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Crosstalk between *S. cerevisiae* SAPKs Hog1 and Mpk1 is mediated by glycerol accumulation

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Abstract

Two stress-activated MAP kinase (SAPK) pathways in *Saccharomyces cerevisiae* respond to osmotic imbalances. The High Osmolarity Glycerol (HOG) pathway is activated in response to hyper-osmotic stress, whereas the Cell Wall Integrity (CWI) pathway is activated in response to hypo-osmotic stress. However, there is also evidence of complex interplay and crosstalk between the two pathways. For example, treatment with zymolyase, a mixture of cell wall degrading enzymes, is known to activate the SAPK Hog1 of the HOG pathway and the SAPK Mpk1 of the CWI pathway sequentially, with Mpk1 activation dependent upon Hog1. Additionally, the *PTP2*- and *PTP3*-encoded tyrosine-specific protein phosphatases play a key role in downregulation of Hog1, but may also down-regulate Mpk1. In this study, we show that hyperactivation of Mpk1 in a *ptp2 ptp3* null mutant is an indirect consequence of Hog1 hyperactivation, which induces accumulation of intracellular glycerol and an attendant hypo-osmotic stress. Mpk1 hyperactivity in the absence of *PTP2* and *PTP3* was suppressed by a *hog1* null mutation, or by restoration of osmotic balance with a constitutive form of the glycerol channel Fps1. We found similarly that activation of Mpk1 in response to zymolyase treatment is partly a consequence of Hog1-driven glycerol accumulation. Thus, we have identified two conditions in which glycerol serves as a mediator of crosstalk between the HOG and CWI pathways.

Keywords

Stress-activated MAPK; HOG pathway; CWI pathway; osmotic imbalance; crosstalk; *S. cerevisiae*

1 Introduction

Saccharomyces cerevisiae encodes two stress-activated MAP kinases (SAPKs). Hog1 is the SAPK of the High Osmolarity Glycerol (HOG) pathway, which responds to hyperosmotic

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

shock by inducing the production and retention of intracellular glycerol as a compatible solute (Saito and Posas, 2012). Mpk1 (Slt2) is the SAPK of the Cell Wall Integrity (CWI) pathway, which responds to cell wall stresses by driving fortification of the wall (Levin, 2011). These two stress pathways have been viewed generally as acting antagonistically to each other (Davenport et al., 1995). When cells are exposed to hyper-osmotic shock, Hog1 is activated rapidly and Mpk1 is inactivated. Conversely, Mpk1 is activated in response to hypo-osmotic stress, a form of cell wall stress, whereas Hog1 is inactivated under such conditions. However, later work demonstrated a more complex relationship of crosstalk between the two pathways. For example, Mpk1 is activated transiently by hyper-osmotic stress after a considerable delay, as Hog1 activity returns to basal levels (Garcia-Rodrigues et al., 2005). The delayed Mpk1 stimulation is dependent upon Hog1 activation and may be the consequence of overshoot of the intracellular glycerol concentration, which would increase turgor pressure and induce cell wall stress.

Another example of crosstalk between Hog1 and Mpk1 is evident from treatment of cells with zymolyase, a mixture of cell wall-digesting enzymes (Bermejo et al., 2008). Zymolyase treatment induces sequential activation of Hog1 and Mpk1 and, in this setting, activation of Mpk1 requires prior activation of Hog1. Although signaling to Hog1 in response to zymolyase is likely stimulated by the osmosensor Hkr1:Sho1 (Rodriguez-Pena et al., 2013), the link between activation of Hog1 and activation of Mpk1 remains unknown.

Finally, another connection between the SAPKs of the HOG pathway and the CWI pathway are the protein phosphatases that negatively regulate their activity. Several protein phosphatases control both the basal level of SAPK phosphorylation and their return to a low-activity state after stimulation. With regard to Hog1, these are the Tyr-specific phosphatases, Ptp2 and Ptp3, and Ser/Thr phosphatase, Ptc1 (Saito and Posas, 2012, and references therein). Deletion of *PTP2* and *PTP3* together elevates basal Hog1 activity (Wurgler-Murphy et al., 2000). Two other protein phosphatases act as key regulators of Mpk1. Msg5 is a dual-specific phosphatase (DSP) that associates stably with Mpk1 (Flandez et al., 2004) and maintains the SAPK in a low-activity state in the absence of stress (Martin et al., 2000; Liu and Levin, 2018). A paralog of Msg5, Sdp1, acts to downregulate Mpk1 after stimulation (Hahn and Theile, 2002). Additionally, Ptp2 and Ptp3 can dephosphorylate Mpk1 in vitro and their absence results in elevated Mpk1 activity in vivo (Mattison et al., 1999). However, it is not clear whether elevated Mpk1 activity observed in a *ptp2*Δ *ptp3*Δ mutant is due to the loss of activity of these phosphatases on Mpk1, or an indirect effect of hyperactive Hog1. In this study, we used a *ptp2*Δ *ptp3*Δ mutant as a starting point to investigate the relationships among active Hog1, osmotic imbalance, and the activation state of Mpk1. We find that Hog1-driven glycerol accumulation in the absence of hyper-osmotic stress causes secondary Mpk1 activation. Glycerol accumulation largely explains Mpk1 activation in both a *ptp2*Δ *ptp3*Δ mutant and in response to zymolyase treatment.

2 Material and Methods

2.1 Strains, growth conditions, plasmids and transformations

The *S. cerevisiae* strains used in this study were derived from strains BY4742 (*MATα* S288c *his3*Δ *leu2*Δ *ura3*Δ *lys2*Δ) and BY4741 (*MATa* S288c *his3*Δ *leu2*Δ *ura3*Δ *met15*Δ; Research

Genetics, Inc.; Huntsville, AL). These were DL3226 (*MATa fps1*Δ*::KanMX*), DL3229 (*MATα fps1*Δ*::KanMX*), DL4285 (*MATa gpd1*Δ*::KanMX gpd2*Δ*::HPHMX4*; Lee and Levin 2019), DL4299 (*MATα ptp2*Δ*::KanMX ptp3*Δ*::HPHMX4*; Lee and Levin, 2018), DL4424 (*MATα ptp2*Δ*::KanMX ptp3*Δ*::HPHMX4 hog1*Δ*::HIS3*; described below),. Yeast cultures were grown in YPD (1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose) or minimal selective medium, SD (0.67% Yeast nitrogen base, 2% glucose), or SG (0.67% Yeast nitrogen base, 2% galactose) supplemented with the appropriate nutrients to select for plasmids. Plasmids used in this study are presented in Table 1. Yeast cultures were transformed according to Geitz et al. (1995).

2.2 Generation of ptp2Δ ptp3Δ hog1Δ strain

Chromosomal deletion of *HOG1* in *ptp2*Δ::*KanMX ptp3*Δ::*HPHMX4* strain (DL4299) was created by homologous recombination. The *HIS3* gene from pFA6a-His3MX6 (Longtine et al, 1998) was amplified by high-fidelity PCR (PrimeSTAR Max DNA polymerase, Clontech) using primers containing the upstream region (45 base pairs immediately before the starting ATG) and downstream region (45 base pairs immediately after the stop codon) of the *HOG1* gene. The PCR product was transformed in DL4299 strain, and the chromosomal integrants were selected for histidine prototrophy, yielding *ptp2*Δ::*KanMX ptp3*Δ::*HPHMX4 hog1*Δ::*HIS3* (DL4424). The gene replacement was validated by PCR analysis across both integration junctions.

2.3 Measurement of β-galactosidase activity and intracellular glycerol concentrations

Measurement of *β*-galactosidase activity from *GPD1-lacZ* and *MLP1-lacZ* expression experiments were conducted in triplicate or quadruplicate and carried out as described in Zhao et al. (1998). Specific activities were given in nanomoles of *o*-nitrophenol produced from *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein. Intracellular glycerol concentrations, were measured in triplicate from whole cells grown in YPD, hyper-osmotically shocked for 10 min with 1 M sorbitol, or treated with zymolyase 100T (US Biological) for 1–2 hours and centrifuged briefly to remove the culture supernatant. Enzymatic assays for glycerol were carried out using a kit from Boehringer Mannheim and normalized to A_{600} of the culture.

2.4 Protein extraction and co-immuneprecipitation

Extracts for co-IP experiments were prepared as described previously (Lee et al., 2013). Cultures co-expressing Rgc2-HA and Fps1-Myc were grown to mid-log phase in selective medium and starved for methionine for two hours to induce expression of Rgc2 and Fps1, which were expressed under the control of the conditional *MET25* promoter. Extracts (100 μg of protein) were incubated with mouse monoclonal α-Myc antibody (1 μg, 9E10; Pierce), for 1 hour at 4°C and precipitated with protein A affinity beads for 1 hour at 4°C. Samples were washed with IP buffer three times and boiled in SDS-PAGE buffer. Protein extraction for immunoblots were carried out as described by Martin et al. (2000), except that protease inhibitor cocktail (cOmplete; Sigma) and phosphatase inhibitor cocktail (PhosSTOP; Sigma) were used.

2.5 SDS-PAGE electrophoresis and immunoblot analysis

Proteins were separated by SDS-PAGE (10% gels) followed by immunoblot analysis using mouse monoclonal antibodies α-Myc (9E10; Santa Cruz), α-HA (16B12; BioLegend), α-Carboxypeptidase Y (CPY; 10A5B5; Abcam), or goat polyclonal α-Mpk1 (yC-20; Santa Cruz) at a dilution of 1:10,000. Rabbit polyclonal α-phospho-p44/42 MAPK (Thr202/Tyr204; Cell Signaling), was used at a dilution of 1:2,000 to detect phosphorylated Mpk1. Goat polyclonal α-Hog1 (yC-20; Santa Cruz) at a dilution of 1:10,000, or rabbit polyclonal α-phospho-p38 (T180/Y182, Cell Signaling) at a dilution of 1:2,000 were used to detect Hog1 and phosphorylated Hog1, respectively. Secondary goat anti-mouse (Jackson ImmunoResearch Labs), donkey anti-rabbit (GE Healthcare), or mouse anti-goat (Santa Cruz) antibodies were used at a dilution of 1:10,000. All results involving immunoblot analyses were replicated at least once and representative blots are shown.

2.6 Microscopy

Cells were visualized by differential interference contrast (DIC) with a Zeiss Axio Observer Z1 with a 20X objective.

2.7 Notes on reproducibility

All reporter assays and glycerol accumulation experiments were reproduced at least once in independent experiments, representatives of which are shown. Similarly, all immunoblots and co-IPs were reproduced at least once in independent experiments with representative images shown. Quantitation of signals from immunoblots was done using a GE LAS4000 imager with ImageQuant TL software (GE Healthcare Life Sciences).

3 Results and Discussion

3.1 Mpk1 is activated by glycerol accumulation in a *ptp2*Δ *ptp3*Δ mutant

A *ptp2*Δ *ptp3*Δ mutant displays a moderate increase in both Hog1 and Mpk1 activity (Wurgler-Murphy et al., 1997; Mattison et al., 1999; Mattison and Ota, 2000; Lee and Levin, 2018). To investigate the relationship between hyperactive Hog1 and hyperactive Mpk1 in the absence of Ptp2 and Ptp3, we explored the physiological consequences of the combined loss of these phosphatases on glycerol production and retention and consequent cell wall stress. First, we found that a *ptp2*Δ *ptp3*Δ mutant retains approximately three times the normal level of intracellular glycerol, an amount roughly equivalent to that induced by a hyper-osmotic shock with 1 M sorbitol (Figure 1A) and approximately 75% of that accumulated in the absence of plasma membrane glycerol channel Fps1 (Lee et al., 2013), a protein that is critical for glycerol efflux. Hog1 that has been activated by hyper-osmotic shock induces glycerol accumulation both through transcriptional induction of genes required for glycerol production (e.g. *GPD1* and *GPP1*; Hohmann, 2009; Saito and Posas, 2012) and through closure of Fps1 (Hohmann, 2009; Lee et al., 2013). Therefore, we examined these functions in the *ptp2*Δ *ptp3*Δ mutant. We found that the basal activity of a *GPD1-lacZ* transcriptional reporter that is responsive to hyperosmotic stress was elevated approximately four-fold in the *ptp2*Δ *ptp3*Δ mutant as compared to wild-type (Figure 1B). This was greater than the response to a hyper-osmotic shock with 1M sorbitol for 1 hour,

and this increase in expression was entirely dependent upon Hog1. In fact, the *GPD1-lacZ* activity was lower in the *ptp2*Δ *ptp3*Δ *hog1*Δ mutant than in wild-type. These results indicate that the absence of the Ptp phosphatases drives *GPD1* expression in a manner similar to a hyper-osmotic shock condition.

To assess glycerol channel status, we tested the association of Fps1 with its positive regulator Rgc2 by co-immunoprecipitation (co-IP) (Lee et al., 2013). Loss of association between these proteins is an indication of Hog1-catalyzed channel closure. We found that Rgc2 was dissociated from Fps1 in the *ptp2*Δ *ptp3*Δ mutant (Figure 1C). Taken together, these results reveal that the absence of Ptp2 and Ptp3 causes Hog1-driven glycerol accumulation both through transcriptional induction of glycerol biosynthetic genes and through closure of Fps1, and suggests that the *ptp2*Δ *ptp3*Δ mutant is in a perpetual state of hyper-osmotic stress response.

As noted above, Mpk1 exhibits elevated basal activity in the absence of Ptp2 and Ptp3, which may be due either to a loss of direct action of these phosphatases on Mpk1 (Mattison et al., 1999), or an indirect effect of increased glycerol concentration from hyperactive Hog1. Therefore, we next asked if activation of Mpk1 in the *ptp2*Δ *ptp3*Δ mutant requires Hog1. We found that the elevated basal Mpk1 activity in a *ptp2*Δ *ptp3*Δ mutant was suppressed in a *ptp2*Δ *ptp3*Δ *hog1*Δ strain (Figure 2A), suggesting that the increased Mpk1 activity is an indirect consequence of hyperactive Hog1, rather than a direct consequence of lost Ptp activity on Mpk1. It is interesting to note that we detected a reproducible increase in Mpk1 protein level in the *ptp2*Δ *ptp3*Δ mutant relative to wildtype cells. This is because expression of Mpk1 is auto-regulated by Mpk1 activity (Jung and Levin, 1999), and suggests that active Mpk1 in this setting engages in cell wall stress transcription. To test this directly, we examined the activity of a cell wall stress transcription reporter (*MLP1-lacZ*; Jung, et al., 2002). We detected greatly elevated transcriptional activity of *MLP1-lacZ* in the *ptp2*Δ *ptp3*Δ mutant relative to wild-type (Figure 2B). Here again, deletion of *HOG1* in the *ptp2*Δ *ptp3*Δ mutant completely suppressed this activity, revealing that cell wall stress transcription in this mutant is an indirect consequence of hyperactive Hog1. These results suggest that the observed hyperactivity of Mpk1 in the absence of *PTP2* and *PTP3* reflects cell wall stress experienced as a consequence of intracellular glycerol accumulation.

To test directly the role of Hog1-driven glycerol accumulation in the activation of Mpk1, we introduced into the *ptp2*Δ *ptp3*Δ mutant an allele of *FPS1* that expresses a constitutively open glycerol channel (Fps1-IV/AA; Lee et al., 2013). The Fps1-IV/AA protein is an open-channel form by virtue of its inability to bind Hog1. Because Hog1 is not recruited to this form of Fps1, it does not phosphorylate and evict the glycerol channel regulators Rgc1 and Rgc2, which maintain Fps1 in an open state (Lee et al., 2013). We found that the *FPS1-IV/AA* allele reduced both the activity level of Mpk1 in the *ptp2*Δ *ptp3*Δ mutant (Figure 3A) and the consequent cell wall stress transcription (Figure 3B), thereby confirming that activation of Mpk1 in the absence of Ptp2 and Ptp3 is largely the result of Hog1-driven glycerol accumulation. We note, however, that expression of the *FPS1-IV/AA* allele did not reduce Mpk1 activity, cell wall stress transcription, to the level of wild-type cells. This observation will be addressed further in section 3.3.

3.2 The temperature-sensitive growth defect of the *ptp2Δ ptp3Δ* mutant is caused by cell lysis due to glycerol accumulation

It has been reported that a *ptp2*Δ *ptp3*Δ mutant displays a growth defect at elevated temperature, which is dependent upon Hog1 activity (Winkler et al., 2002). Mutants defective in Fps1 function also fail to grow at elevated temperature (Beese, et al., 2009), which causes cell wall stress (Kamada, et al., 1995). Elevated turgor pressure from accumulated glycerol in mutants blocked for Fps1 function combined with cell wall stress during growth at elevated temperature results in osmotic-remedial cell lysis (Beese et al., 2009). Therefore, we asked if the growth defect of the *ptp2*Δ *ptp3*Δ mutant at high temperature was similarly the result of accumulated glycerol. We first tested for the suppression of this defect by the inclusion of sorbitol in the growth medium for osmotic support. Figure 4A shows that 5% sorbitol partially suppressed the growth defect at 39°C, revealing that osmotic imbalance in this mutant was partly responsible for its growth defect. Inclusion of 10% sorbitol did not confer greater suppression (data not shown), suggesting that the residual growth defect is not a consequence of osmotic imbalance. We next examined the ability of the *FPS1-IV/AA* allele to suppress the growth defect of the *ptp2*Δ *ptp3*Δ mutant at high temperature. We found that expression of the open-channel form of Fps1 also partially suppressed the temperature sensitivity of the *ptp2*Δ *ptp3*Δ mutant (Figure 4B), which confirms that the growth defect of this mutant at elevated temperature is partly the result of accumulated glycerol. Finally, microscopic examination of *ptp2*Δ *ptp3*Δ cells streaked for growth at elevated temperature for 18 h revealed cell debris and non-refractile “ghosts” that are indicative of cell lysis, similar to that observed in an *fps1*Δ mutant shifted to high temperature (Figure 4C). We conclude from these results that the *ptp2*Δ *ptp3*Δ mutant undergoes cell lysis at elevated temperature due to the accumulation of glycerol.

Thus, we have shown that Mpk1 activation and cell wall stress transcription in a *ptp2*Δ *ptp3*Δ mutant is an indirect consequence of Hog1-driven glycerol accumulation, rather than due to the absence of Ptp phosphatase activity against Mpk1. Similarly, the growth defect of this mutant at elevated temperature is largely, but not entirely, a consequence of a Hog1-driven osmotic imbalance. In addition to glycerol accumulation, activation of Hog1 by hyper-osmotic shock causes a G1 cell cycle delay (Belli, et al., 2001) through interactions of Hog1 with the cell cycle machinery (Saito and Posas, 2012). Similarly, constitutive activation of Hog1 by mutations in the osmosensor Sln1 or the MEK Pbs2 causes cell cycle arrest in G1 that is due, in part, to direct action of Hog1 on the cell cycle inhibitor Sic1 (Escote, et al., 2007). Moderate hyperactivation of Hog1 in the *ptp2*Δ *ptp3*Δ mutant is not sufficient to induce cell cycle arrest but might, nevertheless, influence cell cycle progression. Therefore, we suggest that the growth defect of the *ptp2*Δ *ptp3*Δ mutant at elevated temperature is a consequence of direct and indirect effects of Hog1 hyperactivation. First, cell lysis results from the combined cell wall stress of elevated growth temperature and increased turgor pressure, a phenotype that is suppressed by correction of the osmotic imbalance. Second, a residual growth deficit remains that is likely due to the direct effect of Hog1 hyperactivation on cell cycle progression.

3.3 Crosstalk between Hog1 and Mpk1 in response to zymolyase results from the accumulation of glycerol

The experiments described in section 3.1 demonstrate that Mpk1 is stimulated in response to glycerol accumulation caused by activation of Hog1 in the absence of a hyper-osmotic stress. Therefore, we asked if the previously reported Hog1-dependent activation of Mpk1 by zymolyase treatment (Bermejo et al., 2008) was similarly the result of accumulated glycerol. First, we measured glycerol accumulation in wild-type cells treated with 0.4 U/ml zymolyase, a condition shown previously to activate both Hog1 and Mpk1 (Bermejo et al., 2008). We found that intracellular glycerol concentration increased over a period of 2 hours in response to zymolyase treatment (Figure 5A). Treatment with 0.8 U/ml zymolyase increased glycerol accumulation further. Although this accumulation was less than that observed after a 10-min hyperosmotic shock with 1 M sorbitol, it nevertheless compelled us to ask if glycerol accumulation was responsible for Mpk1 activation in this setting. Therefore, we tested Mpk1 and Hog1 activation in response to zymolyase treatment in the context of the constitutively open form of Fps1 (Fps1-IV/AA). Figure 5B shows that, in both wild-type and *FPS1-IV/AA* cells, Hog1 is activated over time in response to treatment with 0.4 U/ml zymolyase. We note that basal Hog1 activity is higher in cells expressing the open-channel Fps1 form as compared to wild-type, presumably because they experience mild hyper-osmotic stress caused by loss of glycerol. Similarly, Mpk1 activation occurs slowly, rising steadily over the 2-hour time course. However, as was the case for the *ptp2*Δ *ptp3*Δ mutant, Mpk1 activation in *FPS1-IV/AA* cells was diminished relative to wild-type. This result supports the conclusion that the activation of Mpk1 in response to zymolyase treatment is, at least partly, a consequence of Hog1-driven glycerol accumulation. Although the observed increase in intracellular glycerol induced by zymolyase treatment was modest, it was accompanied by a considerable increase in Mpk1 activity. It may be that the glycerol levels were underestimated because the zymolyase treatment ultimately causes cell lysis, and glycerol content was assessed relative to the initial culture OD.

We were interested to know if the residual Mpk1 activation detected in the *FPS1-IV/AA* cells in response to zymolyase treatment would be eliminated in the complete absence of glycerol. To test this, we repeated the zymolyase treatment in a *gpd1*Δ *gpd2*Δ mutant, which is blocked for glycerol production (Hohmann, 2009; Lee and Levin, 2019). Mpk1 activation was diminished in the mutant strain relative to wild-type, but a residual level of Mpk1 activation remained similar to that observed for *FPS1-IV/AA* cells (Figure 5C). Therefore, glycerol accumulation cannot account for the entirety of the Hog1-driven Mpk1 activation. Other compatible solutes are induced in response to Hog1 activation, most notably trehalose (Saito and Posas, 2012), which may account for the residual Hog1-driven activation of Mpk1 observed in both the *gpd1*Δ *gpd2*Δ mutant and in the presence of the open glycerol channel (Fps1-IV/AA).

Many cell wall antagonists act by interfering with cell wall biosynthesis (e.g. echinocandins), whereas zymolyase degrades the yeast cell wall from the outside. This mechanism of action may pose a special problem for cell wall stress perception and hence, CWI pathway activation. Although it is not clear how zymolyase treatment initiates signaling through the osmosensor Hkr1:Sho1, both the protease and glucanase activities of

zymolyase are required (Rodriguez-Pena et al., 2013). Thus, it is possible that signaling is initiated through partial degradation of Hkr1 by zymolyase proteases, although there is no experimental evidence to support this conclusion. The indirect route to CWI pathway activation through the HOG pathway described here may be the only mechanism available to the cell in response to the specialized cell wall challenge posed by zymolyase treatment.

Our findings suggest that whenever Hog1 activity induces glycerol accumulation in the absence of high external osmolarity, this will cause cell wall stress and Mpk1 activation. Because Hog1 is known to be activated by a multitude of stresses other than hyper-osmolarity (Lee et al., 2019), it seems likely that mechanisms exist to prevent the inappropriate accumulation of glycerol and consequent cell wall stress under these Hog1-activating conditions. Indeed, we have identified such a mechanism recently with regard to arsenite activation of Hog1, (Lee and Levin, 2019). In this case, the cell uses a metabolite of the stressor itself to block glycerol production through inhibition of the glycerol-3-phosphate dehydrogenase. We expect that additional mechanisms exist to maintain osmotic homeostasis when Hog1 is activated in response to other stressors.

4 Conclusions

In conclusion, we have identified two circumstances in which crosstalk between the HOG pathway and the CWI pathway is mediated largely by Hog1-driven accumulation of glycerol, which induces cell wall stress and consequent activation of Mpk1. First, in the case of a *ptp2*Δ *ptp3*Δ mutant, we conclude that the loss of tyrosine-specific protein phosphatase activity directed against Hog1 results in its artificial activation and that Mpk1 activation is a secondary event that is largely dependent upon the accumulation of glycerol. Second, Hog1 activation in response to zymolyase treatment activates Mpk1 in a manner that is also largely dependent upon glycerol accumulation. Thus, it seems likely that Hog1-driven accumulation of glycerol is a common link between these two SAPK pathways

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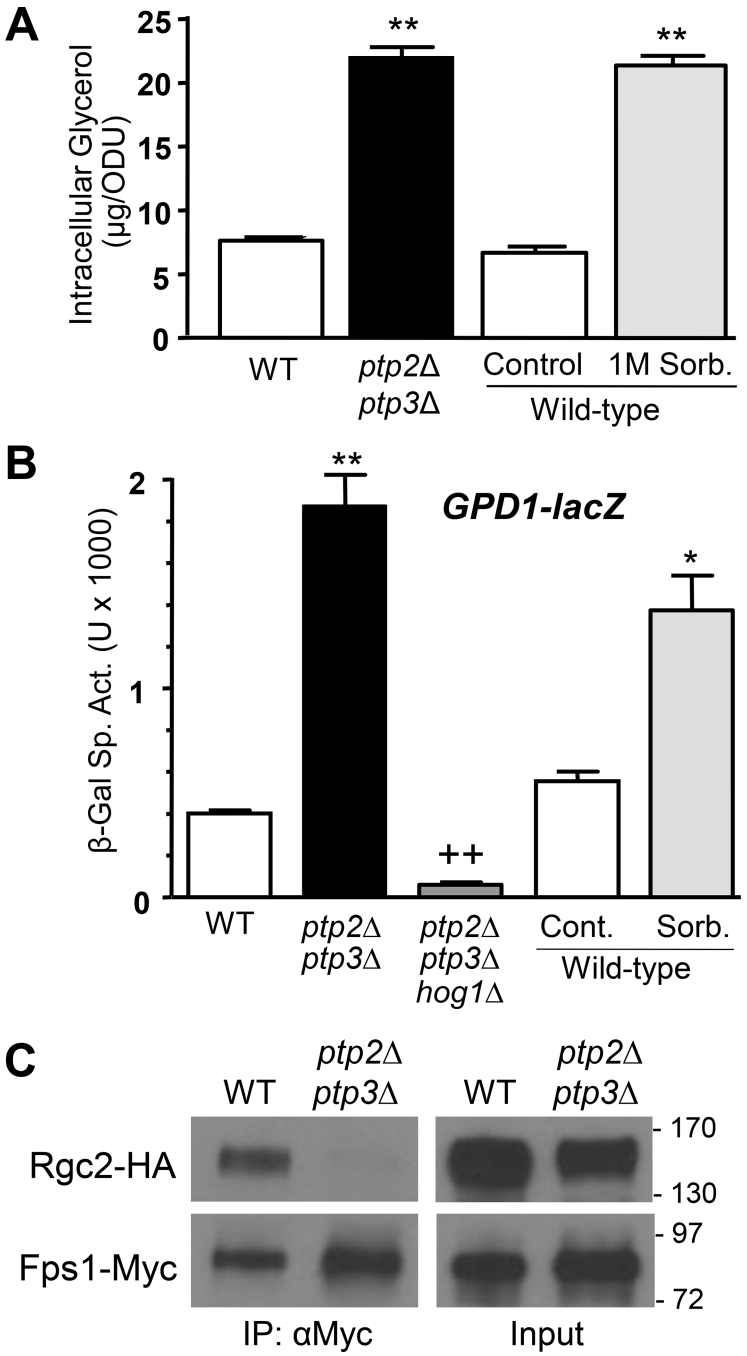

Figure 1.

Glycerol production and accumulation in the *ptp2*Δ *ptp3*Δ mutant. **A.** Intracellular glycerol accumulation was measured in a wild-type strain (BY4742) and the *ptp2*Δ *ptp3*Δ mutant (DL4299) in the absence of stress, or after a 10-min hyperosmotic shock with 1M sorbitol. ODU; Optical Density Unit at A_{600}. **B.** Elevated levels of *GPD1-lacZ* expression in the *ptp2*Δ *ptp3*Δ mutant. The same strains from **A**, plus a *ptp2*Δ *ptp3*Δ *hog1*Δ mutant (DL4424) were transformed with a *GPD1-lacZ* reporter plasmid (p2156) to measure differences in the basal level of *GPD1* expression and for comparison to the response to hyperosmotic shock

of the wild-type strain with 1M sorbitol for 1 hour. For **A** and **B**, values are the mean and standard deviation from three or four independent cultures. Statistical analysis was carried out by *t*-test: ** and *, $P<0.0005$ and $P<0.002$, respectively, for wild-type *vs. ptp2*Δ *ptp3*Δ mutant strain comparison or for untreated vs. sorbitol-treated cultures comparison; ++, $P<0.0005$ for *ptp2*Δ *ptp3*Δ *vs ptp2*Δ *ptp3*Δ *hog1*Δ strain comparison. **C.** Association between glycerol channel Fps1 and its regulator Rgc2 is lost in the *ptp2*Δ *ptp3*Δ mutant. Rgc2-HA was tested for co-IP with Fps1-Myc from extracts of wild-type (BY4742) and *ptp2*Δ *ptp3*Δ mutant (DL4299) cells coexpressing the differentially tagged proteins from plasmids (p3121 and p3151). Immunoprecipitates were separated by SDS-PAGE and subjected to immunoblot analysis. Molecular mass markers (in kilodaltons) are on the right.

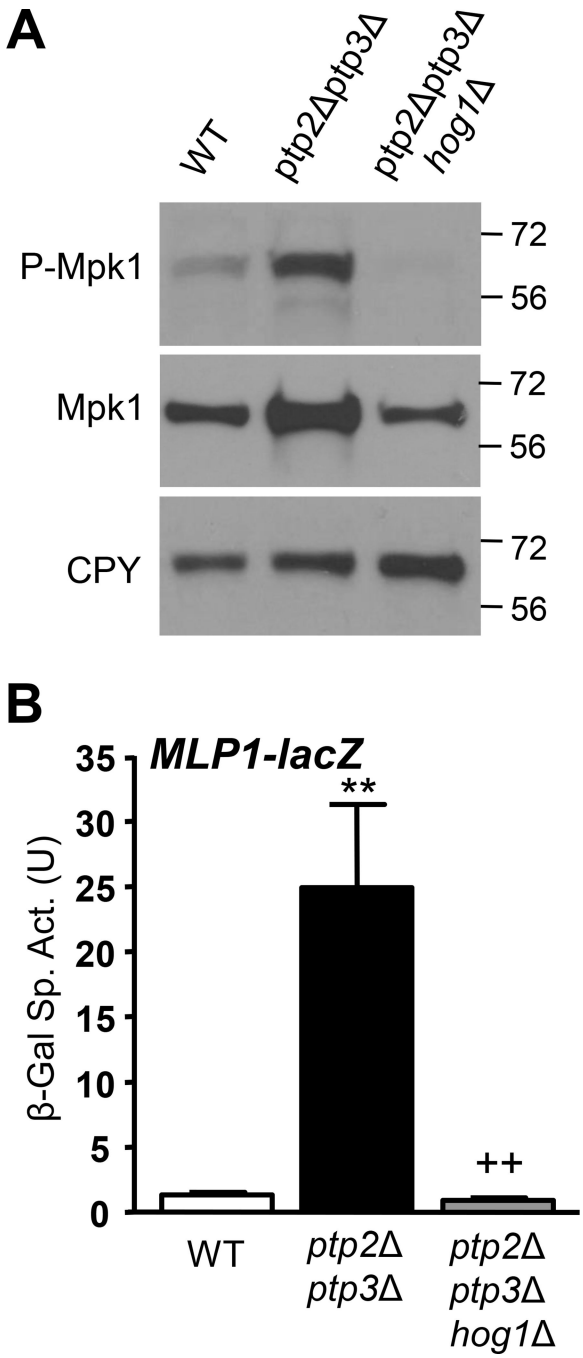

Figure 2.

The *ptp2*Δ *ptp3*Δ mutant experiences Hog1-dependent cell wall stress. **A.** Mpk1 is constitutively phosphorylated in the *ptp2*Δ *ptp3*Δ mutant. Extracts from cultures of wild-type cells (BY4742), *ptp2*Δ *ptp3*Δ (DL4299), and *ptp2*Δ *ptp3*Δ *hog1*Δ (DL4424) mutants were separated by SDS-PAGE and subjected to immunoblot analysis for activated Mpk1 (P-Mpk1), total Mpk1, and CPY as a loading control. Molecular mass markers (in kilodaltons) are on the right. **B.** The same strains were transformed with the *MLP1-lacZ* reporter plasmid (p1368) to measure differences in the basal level of *MLP1* expression. Values are the mean

and standard deviation from four independent cultures. Statistical analysis was carried out by *t*-test: ** and ++, $P<0.0005$ for wild-type *vs. ptp2*Δ *ptp3*Δ mutant strain comparison and for *ptp2*Δ *ptp3*Δ *vs ptp2*Δ *ptp3*Δ *hog1*Δ strain comparison, respectively.

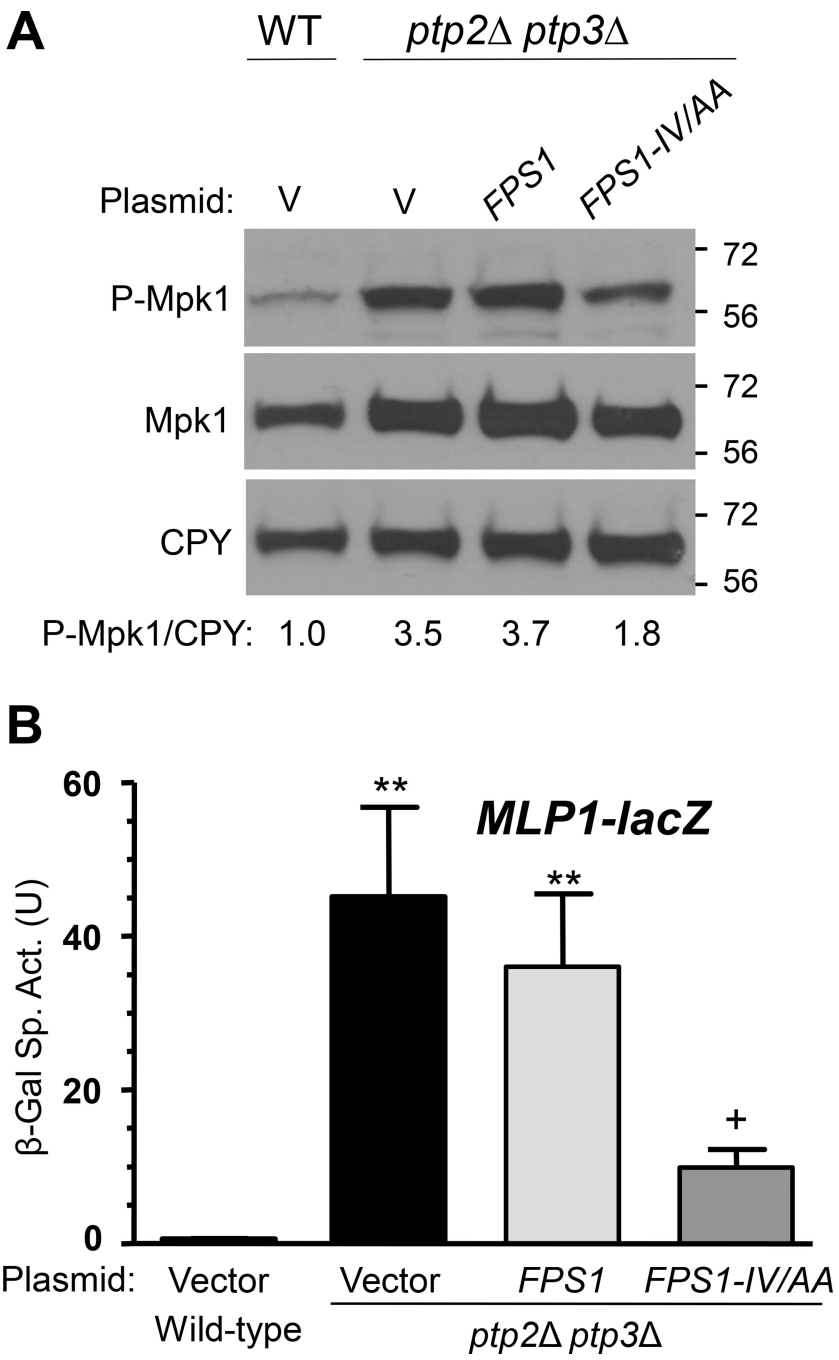

Figure 3.

Venting glycerol relieves the cell wall stress experienced by the *ptp2*Δ *ptp3*Δ mutant. **A.** Wild-type (BY4742) and *ptp2*Δ *ptp3*Δ (DL4299) cells were transformed with plasmids that express Fps1 (p3121), its open-channel form Fps1-IV/AA (p3260), or vector (V; p120). Extracts were separated by SDS-PAGE and subjected to immunoblot analysis for activated Mpk1 (P-Mpk1), total Mpk1, and CPY as a loading control. Molecular mass markers (in kilodaltons) are on the right. Quantitation of the P-Mpk1/CPY ratio was normalized to 1 in the wild-type strain. **B.** The same strains were tested for cell wall stress transcription after

transformation with the *MLP1-lacZ* reporter plasmid (p1368). Values are the mean and standard deviation from four independent cultures. Statistical analysis was carried out by *t*-test: **, $P<0.0005$ for comparison of wild-type strain transformed with vector *vs. ptp2*Δ *ptp3*Δ mutant strain transformed with vector or *FPS1*; +, $P<0.002$ for comparison of *ptp2*Δ *ptp3*Δ strain transformed with the *FPS1* plasmid vs the same strain transformed with the *FPS1-IV/AA* plasmid.

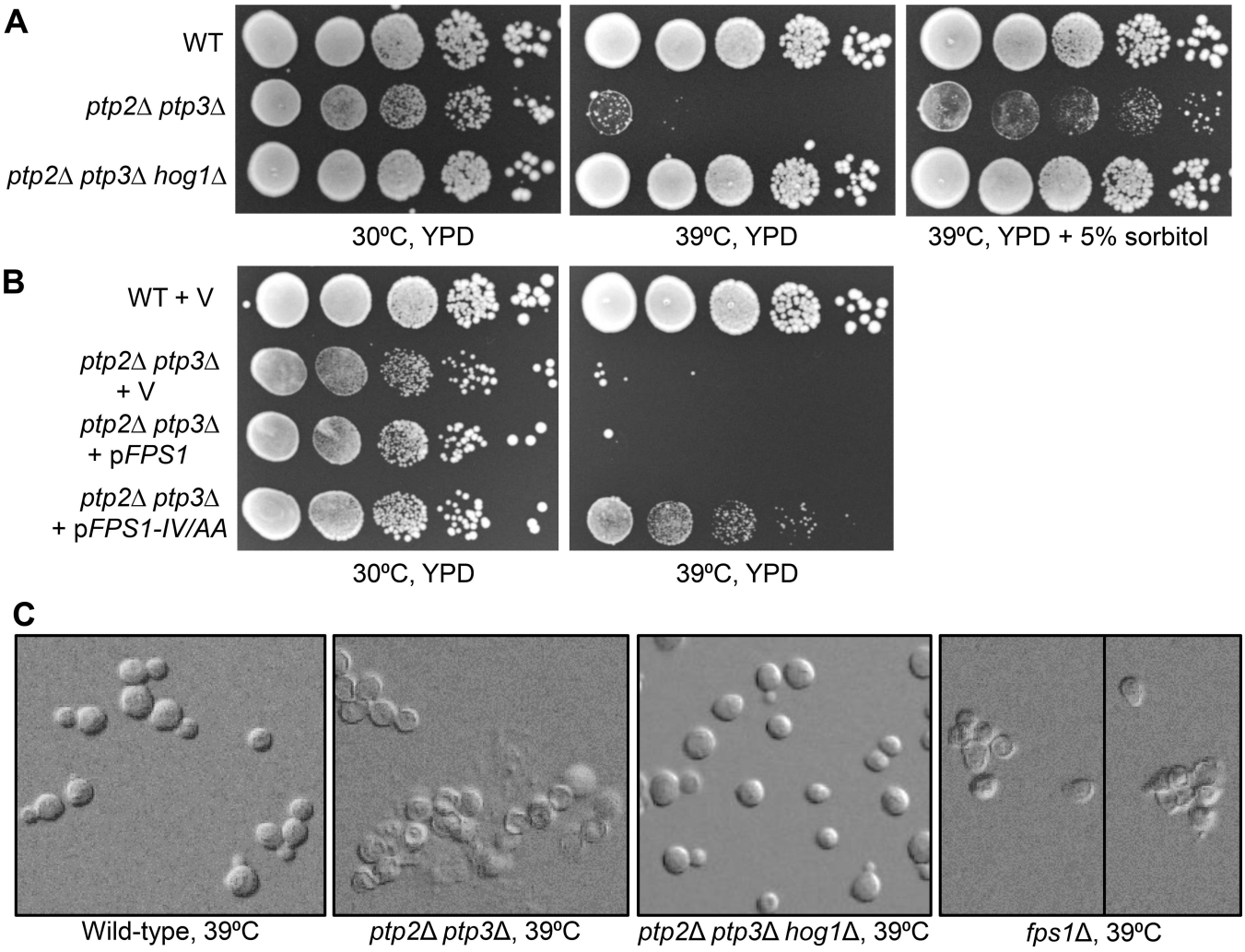

Figure 4.

The *HOG1*-dependent growth arrest of the *ptp2*Δ *ptp3*Δ mutant at elevated temperature is suppressed by correction of its osmotic imbalance. **A.** Wild-type cells (BY4742), *ptp2*Δ *ptp3*Δ (DL4299), and *ptp2*Δ *ptp3*Δ *hog1*Δ (DL4424) mutants were spotted onto YPD plates or YPD plus 5% sorbitol for osmotic support at serial 10-fold dilutions (from left to right) and incubated at the indicated temperatures for 3 days. **B.** Wild-type (BY4742) and *ptp2*Δ *ptp3*Δ (DL4299) cells were transformed with plasmids that express Fps1 (p3121), its open-channel form Fps1-IV/AA (p3260), or vector (V; p120). Transformants were spotted as above and incubated at the indicated temperature for 3 days. **C.** Wild-type (BY4742), *ptp2*Δ *ptp3*Δ (DL4299), *ptp2*Δ *ptp3*Δ *hog1*Δ (DL4424), and *fps1*Δ (DL3229) strains were streaked onto YPD plates and incubated at 39°C for 18 h. Cells were taken from plates for photomicroscopy.

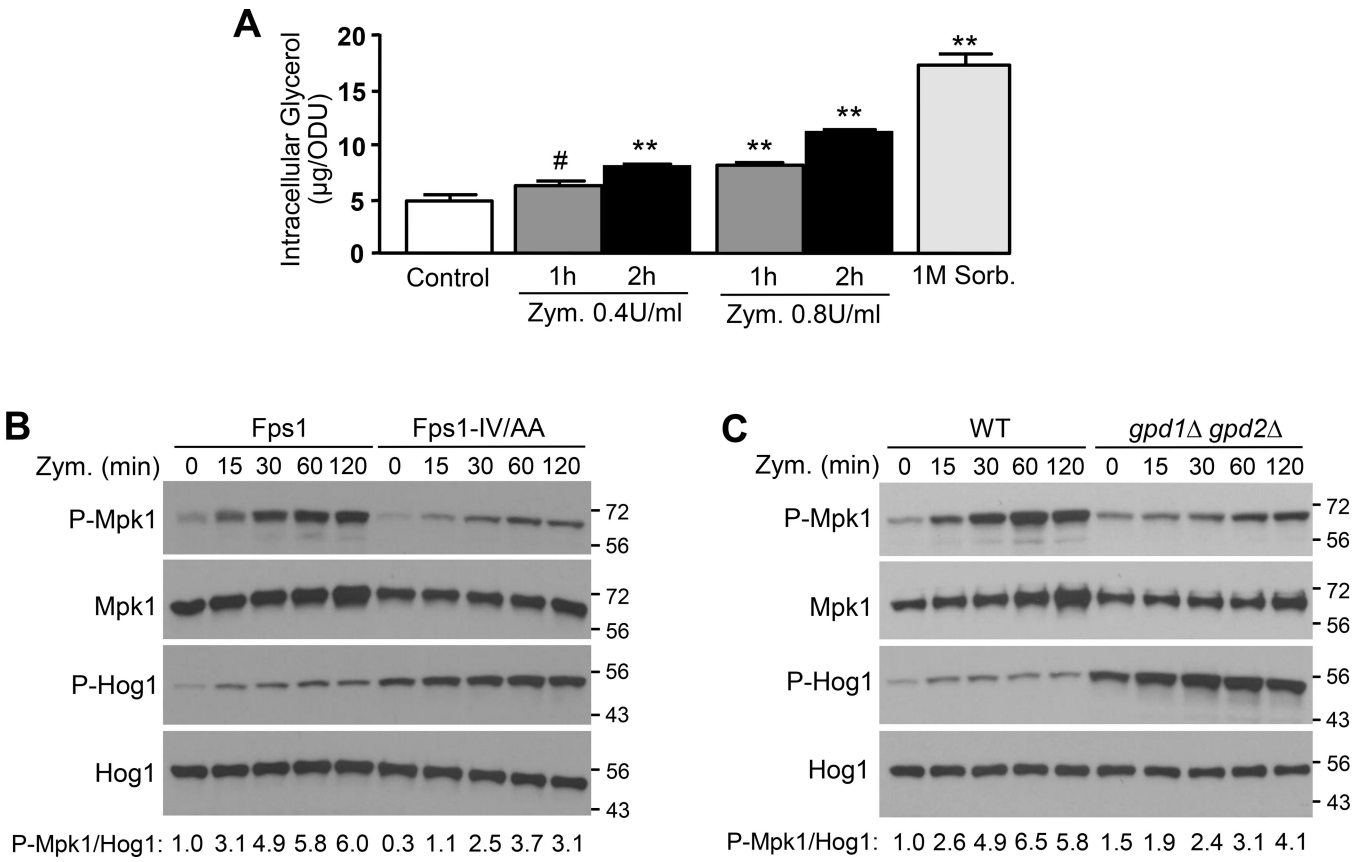

Figure 5.

Treatment with zymolyase induces glycerol accumulation. **A.** Intracellular glycerol accumulation was measured in wild-type cells (BY4742) that were treated with zymolyase 100T for 1 or 2 hours, or a 10-min hyperosmotic shock with 1M sorbitol. Values are the mean and standard deviation from three independent cultures. Statistical analysis was carried out by *t*-test: ** and #, $P<0.0005$ and $P<0.01$, respectively, for zymolyase- or sorbitol-treated vs untreated cultures comparisons. **B and C.** Activation of Mpk1 in response to zymolyase treatment partially depends on glycerol accumulation. **B.** An *fps1*Δ (DL3226) strain, transformed with plasmids that express Fps1 (p3121), its open-channel form Fps1-IV/AA (p3260), were subjected to treatment with 0.4U/ml zymolase for the indicated times. Extracts were separated by SDS-PAGE and subjected to immunoblot analysis for activated Mpk1 (P-Mpk1), total Mpk1, activated Hog1 (P-Hog1), and total Hog1. Molecular mass markers (in kilodaltons) are on the right. Quantitation of the P-Mpk1/total Hog1 ratio was normalized to 1. Hog1 was used as an input control because, in contrast to Mpk1, its level does not vary in response to activating stress. **C.** Wild-type cells (BY4741) and a *gpd1*Δ *gpd2*Δ strain (DL4285) were subjected to treatment with 0.4U/ml zymolase for the indicated times. Extracts were treated as in B. Quantitation of the P-Mpk1/total Hog1 ratio was normalized to 1.

Table 1.

Plasmids

Plasmid	Description	Source or Reference
p115	pRS316	Sikorski & Hieter, 1989
p120	YEp351	Hill, Myers, Koerner, & Tzagoloff, 1986
p1368	pLG178-*MLP1 [YKL161C] (−949 to +1)-lacZ*	Jung et al., 2002
p2156	pLG178-*GPD1 (−1000 to +1)-lacZ*	Lee et al., 2013
p3121	pYEP181-*MET25^{p}-FPS1-Myc*	Beese-Sims, Lee, & Levin, 2011
p3151	pRS316-*MET25^{p}-RGC2–3HA*	Lee et al., 2013
p3260	pYEP181-*MET25^{p}-FPS1-I218A, V220A-Myc* (IV/AA)	Lee et al., 2013