by the observations that the levels of activated GTP-bound Rho1 are reduced in these mutants and that genetic interactions exist between genes encoding the Cdc34/SCF complex and the Rho1-GAPs *Lrg1* and *Sac7*. Inhibition of Rho1 activity via overexpression of these negative regulators is severely toxic to the *cdc53-1* and *cdc34-2* mutants, demonstrating the importance of properly controlled Rho1 activity in these cells. Interestingly, these GAPs affect the Cdc34/SCF mutants to different degrees, with overexpression of *LRG1* causing a stronger growth defect as compared to *SAC7*. Although both GAPs negatively affect Rho1 activity, they have been shown to affect distinct roles for Rho1. For example, *Lrg1* has been observed to regulate the 1,3-β-glucan synthesis activity of Rho1, whereas *Sac7* predominately regulates the actin organization and MAPK-regulating functions of Rho1. This suggests that the decrease in active GTP-Rho1 levels that is observed in the Cdc34/SCF mutants likely exists in the pool of Rho1 that is regulated by *Lrg1*, possibly functioning to regulate 1,3-β-glucan synthesis. This possibility is supported by the elevated expression of *FKS2*, a gene encoding a component of the 1,3-β-glucan synthesis enzyme, in the *cdc34-2* and *cdc53-1* mutants. Furthermore, deletion of *LRG1* suppresses both growth and cell integrity defects associated with *cdc53-1* and *cdc34-2* mutants. This suppressive effect is specific to cell integrity defects, given that at elevated temperatures *cdc34-2 lrg1Δ* and *cdc53-1 lrg1Δ* retain an elongated multi-budded morphology associated with a defect in Sic1 degradation.

Our observations indicate that the balance and specificity of Rho1 activity is crucial in Cdc34/SCF mutants. This is evident when *SAC7* is deleted, which likely shifts the active pool of GTP-Rho1 from its role in 1,3-β-glucan synthesis toward its roles in actin polymerization. This results in severe cell integrity defects, particularly with respect to SDS sensitivity. It has been previously reported that disturbing the cell wall with low amounts of SDS results in an increased GDP/GTP exchange activity of Rho1 (BICKLE *et al.* 1998), a similar effect to the deletion of *SAC7*. As such, deletion of *SAC7* combined with the presence of SDS likely leads to increased Rho1 activity in a certain function that the *cdc53-1* and *cdc34-2* mutants are not capable of coping with, therefore resulting in the severe defects observed. *Sac7* has been shown to have an important role in actin polymerization and Pkc1-Slt2 pathway activation, suggesting that the proper regulation of these activities is essential for maintaining cell viability in the *cdc53-1* and *cdc34-2* mutants.

The analysis of signaling via the Slt2 pathway confirms that defects in Slt2 phosphorylation do exist in these

mutants and suggests that the cell wall integrity defects observed in these cells are, in part, a consequence of defective signaling through this pathway. The misregulation of Slt2 phosphorylation is far more pronounced in the *cdc53-1* mutant than in the *cdc34-2* mutant, correlating with the extreme sensitivity that the *cdc53-1* mutant displays toward SDS and to its more reduced levels of GTP-bound Rho1. The importance of Slt2 pathway activity in these mutants is highlighted by the fact that the deletion of *BCK1* or *SLT2* results in severe lysis defects. These defects are likely due to cell wall disruption, as they can be suppressed by the presence of 1 M sorbitol. This indicates that the expression of downstream target genes of the Slt2 pathway is important for maintaining the integrity of the cell wall in these mutants and is consistent with the enrichment of cell wall biogenesis genes observed in our microarray profiling. However, the defects observed in the Cdc34/SCF mutants are not solely due to the misregulation of the Slt2 pathway, since other cell integrity defects are observed, such as SDS sensitivity. A substantial overlap (see supplemental Figure 1 at <http://www.genetics.org/supplemental/>) of gene regulation in *cdc53-1* and *cdc34-2* mutants as compared to hyperactive *rho1* and *pkc1* mutants lends further support to the idea that the misregulation of Slt2 signaling may occur as a consequence of upstream defects at the level of Rho1 (ROBERTS *et al.* 2000). In fact several clusters of similarly regulated genes are evident between these mutants, including a cluster of genes enriched for mediators of cell wall organization and biogenesis ( $P = 5.66e-09$ ). Interestingly, similar clusters are also observed when the data sets from the *cdc53-1* and *cdc34-2* mutants are compared with those from cells that have undergone cell wall damage either from mutations in key cell wall regulators (LAGORCE *et al.* 2003) or from cell-wall-disrupting reagents (GARCIA *et al.* 2004) (see supplemental Figure 1 at <http://www.genetics.org/supplemental/>). This correlation suggests that a large number of genes involved in the regulation of cell wall organization and biosynthesis may be affected in the *cdc53-1* and *cdc34-2* mutants (more than only the nine genes that met our cutoff of being induced by twofold or greater), further supporting our observations that the Cdc34/SCF complex mediates aspects of yeast cell wall integrity.

As the Cdc34/SCF complex functions to ubiquitinate proteins, its cell integrity function likely reflects this activity. Candidate targets include negative regulators of cell wall synthesis or regulators Rho1 activity. An appealing possibility is that the direct regulation of the Rho1-GAPs (Figure 6), particularly Lrg1, is influenced by Cdc34/SCF-dependent ubiquitination and subsequent degradation. This would provide a logical explanation of our data since Rho1-GAPs have the potential to downregulate both the MAPK cascade and cell wall synthesis. Alternatively, Rho1 itself may be targeted for ubiquitination by the Cdc34/SCF complex

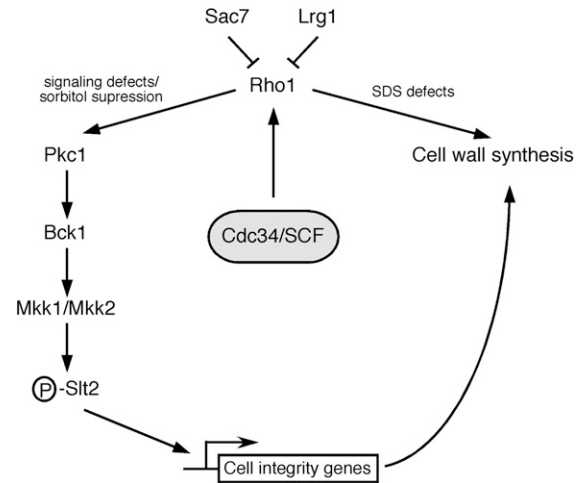


FIGURE 6.—Model for the role of the Cdc34/SCF complex in the maintenance of cell wall integrity. Depicted is our model for the role of the Cdc34/SCF complex in the regulation of cell wall integrity. In this model, the Cdc34/SCF complex regulates Rho1 function, thereby affecting several pathways, including the Slt2 signaling pathway as well as 1,3- $\beta$ -glucan synthesis. This would result in transcriptional variation of genes required for cell wall biogenesis and organization. Several potential Cdc34/SCF targets exist, which could include Rho1 or negative regulators of Rho1 such as the Rho1-GAPs.

in a context-dependent manner. Supporting this possibility, Rho1 has been identified as a ubiquitinated protein in yeast (PENG *et al.* 2003). Furthermore, its mammalian homolog, RhoA, is ubiquitinated and targeted for degradation at distinct subcellular locations (WANG *et al.* 2003a).

Taken together, these observations identify a novel role for the Cdc34/SCF ubiquitination complex in the mediation of yeast cell integrity. Future studies not only should identify specific cell integrity targets, but also should provide insight into the extent to which the cell integrity functions of Cdc34/SCF overlap or are coordinated with its role in cell cycle regulation. Furthermore, signaling from the yeast cell wall and the role of the cell integrity MAPK pathway in mediating cell proliferation show similarities with pathways involved in signaling from the extracellular matrix of mammalian cells. Thus, it will be of interest to determine if mammalian homologs of the yeast Cdc34/SCF complex regulate signaling from the extracellular matrix in a similar fashion.

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