Published in final edited form as:
Yeast. 2007 April ; 24(4): 343–355. doi:10.1002/yea.1481.

Dosage rescue by *UBC4* restores cell wall integrity in *Saccharomyces cerevisiae* lacking the myosin type II gene *MYO1*

Nitza L. Díaz-Blanco and **José R. Rodríguez-Medina**[*]
Department of Biochemistry, School of Medicine, University of Puerto Rico, Medical Sciences Campus, PO Box 365067, San Juan, PR 00936-5067, Puerto Rico

Abstract

Myosin II is important for normal cytokinesis and cell wall maintenance in yeast cells. Myosin II-deficient (*myo1*) strains of the budding yeast *Saccharomyces cerevisiae* are hypersensitive to nikkomycin Z (NZ), a competitive inhibitor of chitin synthase III (Chs3p), a phenotype that is consistent with compromised cell wall integrity in this mutant. To explain this observation, we hypothesized that the absence of myosin type II will alter the normal levels of proteins that regulate cell wall integrity and that this deficiency can be overcome by the overexpression of their corresponding genes. We further hypothesized that such genes would restore normal (wild-type) NZ resistance. A haploid *myo1* strain was transformed with a yeast pRS316-*GAL1*-cDNA expression library and the cells were positively selected with an inhibitory dose of NZ. We found that high expression of the ubiquitin-conjugating protein cDNA, *UBC4*, restores NZ resistance to *myo1* cells. Downregulation of the cell wall stress pathway and changes in cell wall properties in these cells suggested that changes in cell wall architecture were induced by overexpression of *UBC4. UBC4*-dependent resistance to NZ in *myo1* cells was not prevented by the proteasome inhibitor *clasto*-lactacystin-*β*-lactone and required the expression of the vacuolar protein sorting gene *VPS4*, suggesting that rescue of cell wall integrity involves sorting of ubiquitinated proteins to the PVC/LE–vacuole pathway. These results point to Ubc4p as an important enzyme in the process of cell wall remodelling in *myo1* cells.

Keywords

yeast; MYO1; UBC4; CHS3; nikkomycin Z; ubiquitin

Introduction

Cell wall composition and architecture in the yeast *Saccharomyces cerevisiae* is significantly altered by Myo1p deficiency (Cruz *et al.*, 2000; Rios Munoz *et al.*, 2003; Rodriguez and Paterson, 1990; Rodriguez-Medina *et al.*, 1998). Absence of the actomyosin ring in yeast caused by a deletion of the *MYO1* gene activates a functionally redundant cytokinesis pathway, regulated by a cytoskeletal binding protein Hof1p (Korinek *et al.*, 2000; Vallen *et al.*, 2000). This mechanism requires increased deposition of chitin at the bud neck by chitin synthase III (CSIII) (Schmidt *et al.*, 2002; Tolliday *et al.*, 2003). CSIII (also called Chs3p, for its catalytic subunit) is also required for maintenance of lateral cell wall chitin synthesis and for chitin ring formation at the bud site during G_1 phase (Shaw *et al.*, 1991).

[*]Correspondence to: José R. Rodríguez-Medina, Department of Biochemistry, School of Medicine, University of Puerto Rico, Medical Sciences Campus, PO Box 365067, San Juan, PR 00936-5067, Puerto Rico. jorrodriguez@rcm.upr.edu.

Consequently, a deletion of *CHS3* causes a synthetic growth defect in most *myo1* strains (Schmidt *et al.*, 2002) and hypersensitivity to low doses of nikkomycin Z (NZ), an irreversible competitive inhibitor of CSIII (Rivera-Molina *et al.*, 2006).

To better understand this relation between CSIII and cell wall integrity in *myo1* strains, a positive screen was conducted to identify cDNAs that could suppress the hypersensitivity to NZ. A total of 14 cDNAs were identified that conferred high levels (80–100%) of NZ resistance (see Table 1). In anticipation that interactions might already be described between *myo1* and/or *chs3* mutations and genes identified by our screen, we searched in the *Saccharomyces cerevisiae* Genome Database (SGD) (http://www.yeastgenome.org/). We found that a total of 10 synthetic lethal interactions (Breton and Aigle, 1998; Davierwala *et al.*, 2005; Roumanie *et al.*, 2000; Vallen *et al.*, 2000; Wang and Bretscher, 1995) and two synthetic growth interactions (Lillie and Brown, 1998) have been previously described for *myo1*, while 76 synthetic lethal (Friesen *et al.*, 2006; Goehring *et al.*, 2003; Lesage *et al.*, 2005; Tong *et al.*, 2004) and two synthetic growth interactions (Castrejon *et al.*, 2006; Sobering *et al.*, 2004) have been previously described for *chs3*. There were no common synthetic lethal or synthetic growth interactions described between *myo1* and *chs3* in the SGD, although we and others have reported a synthetic growth interaction (Rivera-Molina *et al.*, 2006; Schmidt *et al.*, 2002). Deletions of *smi1* or *fks1* were found to have synthetic lethal interactions with *chs3* and *ubc4* (Lesage *et al.*, 2005; Tong *et al.*, 2004). The latter is a gene encoding a ubiquitin-conjugating enzyme (E2) that was isolated by our genetic screen. No interactions have been previously reported between Myo1p and Ubc4p.

The ubiquitin (Ub) pathway has been shown to be involved in targeting of most plasma membrane (PM) proteins for entrance to the endocytic pathway in *Saccharomyces cerevisiae* (Hicke, 1997; Hicke *et al.*, 1997). Yeast cells can employ both poly-Ub and mono-Ub signals to regulate traffic of transmembrane proteins. For example, yeast cells can use mono-ubiquitination to regulate entry of an integral membrane protein at the plasma membrane to the endocytic pathway and, once in the early endosome, it may determine whether the protein will be recycled to the PM or directed to the vacuole for proteolysis (Kolling and Losko, 1997; Strous and Govers, 1999).

Ubc4p has a key function as a stress-related Ub-conjugating enzyme in *Saccharomyces cerevisiae* (Arnason and Ellison, 1994; Chuang and Madura, 2005) by mediating the selective proteasomal degradation of short-lived and abnormal damaged proteins (Chuang and Madura, 2005) and the ubiquitination of multiple vesicular body (MVB) cargoes for degradation in the vacuole (Seufert and Jentsch, 1990). Requirement of Ubc4p for sorting of membrane proteins into multivesicular bodies [named pre-vacuolar compartment/late endosome (PVC/LE) in yeast] is of particular interest because it has been proposed that Chs3p traffic may be deviated from the recycling compartment to the vacuole in *myo1* cells (Rivera-Molina, 2005). Ubc4p also interacts with other cell wall-related proteins, such as Fks1p and Smi1p (Tongaonkar *et al.*, 2000), further supporting the involvement of Ubc4p in cell wall biogenesis. Another interesting connection between Ubc4p and cell wall biogenesis is its physical interaction with the E3 ligase *UFD4* (Ho *et al.*, 2002). *UFD4* has a synthetic interaction with *CHS5* (Lesage *et al.*, 2004) that encodes a protein that regulates Chs3p traffic (Santos and Snyder, 1997). Of the proteins identified to have ubiquitination sites in *Saccharomyces cerevisiae* (Ho *et al.*, 2002), several are related to functions in cell wall biogenesis (Chs3p, Gsc2p, Lsb1p, Sna3p), endocytosis (Akl1p, Rvs167p, Snc1/2p), exocytosis (Ddi1p, Exo84p), ubiquitination (Cue5p, Ubr2p), and vacuolar import of proteins (Ssa2p). This relationship further emphasizes the importance of studying Ubc4p as a central stress-related E2 enzyme that may interact with these and other cell wall stress-related proteins to restore cell wall integrity in *myo1* strains.

In this study we investigated the relation of Ubc4p to the restoration of normal resistance to NZ in a hypersensitive *myo1* strain of *Saccharomyces cerevisiae*. We found that Ubc4p overexpression produced a downregulation of the Pkc1p-mediated cell wall integrity pathway (CWIP). However, it did not restore the normal β-1,3-glucanase sensitivity profile or the wild-type pattern of chitin distribution to the *myo1* strains, suggesting that the restoration of cell wall integrity involves other changes in the cell wall structure and/or composition not related to chitin synthesis. We demonstrate that these proposed cell wall changes require the *VPS4* gene product, suggesting that the PVC-to-vacuole targeting of ubiquitinated proteins is important for this process. These results point to Ubc4p as an important enzyme in the process of cell wall remodelling in *myo1* cells.

Materials and methods

Construction of strains, plasmids, media, and drugs

myo1 Δ::*HIS5* strain YJR6 was generated from the parental haploid wild-type (wt) strain MGD353-46D by homologous recombination, using a PCR-based method (Longtine *et al.*, 1998). The YJR6 and BY4741 (ATCC #201 388) strains were crossed to generate a heterozygous diploid strain from which wt (YJR12) and *myo1* (YJR13) spores were germinated. All strains used in this study (Table 2) were transformed using the LiOAc method (Ito *et al.*, 1983). The *vps4* strain (YJR44) was generated by transforming the YJR12 strain with a KANMX4 selectable marker to replace the chromosomal *VPS4* gene by homologous recombination (Longtine *et al.*, 1998).

To obtain the *myo1 vps4* strain (YJR45), the *vps4* strain was transformed with a *myo1* deletion cassette containing the *Schizosaccharomyces pombe HIS5* gene flanked by *MYO1* sequences that were obtained, using primers FDMYO1 (5′-GGAAAAATATTCACAGGGTAATG-3′) and RDMYO1 (5′-TGGTTTTGAACAACTTTAGCA-GT-3′). A *UBC4* deletion cassette was obtained from *ubc4* strain (ATCC 4 003 219), using primers UBC4F (5′-AGGGTAACTGCACTATTCATATG-TC-′) and UBC4R (5′-TTGATTACATATACTAG-CTATGCGT-3′) and used to transform the *myo1* strain YJR13*pRS316-MYO1*. The *myo1 ubc4 pRS316-MYO1* strain obtained was incubated on CSM glucose plates with 1 mg/ml 5FOA (Zymo Research), from which the *myo1 ubc4* strain was selected and subsequently confirmed by PCR.

Experiments were carried out in complete synthetic media (CSM) or selective media (BIO101) or YPD (yeast extract/peptone/dextrose) (Becton Dickinson and Co.). Culture media were supplemented with 2% glucose (glu) or 2% galactose (gal) (Sigma) and 1× nitrogen base without amino acids (Difco, Becton Dickinson and Co.) at 25 °C. The proteasome inhibitor *clasto*-lactacystin β-lactone (Calbiochem) was reconstituted in DMSO and used at a concentration of 10 μM. Nikkomycin Z (Sigma) was used at concentrations previously determined for agar (30 μg/ml) and broth (6.25 μM) media (Rivera-Molina *et al.*, 2006).

Positive screen for suppression of NZ-induced lethality in *myo1* cells

Haploid *myo1* cells transformed with a galactose-inducible expression library (*pRS316-GAL1-cDNA*; Liu *et al.*, 1992) were positively selected for growth in selective agar plates containing 30 μg/ml NZ. NZ resistance was then verified in a liquid culture assay format in which 1×10^5 cells/ml transformed cells and their controls were incubated in ura$^-$ glu, ura$^-$ glu NZ (6.25 μM), ura$^-$ gal, and ura$^-$ gal NZ (6.25 μM) broth medium at 26 °C and 225 r.p.m. The OD_{600nm} was determined at 24 and 48 h. Resistant strains were scored as those with 80% or greater resistance to 6.25 μM NZ, calculated as: ($OD_{600nm\ treated}$/

$OD_{600nm\ untreated}$) × 100. The transformed strains were 'cured' of their plasmids by incubation in YPD liquid media for 48 h and then in CSM with 1 mg/ml 5FOA (Zymo Research) agar plates at 26 °C for 3 days, to confirm that NZ resistance was dependent on plasmid expression. To assess the integrity of each cDNA clone, they were isolated from *Escherichia coli* cultures for identification by DNA sequencing (ABI PRISM 310 Genetic Analyser, Perkin-Elmer, Foster City, CA).

Determination of chitin content

Cell wall chitin content of wt, wt*pUBC4, myo1, myo1 ubc4* and *myo1pUBC4* strains grown in ura$^-$ gal medium at 26 °C and 225 r.p.m. for 48 h, was determined through a modified Morgan–Elson assay, as described elsewhere (Wheat, 1966). The *UBC4* mutants were fixed in 3.7% formaldehyde and stained with Calcofluor white (Fluorescent Brightener 28, Sigma) for a qualitative assay of their cell wall chitin distribution compared to *myo1* and wt strains grown in ura$^-$ gal medium. Microscopic analysis was performed using an Olympus UV fluorescence microscope equipped with a UV filter (Set I. D. 31 001, Ex. D480 nm/30×, Em. D535 nm/40 m; Chroma Technology) and images captured with a Spot RT-cooled CCD camera.

Western blot analysis of phospho-Slt2p and Slt2p

wt, wt*pUBC4, myo1, myo1 ubc4* and *myo1pUBC4* strains were grown in selective media with 2% glucose or 2% galactose and harvested at OD_{600nm} = 0.5–0.8 absorbance units (AU) for total protein extraction. Aliquots equivalent to 12 AU were centrifuged and washed with ice-cold water, recentrifuged and resuspended with 350 ml lysis buffer (50 mM Tris–HCl, pH 7.5, 10% glycerol, 1% Triton X100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 5× Protease Inhibitor Cocktail (50× stock; Roche), 5× PMSF (100× stock; EtOH), 1× Phosphatase Inhibitor Cocktail I (100× stock; Sigma), and 1× Phosphatase Inhibitor Cocktail II (100× stock; Sigma). The cells were broken by vortexing three times with acid-washed glass beads for 20 s while maintaining the tubes on ice between intervals. The extracts were centrifuged at 13 000 × *g* for 10 min at 4 °C. The supernatant was removed to a new microcentrifuge tube and protein concentrations were determined by the DC Protein Assay method (BioRad). Samples containing 75 μg total protein were separated in a 10% SDS–PAGE gel and transferred to a nitrocellulose membrane at 130 mA for 16 h at 4 °C (Bio-Rad Trans-blot). Mouse monoclonal antibodies against human phospho-p44/42 MAP kinase (recognizing Yeast phospho Slt2p) (Cell Signalling Technology) and rabbit polyclonal antibodies against Yeast MAP kinase 1 (Slt2p/Mpk1p) (Santa Cruz Biotechnology) were used at a dilution of 1/1000. Alternatively, a mouse monoclonal antibody against Pgk1p (Molecular Probes, Invitrogen) was also used at a 1/125 dilution to ascertain equal loading.

β-1,3-glucanase assays

wt (YJR12), wt*pMETUBC4, myo1* (YJR13), *myo1 ubc4*, and *myo1pMETUBC4* cells were cultured respectively in selective glucose medium to OD_{600nm} = 0.5–0.8 AU for a modified β-1,3-glucanase sensitivity assay (Yin *et al.*, 2005). Culture aliquots equivalent to 1×10^7 cells/ml were centrifuged at 1800 × *g* at 4 °C for 5 min and the cell pellets were washed with 1 ml Tris–HCl, 50 mM, pH 7.4. The pelleted cells were gently resuspended in 1 ml Tris–HCl 50 mM, 40 mM β-mercaptoethanol, pH 7.4, and incubated for 1 h at 25 °C. After determining the initial OD_{600nm} (t_0), 120 U Quantazyme (Quantum Biotechnologies) were added and the sample was incubated at 25 °C. OD_{600nm} measurements were taken at 15 min intervals for a total of 75 min. Cell lysis was determined by following the decrease in turbidity of the cell sample relative to the untreated control over time. The relative β-1,3-glucanase sensitivity was expressed as ($OD_{600nm\ after\ treatment}/OD_{600nm\ before\ treatment}$). The resistance : sensitivity ratio was expressed as (t_{75} OD_{600nm}/t_0 OD_{600nm}).

Zymolyase assay

The same strains as above were incubated in selective glucose medium to OD_{600nm} = 0.5–0.8 AU and culture aliquots equivalent to 1×10^7 cells/ml were used for a zymolyase resistance/sensitivity assay (http://mips.gsf.de/proj/eurofan/eurofan2/n7/protocols.html). The cell pellets were washed and resuspended in 10 mM Tris–HCl, pH 7.4, and zymolyase 20T (ICN Biochemicals) was added to a final concentration of 5 μg/ml. After an initial OD_{600nm} measurement, samples were incubated at 37 °C and OD_{600nm} measurements were taken every hour for up to 4 h. A strain was considered sensitive to zymolyase when the final OD_{600nm} was 51% or less compared to the initial OD_{600nm} and was considered resistant when this value was 95% or higher. Percentages within this range depicted a strain with intermediate resistance. The resistance : sensitivity ratio was expressed as (t_{240} OD_{600nm}/t_0 OD_{600nm}).

Proteasome inhibition

Preliminary assays determined an IC_{50} of 10 μM *clasto*-lactacystin β-lactone (β-lactone) for *myo1* and wt cultures. The effectiveness of the 10 μM β-lactone concentration in partially inhibiting the proteasome was assessed by Western blot to determine poly-ubiquitination levels in *myo1* and wt total protein extracts (data not shown). Cultures containing 1×10^5 cells/ml of strains wt, wt*pUBC4, myo1, myo1 ubc4*, and *myo1pUBC4* were grown in ura$^-$ gal, ura$^-$ gal NZ (6.25 μM), ura$^-$ gal β-lactone (10 μM), and ura$^-$ gal NZ (6.25 μM) plus β-lactone (10 μM) media. Their cell density was determined at OD_{600nm} after 48 h incubation at 26 °C and 225 r.p.m.

NZ resistance/sensitivity assay for a *myo1 vps4 pUBC4* strain

A *myo1 vps4 pUBC4* strain was subjected to a NZ resistance/sensitivity assay along with strains wt, *myo1, myo1pUBC4, myo1 vps4*, and *vps4*. Starting with 1×10^7 cells/ml, aliquots taken from each culture at OD_{600nm} = 0.5–0.8 AU, 5 μl from 1/10 serial dilutions (10^7 - 10^2 cells/ml) were inoculated in their respective selective plates and selective plates containing 30 μg/ml NZ. The plates were incubated at 27 °C for 4 days before assessing growth.

Results

Overexpression of *UBC4* confers NZ resistance to a *myo1* strain

A positive screen for the suppression of NZ-induced lethality in *myo1* cells identified a total of 120 cDNA clones, of which 14 conferred high (80–100%) NZ resistance (Table 1). These were identified by DNA sequencing and classified according to function (Comprehensive Yeast Genome Database: http://mips.gsf.de/genre/proj/yeast/index.jsp). Most of the gene products (10/14) were categorized under protein biogenesis functions related to transcription (*CUP9, MCM1*), protein biosynthesis (*RPP2B, RPS20, RPS24A, RPS30*), translation (*SUI3*), protein transport (*VPS3*) and protein fate (*UBC1, UBC4*).

Of these 14 cDNAs that conferred high NZ resistance, *UBC4* was selected for further studies because of its known interactions with the chitin synthase III and glucan synthase pathways (Firon *et al.*, 2004), two important cell wall components in yeast. Redundant NZ resistance assays for the transformed strain *myo1pUBC4* and the ‘cured’ strain (see Materials and methods) confirmed that overexpression of the *UBC4* cDNA confers NZ resistance to the *myo1* strain (data not shown).

myo1 cells overexpressing *UBC4* maintain the delocalized chitin phenotype

To evaluate the effect that *UBC4* overexpression may have on chitin deposition and budding pattern, the wt, wt*pUBC4, myo1, myo1 ubc4* and *myo1pUBC4* strains were assessed for total cell wall chitin content and chitin distribution. We observed that *myo1* cells overexpressing *UBC4* maintain the same delocalized chitin deposition typical of the *myo1* strain (Figure 1). While the wt cells concentrate the Calcofluor white fluorescence discretely in the bud neck, the fluorescence is more widely distributed along the cell wall in both *myo1* and *myo1pUBC4* cells. These latter strains, as well as the *myo1 ubc4* strain, exhibit thicker chitin depositions in their secondary septa.

The budding pattern of both *myo1* and *myo1p-UBC4* strains was also very similar (Table 3), with a larger percentage of cells exhibiting abnormal non-axial (polar and hyper-polar) budding patterns relative to the wt control cells. These results suggest that overexpression of *UBC4* did not reduce the activity of CSIII or restore its normal localization at the bud neck in the *myo1* mutant. Interestingly, *myo1 ubc4* strains exhibited a relatively large number of single-budded cells compared to its wt and *myo1* counterparts, suggesting that bud growth or cell separation may also be affected in *ubc4* strains. Overexpression of *UBC4* did not affect chitin distribution and budding phenotype in the wt strain.

Quantification of total cell wall chitin content in these strains also showed that CSIII activity was relatively unchanged in the *myo1pUBC4* strain and *myo1* strains but was reduced dramatically in the *myo1 ubc4* strain to levels similar to the wild-type controls (Figure 2). Overexpression of *UBC4* in the wt strain did not affect the typical wild-type chitin content profile. All but the wt*pUBC4* strain exhibited a dramatic reduction in chitin content when treated with NZ 6.25 μM (Figure 2), which demonstrates that NZ can be transported efficiently inside *myo1* strains that overexpress *UBC4* or that contain a *ubc4* deletion.

myo1 cells overexpressing *UBC4* exhibit a downregulation of the Pkc1p-mediated cell wall integrity signalling pathway

In order to determine the CWIP status of *myo1* cells overexpressing *UBC4*, the relative levels of phospho-Slt2p were assessed by Western blot analysis (Figure 3A). The results show a dramatic increase in the dual-phosphorylated Slt2p signal in *myo1* strains when *UBC4* is repressed by glucose. When transcription of the *UBC4* cDNA was induced by galactose, the *myo1pUBC4* strain exhibited a significant ($p = 0.003$) reduction in the dual phosphorylated Slt2p levels relative to the *myo1* cells (Figure 3B). Preliminary results for the *myo1 ubc4* and wt*pUBC4* strains showed similar dual-phosphorylated Slt2p levels relative to the *myo1* and wt strains, respectively (data not shown).

Overexpresssion of *UBC4* does not change sensitivity to β-1,3-glucanase and zymolyase enzymes in *myo1* strains

wt*pMETUBC4, myo1* (YJR13), *myo1 ubc4*, and *myo1pMETUBC4* strains subjected to a β-1,3-glucanase resistance : sensitivity assay (Figure 4A) presented similar levels of sensitivity to the enzyme (ranging from 0.43 to 0.56 resistance : sensitivity ratio), while the control wt exhibited a higher relative sensitivity (0.18). The relative sensitivity of the *myo1* strain (0.43 ratio) comes as a surprise because other chitin mutants, such as *gas1* and *ecm33*, have been shown to have much lower sensitivity to β-1,3-glucanase (Yin *et al.*, 2005). This relative sensitivity to β-1,3-glucanase was not changed in either the *myo1pUBC4* or *myo1 ubc4* strains. Conversely, the relative sensitivity to β-1,3-glucanase was decreased in the wt*pMET-UBC4* strain relative to the wt control strain.

When all strains were subjected to a zymolyase resistance : sensitivity assay, they showed a broad range of resistance : sensitivity ratios, from 0.07 for the wt strain to 0.53 for the *myo1*

ubc4 strain (Figure 4B). It was noted that the *myo1pMETUBC4* strain showed increased sensitivity to the enzyme relative to the *myo1* strain, while the wt*pMETUBC4* strain showed decreased sensitivity relative to the wt strain.

Rescue of NZ resistance by *UBC4* requires the PVC-to-vacuole targeting pathway

In order to ascertain the role of the proteasome in the rescue of NZ resistance, the *myo1pUBC4* strain was assayed for NZ resistance in the presence of the proteasome inhibitor clasto-lactacystin-β-lactone (β-lactone) (Craiu *et al.*, Fenteany and Schreiber, 1998; Lee and Goldberg, 1996). When wt, wt*pUBC4, myo1, myo1 ubc4* and *myo1pUBC4* strains were tested for NZ resistance in the presence of 10 μM β-lactone, the β-lactone alone had a mild effect on growth (growth inhibition ratio = 0.068 ± 0.044) in all strains (Figure 5A). The average results for experiments assaying the effects of 10 μM β-lactone on NZ resistance showed that the *myo1pUBC4* strain maintained high levels of NZ resistance (0.78 resistance : sensitivity ratio) similar to wt control cells (0.89 resistance : sensitivity ratio) while the *myo1* strain continued to exhibit the characteristic hypersensitivity to NZ (0.21 resistance : sensitivity ratio) (Figure 5A). This suggests that partial inhibition of proteasome function by 10 μM β-lactone does not affect the acquired resistance to NZ in *myo1* cells overexpressing *UBC* 4. Surprisingly, resistance to NZ was enhanced by 10 μM β-lactone in the *myo1 ubc4* strain.

We tested the role of the PVC–LE-to-vacuole trafficking pathway in the restoration of cell wall integrity of *myo1* cells by assessing NZ resistance in a *myo1 vps4 pUBC4* strain. A *VPS4* deletion causes a block in the trans-Golgi network (TGN)-to-pre-vacuolar PVC/LE traffic and therefore prevents the downstream traffic between the PVC/LE and vacuole. The results of NZ resistance assays (Figure 5B) revealed that the *myo1 vps4 pUBC4* strain was hypersensitive, as were *myo1 vps4* and *myo1* strains, while the control *vps*4 and wt strains exhibited resistance.

Discussion

The overrepresentation of cDNAs coding for proteins participating in protein synthesis and metabolism functional pathways identified by our NZ resistance screen (Table 1) supports our original hypothesis that the absence of myosin II will alter the normal levels of proteins involved in cell wall integrity. To explain the observed results, we speculate that for a *myo1* strain, the level of these proteins required for the synthesis and metabolism of all cellular proteins could be depleted to an extent that it also produces a decrease in the synthesis of cell wall proteins. Evidently, the restoration of cell wall integrity by *UBC4* dosage rescue (as well as by any of the other proteins listed in Table 1) can produce a compensatory effect at the cell wall level. Indeed, several of these genes that confer 80–100% NZ resistance to the *myo1* strain may be involved directly or indirectly in cell wall protein synthesis and/or its regulation. Moreover, the conjugating enzymes *UBC1* and *UBC4* may also have a regulatory role in restoration of cell wall integrity in the *myo1* strain through their involvement in the cellular stress response (Seufert and Jentsch, 1990). However, we observed that the *UBC4* gene was not essential for cell viability (data not shown).

MYO1 gene disruption causes cells to lose their haploid-specific budding pattern and exhibit abnormal chitin deposition at the mother–bud neck (Rodriguez and Paterson, 1990). Dosage rescue by *UBC4* did not affect the chitin distribution or budding patterns typical of the *myo1* strains, suggesting that the chitin synthesis pathway was not directly involved. A block in the uptake of NZ was excluded as a mechanism for resistance but increased clearance of the drug was not excluded in this study.

It is expected that *myo1* cells will activate a cell wall integrity signalling mechanism that is induced by abnormal cell wall assembly (Cruz *et al.*, 2000; Kapteyn *et al.*, 1997; Osmond *et al.*, 1999; Popolo *et al.*, 1997, 2001; Ram *et al.*, 1998; Valdivieso *et al.*, 2000). The Pkc1p-mediated cell wall integrity signalling pathway (CWIP) is principally responsible for orchestrating changes in the cell wall periodically throughout the cell cycle, as well as in response to various forms of cell wall stress (Heinisch *et al.*, 1999; Levin, 2005; Martin *et al.*, 2005; Vilella *et al.*, 2005). Cell wall perturbation results in the dual phosphorylation of the Slt2p/Mpk1p MAP kinase (de Nobel *et al.*, 2000) and Slt2p dual-phosphorylation has been previously demonstrated in *myo1* cells by Western blot assay (Nishihama *et al.*, 2003; Rivera-Molina, 2005). We observed a decrease in dual-phospho-Slt2 in *myo1pUBC4* strains that suggested a restoration of cell wall integrity. Combined with the observed decrease in the requirement for Chs3p activity in these strains, we believe that this reflects new cell wall modification(s), possibly of the glucans or cell wall mannoproteins that can shut down the CWIP. The physical interaction of *UBC4* with *UFD4* (Ho *et al.*, 2002), a ligase that is physically associated with microtubule subunits *TUB1* and *TUB2* (Gavin *et al.*, 2006), could also be involved in modifying the secretion dynamics of cell wall proteins in our strains.

It has been previously demonstrated by others that cell wall stress can trigger an increase in cross-linking between cell wall proteins via β-1,6-glucan to chitin (Kapteyn *et al.*, 1997) and that a defective incorporation of β-1,3-glucan can lead to an increase in chitin and mannan content in the cell wall (de Nobel *et al.*, 2000; Kapteyn *et al.*, 2001; Yin *et al.*, 2005). These results highlight the importance of glucans, together with chitin and mannoproteins, in preserving cell wall integrity. To explore possible changes in the structure of the glucan layer that may be induced by overexpression of *UBC4*, a β-1,3-glucanase resistance/sensitivity assay was conducted. While the *myo1* strains were considered sensitive by the established quantitative criteria, they were significantly less sensitive to β-1,3-glucanase than the wild-type control strain *UBC4* overexpression did not modify this phenotype in the *myo1* strain, yet it was capable of decreasing the level of sensitivity in a wt strain to a level resembling a *myo1* strain. Therefore, over-expression of *UBC4* can change specific properties of the cell wall that can make the cell wall glucan layer more resistant to β-1,3-glucanase. However, this assay did not resolve whether these changes are related to modification of the glucans themselves or to the limited accessibility of the enzyme to the glucan layer. A greater sensitivity of the *myo1pUBC4* strain to zymolyase (Figure 4B) compared to β-1,3-glucanase (Figure 4A) could reflect the susceptibility of cell wall components other than glucan. Because zymolyase is a mixture of β-1,3-glucanase and other enzymes such as mannase, amylase, xylanase, phosphatase and several proteases, these results were less clear but may reflect a greater sensitivity of the outer protein layer in response to *UBC4* overexpression.

The yeast 20S proteasome mediates ubiquitin-dependent degradation of short-lived proteins in *Saccharomyces cerevisiae* (Seufert and Jentsch, 1990). An alternative to the proteasomal peptide/protein degradation pathway in yeast involves the PVC/LE-to-vacuole trafficking pathway. A *vps4* deletion mutation causes a block in the trans-Golgi network (TGN) to pre-vacuolar compartment/late endosome (PVC/LE) traffic and therefore prevents traffic between the PVC and vacuole. Vps4p is a member of the AAA (ATPase-associated activities) protein superfamily involved in endosomal maturation and exit to the vacuole (Babst *et al.*, 1998; Lemmon and Traub, 2000). Dosage rescue of cell wall integrity in *myo1* strains by *UBC4* required targeting of ubiquitinated proteins to the PVC/LE–vacuole pathway while remaining insensitive to the inhibitor β-lactone, suggesting that proteasome function was not as important. Because plasma membrane proteins are generally targeted for recycling and/or degradation through the PVC/LE–vacuole pathway (Hicke, 1997; Hicke *et al.*, 1997), this raises the possibility that remodelling of the plasma membrane may be involved in the restoration of cell wall integrity.

Concluding remarks

The notion that ubiquitins can be involved in the expression of resistance to antifungal compounds through the association of ubiquitin to receptor-like components at the cell surface of the yeast cell has been proposed to explain acquired resistance to fluconazole (Kano *et al.*, 2001). Because ubiquitination is a major determinant of endocytosis in yeast (Hicke and Riezman, 1996; Kragt *et al.*, 2005; Terrell *et al.*, 1998), we suggest that the restoration of cell wall integrity by overexpression of the E2-conjugating enzyme *UBC4* in *myo1* cells may be explained by a modification in the normal targeting of proteins at the level of the PVC/LE compartment. Such a change may ultimately lead to the enrichment of one or more cell wall components. We propose that the implied changes in cell wall properties may involve the remodelling of glucans and/or a glycoprotein component(s) that can reinforce the cell wall structure and contribute to downregulating the CWIP in the *myo1* strains. The possible enrichment of alkaline-sensitive linkage (ASL) proteins (Yin *et al.*, 2005) in the outer protein layer is of interest in our current studies.

Acknowledgments

We thank Mrs Sahily González for technical assistance and students Glorivee Pagán, Marielis Rivera and Yasdet Maldonado de la Cruz for their support. We are grateful to Anthony Bretscher and Felix Rivera-Molina for providing a cDNA library and plasmids, respectively. Support for this work was provided by a PHS grant from NIGMS-SCORE (S06GM008224), with partial support from NCRR-RCMI (G12RR03051). NLD-B is supported by NIGMS-MARC Grant F34GM69277.

References

Arnason T, Ellison MJ. Stress resistance in *Saccharomyces cerevisiae* is strongly correlated with assembly of a novel type of multiubiquitin chain. Mol Cell Biol. 1994; 14:7876–7883. [PubMed: 7969127]

Babst M, Wendland B, Estepa EJ, Emr SD. The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. EMBO J. 1998; 17:2982–2993. [PubMed: 9606181]

Breton A, Aigle M. Genetic and functional relationship between Rvsp, myosin and actin in *Saccharomyces cerevisiae*. Curr Genet. 1998; 34:280–286. [PubMed: 9799361]

Castrejon F, Gomez A, Sanz M, Duran A, Roncero C. The RIM101 pathway contributes to yeast cell wall assembly and its function becomes essential in the absence of mitogen-activated protein kinase Slt2p. Eukaryot Cell. 2006; 5:507–517. [PubMed: 16524906]

Chuang SM, Madura K. *Saccharomyces cerevisiae* Ub-conjugating enzyme Ubc4 binds the proteasome in the presence of translationally damaged proteins. Genetics. 2005; 171:1477–1484. [PubMed: 16118187]

Craiu A, Gaczynska M, Akopian T, et al. Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. J Biol Chem. 1997; 272:13437–13445. [PubMed: 9148969]

Cruz JA, Garcia R, Rodriguez-Orengo JF, Rodriguez-Medina JR. Increased chitin synthesis in response to type II myosin deficiency in *Saccharomyces cerevisiae*. Mol Cell Biol Res Commun. 2000; 3:20–25. [PubMed: 10683313]

Davierwala AP, Haynes J, Li Z, et al. The synthetic genetic interaction spectrum of essential genes. Nat Genet. 2005; 37:1147–1152. [PubMed: 16155567]

de Nobel H, Ruiz C, Martin H, et al. Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-lacZ expression, glucanase resistance and thermotolerance. Microbiology. 2000; 146(9):2121–2132. [PubMed: 10974100]

Fenteany G, Schreiber SL. Lactacystin, proteasome function, and cell fate. J Biol Chem. 1998; 273:8545–8548. [PubMed: 9535824]

Firon A, Lesage G, Bussey H. Integrative studies put cell wall synthesis on the yeast functional map. Curr Opin Microbiol. 2004; 7:617–623. [PubMed: 15556034]

Friesen H, Humphries C, Ho Y, et al. Characterization of the yeast amphiphysins Rvs161p and Rvs167p reveals roles for the Rvs heterodimer *in vivo*. Mol Biol Cell. 2006; 17:1306–1321. [PubMed: 16394103]

Gavin AC, Aloy P, Grandi P, et al. Proteome survey reveals modularity of the yeast cell machinery. Nature. 2006; 440:631–636. [PubMed: 16429126]

Goehring AS, Mitchell DA, Tong AH, et al. Synthetic lethal analysis implicates Ste20p, a p21-activated protein kinase, in polarisome activation. Mol Biol Cell. 2003; 14:1501–1516. [PubMed: 12686605]

Heinisch JJ, Lorberg A, Schmitz HP, Jacoby JJ. The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*. Mol Microbiol. 1999; 32:671–680. [PubMed: 10361272]

Hicke L. Ubiquitin-dependent internalization and downregulation of plasma membrane proteins. FASEB J. 1997; 11:1215–1226. [PubMed: 9409540]

Hicke L, Riezman H. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. Cell. 1996; 84:277–287. [PubMed: 8565073]

Hicke L, Zanolari B, Pypaert M, Rohrer J, Riezman H. Transport through the yeast endocytic pathway occurs through morphologically distinct compartments and requires an active secretory pathway and Sec18p/N-ethylmaleimide-sensitive fusion protein. Mol Biol Cell. 1997; 8:13–31. [PubMed: 9017592]

Ho Y, Gruhler A, Heilbut A, et al. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature. 2002; 415:180–183. [PubMed: 11805837]

Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. J Bacteriol. 1983; 153:163–168. [PubMed: 6336730]

Kano R, Okabayashi K, Nakamura Y, Watanabe S, Hasegawa A. Expression of ubiquitin gene in *Microsporum canis* and *Trichophyton mentagrophytes* cultured with fluconazole. Antimicrob Agents Chemother. 2001; 45:2559–2562. [PubMed: 11502529]

Kapteyn JC, Ram AF, Groos EM, et al. Altered extent of cross-linking of beta1,6-glucosylated mannoproteins to chitin in *Saccharomyces cerevisiae* mutants with reduced cell wall beta1,3-glucan content. J Bacteriol. 1997; 179:6279–6284. [PubMed: 9335273]

Kapteyn JC, Riet B, Vink E, et al. Low external pH induces HOG1-dependent changes in the organization of the *Saccharomyces cerevisiae* cell wall. Mol Microbiol. 2001; 39:469–479. [PubMed: 11136466]

Kolling R, Losko S. The linker region of the ABC-transporter Ste6 mediates ubiquitination and fast turnover of the protein. EMBO J. 1997; 16:2251–2261. [PubMed: 9171340]

Korinek WS, Bi E, Epp JA, et al. Cyk3, a novel SH3-domain protein, affects cytokinesis in yeast. Curr Biol. 2000; 10:947–950. [PubMed: 10959846]

Kragt A, Voorn-Brouwer T, van den Berg M, Distel B. The *Saccharomyces cerevisiae* peroxisomal import receptor Pex5p is monoubiquitinated in wild-type cells. J Biol Chem. 2005; 280:7867–7874. [PubMed: 15632140]

Lee DH, Goldberg AL. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. J Biol Chem. 1996; 271:27280–27284. [PubMed: 8910302]

Lemmon SK, Traub LM. Sorting in the endosomal system in yeast and animal cells. Curr Opin Cell Biol. 2000; 12:457–466. [PubMed: 10873832]

Lesage G, Sdicu AM, Menard P, et al. Analysis of beta-1,3-glucan assembly in *Saccharomyces cerevisiae* using a synthetic interaction network and altered sensitivity to caspofungin. Genetics. 2004; 167:35–49. [PubMed: 15166135]

Lesage G, Shapiro J, Specht CA, et al. An interactional network of genes involved in chitin synthesis in *Saccharomyces cerevisiae*. BMC Genet. 2005; 6:8. [PubMed: 15715908]

Levin DE. Cell wall integrity signalling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev. 2005; 69:262–291. [PubMed: 15944456]

Lillie SH, Brown SS. Smy1p, a kinesin-related protein that does not require microtubules. J Cell Biol. 1998; 140:873–883. [PubMed: 9472039]

Liu H, Krizek J, Bretscher A. Construction of a GAL1-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. Genetics. 1992; 132:665–673. [PubMed: 1468625]

Longtine MS, McKenzie A III, Demarini DJ, et al. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast. 1998; 14:953–961. [PubMed: 9717241]

Martin H, Flandez M, Nombela C, Molina M. Protein phosphatases in MAPK signalling: we keep learning from yeast. Mol Microbiol. 2005; 58:6–16. [PubMed: 16164545]

Nishihama R, Ko N, Caruso C, Yu I, Pringle J. Septin dependent, actomyosin ring independent cytokinesis in yeast [abstr]. Am Soc Cell Biol. 2003; 43:L81.

Osmond BC, Specht CA, Robbins PW. Chitin synthase III: synthetic lethal mutants and 'stress related' chitin synthesis that bypasses the CSD3/CHS6 localization pathway. Proc Natl Acad Sci USA. 1999; 96:11206–11210. [PubMed: 10500155]

Popolo L, Gilardelli D, Bonfante P, Vai M. Increase in chitin as an essential response to defects in assembly of cell wall polymers in the *ggp1Δ* mutant of *Saccharomyces cerevisiae*. J Bacteriol. 1997; 179:463–469. [PubMed: 8990299]

Popolo L, Gualtieri T, Ragni E. The yeast cell-wall salvage pathway. Med Mycol. 2001; 39(suppl 1): 111–121. [PubMed: 11800265]

Ram AF, Kapteyn JC, Montijn RC, et al. Loss of the plasma membrane-bound protein Gas1p in *Saccharomyces cerevisiae* results in the release of beta1,3-glucan into the medium and induces a compensation mechanism to ensure cell wall integrity. J Bacteriol. 1998; 180:1418–1424. [PubMed: 9515908]

Rios Munoz W, Irizarry Ramirez M, Rivera Molina F, Gonzalez Crespo S, Rodriguez-Medina JR. Myosin II is important for maintaining regulated secretion and asymmetric localization of chitinase 1 in the budding yeast *Saccharomyces cerevisiae*. Arch Biochem Biophys. 2003; 409:411–413. [PubMed: 12504909]

Rivera-Molina, F. PhD Thesis. Department of Biochemistry, University of Puerto Rico; 2005. Biochemical and Genetic Analysis of Myosin II Function in the Trafficking of the Chitin Synthase III Catalytic Subunit; p. 119

Rivera-Molina F, Gonzalez-Crespo S, Maldonado-De la Cruz Y, Ortiz-Betancourt J, Rodriguez-Medina J. 2,3-Butanedione monoxime increases sensitivity to nikkomycin Z in the budding yeast *Saccharomyces cerevisiae*. World J Microbiol Biotechnol. 2006; 22:255–260.

Rodriguez JR, Paterson BM. Yeast myosin heavy chain mutant: maintenance of the cell type specific budding pattern and the normal deposition of chitin and cell wall components requires an intact myosin heavy chain gene. Cell Motil Cytoskel. 1990; 17:301–308.

Rodriguez-Medina JR, Cruz JA, Robbins PW, Bi E, Pringle JR. Elevated expression of chitinase 1 and chitin synthesis in myosin II-deficient *Saccharomyces cerevisiae*. Cell Mol Biol (Noisy-le-grand). 1998; 44:919–925. [PubMed: 9763195]

Roumanie O, Peypouquet MF, Bonneu M, et al. Evidence for the genetic interaction between the actin-binding protein Vrp1 and the RhoGAP Rgd1 mediated through Rho3p and Rho4p in *Saccharomyces cerevisiae*. Mol Microbiol. 2000; 36:1403–1414. [PubMed: 10931290]

Santos B, Snyder M. Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p. J Cell Biol. 1997; 136:95–110. [PubMed: 9008706]

Schmidt M, Bowers B, Varma A, Roh DH, Cabib E. In budding yeast, contraction of the actomyosin ring and formation of the primary septum at cytokinesis depend on each other. J Cell Sci. 2002; 115:293–302. [PubMed: 11839781]

Seufert W, Jentsch S. Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. EMBO J. 1990; 9:543–550. [PubMed: 2154373]

Shaw JA, Mol PC, Bowers B, et al. The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. J Cell Biol. 1991; 114:111–123. [PubMed: 2050738]

Sobering AK, Watanabe R, Romeo MJ, et al. Yeast Ras regulates the complex that catalyzes the first step in GPI-anchor biosynthesis at the ER. Cell. 2004; 117:637–648. [PubMed: 15163411]

Strous GJ, Govers R. The ubiquitin–proteasome system and endocytosis. J Cell Sci. 1999; 112:1417–1423. [PubMed: 10212136]

Terrell J, Shih S, Dunn R, Hicke L. A function for mono-ubiquitination in the internalization of a G protein-coupled receptor. Mol Cell. 1998; 1:193–202. [PubMed: 9659916]

Tolliday N, Pitcher M, Li R. Direct evidence for a critical role of myosin II in budding yeast cytokinesis and the evolvability of new cytokinetic mechanisms in the absence of myosin II. Mol Biol Cell. 2003; 14:798–809. [PubMed: 12589071]

Tong AH, Lesage G, Bader GD, et al. Global mapping of the yeast genetic interaction network. Science. 2004; 303:808–813. [PubMed: 14764870]

Tongaonkar P, Chen L, Lambertson D, Ko B, Madura K. Evidence for an interaction between ubiquitin-conjugating enzymes and the 26S proteasome. Mol Cell Biol. 2000; 20:4691–4698. [PubMed: 10848595]

Valdivieso MH, Ferrario L, Vai M, Duran A, Popolo L. Chitin synthesis in a *gas1* mutant of *Saccharomyces cerevisiae*. J Bacteriol. 2000; 182:4752–4757. [PubMed: 10940014]

Vallen EA, Caviston J, Bi E. Roles of Hof1p, Bni1p, Bnr1p, and myo1p in cytokinesis in *Saccharomyces cerevisiae*. Mol Biol Cell. 2000; 11:593–611. [PubMed: 10679017]

Vilella F, Herrero E, Torres J, de la Torre-Ruiz MA. Pkc1 and the upstream elements of the cell integrity pathway in *Saccharomyces cerevisiae*, Rom2 and Mtl1, are required for cellular responses to oxidative stress. J Biol Chem. 2005; 280:9149–9159. [PubMed: 15637049]

Wang T, Bretscher A. The rho-GAP encoded by *BEM2* regulates cytoskeletal structure in budding yeast. Mol Biol Cell. 1995; 6:1011–1024. [PubMed: 7579704]

Wheat RW. The isolation and characterization of glucosamine and muramic acid from the cell-wall mucopeptide of the Gram-negative bacterium, *Chromobacterium violaceum*. Biochim Biophys Acta. 1966; 121:170–172. [PubMed: 5956935]

Yin QY, de Groot PW, Dekker HL, et al. Comprehensive proteomic analysis of *Saccharomyces cerevisiae* cell walls: identification of proteins covalently attached via glycosylphosphatidylinositol remnants or mild alkali-sensitive linkages. J Biol Chem. 2005; 280:20894–20901. [PubMed: 15781460]

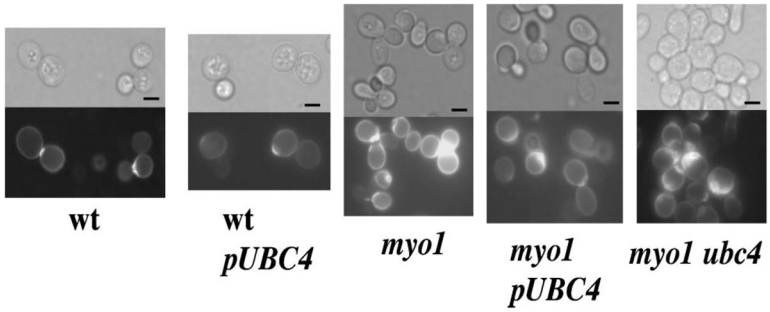

Figure 1.

Visualization of chitin distribution by staining with Calcofluor white. *myo1pUBC4, myo1, myo1 ubc4, wt* and wt*pUBC4* cells were cultured in ura^{-} gal medium, fixed in 3.7% formaldehyde and stained with Calcofluor white. Light microscopy images are presented in the upper panels. Below each bright-field image is the corresponding fluorescence micrograph. Bar = 5 μm

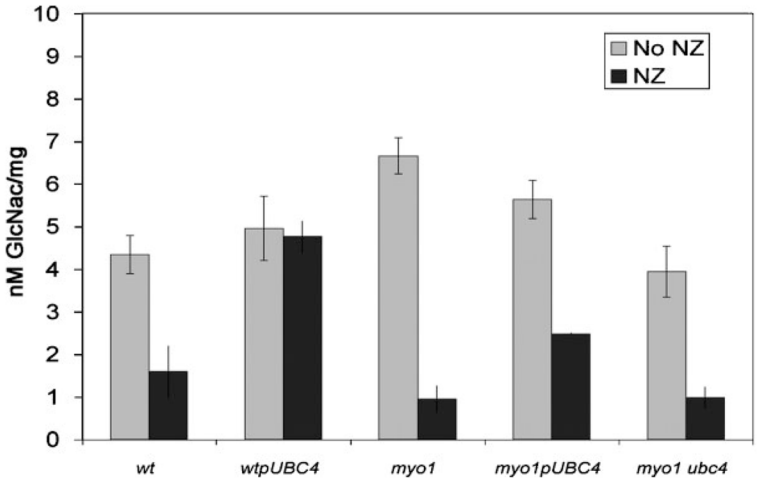

Figure 2.

Measurement of chitin content in *myo1pUBC4* cells. *myo1pUBC4, myo1, myo1 ubc4, wt* and wt*pUBC4* cells were cultured in ura$^-$ gal and ura$^-$ gal NZ 6.25 μM media at 26 °C and 225 r.p.m. for 48 h before chitin content assessment. The results represent the average of three experiments

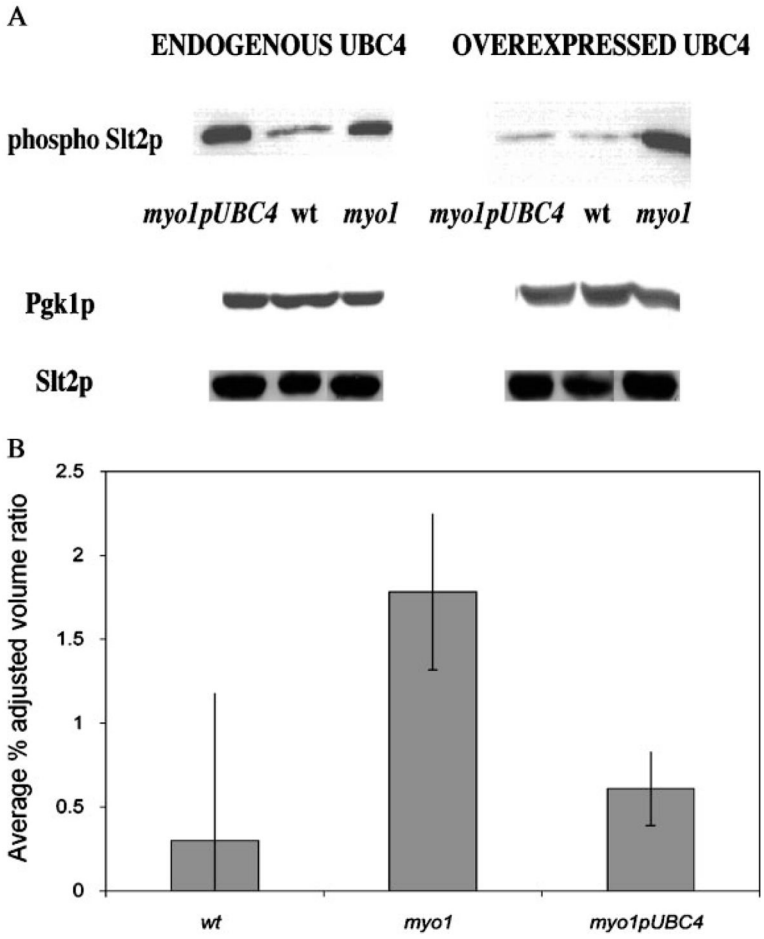

Figure 3.

Analysis of phospho-Slt2p steady-state levels. (A) Equal amounts of protein extracts prepared from *myo1pUBC4*, wt and *myo1* cells cultured in ura$^-$ glu (endogenous *UBC4*) and ura$^-$ gal (overexpressed *UBC4*) media were analysed by Western blot, as described in Materials and methods. The Western blot was probed with a mouse monoclonal antibody against phospho p44/42 MAP kinase (phospho-Slt2p) (first row) and re-probed with a mouse monoclonal antibody against cytoplasmic Pgk1p (middle row) and a rabbit polyclonal antibody against Slt2p (bottom row) for loading controls. (B) Histograms show the average percentage adjusted volume ratio for blot results of strains cultured in ura$^-$ gal (overexpressed *UBC4*), as determined by densitometry analysis. The results represent the average of three experiments

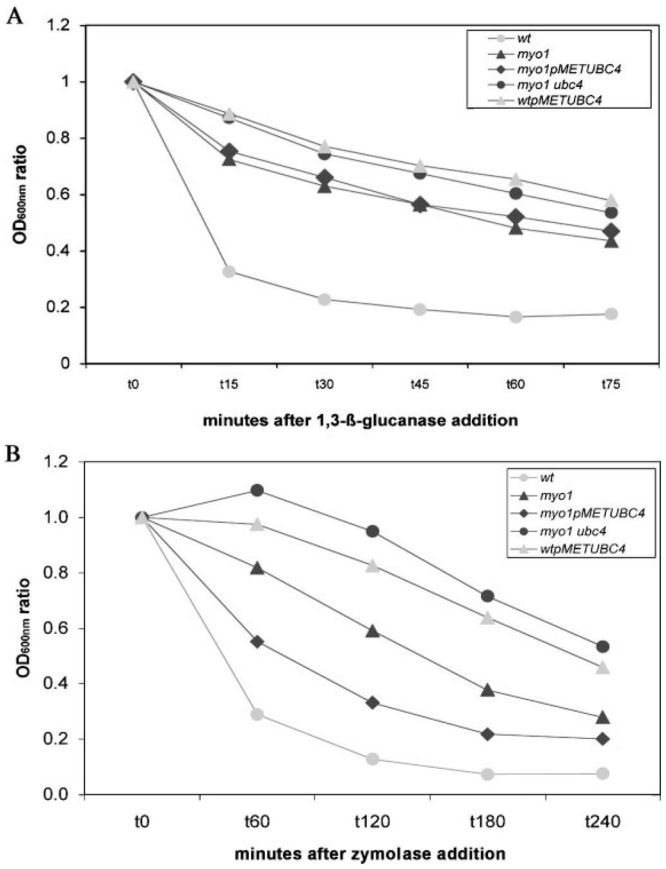

Figure 4.

Determination of sensitivity profile to the enzymes β-1,3-glucanase and zymolyase. (A) *myo1p-METUBC4, myo1* (YJR13), *myo1 ubc4*, wt (YJR12) and wt*pMETUBC4* cells were incubated with 120 U β-1,3-glucanase at 25 °C for up to 75 min, as described in Materials and methods. To obtain their sensitivity profiles, the OD_{600nm} ratio was determined by dividing the absorbance units at any time point by the absorbance units at t_0. Chart presents the average of three experiments. (B) The same strains were incubated with 5 μg/ml zymolyase at 37 °C for up to 4 h, as described in Materials and methods. The OD_{600nm} ratio was determined as decribed above. Chart presents the average data from three experiments

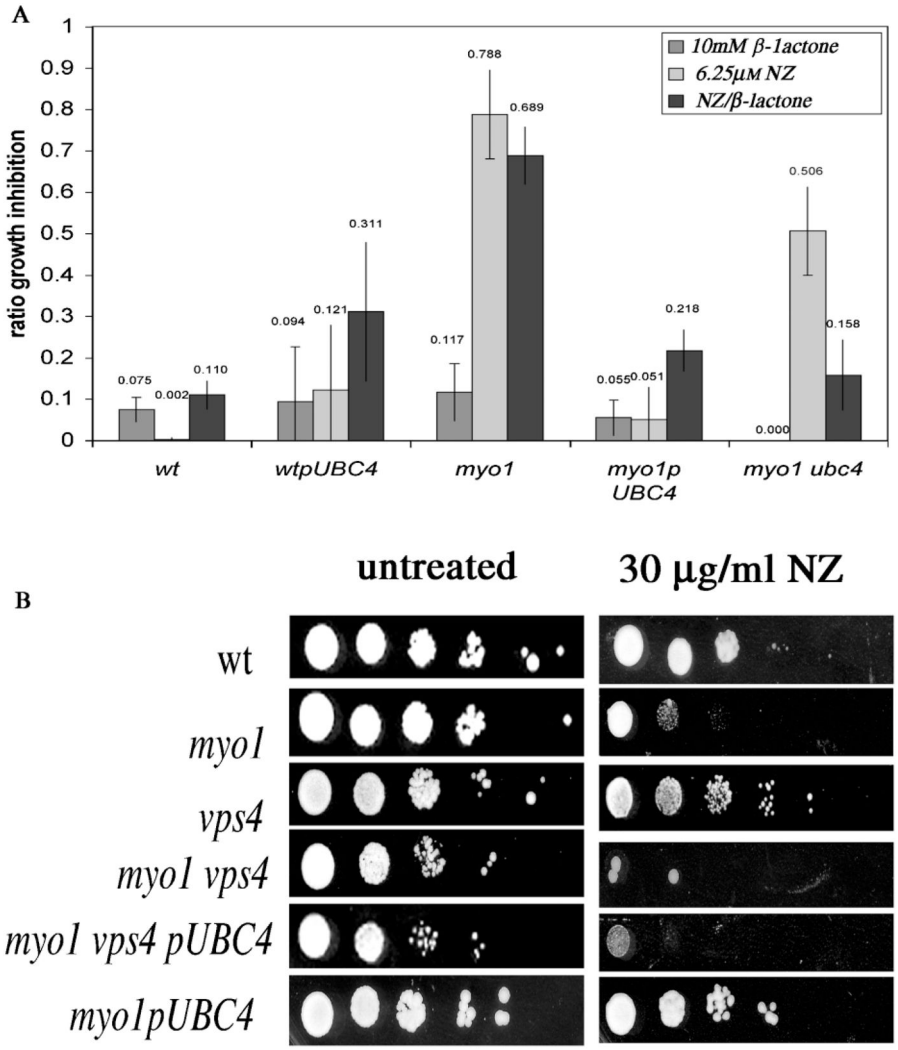

Figure 5.

Assessment of the role of proteasome and PVC/LE pathways in acquired NZ resistance. (A) Effects of 10 μM *β*-lactone on NZ resistance of wt, *myo1, myo1* cells overexpressing *UBC4* (*myo1pUBC4*), *myo1 ubc4* and wt cells overexpressing *UBC4* (*wtpUBC4*). Strains with high NZ resistance show a low growth inhibition ratio and vice versa. The growth inhibition ratio was calculated as follows: $OD_{600nm\ treated}/OD_{600nm\ untreated}$. The chart represents the average of at least three experiments. (B) NZ resistance agar plate assays of the indicated strains were conducted by drop tests (from left to right, $10^7 – 10^2$ cells/ml serial dilutions) on ura$^-$ gal agar plates (untreated) and ura$^-$ gal agar plates with 30 μg/ml NZ. NZ-resistant wild-type (wt) and *myo1pUBC4* cells and sensitive *myo1* cells were included as controls. The plates were incubated at 27 °C for 4 days before assessing growth and photographing

Table 1

Identification of cDNAs whose overexpression confers NZ-resistance to *myo1* cells

Systematic name	Gene	Redundancy (No. of clones)	NZ resistance (%)	Description
YPL177C	*CUP9*	1	84.2	Homeodomain-containing transcriptional repressor of PTR2, which encodes a major peptide transporter; imported peptides activate ubiquitin-dependent proteolysis, resulting in degradation of Cup9p and de-repression of PTR2 transcription
YGR175C	*ERG1*	1	81	Squalene epoxidase, catalyses the epoxidation of squalene to 2,3-oxidosqualene; plays an essential role in the ergosterol-biosynthesis pathway and is the specific target of the antifungal drug terbinafine
YMR043W	*MCM1; FUN80*	1	92.5	Transcription factor involved in cell-type-specific transcription and pheromone response; plays a central role in the formation of both repressor and activator complexes
YOL126C	*MDH2*	1	80	Cytoplasmic malate dehydrogenase, one of the three isozymes that catalyse interconversion of malate and oxaloacetate; involved in gluconeogenesis during growth on ethanol or acetate as carbon source; interacts with Pck1p and Fbp1p
YOL064C	*MET22*	1	100	Bisphosphate-3′-nucleotidase, involved in salt tolerance and methionine biogenesis; dephosphorylates 3′-phosphoadenosine-5′-phosphate and 3′-phosphoadenosine-5′-phosphosulfate, intermediates of the sulphate assimilation pathway
YDR505C	*PSP1; GIN5*	1	91.9	Asn and gln rich protein of unknown function; high-copy suppressor of POL1 (DNA polymerase-α) and partial suppressor of CDC2 (polymerase delta) and CDC6 (pre-RC loading factor) mutations; overexpression results in growth inhibition
YDR382W	*RPP2B; RPL45; YPA1*	3	87.9	Ribosomal protein P2-β, a component of the ribosomal stalk, which is involved in the interaction between translational elongation factors and the ribosome; regulates the accumulation of P1 (Rpp1Ap and Rpp1Bp) in the cytoplasm
YHL015W	*RPS20; URP2*	1	93	Protein component of the small (40S) ribosomal subunit; overproduction suppresses mutations affecting RNA polymerase III-dependent transcription; has similarity to *E. coli* S10 and rat S20 ribosomal proteins
YER074W	RPS24A; RPS24EA	1	89.6	Protein component of the small (40S) ribosomal subunit; identical to Rps24Bp and has similarity to rat S24 ribosomal protein
YOR182C	RPS30B	1	83.6	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps30Ap and has similarity to rat S30 ribosomal protein
YPL237W	SUI3	2	100	β-subunit of the translation initiation factor eIF2, involved in the identification of the start codon; proposed to be involved in mRNA binding
YDR177W	UBC1	1	81.1	Ubiquitin-conjugating enzyme that mediates selective degradation of short-lived and abnormal proteins; plays a role in vesicle biogenesis and ER-associated protein degradation (ERAD); component of the cellular stress response
YBR082C	UBC4	4	87.9	Ubiquitin-conjugating enzyme that mediates degradation of short-lived and abnormal proteins; interacts with E3-CaM in ubiquitinating calmodulin; interacts with many SCF ubiquitin protein ligases; component of the cellular stress response

Systematic name	Gene	Redundancy (No. of clones)	NZ resistance (%)	Description
YDR495C	VPS3; PEP6; VPL3; VPT17	1	96.6	Cytoplasmic protein required for the sorting and processing of soluble vacuolar proteins, acidification of the vacuolar lumen, and assembly of the vacuolar H+-ATPase

Table 2

Strains used in this study

Strain	Name	Genotype	Plasmid source
YJR12		*MAT α trp1 ura3 leu2–3, his3delta1 cyh^R*	
YJR13		*MAT* a *trp1 ura3 leu2–3, his3delta1 cyh^R myo1 Δ::HIS5*	
YJR13pRS316-*GAL1-UBC4*	*myo1pUBC4*	*MAT* a *trp1 ura3 leu2–3, his3delta1 cyh^R myo1 Δ::HIS5*	A. Brestcher
YJR13pFR23-*MET25-UBC4*	*myo1 pMETUBC4*	*MAT* a *trp1 ura3 leu2–3, his3delta1 cyh^R myo1 Δ::HIS5*	This study
YJR13pRS316$_{blank}$	*myo1*	*MAT* a *trp1 ura3 leu2–3, his3delta1 cyh^R myo1 Δ::HIS5*	ATCC #77145
YJR13pRS316-MYO1	wt	*MAT* a *trp1 ura3 leu2–3, his3delta1 cyh^R myo1 Δ::HIS5*	F. Rivera
YJR12pRS316-*GAL1-UBC4*	*wtpUBC4*	*MAT α trp1 ura3 leu2–3, his3delta1 cyh^R*	A. Brestcher
YJR12pFR23-MET25-UBC4	wt *pMETUBC4*	*MAT α trp1 ura3 leu2–3, his3delta1 cyh^R*	This study
YJR47	*myo1 ubc4*	*MAT* a *trp1 ura3 leu2–3, his3delta1 cyh^R myo1 Δ::HIS5 ubc4 Δ::kanMX4*	
YJR45	*myo1 vps4*	*MAT α trp1 ura3 leu2–3, his3delta1 cyh^R myo1 Δ::HIS5 vps4 Δ::kanMX4*	
YJR45pRS316-*GAL1-UBC4*	*myo1 vps4 pUBC4*	*MAT α trp1 ura3 leu2–3, his3delta1 cyh^R myo1 Δ::HIS5 vps4 Δ::kanMX4*	A. Brestcher
ATCC # 4003219	*ubc4*	*MAT* a *his3delta1 leu2delta0 met15delta0 ura3delta0 deltaUBC4*	
YJR44	*vps4*	*MAT α trp1 ura3 leu2–3, his3delta1 cyh^R vps4 Δ::kanMX4*	

Table 3

Budding pattern comparison

Budding pattern	wt	*wt pUBC4*	*myo1*	*myo1 pUBC4*	*myo1 ubc4*
% Single buds	15	9.8	16	13	52.6
% Axial	0.5	0.4	5	2	7.1
% Non-axial	0	0	5.5	3.5	11.9

200 budded cells studied from strains cultured in galactose media.