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The transcription factor Rap1p is required for tolerance to cell-wall perturbing agents and for cell-wall maintenance in *Saccharomyces cerevisiae*

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ABSTRACT

Yeast repressor activator protein (Rap1p) is involved in genomic stability and transcriptional regulation. We explored the function of Rap1p in yeast physiology using Rap1p truncation mutants. Our results revealed that the N-terminal truncation of Rap1p (Rap1ΔN) leads to hypersensitivity towards elevated temperature and cell-wall perturbing agents. Cell wall analysis showed an increase in the chitin and glucan content in Rap1ΔN cells as compared with wild type cells. Accordingly, mutant cells had a twofold thicker cell wall, as observed by electron microscopy. Furthermore, Rap1ΔN cells had increased levels of phosphorylated Slt2p, a MAP kinase of the cell wall integrity pathway. Mutant cells also had elevated levels of cell wall integrity response transcripts. Taken together, our findings suggest a connection between Rap1p and cell wall homeostasis.

1. Introduction

The yeast cell wall is an essential structure that protects the cell from lysis during budding, mating, and environmental stress. It is composed of a complex lattice of proteins and carbohydrates that are deposited onto the plasma membrane. The cell wall is a layered structure consisting of an electron-transparent inner layer and an electron-dense outer layer [1]. The inner layer is comprised of glucan polymers and chitin (N-acetylglucosamine polymers) that is largely responsible for the mechanical strength and elasticity of the cell wall [2]. The outer layer is a lattice of highly glycosylated mannoproteins that protects the inner layer from cell wall-degrading enzymes [3,4].

Because the cell wall is important for survival, stress conditions that alter this structure lead to activation of cell wall integrity (CWI) signaling. Activation of mitogen activated protein kinase (MAPK) typically leads to activation of the downstream transcription factors and subsequent changes in gene expression. The MAPK cascade is a linear pathway comprised of a family of cell-surface sensors and downstream factors such as Pkc1, a MAPK kinase (Bck1p), a pair of redundant, MAPK kinases (Mkk1/2p), and a MAP kinase (Mpk1p/Slt2p) [5,6]. Under cell wall stress conditions, the MAP kinase cascade is activated through phosphorylation of Slt2p, which is required for the regulation of cell wall damage response genes. In fact, the CWI signaling pathway is activated in response to treatment with cell wall-perturbing agents, such as Congo red (CR), calcofluor white (CFW), caffeine, and zymolyase [7–9], as well as mutations that weaken the cell wall, such as *fks1*Δ or *gas1*Δ [8]. Activation of the CWI pathway protects yeast cells from cell wall stress by inducing a well-characterized transcriptional program [10–12], which includes genes related to metabolism, cell wall remodeling, and signaling pathways.

The *Saccharomyces cerevisiae* protein Rap1 plays multiple roles in genome stability and transcriptional regulation. Yeast Rap1p is encoded by a single-copy essential gene [13]. It is a DNA-binding protein that represses transcription at the silent mating-type loci and telomeres. Rap1p also participates in transcriptional repression, telomere length modulation, and chromatin barrier functions [14–19]. Moreover, Rap1p has been shown to act as a key transactivator of over 300 co-regulated genes, including genes encoding ribosomal proteins and glycolytic enzymes [20–22]. Structurally, Rap1p contains 827 amino acids, and it can be divided into three regions, a central DNA-binding domain plus functional N-terminal

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and C-terminal domains of approximately equal size. The N-terminal domain of Rap1p is non-essential and shares similarity with a BRCA1 C-terminal (BRCT) domain known to be involved in the DNA repair process [23]. The C-terminus of Rap1p is the protein–protein interaction domain that is also required for transcriptional activation and repression [24–27]. The functional role of Rap1p in the regulation of transcription remains elusive, mainly because Rap1p mutations that abolish DNA binding are lethal [28], and Rap1p overexpression is toxic [29].

In an attempt to characterize the role of Rap1p in yeast physiology, we generated truncation mutants of Rap1p. Our results demonstrated that the N-terminal domain of Rap1p is required for cell wall homeostasis. An N-terminal deletion mutant of Rap1p (Rap1ΔN) displayed hypersensitivity to cell wall-perturbing agents and altered the cell wall structure and composition. We also detected the phosphorylated form of Slt2p in the Rap1ΔN mutant. The results provide evidence of a new role for Rap1p in the regulation of yeast cell wall integrity.

2. Materials and methods

2.1. Strains, chemicals, and growth media

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich. The *S. cerevisiae* strains used in this study were gifts from Prof. J. C. Reese. The background of strains are as follows: wild type (W303; rap1::LEU2; Ycp50-RAP1), Rap1ΔC (W303; rap1::LEU2; rap1–8, rap1 1–716 [HIS3/CEN]), Rap1ΔN (W303; rap1::LEU2; rap1–6, rap1 Δ43–279 [HIS3/CEN]) and W303; MAT alpha; ade2-11; his3-11,15; leu2-3,112; ura3-1; trp1-1; can1-100. To make synthetic complete (SC) medium containing all amino acids, yeast nitrogen base and ammonium sulfate were mixed together following the standard protocol [30,31]. Yeast cells were grown in SC medium containing 2% dextrose (SCD) at different temperatures as described in the text. To make solid agar plates, 2% agar was added to SCD.

2.2. Growth sensitivity assay

The sensitivity of yeast cells to different cell wall-perturbing agents, such as calcofluor white (CFW), Congo red (CR), and caspofungin (CASP) was analyzed by performing spot tests as previously described [32,33]. Briefly, wild-type and *RAP1* mutant cells were grown to saturation (overnight) in SCD medium at 24 °C, and then tenfold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were made in sterile double-distilled water. Aliquots of these dilutions (3 μL) were then spotted on SCD plates without or with various concentrations of the cell wall-perturbing agents. To analyze the effects of osmolyte, 1 M sorbitol was added to the SCA plates (SCD + 2% Agar) along with the highest concentrations of CFW, CR, and CASP. All plates were incubated at 24 °C, and the growth of the yeast strains at 48 h was recorded by scanning the plates with an HP scanner.

2.3. Measurement of cellular DNA content in yeast cells by FACS analysis

The yeast cells in the exponential phase were harvested, and ethanol was added to the cell pellets as described previously [34,35]. The pellets were then resuspended by vigorous vortexing. The cells were washed once with 50 mM sodium citrate buffer (pH 7.0) and sonicated to remove cell clumps. RNase A was added to the samples and incubated at 37 °C for 1 h. RNase A-treated samples were transferred to BD FACS flow (Becton Dickinson) containing 20 mg/ml propidium iodide. Cellular DNA was detected with a BD FACS Aria III with FACS Diva software (Becton Dickinson).

2.4. Measurement of cell viability and chitin and glucan contents in yeast cells by FACS analysis

2.4.1. Cell viability

Wild-type and *RAP1* mutant cells were grown at 24 °C until they reached the exponential phase. Half of the cell cultures were shifted to an elevated temperature (37 °C) and incubated for 6 h. Aliquots of the cells (1 ml) incubated at both 24 °C and 37 °C were harvested, washed with ice-cold phosphate buffer saline (PBS; pH 7.4), and then resuspended in 1 ml of PBS containing propidium iodide (100 μg/ml). The PI-treated samples were immediately transferred to FACS flow, and PI staining was detected with a FACS Aria III with CELL Quest software (Becton Dickinson). As a positive control, cells were heat killed (70 °C/10 min) and processed for FACS analysis as described above.

2.4.2. Chitin and glucan content

To measure the chitin and glucan content we used fluorescent dye that stains chitin (CFW) and glucan (Aniline Blue) of the yeast cell wall. The method to measure the chitin and glucan through FACS were adopted from previous literature with slight modifications [36–40]. Briefly, exponentially growing cells were washed with PBS (pH 7.4) and incubated for 5 min in 0.01% CFW or 0.5% aniline blue, respectively. Treated samples were immediately transferred to FACS flow, and fluorescence was detected by using a BD FACS Aria III equipped with CELL Quest software using DAPI filters.

2.5. Western blot analysis

Whole cell extracts were prepared using the TCA extraction method [41]. For immunoblotting, extracts were transferred onto PVDF membranes with a mini wet transfer apparatus (Bio-Rad). The membrane was blocked with blocking buffer (2.5% BSA) for 45 min and then incubated with the primary Slt2p-P/Phospho-p42/44 MAPK antibody (# 4370, 1:2000; Cell Signaling), anti-Tbp (Polyclonal antibodies against recombinant yeast TBP was raised in rabbit) and then the IRDyeH 800CW anti-rabbit IgG secondary antibody (1:10000) for 1 h each. Blots were scanned with an Odyssey Infrared imager (LI-CORH Biosciences).

2.6. RNA isolation and real-time PCR

The exponentially growing wild-type and *RAP1* mutant cells were harvested. Total RNA was extracted by the heat/freeze phenol method as previously described [42]. Total RNA (1 μg) was reverse transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (Bio-Rad) according to the manufacturer's instructions. Real-time PCR experiments were performed by using SYBR Green mix (Roche diagnostics) in an ABI real-time PCR instrument. Melting curve analysis was performed for each primer pair, and the relative changes in mRNA levels between the control and treated groups were calculated by using the $2^{-\Delta\Delta CT}$ method. Target gene mRNA levels were normalized to the level of *ACT1* mRNA (the internal control). The primers used in this study are listed in Table 1.

2.7. Transmission electron microscopy (TEM) of yeast cells

TEM of yeast cells were performed following standard method as described previously [43]. Briefly, 30 OD_{600} units of exponentially growing cells (equivalent to 30 ml of culture at an OD_{600} of 1.0) were harvested, sonicated to remove cell clumping and prefixed with 3 × FIX (6% glutaraldehyde, 300 mM Pipes-HCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, and 600 mM sorbitol, pH 6.8) for 5 min at room temperature. Cells were processed for TEM as described earlier [43] and cells were finally resuspended in fresh Epon812, which

Table 1
List of primers used in quantitative real-time PCR.

Gene name	Primer (5′–3′)
ACT1	Forward Primer_CCTTCTGTTTTGGGTTTGGA Reverse Primer_CGGTGATTTCCTTTTGCATT
SLT2	Forward Primer_CCAAAACAGATGGCCACGTC Reverse Primer_GGTCTAGAAGCGTGCCGTTA
CRG1	Forward Primer_GGACTTTCCCGAAGCCTTGA Reverse Primer_GCATTTGGGTCCGAAGGACT
SRL3	Forward Primer_CCACAGCGGTTATCGAGGAA Reverse Primer_ATCTAACGAGTGACAGCGCC
YLR194C	Forward Primer_GCCGCTGCTCAAAAAGACTC Reverse Primer_GGAGCCACAGCATAGGTGTT
KTR2	Forward Primer_AAAGCAACGCTTTCGGGTTC Reverse Primer_GTGCATCTCCCCACCTTTCA
YLR040C	Forward Primer_TCCTTTCTCGACAGCGAACG Reverse Primer_GGGCAACTGCAAAGATTCCG
PIR3	Forward Primer_AGACGCTAACGATCCAGTCG Reverse Primer_TGGACCAACCAGCAGCATAG
BAG7	Forward Primer_ACGTGCACTTTCTGACTCCA Reverse Primer_CTGCTGTTTCCAACGTTCCC
YLR042C	Forward Primer_TTCCAAGCACCACCTCTTCC Reverse Primer_TCATTTCTCGACGAAGAAGGCA
KDX1	Forward Primer_TAAGGTCCGAACAACGCCTG Reverse Primer_AGCCGTCGTTAACCTTGTGG

was polymerized at 60 °C for 72 h. Sections (70-nm thick) were cut with a diamond knife on an ultramicrotome, transferred to single slot grids, stained with 2% uranyl acetate and lead citrate, and examined with a Zeiss TEM 902A electron microscope at 50 kV.

3. Results

3.1. The N-terminal domain of Rap1p is essential for heat stress survival

To determine the role of Rap1p in yeast cell physiology, we generated truncation mutants of Rap1p as shown in Fig. 1A. The Rap1ΔN and Rap1ΔC mutants and the wild-type strain were exposed to different forms of stress (data not shown). Interestingly, we noticed that the Rap1ΔN mutant failed to grow at elevated temperature (37 °C), whereas Rap1ΔC and wild-type cells grew normally at 37 °C (Fig. 1B). This result motivated us to determine the reason for the observed heat sensitivity of the Rap1ΔN mutant. Next, we examined the effect of elevated temperature (37 °C) on the cell viability of Rap1ΔN. After exposure to heat stress (37 °C) for 6 h, cell viability was assessed by propidium iodide (PI) staining and FACS analysis (Fig. 1C). Our results showed that a much higher number of PI-stained cells were observed in Rap1ΔN cell cultures incubated at the elevated temperature, suggesting that this mutant had lower viability at high temperature. These results indicate a novel function for the N-terminal domain of Rap1p in cell survival under heat stress.

3.2. Rap1ΔN showed a cell aggregation phenotype

While attempting to understand the reason for the reduction in cell viability of the Rap1ΔN mutant at elevated temperature, we noticed that this mutant also exhibited an abnormal cell cycle distribution. Wild-type and *RAP1* mutant cells were grown until they

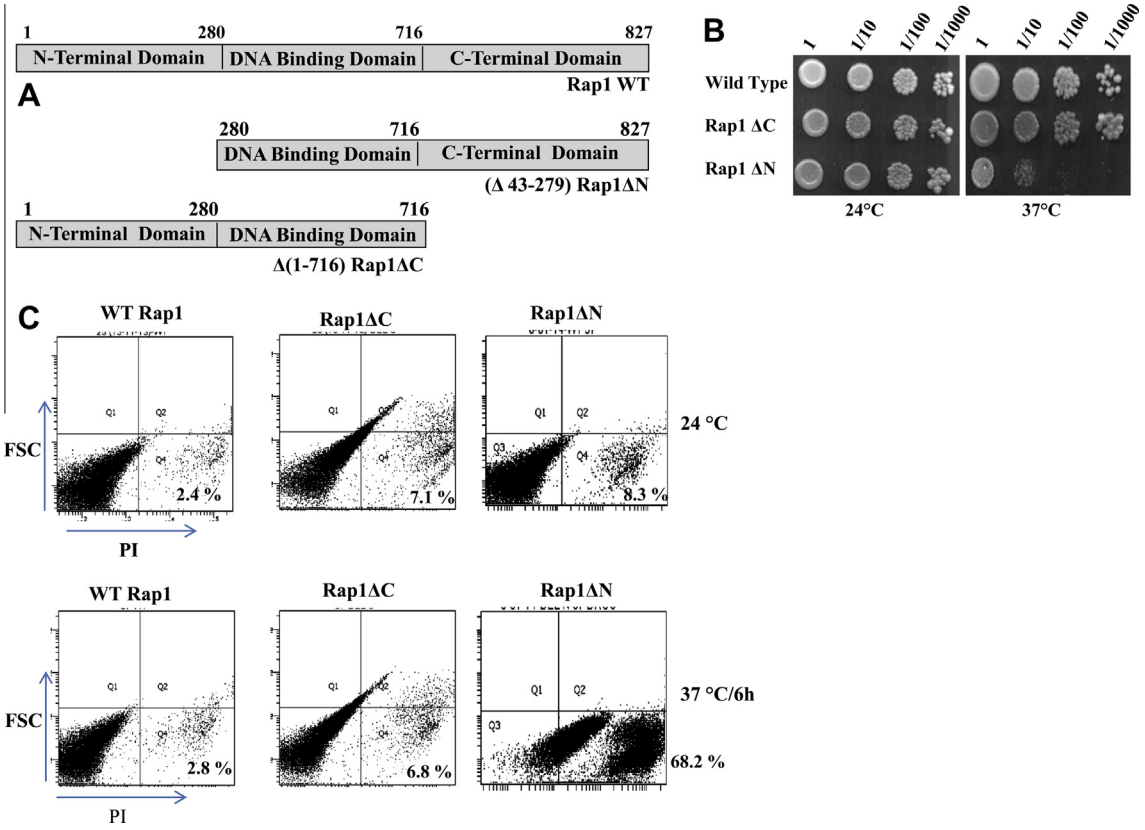

Fig. 1. Rap1p is required for tolerance to heat stress in yeast. (A) A schematic diagram showing the wild-type and Rap1p mutant strains used in this study. (B) The wild-type and *RAP1* mutant strains were grown to saturation, and then serially diluted (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) in 1.0 ml of sterile, double-distilled water. Aliquots of each dilution (3 μL) were spotted on SCA plates and exposed to heat stress (37 °C). After 2–3 days, the growth of the yeast strains was recorded by scanning the plates using an HP scanner. (C) The viability of wild-type and mutant cells under heat stress as determined by PI staining and FACS analysis. Exponential phase cells were exposed to the elevated temperature for 6 h. Small aliquots of cells were processed for FACS analysis. The values written in the graph shows the percentage of cells present in the quadrant 4. Heat-killed cells were used as a control and FACS showed that more than 97% cells were in quadrant 4.

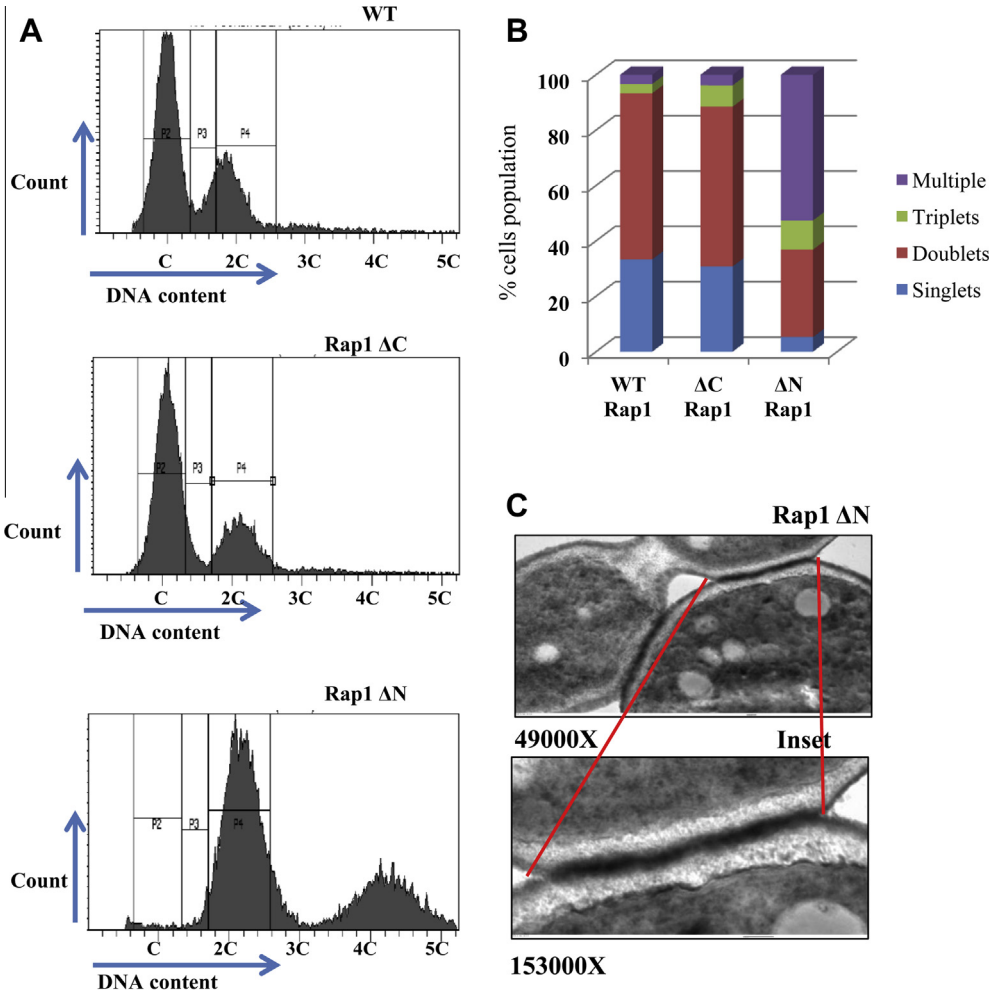

Fig. 2. Truncation of the Rap1p N-terminal domain leads to a cell aggregation phenotype. (A) Exponential phase cells were processed for cell cycle analysis by FACS. The amount of DNA was denoted as C, 2C, 3C, etc. as shown in the figure. (B) DIC images of *RAP1* mutants were taken, and the cells were counted to show the single, double, triple, or multiple arrangements, and the data were plotted in the form of a bar diagram. The error bars represent the SD of three independent experiments. At least 100 cells were counted in each experiment. (C) Rap1ΔN cells were grown to saturation (at 24 °C) and then processed for TEM as described in the Materials and methods. Representative TEM image showing the adherence phenotype of Rap1ΔN cells.

reached the exponential phase, and then the cell cycle distribution was analyzed by FACS analysis as described in the Materials and methods. Our results showed that wild-type and Rap1ΔC cells have similar cell cycle distributions profile (Fig. 2A), and most cells have DNA content values of C or 2C. In contrast, Rap1ΔN cells exhibited abnormal cell cycle profile and FACS revealed that the DNA content values ranges from 2C, 3C, 4C, and 5C (Fig. 2A), possibly by sticking of multiple cells together. These results were further confirmed by visualizing the cells under a microscope. The DIC images revealed that while wild-type and Rap1ΔC cells exhibited normal cell separation, Rap1ΔN cells adhered to each other (Supplementary Fig. 1). Additionally, cell morphology observations revealed that most Rap1ΔN mutant cells (more than 50% of the total number of cells) showed a multiple-cell arrangement with more than three cells attached to each other, while wild-type and Rap1ΔC cells did not exhibit this abnormal phenotype (Fig. 2B). To confirm these results, we performed electron microscopy (EM) to visualize the morphological defects of Rap1ΔN cells. Our EM images of Rap1ΔN cells showed that the cells were adhered to each other (Fig. 2C, magnified image in inset shows two cells adhered to each other). These observations suggest that Rap1ΔN mutant cells exhibit an aggregation phenotype.

3.3. Rap1ΔN displayed hypersensitivity to cell wall-perturbing agents

Our phenotypic observations revealed that Rap1ΔN cells are sensitive to elevated temperature and show morphological defects. Similar phenotypes (temperature sensitivity and cell wall aberrations) are also exhibited by cell wall mutants. Mutants with defective cell wall structures also exhibit sensitivity to cell wall-perturbing agents. To test the possibility that the Rap1ΔN mutant might have a cell wall defect, we analyzed the sensitivity of *RAP1* mutants to known cell wall perturbing agents, including CFW, CR, and CASP. These cell wall-perturbing agents are a powerful tool to identify mutants with defective cell walls [15]. Wild-type and *RAP1* mutant strains were grown till saturation and tenfold serial dilutions were spotted onto SCA plates containing increasing concentrations of cell wall-perturbing agents. Interestingly, Rap1ΔN exhibited hypersensitivity to CFW, CR and CASP (Fig. 3), suggesting that this mutant has a defect in its cell wall. Moreover, in the presence of 1 M sorbitol, an osmotic stabilizer, its sensitivity to these agents was significantly reduced (Fig. 3), suggesting that the sensitivity was due to a defective cell wall. Next, we determined whether osmotic stabilization could rescue the temperature-sensitive phenotype of the Rap1ΔN mutant. Wild-type and *RAP1* mutant

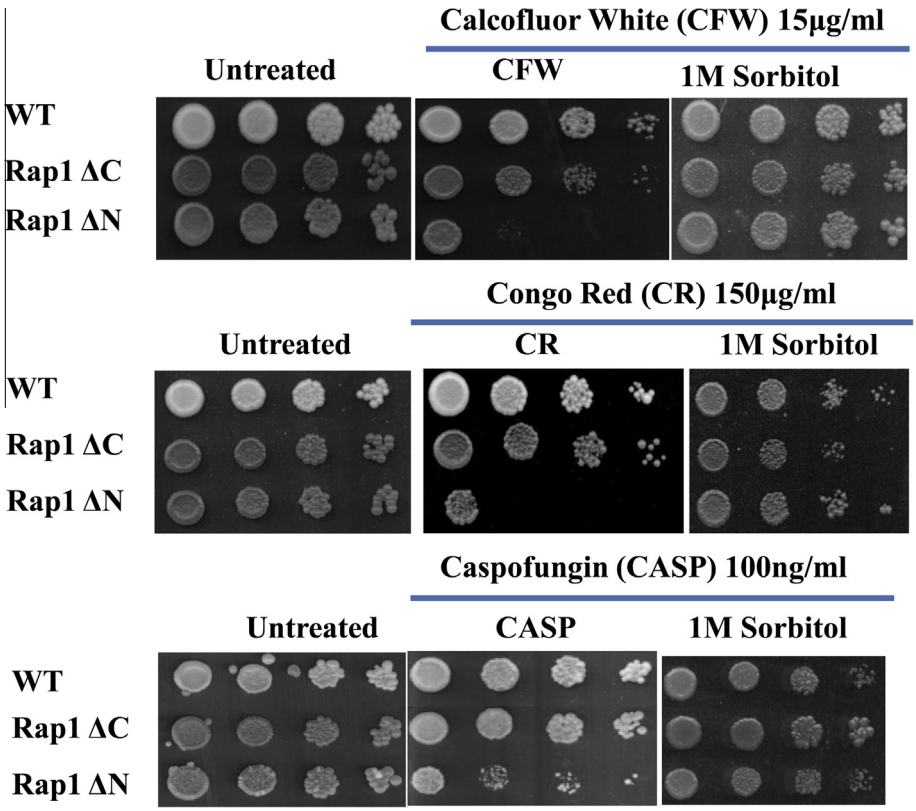

Fig. 3. Effect of cell wall-perturbing agents on *RAP1* mutants. The wild-type and *RAP1* mutant strains were grown to saturation and serially diluted (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) in 1.0 ml of sterile double-distilled water. Aliquots of each dilution (3 µL) were spotted on SCA plates, SCA plates impregnated with various concentrations of cell wall-perturbing agents (Calcofluor white [CFW], Congo red [CR], caspofungin [CASP], and SCA plates containing the above-mentioned cell wall-perturbing agents and 1 M sorbitol (an osmolyte).

strains were grown till saturation and then tenfold serial dilutions were spotted onto SCA plates with or without 1 M sorbitol. The plates were incubated at a high temperature (37 °C) for 2–3 d. Our results showed that stabilization of the cell by the increased osmotic support restored the growth of Rap1ΔN at 37 °C (Supplementary Fig. 2A), suggesting that the cell wall defect is the main reason behind the failure of the Rap1ΔN mutant to grow at elevated temperature (37 °C). Since the Rap1ΔN mutant also displayed a cell aggregation phenotype (Fig 2), we next determined whether supplementation with the osmotic stabilizer could also rescue this phenotype. Wild-type and *RAP1* mutant strains were grown to exponential phase, and then 1 M sorbitol was added. Cells were grown for another 12 h, and then samples were prepared for FACS analysis as described in the materials and methods to check for cell aggregation. Our results showed that sorbitol supplementation failed to rescue the cell aggregation displayed by the Rap1ΔN mutant (Supplementary Fig. 2B).

3.4. The N-terminal truncation of Rap1p alters cell wall composition and structure

To investigate the possible defect in the cell wall of the Rap1ΔN mutant in more detail, we measured two important constituents of the cell wall, chitin and glucan, by FACS. Our results showed higher chitin (approx. 40%) and glucan (approx. 60%) fluorescence in Rap1ΔN than in wild-type and Rap1ΔC cells (Fig. 4A and B). We also performed fluorescence microscopy after staining cells with CFW and our results revealed that Rap1ΔN exhibited enhanced fluorescence (Supplementary Fig. 3) compared to wild type and Rap1ΔC that are consistent with FACS results. These results suggest that the Rap1ΔN mutant has high carbohydrate content in its cell wall. This phenotype, in which increased chitin deposition compensates for the weakened cell wall [44], is often observed in cell wall mutants. To confirm our observation, we analyzed the ultrastructure of cell wall by transmission electron microscopy (TEM). Both the wild-type and *RAP1* mutant strains exhibited characteristic bilayered cell walls with a translucent carbohydrate region sandwiched between two electron-dense mannoprotein layers (Fig. 4C). We measured the cell wall thickness as described previously [45] and our results revealed that the Rap1ΔN strain showed a thicker cell wall (approx. 200 nm) than that of the wild type or Rap1ΔC strain (approx. 100 nm), which is may be due to the accumulation of chitin and glucan in Rap1ΔN cells (Fig. 4C and D). Our observations of cell wall structure and composition revealed that the Rap1ΔN mutant has a thicker cell wall.

3.5. The MAP kinase Slt2p remains activated in Rap1ΔN

Cell wall damage has been shown to trigger activation of the CWI pathway, which is responsible for the maintenance of cell integrity. CWI pathway activation leads to phosphorylation of Slt2p, the MAP kinase that mediates this signaling cascade [46]. Since the Rap1ΔN mutant showed a cell wall defect, we next tested for activation of the CWI pathway by Western blotting with a phospho-p44/p42 MAP kinase antibody that recognizes the activated form of Slt2p [47]. To investigate the CWI pathway, all strains were grown at 24 °C, a temperature at which only a basal level of Slt2p activation is usually detected in a wild-type strain [47]. Under these conditions, the Rap1ΔC mutant was indistinguishable from the wild-type strain; however, the Rap1ΔN mutant strain showed a fivefold increase in the levels of dual-phosphorylated Slt2p compared to the levels in the wild-type strain (Fig. 5A and B). The

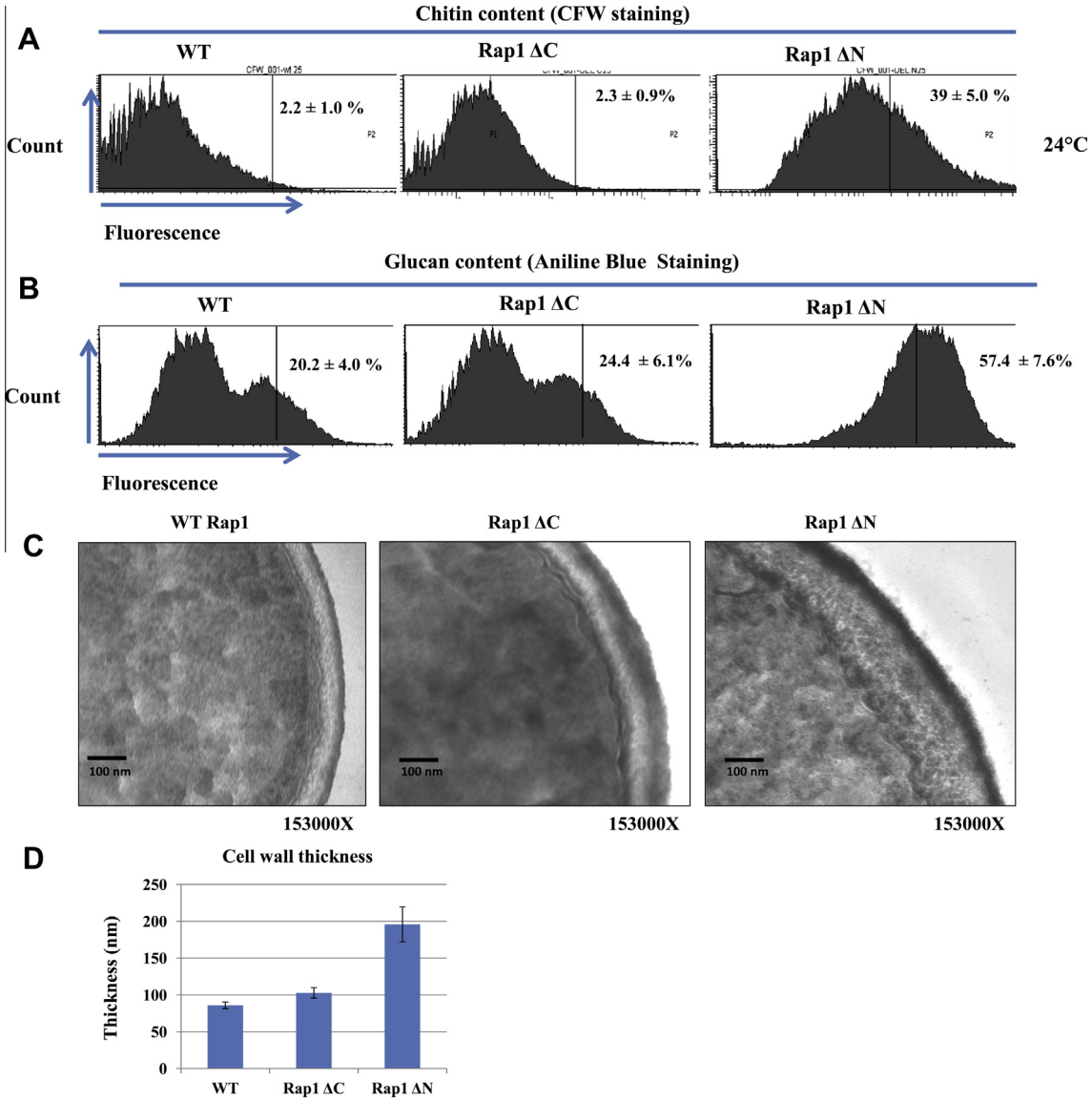

Fig. 4. Analysis of cell wall composition and structure. Rap1 mutants were grown to exponential phase in SC medium. The fluorescence of (A) CFW (chitin content) and (B) Aniline blue (glucan) was analyzed by using FACS with the appropriate filters. The experiments were repeated at least three times and representative images are shown. (C) The cell wall ultrastructure of wild-type, Rap1ΔC, and Rap1ΔN cells as observed by transmission electron microscopy. Bar = 100 nm. (D) The thickness of the cell wall was measured, and the data obtained are shown as a bar diagram. Error bars represent the SD of three independent experiments. The cell walls of at least 20 cells were measured in each experiment.

gas1Δ mutant, which was used as a control for Slt2p activation, showed a tenfold increase in the levels of dual-phosphorylated Slt2p (Fig. 5A and B). We have also analyzed the Slt2p-phosphorylation upon cell wall stress condition and observed increased levels in wild type or Rap1 mutants (Supplementary Fig. 4).

To ensure that the increase in Slt2p phosphorylation was not the result of a possible increase in *SLT2* mRNA levels, we measured the expression of *SLT2* mRNA. Quantitative PCR revealed that *SLT2* mRNA expression was similar in all strains (Table 2). Since Slt2p was phosphorylated in Rap1ΔN cells, we next analyzed a few representative genes whose expression is dependent on Slt2p phosphorylation, *CRG1*, *SRL3*, and *YLR194C*. The wild-type and *RAP1* mutant strains were grown to exponential phase (at 24 °C), and then total RNA was extracted and reverse-transcribed to cDNA. Then, the expression of *CRG1*, *SRL3*, and *YLR194C* was analyzed using real-time PCR. Transcript levels were normalized to the reference gene *ACT1*. We observed a consistent increases in the expression of all three genes in the Rap1ΔN mutant compared to their levels in wild-type or Rap1ΔC cells, indicating that in Rap1ΔN, Slt2p is not only phosphorylated but it also upregulates downstream genes (Table 2). These observations prompted us to analyze the expression of additional genes that are known to be involved in the maintenance of CWI integrity. We analyzed the expression of genes [48], such as *KTR2*, *YLR040C*, *YLR042C*, *PIR3*, *BAG7*, and *KDX1*, which are known to be overexpressed in response to cell wall perturbation. The results showed that these genes were expressed at higher levels in Rap1ΔN cells than in wild-type cells. These results further substantiate our conclusion that in yeast, Rap1p is involved in the maintenance of CWI.

4. Discussion

When exposed to environmental stress, yeast cells respond by activating conserved MAPK pathways that rapidly induce the transcription of stress-responsive genes. Rap1p is one of the most abundant and ubiquitous transcription factors in yeast. Most of

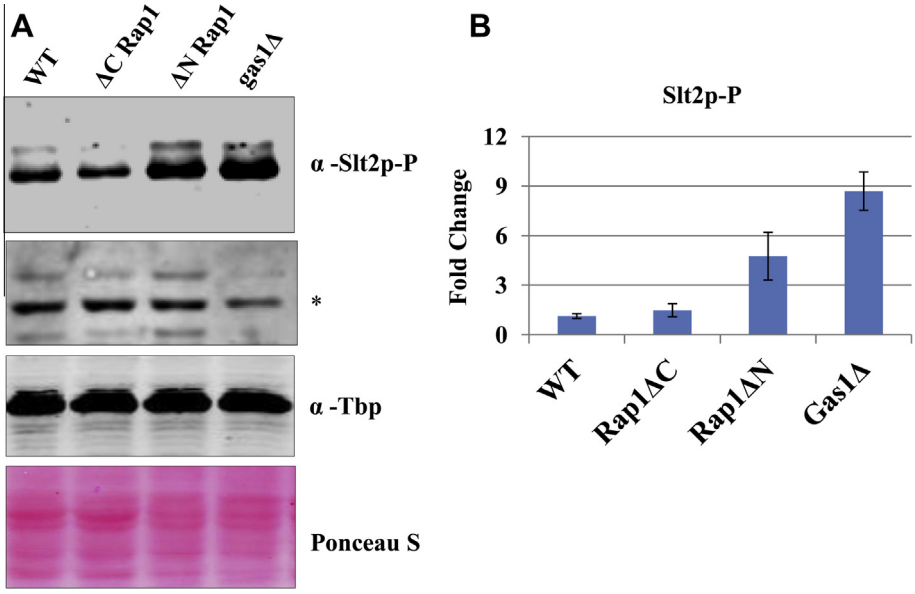

Fig. 5. Activation of the MAP kinase Slt2p (A) *RAP1* mutants were grown to mid-exponential phase (at 24 °C), and then cell extracts were fractionated and analyzed by Western blotting using anti-phospho-p44/p42 MAPK antibodies to detect dually phosphorylated Slt2p. The asterisk indicates a non-specific band detected by the anti-phospho-p44/p42 MAPK antibody. The Tbp Western blotting was used as a loading control along with the Ponceau S staining of the representative blot. (B) The intensity of phosphorylated Slt2p was quantified using Image J software, and the data are presented as a bar diagram. The intensity of the non-specific band detected by the phospho-p44/p42 MAPK antibody was used for normalization. The error bars represent the SD of three independent experiments.

its functions are related to the control of chromatin structure. Here, we showed that Rap1p, a key regulator of more than 300 genes, also plays a role in the maintenance of CWI.

A previous in vivo functional analysis demonstrated that the C terminus of Rap1 is not essential for vegetative growth even though it is the main target for all protein–protein interactions [49–51]. The C terminus contains the transcriptional activation domain [52], and deletion of the C terminus results in slow growth. The N terminus of Rap1 is a large region that is not essential for cell viability [53]; however, it may regulate its activity through a putative BRCT domain. The function of the putative BRCT domain is unclear; however, it has been proposed that it may be involved in bending the DNA immediately flanking the Rap1p recognition site [54]. Here, we provide evidence that the Rap1p N-terminal domain has an expected function that is required to ensure CWI. Our results showed that deletion of the Rap1p N-terminal domain causes hypersensitivity to various cell wall-perturbing agents, including CFW, CR and CASP. Increased susceptibility to these agents has been used to identify cell wall-defective mutants [55–57]. Consistent with these studies, the hypersensitivity to cell wall-perturbing agents exhibited by Rap1ΔN is indicative of a defective cell wall. These results also indicated that Rap1p is required for the yeast cell wall to function against cell wall-perturbing agents. Furthermore, osmotic stabilization of the cell in the presence of a cell wall-perturbing agent can rescue the growth inhibitory defect of Rap1ΔN. We also found that due to the accumulation of chitin and glucan in the Rap1ΔN strain causes a two-fold thicker cell wall (200 nm) than those of the wild-type and Rap1ΔC strains (100 nm). Furthermore, studies have also shown that defects in cell wall structure and composition can also lead to cell aggregation phenotypes [58–60], which is consistent with our observation that Rap1ΔN also displayed a cell aggregation phenotype. Rap1 is known to have pronounced effect on chromatin structure [61] and one of the study revealed that proper nucleosome positioning is required for CWI signaling through Rlm1 [62]. Based on our results we can propose that the truncation of Rap1 N terminal domain might leads to alteration in chromatin structure leading to defect in CWI signaling. Furthermore, recently

Table 2
Real-time quantitative RT-PCR of some selected CWI genes.

Gene	Fold change* (Rap1 ΔN/WT)	Standard deviation
SLT2	1.166	0.216
CRG1	2.223	0.230
SRL3	1.876	0.144
YLR 194C	2.507	0.382
KTR2	2.309	0.390
PIR3	4.451	1.490
BAG7	2.870	0.557
YLR042C	6.147	1.335
KDX1	2.871	1.268

Table showing the expression of CWI genes in Rap1ΔN mutant with respect to its wild type cells.
* The fold change was normalized to ACT1 levels.

it has been shown that cell wall thickness is also regulated through glycolytic pathway [43] and Rap1 is a known activator of glycolytic enzyme genes [22], suggesting that the defect in cell wall structure observed in our study might be linked to defect in glycolytic pathway.

The CWI signaling pathway detects and responds to cell wall stresses that arise during growth or due to mutations in cell wall-related genes or harsh environmental challenges. Transcription of cell wall damage response genes is induced upon activation of the Slt2p-mediated MAP kinase cascade of the CWI pathway [10,63]. This signaling pathway is activated by various cell wall stress conditions, triggering a compensatory mechanism to ensure CWI [47]. Indeed, the Rap1ΔN strain showed increased activation of the Slt2p-mediated cascade, in agreement with the observed cell wall defect. This situation is also encountered in other mutant strains with compromised CWI, namely, *pts1*Δ, *gas1*Δ, *fks1*Δ, and *kre9*Δ [46,64]. We also observed that Rap1ΔN exhibited increased chitin and glucan content, which is consistent with previous reports showing a strong increase in chitin deposition in the cell wall of many cell wall mutants, including *gas1*Δ and *fks1*Δ [65,66], and cells stressed with CFW [7]. To obtain additional

insight into the defects in Rap1ΔN cells, we used an alternative approach: characterization of the transcriptional response of Rap1ΔN cells compared to that of wild-type cells. Our results showed that the transcript levels of genes that are known to be overexpressed upon cell wall perturbation [48], such as *KTR2*, *YLR042C*, *PIR3*, *BAG7*, *CRG1* and *KDX1*, were also upregulated in Rap1ΔN cells. Similarly, the expression of these genes was also found to be upregulated in mutants that have constitutive damage due to mutations in genes, such as *gas1*Δ, *knr4*Δ, *fks1*Δ, *kre6*Δ, and *mnn*9Δ, which are involved in the different steps of cell wall construction [67]. Together, these results clearly show that Rap1ΔN cells have a defect in its cell wall structure, which leads to the activation of the CWI pathway.

Although we have sufficient evidence to conclude that Rap1p is involved in the maintenance of cell wall homeostasis, the exact mechanism through which Rap1p induces the transcription of CWI genes remains to be elucidated. Moreover, the previous study on genome-wide Rap1 binding sites not have any CWI gene [22], but Rap1 is known to bind to several new targets during senescence [68] and low-glucose stress conditions [69]. Therefore, it is possible that under cell wall stress condition Rap1 might bind to promoters of CWI response genes which are still not known. Our study provides indirect evidence that truncation of N terminal domain of Rap1 causes defect in cell wall but the mechanism needs to be elucidated. Given our limited understanding of the role of Rap1p in cell wall homeostasis, it seems likely that there are several other factors that regulate Rap1 action. Identifying the factors that interact with the N-terminal domain of Rap1p and studying the genetic and biochemical interactions between these yet to be determined factors and known CWI-signaling factors, will provide important clues toward understanding how Rap1p functions to maintain CWI.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.11.024.

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