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Research Article

Mechanism of polyamine tolerance in yeast: novel regulators and insights

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Abstract. Polyamines are small charged molecules essential for various cellular functions, but at high levels they are cytotoxic. Two yeast kinases, SKY1 and PTK2, have been demonstrated to regulate polyamine tolerance. Here we report the identification and characterization of additional genes involved in regulating polyamine tolerance: YGL007W, FES1 and AGP2. Deletion of YGL007W, an open reading frame located within the promoter of the membrane proton pump PMA1, decreased Pma1p expression. Deletion of FES1 or AGP2 resulted in reduced polyamine uptake. While high-affinity spermine uptake was practically absent in *agp2Δ* cells, *fes1Δ* cells displayed only reduced affinity towards spermine. Despite the reduced uptake, the resistant strains accumulated significant levels of polyamines and displayed increased ornithine decarboxylase activity, suggesting reduced polyamine sensing. Interestingly, *fes1Δ* cells were highly sensitive to salt ions, suggesting different underlying mechanisms. These results indicate that mechanisms leading to polyamine tolerance are complex, and involve components other than uptake.

Key words. FES1; AGP2; YGL007W; polyamines; ornithine decarboxylase.

The naturally occurring polyamines putrescine, spermidine and spermine are widely distributed in living organisms. They have a low molecular weight, a simple chemical structure (aliphatic amines) and are highly charged cations at physiological conditions. Polyamines are essential for the maintenance of cell growth, survival and for macromolecular biosynthesis, probably by interacting with nucleic acids, proteins and membranes through ionic interactions. They are involved in many cellular functions including chromatin structure, gene expression, transcription, signal transduction, cell growth, cell cycle regulation, proliferation, membrane stability, ion channels and cell signaling [1–8]. Since polyamines can stimulate proliferation and metastasis of cancer cells they have become a target for therapeutic efforts [3, 5, 9–11]. Polyamine levels are tightly regulated, as low levels fail to support cell growth, while excesses appear to be toxic. An important regulatory step is their uptake across the plasma membrane [3, 4, 7, 8]. Many cell types possess a polyamine uptake system that is distinct from those for amino acids. In most mammalian cells, polyamine uptake is carrier mediated, energy dependent and Na^+ activated [3, 8]. However, polyamine-specific transporters from mammalian cells have not yet been purified and characterized. In yeast, polyamine uptake is also energy dependent, and seems to involve at least two transport systems that recognize all three polyamines, although the K_m values for each polyamine are different [8, 12, 13].
The major determinants of the plasma membrane potential in *Saccharomyces cerevisiae* are the Pma1p H^+-ATPase proton pump, generator of the membrane potential,

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and the Trk1,2 major high-affinity K^+ uptake system, a consumer of the membrane potential. These energize secondary transporters such as amino acids and polyamine transporters. Pma1p is positively regulated through phosphorylation by Ptk2p, a serine/threonine protein kinase [14]. The Trk1,2 system is positively regulated by the Hal4p and Hal5p kinases [15], and has been suggested to be negatively regulated by the serine/threonine protein kinase Sky1p [16]. Both Sky1p and Ptk2p were found to be involved in regulating polyamine uptake and tolerance, as cells deleted for these genes can grow in high spermine concentrations [17, 18]. In addition, their deletion increases the tolerance towards high salt concentrations [18, 19].

SKY1 and PTK2 were also identified in a genome-wide screen for genes whose deletion conferred resistance to bleomycin, an anti-cancer drug containing a spermidine moiety [20]. In addition, three other genes, AGP2, FES1 and YGL007w, were identified in this screen, which were not previously associated with polyamine transport [20]. AGP2 is a plasma membrane carnitine transporter also functioning as a low-affinity amino acid permease, and its expression is down-regulated by osmotic stress [21, 22]. FES1 is a nucleotide exchange factor for the yeast 70-kDa heat shock protein (Hsp70), Ssa1p [23]. Fes1p was shown to promote ADP release from Ssa1p and its deletion resulted in a thermosensitive phenotype. Fes1p has an important role in protein translation, but not in protein translocation or folding [23]. YGL007W is an uncharacterized small open reading frame (ORF) with unknown function.

We show here that deletion of these genes confers spermine resistance. The spermine tolerance of the YGL007W deleted strain is mediated by decreased activity of the proton pump PMA1. Deletion of FES1 or AGP2 resulted in reduced polyamine uptake. Despite the reduced uptake activity, all resistant strains accumulated significant levels of polyamines and displayed increased ornithine decarboxylase (ODC) activity, suggesting less sensing of polyamines, perhaps due to their sequestration to a non-accessible subcellular location. While tolerating high spermine concentration, FES1 deleted cells were very sensitive to salt ions, suggesting a different underlying mechanism. While we identify here new genes involved in regulating polyamine transport and tolerance, our data suggests that polyamine uptake is not the sole mechanism leading to polyamine tolerance.

Materials and methods

Strains and media. The *S. cerevisiae* strains used in the present work are listed in table 1. These strains were routinely maintained in YPD medium (1 % yeast extract, 2 % peptone, 2 % D-glucose) or in Mg^{2+}-limited synthetic complete (LMSC) medium supplemented with 50 mM $MgSO_4$ as described elsewhere [24] and with the required essential amino acids. Yeast cells were transformed by the lithium acetate method [25].

Table 1. Yeast strains used in this study.

Strain	Genotype	Source
BY4741	MATa, *leu2, ura3, his3, lys2*	EUROSCARF
sky1Δ	BY4741, SKY1::KanMX4	EUROSCARF
ygl007wΔ	BY4741, YGL007W::KanMX4	EUROSCARF
agp2Δ	BY4741, AGP2::KanMX4	EUROSCARF
agp2Δ sky1Δ	*agp2Δ*, SKY1::Leu2	this study
fes1Δ	BY4741, FES1::KanMX4	EUROSCARF
fes1Δ sky1Δ	*fes1Δ*, SKY1::Leu2	this study
npr2Δ	BY4741, NPR2::KanMX4	EUROSCARF
stp1Δ	BY4741, STP1::KanMX4	EUROSCARF
stp2Δ	BY4741, STP2::KanMX4	EUROSCARF

Plasmids and gene disruption. The SKY1 gene was cloned onto the pGEM-T Easy vector (Promega). To disrupt the SKY1 gene, the corresponding DNA was digested with *Kpn*I. The resulting *Kpn*I sites were filled with T4 polymerase to yield blunt ends, and religated with a *Bam*HI linker. Then a *Bam*HI fragment encompassing the LEU2 gene was cloned into the implanted *Bam*HI site. Following verification by PCR and sequencing, the construct was used to transform *agp2Δ* and *fes1Δ* cells. Positive yeast colonies were identified by PCR. The ORF YGL007W was amplified by PCR from yeast genomic DNA using the primers: CTATGTCGACAATGGAAAAGGAAGGAAAA (5' primer) and CTACGTCGACAGCTAAAGTGCAAAAAGTCGTT (3' primer) and cloned between the *Sal*I (5') and *Sac*I (3') sites of the yeast expression vector pAD54 [26] downstream to an HA tag.

Membrane preparation and ATPase activity determination. Total membrane fractions were prepared essentially as described elsewhere [27]. Briefly, log phase cells from 100 ml of culture were pelleted, washed and resuspended in ice-cold lysis buffer (10 mM Tris pH 7.4, 0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$ and protease inhibitors). Cells were then lysed at 4 °C using glass beads, and the lysate was centrifuged at 700 g for 5 min to remove unbroken cells. Membranes were then pelleted at 4 °C by centrifugation for 1.5 h at 100,000 g. The membranes were washed twice and stored at –80 °C in storage buffer (10 mM Tris pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol, 20 % glycerol). Vanadate-sensitive ATPase activity was measured in the presence or absence of 100 mM sodium orthovanadate. Equal amounts of protein from the membrane fractions were added to the

assay mixture containing 10 mM MOPS-Tris (pH 6.5), 5 mM disodium ATP, 5 mM $MgCl_2$, 5 mM NaN_3, 5 mM phosphoenolpyruvate and 25 μg of pyruvate kinase. The reaction was carried out for 10 to 30 min at 30 °C. Free phosphate was determined according to the Fiske-Subbarow procedure [28]. Activity is expressed as arbitrary units based on absorption at 820 nm.

Western blot analysis. Cells from 1.5 ml of cultures were collected by centrifugation and resuspended in 50 μl of sample buffer (125 mM Tris-HCl pH 6.8, 4 % sodium dodecyl sulfate (SDS), 20 % glycerol, 1.4 M -mercaptoethanol and bromophenol blue). Following vortex and 5 min of boiling, the samples were fractionated by SDS-10 % polyacrylamide gel electrophoresis (PAGE), and blotted onto a nitrocellulose membrane. The blots were probed with anti-HA monoclonal antibody (1:2000; BabCo) and goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (1:10,000; Jackson) was added as a secondary antibody. Signals were detected using the enhanced chemiluminescence system (Pierce). The protein concentration of the wild-type (WT) and *ygl007wΔ* membrane extracts was determined by the Bradford assay (BIO-RAD), and 35 μg of protein was fractionated on a polyacrylamide gel. In this case, the blot was probed independently with two different antibodies, anti-Pma1p polyclonal antibody (1:2000; kindly provided by C. W. Slayman and K. Allen) and anti-Sso1p polyclonal antibody (1:4000; a gift from J. Gerst), and after the addition of each, goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate was added as a secondary antibody (1:10,000; Jackson). Differences in Pma1p expression were evaluated by pixel densitometer measurements of the corresponding bands in the western blot.

Polyamine uptake assay. Cells were grown to the mid-logarithmic phase at an optical density at 600 nm (OD_{600}) of 1.1–1.3, washed three times in glucose-citrate buffer (50 mM sodium citrate pH 5.5, 2 % D-glucose), and resuspended in the same buffer at a concentration of 10^8 cells/ml. Transport was initiated by adding 0.2 volumes [^{14}C]spermine (10 Ci/mol at 100 mM; from Amersham Pharmacia), and the cells were incubated at 30 °C with mild shaking. Uptake was stopped by transferring 100-μl aliquots into 1 ml of ice-cold stop buffer (glucose-citrate buffer containing 2 mM spermine). The cells were then layered on cellulose-acetate filters (0.45 mm pore size) that had been washed with stop buffer. The filters were washed three times with stop buffer, and the retained radioactivity was determined by liquid scintillation spectrometry.

Growth assays. The growth of yeast strains on YPD, or LMSC-Leu plates containing different additives was performed by spotting 2 μl from five-fold dilutions of cultures at OD_{600} = 1.0. Growth curves were generated by diluting overnight cultures of yeast strains to OD_{600} = 0.05, and growing them in a microplate optical reader (GENios, Tecan) at 30 °C with shaking. The OD_{600} was measured every 10 min.

Polyamine assay. The assay was performed according to Madhubala [29]. Briefly, yeast strain cultures were grown overnight, and 15 optical density units were collected from each sample. The samples were centrifuged for 30 s at 12,000 g, washed with phosphate-buffered saline (PBS) and centrifuged again. The supernatant was removed and the cells were resuspended in 100 μl PBS. Then, 4.5 μl of 70 % perchloric acid was added, and the samples were vortexed and centrifuged for 5 min at 16,000 g. Markers were prepared using equal amounts of putrescine, spermidine and spermine. Dansyl chloride, 200 μl (3 mg/ml acetone) and 10 mg of Na_2CO_3 were added to 100 μl of each sample. The samples were incubated overnight in the dark. Then, 10 mg proline was added, and the samples were incubated for 1 h. After this incubation period, 250 μl toluene was added, and samples were centrifuged for 30 s at 16,000 g. Marker (5 μl) and 100 μl of each sample were spotted on a thin layer chromatography (TLC) plate. The TLC was then developed in a glass chamber containing ethyl acetate and cyclohexane at a 2:3 ratio. The results were visualized and analyzed, using a gel imager with a UV filter. Relative polyamine amounts were evaluated by densitometer pixel measurements.

ODC activity assay. Yeast cultures (2 ml) were grown overnight. The cells were washed with double-distilled water (DDW) once before adding similar volumes of glass beads and 200 μl of ODC activity buffer [25 mM Tris HCl pH 7.5, 2.5 mM DTT, 0.2 mM pyridoxal-5'-phosphate (PLP)]. The samples were vortexed for 30 min at 4 °C, the resulting solution was then centrifuged at 16,000 g for 10 min and the protein content was measured using the Bradford reagent. Into each well, 200 μg of protein was placed, the volume was completed to 200 μl with ODC activity buffer and 1 μl [^{14}C]ornithine (52 mCi/mmol; from Amersham Pharmacia). A 3MM paper was cut to the shape of the microplate and soaked in a saturated BaOH solution. The paper was dried, two more 3MM papers were added, and the microplate was closed and incubated for 4 h at 30 °C. Finally, the 3MM paper was washed with acetone, dried, exposed to a fluorescent screen overnight, and examined using a phosphoimager.

ODC activity decay rates were determined by adding cycloheximide (0.1 mg/ml) to logarithmically growing yeast cultures. Aliquots were then removed at 0, 30 and 60 min. ODC activity was measured as describe above.

Results

Cells lacking FES1, AGP2 or YGL007w are more resistant to spermine. A genome-wide screen identified five yeast genes, SKY1, PTK2, AGP2, FES1 and YGL007W, whose deletion conferred resistance to bleomycin [20]. Two of these genes, SKY1 and PTK2, were demonstrated previously to be also involved in regulating transport of polyamines and salt ions across the plasma membrane [17–19]. To determine whether these genes are also involved in regulating polyamine tolerance, we assessed the growth of yeast strains deleted for AGP2, FES1 or YGL007W in YPD medium supplemented with toxic amounts of spermine. *agp2Δ* and *fes1Δ* cells grew efficiently in the presence of spermine concentration as high as 10 mM (fig. 1 and data not shown). *agp2Δ* cells were slightly less resistant than *fes1Δ* and *sky1Δ* cells, but they were more resistant then *ygl007wΔ* cells (fig. 1).

The ability of YGL007W to regulate spermine uptake is strictly dependent on it being part of the PMA1 promoter. A genomic fragment was isolated due to its ability to restore growth sensitivity to added spermine in an otherwise spermine-resistant mutant [described in ref. 18]. This genomic fragment was demonstrated to contain three ORFs: the yeast plasma membrane H^+-ATPase PMA1, a vacuolar $Ca2^+$-ATPase termed PMC1 and a short putative ORF termed YGL007W. YGL007W is a 378 bp fragment located in the promoter of PMA1 and is important for promoter function because it contains two upstream activating sequences that are important for the expression of the PMA1 gene [30]. As Pma1p provides the driving force required for the transport of various solutes into the cell [31], the restoration of spermine sensitivity is likely a result of PMA1 overexpression. However, since YGL007W is a putative ORF, we tested the possibility that this putative ORF encodes a polypeptide involved in regulating polyamine uptake. Deletion of YGL007W conferred increased tolerance to spermine (fig. 1), though to a lesser extent than the other resistant strains. We first tested whether deletion of YGL007W affects PMA1 protein and activity. We measured Pma1p protein level and activity in plasma membranes isolated from WT and ***ygl007wΔ*** cells. Deletion of the YGL007W gene resulted in an ~50 % reduction in Pma1p levels and in ATPase activity (fig. 2A, B). Next, to determine whether the YGL007W ORF encodes a polypeptide that is directly involved in regulating polyamine uptake, this polypeptide was expressed in WT and ***ygl007wΔ*** cells from a transfected expression construct (fig. 2C). As shown in figure 2D, overexpressed YGL007W did not result in increased spermine sensitivity. We therefore concluded that YGL007W does not function independently of its PMA1 promoter function in regulating spermine uptake. We could not exclude, however, that the encoded protein may be involved in another cellular function.

***fes1Δ* and *agp2Δ* cells show decreased spermine uptake.** We have demonstrated that the polyamine resistance phenotype of *sky1Δ* cells is correlated with re duced polyamine uptake [18]. Therefore, we set out to determine whether reduced uptake also underlines the polyamine resistance of *fes1Δ* and *agp2Δ* cells. To that end, we measured the uptake of [^{14}C]spermine by *fes1Δ* and *agp2Δ* cells. Both strains showed a slower spermine accumulation rate compared to WT cells (fig. 3A). Accumulation of spermine in *agp2Δ* cells was slower than in *fes1Δ* cells. Kinetics analysis demonstrated that disruption of AGP2 resulted in a linear dependence between the concentration of spermine and its uptake by the cells (fig. 3B), being compatible with low-affinity uptake [19]. Similar results for spermidine uptake by *agp2Δ* cells were recently reported [32]. In contrast to the linear curve in *agp2Δ* cells, the kinetics of spermine uptake by *fes1Δ* cells were saturable as in WT cells. Interestingly, while the V_{max} in *fes1Δ* cells was similar to that of WT cells (31 ± 3 and 34 ± 3 pmol/10^7 cells per minute, respectively), the affinity for spermine was lower in *fes1Δ* cells than in WT cells (K_m = 58±5 and 23 ± 1.5 μM, respectively).
We therefore concluded that as previously demonstrated for PTK2 [14, 17, 19] and SKY1 [18], AGP2 and FES1 are also involved in regulating polyamine transport. While deletion of AGP2 practically abolishes the high-affinity spermine uptake, deletion of FES1 results only in reduced affinity toward spermine.

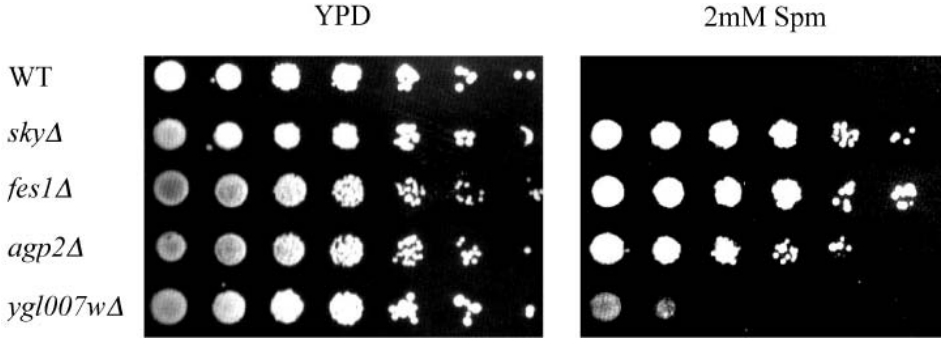

Figure 1. Cells deleted for AGP2, FES1 or YGL007w are more resistant to spermine than WT cells. Yeast overnight cultures were plated in five-fold dilutions on YPD plates or YPD supplemented with 2 mM spermine. The plates were grown at 30 °C for 48 h.

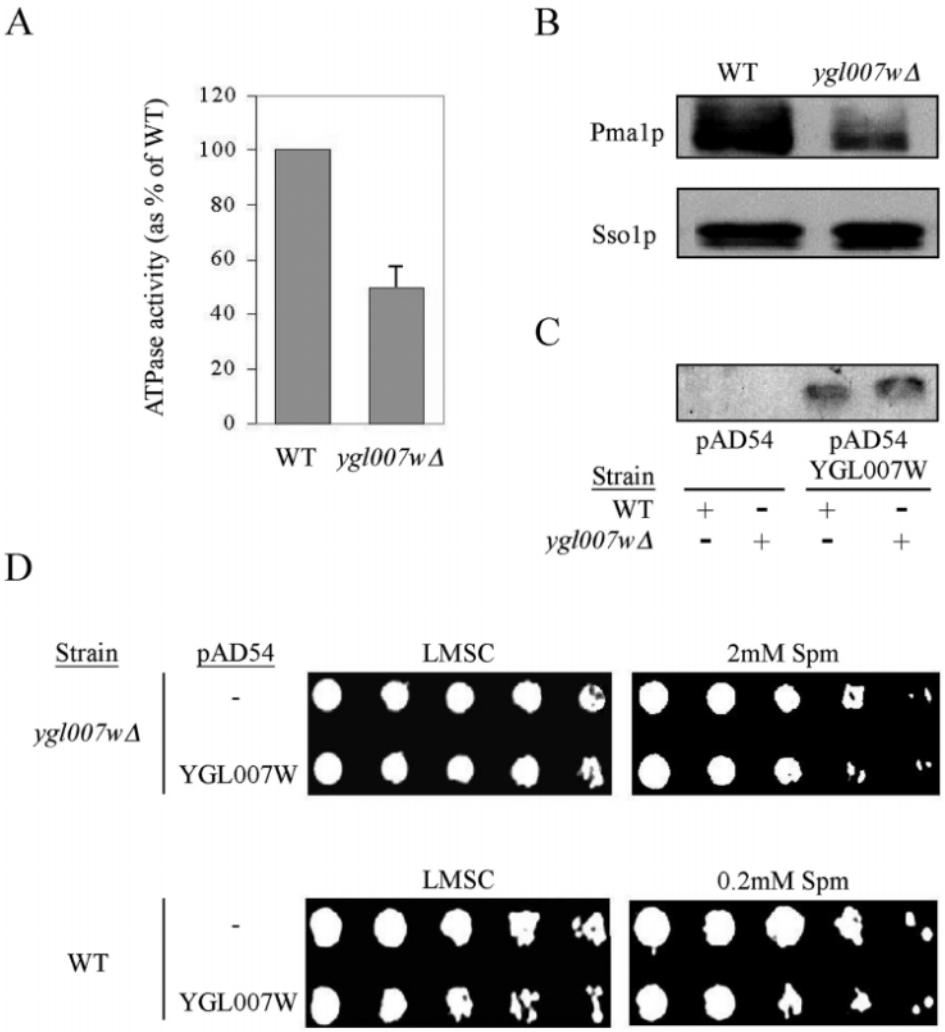

Figure 2. YGL007W does not function as an independent ORF involved in the uptake of spermine. (*A*) Plasma membranes were prepared from WT and *ygl007wΔ* cells and ATPase activity was determined. (*B*) The amount of Pma1p and Sso1p that served as normalizer was determined as described under Materials and methods. (*C*) YGL007W was cloned into the yeast expression vector pAD54 downstream to an HA tag, expressed in *ygl007wΔ* and WT cells and verified by Western blot using anti-HA antibody. (*D*) Fivefold dilutions of the resulting transformants were spotted on LMSC-Leu plates with and without 2.0 mM spermine (for *ygl007wΔ* cells) or 0.2 mM spermine (for WT cells).

Spermine-resistant cells have altered polyamine content and higher ODC activity. As the uptake measurements reflect accumulation over a short period of time, we set out to determine whether the various polyamine-resistant cells accumulate less polyamines over a longer time period. To this end, overnight cultures of WT, *sky1Δ*, *fes1Δ*, *agp2Δ* and the double-deletion strains *sky1Δagp2Δ* and *sky1Δfes1Δ* were grown in YPD with or without the addition of 0.5 mM spermine and their polyamine content was determined. Spermidine was the main polyamine observed in cells that were grown in YPD medium without added spermine (fig. 4A). Interestingly, yeast strains that resisted toxic levels of spermine contained higher putrescine levels than WT cells, a phenomenon that was most prominent in *sky1Δ* cells (fig. 4A). Upon growth in spermine-supplemented medium, WT cells accumulated significant amounts of spermine, and showed decreased levels of spermidine, while *sky1Δ* cells accumulated comparable amounts of spermine but showed higher spermidine levels. *agp2Δ* cells accumulated less spermine, and their spermidine concentration was similar to that of WT cells. *fes1Δ* cells accumulated slightly less spermine than WT cells, and had similar amounts of spermidine. The double-deletion strains showed a polyamine profile intermediate to the corresponding single deletions. This result indicates that despite having slower polyamine uptake rates, the different polyamine-resistant strains accumulate significant amounts of spermine and there is no direct correlation between the amount of accumulated spermine and their ability to grow in the presence of a toxic amount of spermine. Since all resistant strains displayed a higher spermidine to spermine ratio (fig. 4A), we hypothesized that the spermine tolerance results from increased conversion of spermine to spermidine.

To test this hypothesis we deleted FMS1, the only known polyamine oxidase of yeast that is essential for converting spermine to spermidine [33] from *sky1Δ* cells. Since *sky1Δfms1Δ* and *sky1Δ* cells were equally resistant to spermine, and did not contain significantly less spermi-

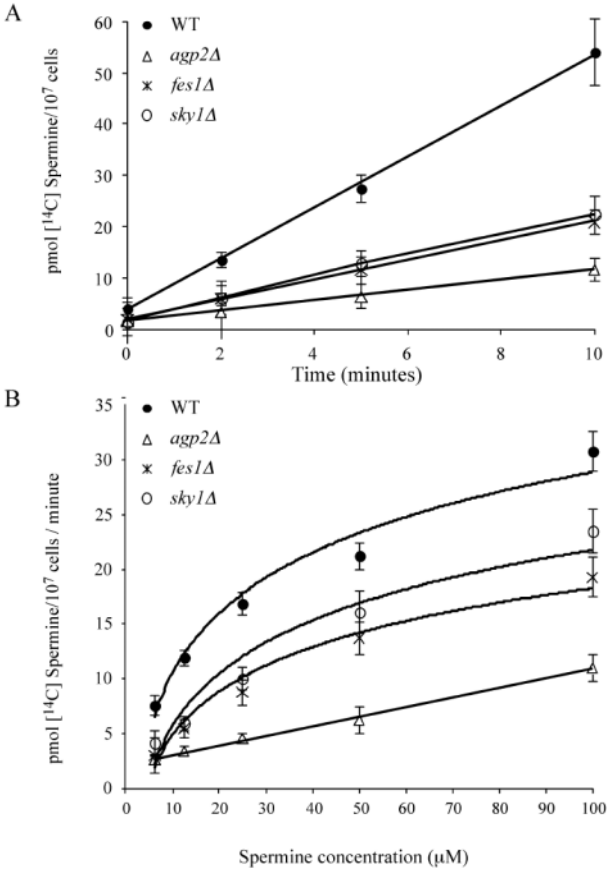

Figure 3. Cells deleted for FES1 or AGP2 show reduced spermine uptake rate. Logarithmically growing cultures of WT, *fes1Δ*, *agp2Δ* and *sky1Δ* cells were evaluated for uptake of [^{14}C]spermine. (*A*) Uptake of spermine (20 μM) was determined at the indicated time points. (*B*) Uptake of different spermine concentrations was determined for 1.5-min periods. The experiment was repeated at least four times with similar results.

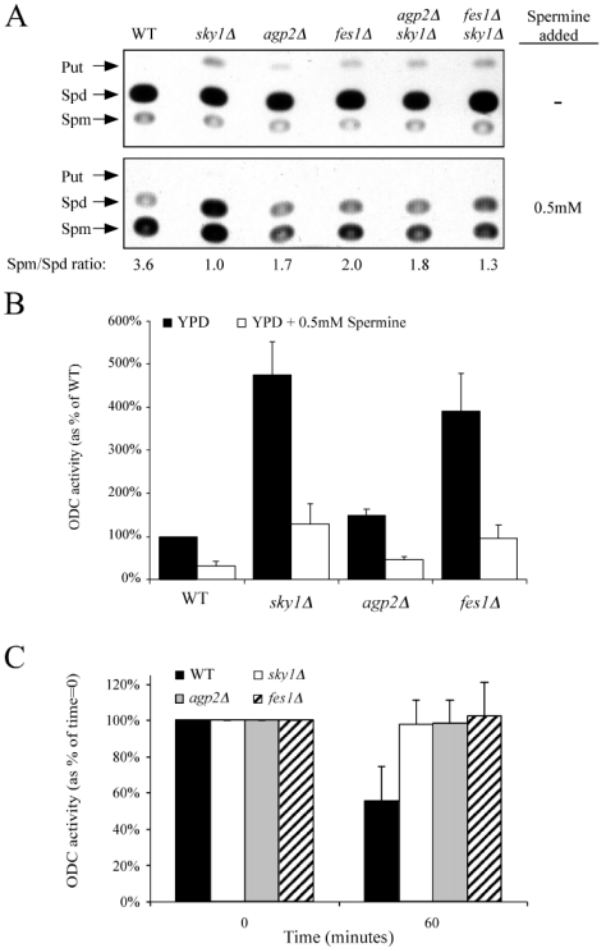

Figure 4. Cells deleted for SKY1, AGP2 or FES1 show an altered polyamine profile and higher ODC activity. (*A*) WT, *fes1Δ*, *agp2Δ* and *sky1Δ* and the double-deleted *sky1Δagp2Δ* and *sky1Δfes1Δ* strains were grown in YPD with or without 0.5 mM spermine, and their polyamine content was determined using the dansyl chloride method (see Materials and methods). The relative amounts of polyamine were quantified using pixel densitometry analysis and the ratio of spermine to spermidine (for spermine-treated cells) is shown below. The presented experiment is a representative of three independent experiments. Spm, spermine; Spd, spermidine; Put, putrescine. (*B*) Yeast overnight cultures were grown in YPD, with or without 0.5 mM spermine. Cells were lysed and ODC activity was determined in duplicates. The ODC activity, expressed as percent of the activity of WT cells represents an average of three independent experiments. (*C*) Logarithmically growing cultures of WT, *sky1Δ*, *fes1Δ* and *agp2Δ* cells were supplemented with cycloheximide and the ODC activity was determined at the indicated time points, in duplicates. This experiment was repeated at least three times with similar results.

dine (data not shown), we concluded that increased conversion of spermine to spermidine is not the mechanism accounting for spermine resistance, at least for *sky1Δ* cells.

As mentioned above, we noticed that the spermine-resistant strains, *sky1Δ* , *agp2Δ* and *fes1Δ*, contained elevated amounts of putrescine, which is not detected in WT cells. Since in eukaryotic cells elevated putrescine is typical of cells overproducing ODC [34], we set out to determine ODC activity in WT and in the spermine-resistant cells. Indeed, elevated ODC activity was noted in extracts of the spermine-resistant cells, being highest in *sky1Δ* cells and lowest in *agp2Δ* cells (fig. 4B). To examine whether the higher ODC activity results from reduced degradation, we examined decay rates of ODC activity in WT, *sky1Δ*, *agp2Δ* and *fes1Δ* cells. While a significant decrease in ODC activity was noted in WT cells over a 1-h-period, ODC activity remained stable in all the resistant strains (fig. 4C). We therefore conclude that the increased ODC activity is brought about at least in part by its stabilization in the spermine-resistant cells.

***agp2Δ* and *fes1Δ* cells display altered salt tolerance.** In addition to tolerating toxic spermine concentrations, *sky1Δ* and *ptk2Δ* cells also demonstrated salt tolerance [14, 18, 19]. We therefore set out to determine whether

the polyamine tolerance of *agp2Δ* and *fes1Δ* cells is also accompanied by altered salt sensitivity. For this purpose, the growth rate of *sky1Δ*, *agp2Δ* and *fes1Δ* cells was determined in YPD medium supplemented with 1.5 M KCl, 1.2 M NaCl or 0.4 M LiCl. As previously reported, *sky1Δ* cells were more resistant to NaCl and LiCl, and slightly less resistant to KCl [18]. *agp2Δ* cells were more sensitive to all cations (fig. 5). It should be noted, however, that *agp2Δ* cells showed slight growth inhibition also when grown in unsupplemented rich medium. Surprisingly, despite being very resistant to spermine, *fes1Δ* cells were highly sensitive to the cations examined, demonstrating significant growth inhibition. The observed growth sensitivity could result either from an osmotic stress or from the toxic effects of the tested cations. To distinguish between these possibilities, we examined the growth of the tested strains in medium containing sorbitol (1.5 M), which provides only osmotic stress. Since all strains showed growth inhibition similar to that of WT cells (data not shown), we concluded that the observed sensitivity was specific for these cations and not caused by the osmotic stress.

Epistatic relationships between AGP2, FES1 and SKY1. We have previously demonstrated that the spermine tolerance of the *sky1Δptk2Δ* double-mutant cells was greater than additive, implying that these two kinases act in parallel pathways [18]. We therefore next tested for possible relationships between AGP2, FES1 and SKY1, by constructing the double deletions *sky1Δagp2Δ* and *sky1Δfes1Δ*. When grown in rich medium, the polyamine content of the double deletions was intermediate to their respective single deletions (fig. 4A), and the spermine/spermidine ratio was either intermediate (for *sky1Δfes1Δ*) or similar to *agp2Δ* (for *sky1Δagp2Δ*). We demonstrated that the growth rate of *sky1Δagp2Δ* cells was intermediate to the growth rates of *sky1Δ* or *agp2Δ* cells, while *sky1Δfes1Δ* cells showed a growth phenotype that was either similar to that of *fes1Δ* cells or intermediate to the corresponding single deletions (fig. 6 and data not shown). Interestingly, *sky1Δfes1Δ* cells displayed a sensitivity to NaCl and LiCl like *fes1Δ* cells, in contrast to the tolerance of *sky1Δ* cells (fig. 5)*Δ*. Compared with their single deletions, *sky1Δagp2Δ* cells were more sensitive to KCl and also slightly more sensi-

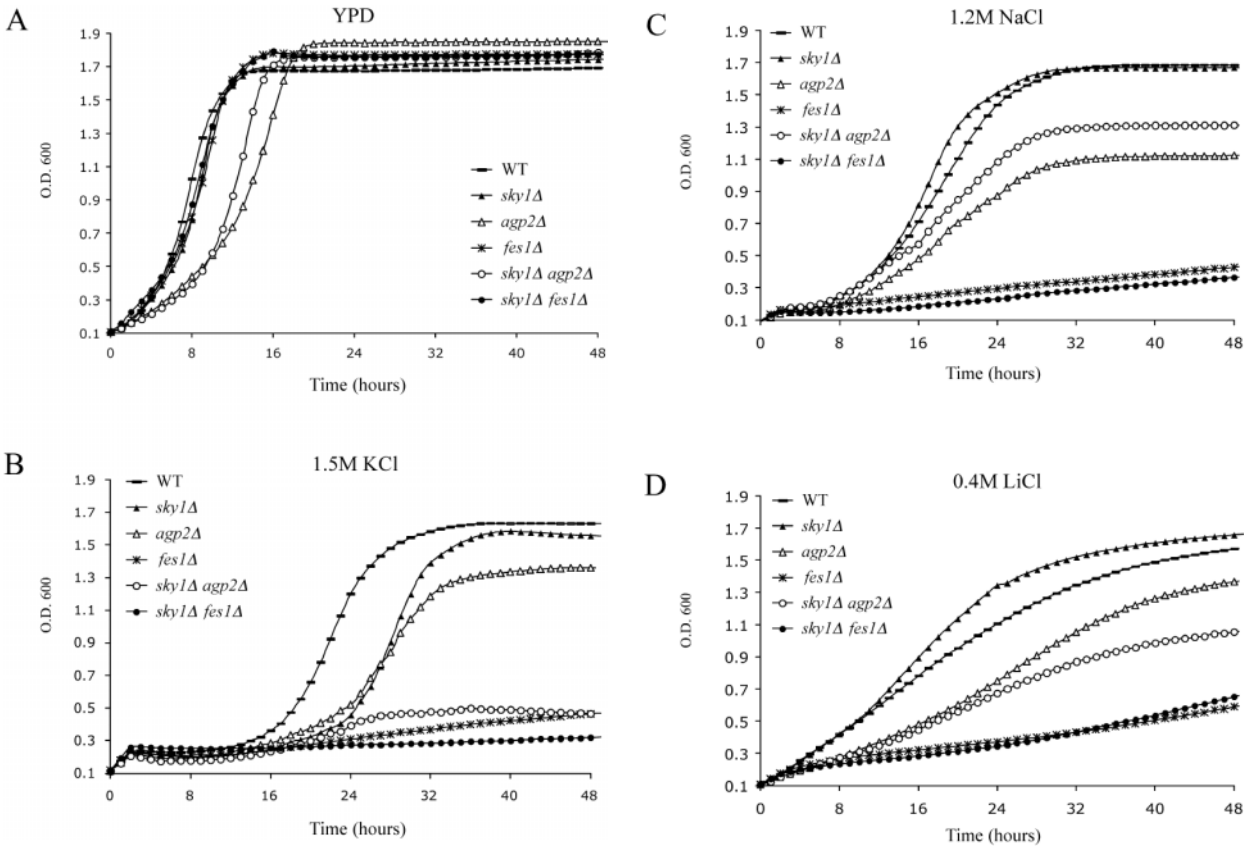

Figure 5. *agp2Δ, fes1Δ* and the double deletions *agp2Δsky1Δ* and *fes1Δsky1Δ* show alered salt tolerance. Yeast overnight cultures were diluted to OD_{600} = 0.05 and plated in triplicate in a 96-well plate in YPD medium, either unsupplemented (*A*) or supplemented with 1.5 M KCl (*B*), 1.2 M NaCl (*C*) or 0.4 M LiCl (*D*). The plates were placed in a GENius microplate optical reader for 48 h at 30 °C with constant shaking, and the OD_{600} was measured every 10 min. The experiment was repeated three times with similar results.

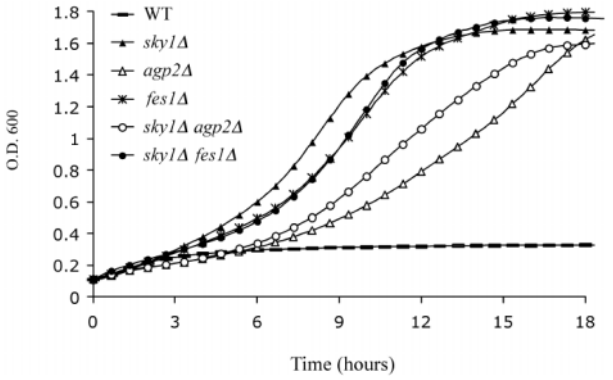

Figure 6. The double deletions *agp2Δsky1Δ* and *fes1Δsky1Δ* show intermediate spermine resistance compared with the single deletions. Yeast overnight cultures were diluted to OD_{600} = 0.05 and plated in triplicate in a 96-well plate in YPD supplemented with 3 mM spermine. The plates were incubated in a GENius microplate optical reader for 24 h in 30 °C with constant shaking, and the OD_{600} was measured every 10 min.

tive to LiCl. Their growth rate in the presence of NaCl was intermediate to that of the respective single deletions (fig. 5).

We therefore concluded that the polyamine-resistant phenotype of *fes1Δsky1Δ* was intermediate to the corresponding single deletions, and the *agp2Δsky1Δ* double mutant was closer to the *agp2Δ* mutant, suggesting that they either operate in the same pathway, or that they are epistatic. Interestingly, the salt sensitivity of *fes1Δ* cells could not be reverted by deleting SKY1, whose deletion confers salt resistance.

Deletion of STP1, STP2 and NPR2 confers intermediate spermine resistance. AGP2 may mediate spermine uptake either directly or by sensing polyamines, thus mediating their uptake by other transporters. As AGP2 was previously identified as a low-affinity amino acid permease [22], we set out to examine whether other components of amino acid sensing are involved in regulating polyamine tolerance. The sensing mechanism for amino acids involves a membrane complex, which upon activation regulates the transcription factors STP1 and STP2 [35]. To examine whether STP1 or STP2 are involved in the manifestation of polyamine resistance, the growth of *stp1Δ* and *stp2Δ* cells was tested in rich medium supplemented with spermine. *stp1Δ* cells, and to a smaller extent also *stp2Δ* cells, showed spermine tolerance that was intermediate to that observed for WT and *sky1Δ* cells (fig. 7). The nitrogen permease regulator NPR2, involved in urea and proline uptake [36], was shown to be epistatic with SKY1 in mediating cisplatin resistance [37]. Interestingly, deletion of NPR2 also resulted in intermediate polyamine tolerance (fig. 7). These results provide an interesting correlation between amino acid sensing, nitrogen permease and polyamine resistance.

Discussion

In the present study we identified and characterized additional yeast genes involved in regulating polyamine transport and tolerance. The first is the uncharacterized ORF, YGL007W, that was present in a DNA fragment that restored spermine sensitivity upon its transfection into an otherwise resistant mutant. We demonstrated that deletion of YGL007W lead to polyamine resistance, while over-expression of YGL007W did not restore sensitivity to a toxic spermine concentration. YGL007W is located within the 5' upstream region of PMA1, which contains two upstream activating sequences ($UAS1_{PMA1}$ and $UAS2_{PMA1}$) that are recognized by the DNA-binding protein TUF (RAP/GRF1) that regulates PMA1 transcription [30]. Our results demonstrate that the polyamine resistance phenotype of *ygl007wΔ* cells is a result of down-regulation of PMA1 expression, and not of the activity of a protein encoded by the YGL007W ORF. Interestingly, reduction of Pma1p activity to below 20 % results in arrested growth of haploid cells [38], while cells deleted for YGL007W grow normally. Apart from differences in experimental conditions (strain, medium, growth periods and measurement methods), this could be explained as a threshold effect, as 50 % activity enables normal growth rates while lower levels interfere with cell growth. This could provide a useful tool for investigating PMA1, and for reducing membrane potential without affecting cell growth.

The other two genes, AGP2 and FES1, were selected for investigation based on the resistance of their deletion

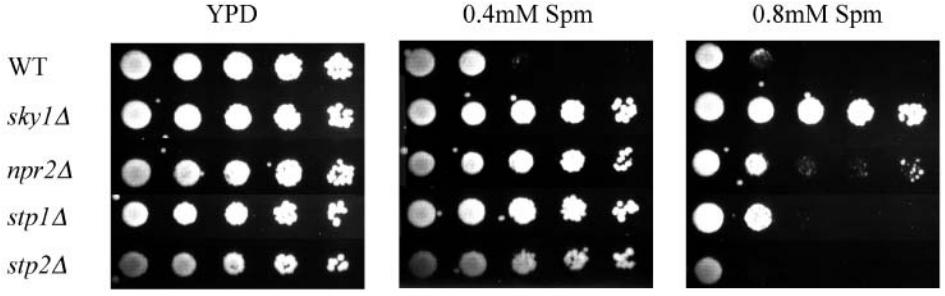

Figure 7. *npr2Δ, stp1Δ* and *stp2Δ* cells show intermediate resistance to spermine compared with WT and *sky1Δ* cells. Yeast overnight cultures were plated at five-fold dilutions on YPD plates or YPD supplemented with 0.4 mM or 0.8 mM spermine. The plates were grown at 30 °C for 48 h.

mutants to the anti cancer drug bleomycin [20]. We show here that these two strains have a slower spermine uptake rate. These results are in agreement with a recent report demonstrating reduced uptake of spermidine in *agp2Δ* cells [32]. The spermine uptake rate in *agp2Δ* cells was linear with spermine concentrations, as described for spermidine [32], in accordance with Agp2p being a low-affinity uptake component [19]. Interestingly, *fes1Δ* cells displayed uptake kinetics similar to that of WT cells, having similar V_{max} values, but the K_m was increased by ~2.5-fold. Therefore, our data and that of Aouida et al. [32] strongly suggest an important role for AGP2 in polyamine high-affinity uptake. As AGP2 was originally discovered as a transporter of L-carnitine [21], AGP2 was suggested to function either as a polyamine transporter or as a polyamine sensor [32]. We show here that STP1 and STP2, transcription factors involved in amino acid sensing, exert some role in regulating polyamine tolerance, possibly suggesting a connection between amino acid sensing and polyamine tolerance. Since its role in regulating L-carnitine uptake is independent of its effect on polyamine uptake [32], AGP2 may be involved in polyamine sensing rather than in polyamine uptake per se. However, as there is no direct evidence for the exact role of Agp2p, further experiments are required to distinguish between these two possibilities.

The polyamine-resistant phenotype of *agp2Δ* , *fes1Δ* and *sky1Δ* is correlated with reduced polyamine uptake [18, 32] (fig. 3). It was therefore surprising that cells with slower spermine uptake rate eventually accumulate significant amounts of spermine (fig. 4A) without affecting cell growth. One plausible explanation could be that the slower uptake rate enables the cells to cope better with the toxicity of spermine. This is probably not done by its acetylation and excretion or degradation, as this should have resulted in a decrease in spermine levels. In addition, cells deleted for both SKY1 and FMS1, the enzyme converting spermine to spermidine, are as resistant to spermine as cells deleted for SKY1 alone [Z. Porat, unpublished results]. Therefore, the excess spermine is possibly stored in cellular compartments that render it less harmful to the cell. A possible candidate for such a storage location could have been the vacuole. However, the spermine-resistant strains accumulate less polyamines in their vacuoles [32; our unpublished results]. The slower uptake rate may enable the cells to direct the excessive polyamines to a different cellular compartment.

An important regulation of polyamine levels is through their production by the rate-limiting enzyme ODC [reviewed in ref. 39]. We observed an increase in putrescine levels in the resistant strains that correlated with increased ODC activity (fig. 4B). Increased polyamine production may compensate for the decreased uptake rates and prevent depletion of cellular polyamine reservoirs. Interestingly, however, this is probably not essential for the spermine-resistant phenotype, as cells deleted for both SKY1 and SPE1 (encoding yeast ODC) do not show any growth inhibition upon growth in rich medium supplemented with toxic spermine levels [Z. Porat, unpublished results]. Upon addition of spermine to the growth medium, the ODC activity level drops to 24–31 % in all strains (fig. 4B). Nevertheless, the absolute levels of ODC activity in the resistant strains remain higher than those of WT cells. ODC is regulated by antizyme, a small protein that is induced by polyamines and targets ODC to degradation [40]. Indeed, the higher ODC activity is most likely a result of reduced ODC degradation, as the decay of ODC activity was slower in all resistant strains (fig. 4C). This indicates that induction of antizyme in the resistant cells requires higher polyamine levels, further supporting the notion that in the resistant cells, the polyamines are less available for antizyme induction. Nevertheless, we cannot rule out other factors, such as decreased antizyme frameshift efficiency or its reduced transcription.

Since there is no direct correlation between polyamine resistance and polyamine cellular content, the observed resistance might actually reflect the ability of the resistant strains to cope with the polyamine-imposed toxicity, rather than being a direct result of the polyamine content. In line with this, *sky1Δ* cells show resistance to cisplatin and doxorubicin, even though their accumulation and DNA platination is similar to that observed in WT cells [41]. This was suggested to be a result of reduced mismatch repair activity, as *sky1Δ* cells display a mutator phenotype [41]. This indicates that additional mechanisms other than reduced uptake underlie the observed resistance. Interestingly, in addition to SKY1 deletion, deletion of the nitrogen permease regulator NPR2, and of the transcription factors STP1 and STP2, also results in resistance to both cisplatin [37, 41, 42] and polyamines (fig. 7). The other genes involved in mediating polyamine resistance, FES1 and AGP2, were not identified in several genome-wide screens for cisplatin resistance, further suggesting that different mechanisms lead to polyamine resistance. This is supported by the notion that both FES1 and SKY1 are involved in cellular processes that are not directly related to polyamines. Sky1p was originally identified as the yeast SR protein kinase, being involved in splicing [43], mRNA shuttling and processing [44]. FES1 is a nucleotide exchange factor of Hsp70p that was demonstrated to be important for protein translation activity. Its deletion was shown to result in general translation defects as well as cycloheximide sensitivity and thermosensitivity [23], but it was not associated with polyamine or salt tolerance until now. It is therefore possible that effectors operating downstream to SKY1 or FES1 mediate the ability of cells to cope with polyamine toxicity.

Altered polyamine and salt tolerance can result from an altered membrane potential, as demonstrated in the cases

of SKY1 and PTK2 [14, 18]. A decrease in membrane potential affects the secondary transporters (energized by the membrane potential) for cations or polyamines and increases the tolerance toward them [14]. It is therefore intriguing as to why *fes1Δ* cells show on the one hand polyamine resistance and on the other, significant salt sensitivity (figs 1, 5). FES1 deletion may alter the expression profile of several proteins resulting in various cellular consequences, including altered salt and polyamine tolerance, possibly via different pathways. Interestingly, while deletion of SKY1 results in salt tolerance, *sky1Δfes1Δ* cells are as sensitive as *fes1Δ* cells. This may suggest that FES1 is essential for the salt-tolerant phenotype of *sky1Δ* cells, as its deletion abolishes it. However, Sky1p is not necessarily involved in the salt sensitivity phenotype, because its deletion does not diminish the sensitivity of *fes1Δ* cells. It is also possible that downstream effectors are involved in establishing these phenotypes, rather than Sky1p and Fes1p themselves. Presently we cannot distinguish between these possibilities.

Our data support the notion that reduced uptake is not the sole mechanism leading to polyamine tolerance. We suggest that other activities of Sky1p or Fes1p are involved in mediating the polyamine-resistant phenotype, either independently or together with the altered uptake. Identification of cellular effectors located downstream to Fes1p and Sky1p will hopefully shed more light on the mechanisms that mediate altered polyamine and salt tolerance.

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