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Rsp5 ubiquitin ligase affects isoprenoid pathway and cell wall organization in *S. cerevisiae*★✪

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Dimethylallyl diphosphate, an isomer of isopentenyl diphosphate, is a common substrate of Mod5p, a tRNA modifying enzyme, and the farnesyl diphosphate synthase Erg20p, the key enzyme of the isoprenoid pathway. *rsp5* mutants, defective in the Rsp5 ubiquitin-protein ligase, were isolated and characterized as altering the mitochondrial/cytosolic distribution of Mod5p. To understand better how competition for the substrate determines the regulation at the molecular level, we analyzed the effect of the *rsp5-13* mutation on Erg20p expression. The level of Erg20p was three times lower in *rsp5-13* compared to the wild type strain and this effect was dependent on active Mod5p. Northern blot analysis indicated a regulatory role of Rsp5p in *ERG20* transcription. *ERG20* expression was also impaired in *pkc1*Δ lacking a component of the cell wall integrity signaling pathway. Low expression of Erg20p in *rsp5* cells was accompanied by low level of ergosterol, the main end product of the isoprenoid path-

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Abbreviations: DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; SRE, sterol regulatory element; SREBP, SRE-binding protein.

way. Additionally, *rsp5* strains were resistant to nystatin, which binds to ergosterol present in the plasma membrane, and sensitive to calcofluor white, a drug destabilizing cell wall integrity by binding to chitin. Furthermore, the cell wall structure appeared abnormal in most *rsp5-13* cells investigated by electron microscopy and chitin level in the cell wall was increased two-fold. These results indicate that Rsp5p affects the isoprenoid pathway which has important roles in ergosterol biosynthesis, protein glycosylation and transport and in this way may influence the composition of the plasma membrane and cell wall.

The isoprenoid pathway supplies the cell with isoprenoid lipids, which are used to produce dolichols, prenylated proteins, ubiquinon and heme. However, the most prevailing product of the pathway is the ergosterol in yeast and cholesterol in humans. Ergosterol is an important component of the plasma membrane and organellar membranes which affects their fluidity, permeability and other features. Cells lacking normal ergosterol biosynthesis show defects in endocytosis (Munn *et al.*, 1999; Heese-Peck *et al.*, 2002), vacuole fusion (Kato & Wickner, 2001), and mitochondrial respiration (Parks & Casey, 1995). The branch point enzyme of the isoprenoid pathway is an essential farnesyl diphosphate synthase (Erg20p) which catalyses the sequential condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) to form geranyl diphosphate (GPP) and further farnesyl diphosphate (FPP) (Song & Poulter, 1994). FPP, besides being a precursor for several isoprenoid compounds, also serves a regulatory role in the isoprenoid pathway (see review by Grabińska & Palamarczyk, 2002). DMAPP used by Erg20p is also a common substrate for Mod5p, a tRNA isopentenyltransferase (Dihanich *et al.*, 1987). Both enzymes compete for the DMAPP pool as indicated by the finding that overexpression of the *ERG20* gene causes a decrease in isopentenylation of tRNAs (Benko *et al.*, 2000). This was confirmed further by the result showing that an increase in tRNA biosynthesis was accompanied by lower ergosterol production if active Mod5p was present (Kamińska *et al.*, 2002a).

Sterol depletion in mammalian cells causes activation of the transcription factors known as sterol regulatory element (SRE) binding proteins (SREBPs) (reviewed in Edwards *et al.*, 2000). When sterols are abundant the SREBPs are inactive and tethered to the endoplasmic reticulum membrane by their transmembrane domains. When sterol level drops, regulated proteolysis releases the transcrpitional activation domain of SREBPs allowing its nuclear transport. SREBPs activate transcription of genes involved in sterol and fatty acid synthesis. The human gene encoding FPP synthase contains SRE sequence (Sato *et al.*, 1996). However, less is known about this regulatory mechanism in yeast. Many genes of the isoprenoid pathway are transcriptionaly regulated in response to *erg* mutations, inhibitors of isoprenoid pathway and anaerobiosis as determined by genome-wide expression profile analysis (Dimster-Denk *et al.*, 1999; Bammert & Fostel, 2000; Kwast *et al.*, 2002; Agarwal *et al.*, 2003). On the other hand, *ERG20* is constitutively expressed after inhibition of the downstream part of the ergosterol synthesis pathway by azoles (Bammert & Fostel, 2000; Agarwal *et al.*, 2003) and in anaerobiosis (Kwast *et al.*, 2002), and only 2–3-fold upregulated by inhibition by lovastatin of HMG-CoA reductase from the upstream part of the pathway (Dimster-Denk *et al.*, 1999). An about two-fold increase of *ERG20* expression was also observed in an RNA polymerase III regulatory mutant that has enhanced tRNA synthesis (Kamińska *et al.*, 2002a). Many *ERG* genes are activated by the Upc2p and Ecm22p transcription factors which bind yeast SRE (Vik & Rine, 2001) and repressed by Mot3p and Rox1p (Kwast *et al.*, 2002; Henry *et al.*, 2002). However, *ERG20* was not found among those *ERG* genes and the mech-

anism of transcriptional regulation of *ERG20* remains unknown.

One of the enzymes of the isoprenoid pathway, HMG-CoA reductase (Hmg2p), is physiologically regulated by ubiquitination and degradation in proteasomes (Gardner *et al.*, 2001). Ubiquitination is a posttranslational modification of proteins by the polypeptide ubiquitin, which is achieved by the action of a cascade of enzymatic reactions involving ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) which has an important role in substrate selection. Polyubiquitination serves to direct short-lived and abnormal proteins for proteasomal degradation but mono- and multiubiquitination does not involve proteasomes and has regulatory roles in several processes (reviewed in Weissman, 2001; Hershko & Ciechanover, 1998; Hicke & Dunn, 2003; Muratani & Tansey, 2003). One of the yeast ubiquitin ligases is Rsp5p (Huibregtse *et al.*, 1995). The best studied is Rsp5p involvement in ubiquitination and endocytosis of plasma membrane transporters and receptors (reviewed in Rotin & Haguenauer-Tsapis, 2000; see also Gajewska *et al.*, 2001; Kamińska *et al.*, 2002b; Stamenova *et al.*, 2004) but it affects also other steps of vesicular traffic (Katzmann *et al.*, 2004; Morvan *et al.*, 2004), transcription (Beaudenon *et al.*, 1999), nuclear export of mRNAs and tRNAs (Rodriguez *et al.*, 2003; Neumann *et al.*, 2003; and our unpublished observations), biosynthesis of unsaturated fatty acids (Hoppe *et al.*, 2000; Shcherbik *et al.*, 2003; 2004), regulation of cellular pH (De la Fuente *et al.*, 1997; Kamińska *et al.*, 2000) and other processes. Genetic links were found between *RSP5* gene and cellular signaling pathways. *RSP5* is a multicopy suppressor of the temperature-sensitive phenotype of the *rlm1 slt1*(*ssd1*) double mutant (Watanabe *et al.*, 1995) which contains mutations in the *RLM1* gene encoding a transcription factor regulated by the Pkc1p signaling pathway and in the *SSD1* gene encoding a signaling protein involved in cell wall integrity independent of Pkc1p (Kaeberleine & Guarente, 2002). Moreover, the *WSC2* gene encoding one of the plasma membrane sensors of the Pkc1p-dependent pathway was identified as multicopy suppressor of *rsp5* mutant phenotypes (De la Fuente & Portillo, 2000).

In this work the *rsp5-13* mutant with deficiency of ubiquitin ligase was studied to investigate a possible link between the ubiquitination pathway, the expression of *ERG20* gene encoding the branch point enzyme of the isoprenoid pathway, and sterol biosynthesis. This connection was suggested by isolating *rsp5* mutants as affecting subcellular distribution of Mod5p, an enzyme using an isoprenoid substrate (Żołądek *et al.*, 1995; 1997). Seeking for the consequences of this link we also studied the effect of the *rsp5-13* mutation on cell wall structure and function. We show here a correlation between the *rsp5-13* mutation, lower expression of *ERG20* and lower synthesis of ergosterol. As a consequence of mutated Rsp5p the cell wall structure, chitin content and sensitivity to calcofluor white, a chitin-binding drug, was also changed.

MATERIALS AND METHODS

Yeast strains, genetics methods and media. *Saccharomyces cerevisiae* strains used are listed in Table 1. Standard genetic manipulations were performed as described by Sherman (2002). Yeast were grown on rich media containing 2% glucose (YEPD), or on SC-ura and SC-ura-leu selective media containing 2% glucose which were supplemented with required amino acids. Strain *pkc1*Δ (Y23133-1D) was grown in media additionally supplemented with 1 M sorbitol. In growth assays YEPD with addition of nystatin (Sigma, 6 U/ml) or calcofluor white (Sigma, 1 μg/ml) was also used. Cells were grown overnight in YEPD at 30^{o}C to equal absorbance, then ten-fold serially diluted and spotted on plates.

Plasmids and plasmid constructions. Plasmid pJK1 contains the *ProtA-ERG20* fusion gene expressed from the *ERG20* promoter on the centromeric vector pRS316 (*URA3*) (Kamińska *et al.*, 2002a). Also plasmids pLB2 (containing *ERG20*, Blanchard & Karst, 1993), YCp-mod5-M2 (Gillman *et al.*, 1991) and YEp-SPT23 (from D. Haines, Temple University, Philadelphia, U.S.A.) and YEp-RLM1 (from J. M. Francois, Toulouse, France), were used.

Ttoal protein extracts and Western blot analysis. **P**rotein extracts were prepared by disrupting cells by alkaline lysis (Kamińska *et al.*, 2002a). Samples were analyzed by standard Western blot method using polyclonal rabbit anti-ferrochelatase antibodies (kind gift of J. Rytka, IBB PAS, Warszawa, Poland) or rabbit anti-Mdm1p antibodies (from M. Yaffe, University of California, U.S.A.) and secondary anti-rabbit horseradish peroxidase-conjugated antibody followed by chemiluminescence (ECL Plus, Amersham). The level of proteins was quantified using Image Quant V.1.1 software with local average background subtraction.

Preparation of total RNA and Northern analysis. Preparation of total RNA and Northern analysis were performed using specific probes for *ERG20* and *ACT1* as described (Kamińska *et al.*, 2002a). The levels of RNA were quantified using Gene Tools 3.0 (Syngene).

Determination of ergosterol levels. Sterols were extracted from yeast cells with n-heptane after alkaline lysis and the amount of ergosterol was determined from the absorbance at 281.5 nm (Servouse & Karst, 1986).

Transmission electron microscopy. Cells grown on YEPD to logarithmic phase were fixed with 2% glutaraldehyde followed by treatment with 2% OsO_4 for 2 h. Thin sections were cut from Epon blocks and post-stained with 2% uranyl acetate followed by lead citrate (Reynolds, 1963). Micrographs were

Table 1. List of *S. cerevisiae* strains

Strain	Genotype	Source or Reference
T8-1D	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519*	Żołądek *et al.*, 1995
T8-1D/1	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519* [YCf-mod5-M2]	This work
TZ23	*MATα rsp5-13 SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3 112 his4-519*	Żołądek *et al.*, 1997
TZ23/1	*MATα rsp5-13 SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3 112 his4-519* [YCf-mod5-M2]	This work
MK1	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 rsp5::HA-RSP5*	Kamińska *et al.*, 2002
MK5	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 rsp5::HA-rsp5-13*	Kamińska *et al.*, 2002
BY4742	*MAT***a** *his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0*	Euroscarf
Y14869	*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 spt23::kanMX4*	Euroscarf
Y12739	*MAT***a** *his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 rlm1::kanMX4*	Euroscarf
Y10249	*MAT***a** *his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 gcn4::kanMX4*	Euroscarf
Y23133-1D	*MATα his3-Δ1 leu2-Δ1 lys2-Δ1 ura3-Δ1 met15-Δ0 pkc1::kanMX4*	Derivative of Y23133 Euroscarf

taken on a JEOL transmission electron microscope JEM1220.

Analysis of cell wall composition. To determine chitin and glucan levels cells were grown overnight in SC media supplemented with required amino acids, collected and disrupted with glass beads. Cell walls were separated from cell extracts by 5 000 × ***g*** centrifugation. For chitin measurements alkaline lysis of cell walls was performed in 6% KOH for 90 min at 80°C to release cell wall proteins. After neutralization with acetic acid the cell walls were washed with phosphate-buffered saline and chitinase buffer pH 6.0, containing 18 mM citric acid and 60 mM dibasic sodium phosphate, and then treated with chitinase C (InterSpex Products) for 3 h at 37°C. Chitin was measured as described (Bulik *et al.,* 2003). For glucan determination cell walls were hydrolysed similarly as described for *Trichoderma viride* (Nemčovič & Farkaš, 2001) and glucose in the hydrolysate was measured by a published method (Wood & Bhat, 1988).

Zymolyase assay. Sensitivity to zymolyase was determined based on the method described by De Groot *et al.* (2001). Cells grown overnight in YEPD were resuspended in 10 mM Tris/HCl, pH 7.4, than incubated at 37°C with zymolyase 20T (ICN) 50 μg/ml and A_{600} was measured in 15 min intervals.

RESULTS

The *rsp5-13* mutation affects expression of *ERG20* gene

Erg20p, a farnesyl diphosphate synthase, competes for the substrate, dimethylallyl diphosphate, with Mod5p (Benko *et al.*, 2000), a tRNA isopentenyltransferase which modifies cytosolic and mitochondrial tRNAs (Dihanich *et al.*, 1987). On the other hand, distribution of the cellular pool of Mod5p is dependent on the activity of Rsp5p, a protein-ubiquitin ligase (Żołądek *et al.*, 1995; 1997). These two findings persuaded us to assess if Rsp5p affects the ergosterol pathway. For these studies we used the *rsp5-13* mutant with the G707D substitution in the catalytic domain of Rsp5p (Żołądek *et al.*, 1997) which confers a temperature-sensitivity phenotype.

The level of Erg20 protein was determined in isogenic strains *rsp5-13, rsp5-13 mod5-1* and wild type transformed with pJK1 centromeric plasmid bearing the *ProtA-ERG20* gene fusion. Cellular extracts prepared from cells grown at 28°C and shifted to the restrictive temperature of 37°C for 2.5 h were analyzed by Western blot using anti-ferrochelatase antibody. This antibody recognized ProtA and endogenous ferrochelatase, which served as internal control for quantification of ProtA-Erg20p bands. In *rsp5-13* the level of Erg20p was decreased three fold as compared to wild type cells, both grown at 28°C and shifted to the restrictive temperature (Fig. 1). However, this difference was dependent on the pres-

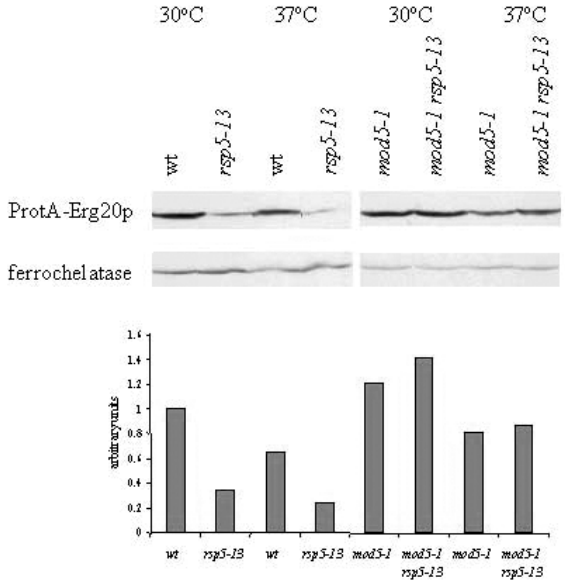

Figure 1. The level of Erg20p is lower in *rsp5-13* and depends on Mod5p.

Yeast strains: wt (T8-1D/1, pJK1), *rsp5-13* (TZ23/1, pJK1), *mod5-1* (T8-1D, pJK1) and *mod5-1 rsp5-13* (TZ23, pJK1) were grown in SC-ura-leu at permissive temperature followed by a shift to 37°C for 2.5 hours. Protein extracts were analyzed by Western blot with anti-ferrochelatase antibody. Quantification of ProtA-Erg20p level after correction for ferrochelatase as a control is shown below.

ence of active Mod5p. *rsp5-13* had no effect on Erg20p level in the background of *mod5-1* mutation which was probably due to the lack of competition of mutant Mod5-1p with Erg20p for the substrate. Moreover, an about 40% decrease of Erg20p level in response to temperature elevation was consistently observed in all strains studied.

Since the Erg20p cellular content was decreased in the *rsp5-13* mutant strain, we determined if the reason for this effect was the difference in *ERG20* transcript level. Total RNA isolated from *rsp5-13* and wild type strain transformed with pJK1, grown at 28°C and shifted to 37°C for 2 h, was analyzed by Northern blot using *ERG20* specific probe. As shown in Fig. 2, the level of both genomic *ERG20* transcript and plasmid *ProtA-ERG20* transcript were decreased 3–4-fold in the *rsp5-13* mutant. In cells shifted to the elevated temperature the decrease of *ERG20* transcript level was observed irrespective of the presence of the *rsp5-13* mutation. Therefore, changes in *ProtA-ERG20* and *ERG20* transcript levels in response to the *rsp5-13* mutation or temperature elevation exactly paralleled the changes observed in Erg20p level. From these results we concluded that Rsp5p is involved in regulation of *ERG20* expression and this regulation occurs at the level of *ERG20* transcript synthesis or degradation.

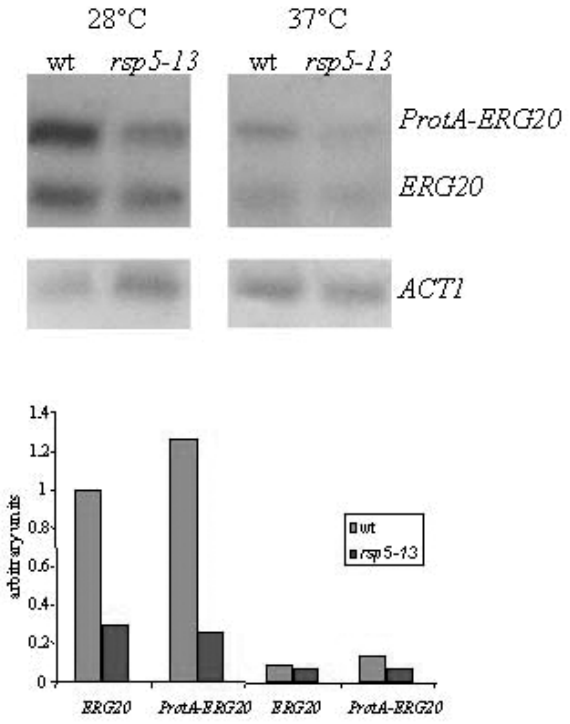

Figure 2. The level of *ERG20* mRNA is lower in *rsp5-13* compared to wild type.

Northern analysis of RNA isolated from wt (T8-1D/1, pJK1) and *rsp5-13* (TZ23/1, pJK1) strains grown in SC-ura-leu at permissive temperature followed by a shift to 37°C for 2.5 h. Radioactive probes specific for *ERG20* or *ACT1* were used. Quantification of *ERG20* mRNA specific signals after correction for *ACT1* mRNA is shown below.

Transcription factors involved in expression of *ERG20*

Since the control of *ERG20* expression by Rsp5p possibly occurred at the transcriptional level, the question arose which transcription factor was involved in this regulation. *ERG20* promoter had not been experimentally analyzed and transcription factors regulating *ERG20* were not described so far. In mammalian cells ergosterol biosynthesis and fatty acid biosynthesis pathways are to some extent commonly regulated (Edwards *et al.*, 2000). We investigated if *ERG20* is regulated by Rsp5p in common with genes involved in unsaturated fatty acid biosynthesis. It is known that transcription of the *OLE1* gene encoding fatty acid desaturase is under the control of the Spt23p and Mga2p transcriptional regulators. To be active, Spt23p and Mga2p require Rsp5p-dependent ubiquitination, proteolytic processing and release from endoplasmic reticulum membranes (Hoppe *et al.*, 2000; Shcherbik *et al.*, 2003). To test if Spt23p affects expression of *ERG20* we analyzed the level of Erg20p in strains with different *SPT23* gene copy number. For this purpose we used a wild type strain transformed with vector alone or transformed with a multicopy plasmid expressing *SPT23,* and a strain with *SPT23* deletion, all

expressing ProtA-Erg20p from the pJK1 plasmid. The results presented in Fig. 3

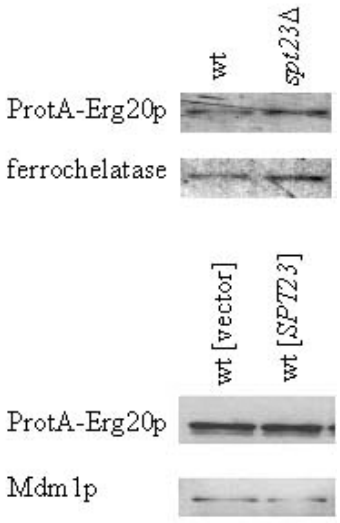

Figure 3. Spt23p does not affect *ERG20* expression.

Western blot analysis of wt (BY4742) and *spt23*Δ (Y14869) and wt (BY4742) transformed with YEp-SPT23, all bearing pJK1 plasmid. Antibodies against ferrochelatase or Mdm1p were used.

showed that neither *SPT23* deletion nor *SPT23* overexpression had an effect on Erg20p level.

Because of the genetic links between *RSP5* and the cell wall integrity pathway we tested if *ERG20* expression is under the regulation of protein kinase C (Pkc1p), an important component of this signaling pathway. Northern analysis performed in *pkc1*Δ and wild type strain showed indeed lower expression (0.6 of wild type) of *ERG20*, both from the genomic copy and a copy expressed from a multicopy plasmid, when Pkc1p was absent (Fig. 4). This result indicates that *ERG20* and the cell wall integrity pathway use at least one common signal. Computer analysis of the *ERG20* promoter by Mat Inspector (http://imgt.cines.fr) revealed potential binding sites for several transcription factors, including Rlm1p (Jung *et al.*, 2002) and Gcn4p (Natarajan *et al.*, 2001). However, our studies indicate that neither Rlm1p nor Gcn4p regulate expression of *ERG20* (not shown). Since the presence of other transcription factors, besides Rlm1p, controlled by the Pkc1p-dependent pathway has been postulated (see a review by Smits *et al.*, 2001) these so far unidentified factors might be responsible for the regulation of *ERG20*.

Cellular ergosterol content is low in *rsp5-13* mutant

Low expression of Erg20p in *rsp5-13* mutant could result in changes of the amounts of the end products of the isoprenoid pathway. Since most of farnesyl diphosphate produced by Erg20p is used for ergosterol biosynthesis, we measured the level of ergosterol in *rsp5-13* cells and in wild type cells grown in YEPD at 30°C. The amount of ergosterol was lower by about 21% in *rsp5-13* cells than in wild type cells (Table 2). This difference was statistically significant. The presented results indicate that Rsp5p indirectly affects the cellular ergosterol level.

Table 2. Effect of *rsp5-13* on cellular level of ergosterol.

Cells were grown overnight on YEPD at 30°C. Cellular ergosterol content was determined in eight independent samples of wild type (MK1) and *rsp5-13* (MK5) strains.

Strain	Ergosterol [% of dry weight]
wt	0.425 ± 0.026
rsp5-13	0.335 ± 0.027

Nystatin is an antifungal drug that interacts selectively with membrane ergosterol but not with other sterols (Lees *et al.*, 1995) and absence of ergosterol is known to confer nystatin-resistant growth. Therefore, we determined if the *rsp5-13* mutation affects growth of cells in the presence of nystatin. Growth of *rsp5-13* and wild type strains was tested by drop test on YEPD medium containing nystatin at 30°C. The *rsp5-13* mutant cells were slightly resistant to nystatin (Fig. 5).

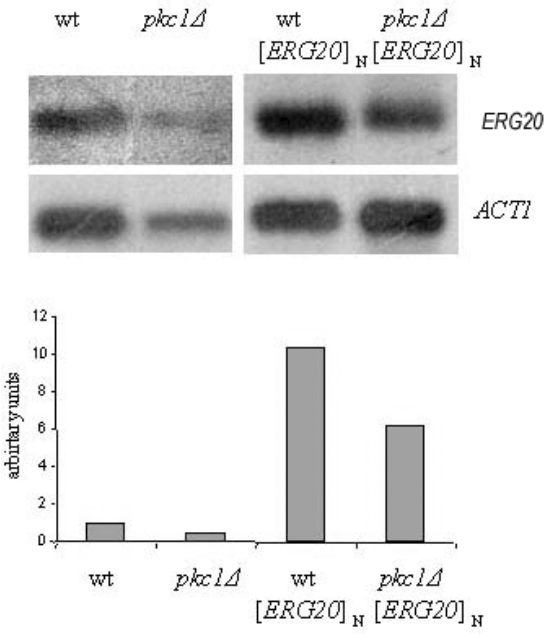

Figure 4. Transcription of *ERG20* is controlled by cell wall integrity signaling pathway.

Northern blot analysis of RNA isolated from wt (BY4742) and *pkc1*Δ (Y23133-1D) strains bearing vector or pLB2 grown on SC-ura at 30°C. Radioactive probes specific for *ERG20* or *ACT1* were used. Quantification of *ERG20* mRNA specific signals after correction for *ACT1* mRNA is shown below.

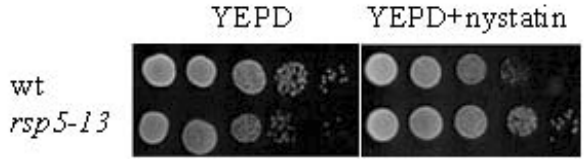

Figure 5. *rsp5-13* cells are resistant to nystatin.

Growth of wild type (MK1) and *rsp5-13* (MK5) strains on YEPD and YEPD containing nystatin.

This result is in agreement with the lower content of ergosterol in the *rsp5-13* mutant.

Cell wall is defective in *rsp5-13* mutant

Since *rsp5* mutant cells showed lower *ERG20* expression and in consequence a decreased flux of the isoprenoid pathway, we assumed that also other products of isoprenoid pathway besides ergosterol might be synthesized with lower efficiency. Such products as dolichols are very important for protein glycosylation in the endoplasmic reticulum and disturbances in their synthesis cause defects of maturation and transport of cell wall proteins and in consequence in cell wall biogenesis. Therefore, we studied cell wall morphology and resistance to treatments which disturb its function. Electron microscopy was used to investigate cell wall morphology in wild type and *rsp5-13* cells grown on YEPD at 30°C. Our control cells displayed a single thin layer cell wall (Fig. 6A), but some cells (about 6%) exhibited aberrant cell wall morphology. The proportion was different in the *rsp5-13* mutant as only 42% of cells

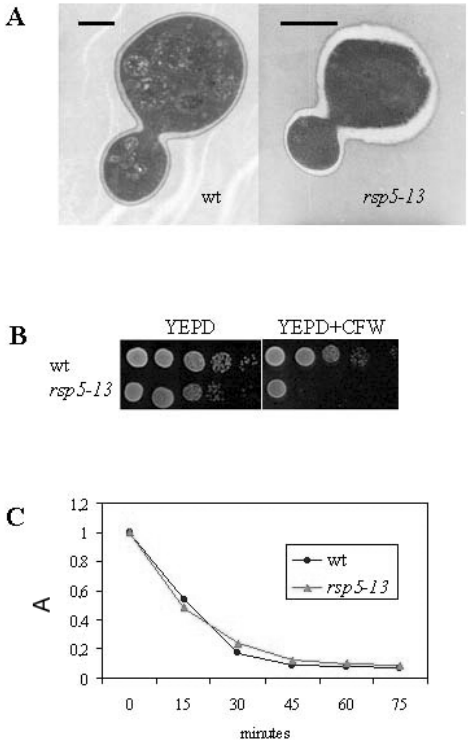

Figure 6. Cell wall is defective in *rsp5-13* strain.

A. Cell wall structure is abnormal in *rsp5-13* strain. Electron microscopy was performed on wild type (MK1) and *rsp5-13* (MK5) strains grown on YEPD at 30°C. Bar represents 1 μm. **B.** *rsp5-13* strain is sensitive to calcofluor white. Growth of wild type (MK1) and *rsp5-13* (MK5) strains on YEPD and YEPD with calcofluor white (YEPD+CFW) **C.** *rsp5-13* strain is sensitive to zymolyase treatment similarly as wild type strain. A_{600} of cell suspensions was measured at indicated time intervals after zymolyase addition.

showed normal morphology, while the cell wall of the mother cell was thicker than that of the bud in 58% of dividing cells. As shown in Fig. 6A, the structure of the thick cell wall in *rsp5-13* was uniform, with no visible additional layers, but the thickness differed around the cell.

Calcofluor white is a drug which destabilizes cell wall by binding to chitin. Changes in resistance to this drug are often indicative of cell wall abnormalities and are accompanied by changes in chitin level (De Nobel *et al.*, 2001). Growth of wild type and *rsp5-13* strains was compared on YEPD containing calcofluor white. The *rsp5-13* strain appeared much more sensitive than wild type to this drug (Fig. 6B) confirming that cell wall function is disturbed in *rsp5-13*. To find out if the sensitivity to calcofluor white is a result of abnormal call wall composition the content of chitin in the cell wall was measured. The cell wall of *rsp5-13* strain contained almost twice as much chitin as the wild type (Table 3) and this might be the reason for the observed calcofluor white sensitivity. We also tested other parameters of the cell wall, the content of glucan, the main component of the cell wall, and sensitivity to zymolyase, a mixture of enzymes comprising β-1,3-glucanase and protease activities. There was only a slightly higher level of glucan content in *rsp5-13* than in the wild type strain (Table 3) and the sensitivity to zymolyase was comparable in these two strains (Fig. 6C).

Table 3. Effect of *rsp5-13* on chitin and glucan content in cell wall.

Cells were grown overnight on YEPD at 30°C. Content of chitin and glucan was determined in cell walls of wild type (MK1) and *rsp5-13* (MK5) strains. Data are averages of two chitin and glucan determinations for each strain.

Strain	Chitin [% GlcNAc/dry weight]	Glucan [% glucose/dry weight]
wt	1.14	52.76
rsp5-13	2.19	59.17

These results document that defects of the Rsp5 ubiquitin ligase result in thickening of the cell wall and this correlates with a higher content of chitin and increased sensitivity to a chitin-binding drug. In the same cells the β-1,3-glucan layer and sensitivity to cell wall degrading enzymes are not altered.

DISCUSSION

Regulation of the isoprenoid pathway is intensively studied because products of this pathway have great impact on cell physiology. Erg20p is a branch point enzyme of this pathway and farnesol deriving from the Erg20p product, FPP, has been shown to be a non-sterol regulator in the synthesis of ergosterol in yeast and cholesterol in human (for a review see Grabińska & Palamarczyk, 2002). In this report we provide data that *ERG20* gene expression is regulated at the level of *ERG20* transcript synthesis or degradation, and that this regulation is dependent on the ubiquitin system and the cell wall integrity pathway. In particular, the Rsp5 ubiquitin protein ligase affects greatly the *ERG20* transcript level. In consequence, Rsp5p affects also cellular content of ergosterol and cell wall structure and composition.

Changes in transcriptional regulation of genes in response to altered activity of Rsp5p have been reported previously. *RSP5* affects transcriptional activation of heat shock element (HSE)-dependent targets (Kaida *et al.*, 2003) and transcriptional regulation of *OLE1* gene, encoding fatty acid desaturase (Hoppe *et al.*, 2000). This latter regulation is known in detail and it involves the Spt23p and Mga2p transcriptional regulators. They are docked *via* transmembrane domain in the endoplasmic reticulum and must be processed and released, depending on Rsp5p, to perform their action in the nucleus (Hoppe *et*

al., 2000; Shcherbik *et al.*, 2003; 2004). However, we did not find evidence that this mechanism operates in the regulation of *ERG20* expression. It has been demonstrated that Rsp5p also ubiquitinates and directs for degradation the large subunit of RNA polymerase II (Huibregtse *et al.*, 1997; Beaudenon *et al.*, 1999) and in this way may affect transcription of some genes. Transcriptional downregulation of *ERG20* in *rsp5-13* might be also a more indirect effect of changes in cellular physiology resulting from defects in Rsp5p. In theory it is also possible that Rsp5p affects *ERG20* transcript stability, not the synthesis. We found a strict (three fold decrease) correlation between low *ERG20* transcript level and diminished levels of Erg20p in *rsp5-13*. Thus, a translational regulation of *ERG20* expression was probably not involved. However, Erg20p level was not quantitatively related to the sterol content, which was diminished only by about 21%, indicating for possible regulation of Erg20p at the level of enzyme activity (Szkopińska *et al.*, 2000). Ergosterol is mainly present in the plasma membrane (Zinser *et al.*, 1993). A regulatory role in the transport of ergosterol to this destination is played by lipid particles and sterol/steryl esters homeostasis through an esterification/hydrolysis mechanism (Mullner & Daum, 2004) so the total level of ergosterol does not necessarily reflect the plasma membrane level. Resistance of *rsp5-13* to nystatin, however, indicates that the plasma membrane level of ergosterol must be lower in this mutant. Since ergosterol is necessary for several steps of endocytosis (Munn *et al.*, 1999; Heese-Peck *et al.*, 2002) this may possibly be an additional reason why endocytosis is impaired in *rsp5* mutants (Żołądek *et al.*, 1997; Gajewska *et al.*, 2001).

Dolichol, one of the products of the isoprenoid pathway, is used as a sugar carrier lipid or sugar donor in N-glycosylation, O-mannosylation and GPI-anchoring, all important for cell wall synthesis and secretion. The cell wall is a complex and dynamic structure which is remodeled in response to different environmental conditions like osmotic or temperature stress, nutritional factors and pheromones (Smits *et al.*, 2001). Cell wall remodeling is regulated by the cell wall integrity pathway comprising Pkc1p and the MAP kinase cascade. We have found downregulation of *ERG20* in *pkc1*Δ but it was only by about 40% suggesting that the cell wall integrity pathway is involved and also other factors contribute to the transcriptional regulation of this gene. The *pkc1*Δ dependent regulation of *ERG20* was not mediated *via* the transcriptional factor Rlm1p and other factors must be sought. The presence of Pkc1p-dependent factors other than Rlm1p has long been suspected (Smits *et al.*, 2001).

The yeast cell wall has a layered structure and is composed mainly of glucan (60%), mannoproteins (40%) and chitin (1%). About 90% of chitin in *Saccharomyces cerevisiae* is synthesized by chitin synthase 3 (Chs3p), one of the three yeast chitin synthases. Chs3p transits through the endoplasmic reticulum/Golgi secretory pathway to the plasma membrane and later is retrieved by enodocytosis. The cycling between the plasma membrane and endosomes is highly regulated by several proteins (Smits *et al.*, 2001). Deregulation results in abnormal chitin content in the cell wall. It is apparent from recent studies that deletion of genes that influence protein glycosylation or endocytosis yields strains that have high chitin content (Bulik *et al.*, 2003). Thus, the observed changes of cell wall structure and higher chitin content in *rsp5* mutant may reflect defects in two processes: possible defects in glycosylation of cell wall proteins due to downregulation of the isoprenoid pathway and slower endocytosis affecting chitin synthase cycling.

Understanding how cells regulate expression of key enzymes of the isoprenoid pathway is critical for the description of events that regulate cellular levels of sterols, dolichols and other products important for vital cell functions. A role of the cell wall integ-

rity pathway and the ubiquitin system in regulation of *ERG20* gene is now established but further work is needed to understand the molecular mechanism.

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