The SMuSh pathway is essential for survival during growth-induced compressive mechanical stress

Morgan Delarue[1,2], Gregory Poterewicz[2], Ori Hoxha[1], Jessica Choi[1], Wonjung Yoo[1], Jona Kayser[1], Liam Holt[2,†] and Oskar Hallatschek[1,† ‡]

June 15, 2017

Cells that proliferate in a confined environment eventually build up mechanical compressive stress. For example, mechanical pressure can emerge from the growth of cancer cells or microbes in their natural settings [1–4]. However, while the biological effects of tensile stress have been extensively studied [5–10], little is known about how cells sense and respond to mechanical compression. By combining genetic analysis with microfluidic approaches, we discovered that compressive stress is sensed through a module consisting of the mucin Msb2 and Sho1, which is one of the two osmosensing pathways in budding yeast [11]. This signal is transmitted via the MAP kinase Ste11 to the cell wall integrity pathway. We term this mechanosensitive pathway the *SMuSh* pathway, for *S*te11 through *Mu*cin / *Sh*o1 pathway. The SMuSh pathway is necessary for G1 arrest and cell survival in response to growth-induced pressure. Our study demonstrates the ability of budding yeast to specifically respond to mechanical compressive stress raising the question of whether homologous pathways confer mechano-sensitivity in higher eukaryotes.

Recent experiments suggest that the fungus *S. cerevisiae* senses and adapts to compressive mechanical stress [4]. To discover the molecular basis of this mechanosensing, we improved the design of a previously developed confining microfluidic device to enable higher statistics in an easier-to-handle device [4] (Fig. S1). Unconstrained cell proliferation occurred in the chamber until cells filled it, at which point further proliferation resulted in the progressive build-up of growth-induced pressure with a typical timescale of $\sim$ 10h (Fig. 1a and Movie S1). Pressure was calculated by quantifying the deformation of the PDMS walls of the chamber (Fig. S2). We chose a pressure of $\sim$ 0.4 MPa as the set-point for our analysis, which is about half of the pressure at which wild-type cells stall growth [4]. Importantly, all mutant strains were able to generate this pressure, thus enabling direct phenotypic comparison.

In contrast to osmotic stress, which causes isotropic reduction of cell volume without major shape changes [12], contact forces imposed by growth in confinement led to severe cell deformation (Fig. 1b), cell size reduction (Fig. S3), and decreased cell proliferation. Using a *WHI5-mCherry* strain, we observed an accumulation of cells delayed in G1 as pressure built up (Fig. 1b), as indicated by a nuclear Whi5 signal [13]. We also noticed that about 10% of the cell population died when grown to 0.4 MPa of pressure, as evidenced by accumulation of autofluorescent cell debris (Fig. 1b, Fig 2a).

The progressive enrichment of cells in the G1 phase of the cell cycle, together with the occurrence of cell death suggested that inhibition of proliferation under mechanical stress could be an adaptation to increase survival in this challenging environment. This model implies the existence of molecular pathways that sense and respond to compressive stress. We hypothesized that this mechanosensing could employ elements of the osmosensing machinery, because both osmotic and mechanical can result in water efflux. In budding yeast, two overlapping osmosensing pathways have been identified, both of which activate MAP kinase (MAPK) cascades [12]. The SLN1 pathway regulates the activity of the MAPKKKs Ssk2 and Ssk22 under osmotic stress, whereas the SHO1 pathway activates the MAPKKK Ste11. Both pathways converge to activate the MAPK Hog1, which is thought to be the primary effector of the osmotic stress response.

We explored whether genetic alterations to these pathways would lead to differential cell survival between a mechanical and an osmotic stress. We found that, while both pathways respond to and promote cell survival under osmotic stress (Fig. S4), disrupting the SLN1 pathway by deletion of *SSK1* did not affect cell survival under mechanical stress (Fig. 2a), suggesting that the SLN1 pathway is dispensable for survival under compressive mechanical stress. In clear contrast, deletion of *SHO1* led to dramatic cell death: close to 50% of the cells died within the 10h of mechanical stress build-up from 0 MPa to 0.4 MPa (Fig. 2a-b). Thus, the SHO1 pathway is required for

*[1] Department of Physics and Integrative Biology, University of California, Berkeley, USA

†[2] Institute for Systems Genetics, New York University Langone Medical Center, New York, USA

‡† These authors equally contributed to the work.

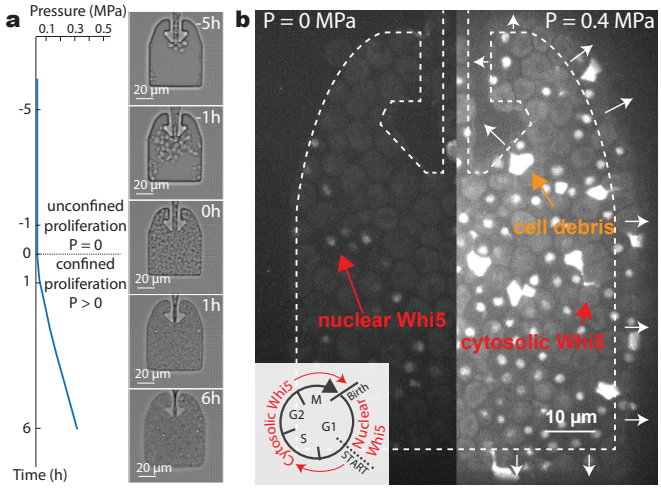

Figure 1: **Growth-induced mechanical pressure leads to cell cycle arrest in G1**. **a**. Cells initially proliferate unconfined, developing no pressure, until they fill the confining chamber. At this point, proliferation leads to build-up of compressive pressure within hours. We calculate the pressure developed by the cells through the deformation of the PDMS chamber (Fig. S2). **b**. Nuclear accumulation of *WHI5-mCherry* indicates a delay in the G1 phase of the cell cycle. The contour of the chamber is outlined in dash, white arrows indicating chamber deformation. Red arrows point to cytosolic Whi5 and nuclear Whi5, while the orange arrow points to a cell debris.

survival during growth-induced compressive stress.

The Sho1 protein has two different mucin-like co-activators: Msb2p and Hkr1p, high molecular weight, membrane bound glycoproteins that communicate with Sho1p through a poorly understood mechanism [14, 15]. Deletion of the *HKR1* co-activator only had a mild effect on cell survival, but deletion of *MSB2* showed the same dramatic cell death phenotype under mechanical stress as *SHO1* deletion (Fig. 2a). This result is in contrast with zymolyase treatment, a model cell wall stress, that requires HKR1 for optimal survival, but not MSB2 [16]. Furthermore, deletion of *STE11* also led to dramatic cell death under pressure. Together, these results suggest that Msb2 / Sho1 senses compressive mechanical stress and activates the MAPKKK Ste11 (Fig. 2c). Thus, we have defined a pathway essential for cell survival under compressive mechanical stress, which we term the *SMuSh* pathway, for *S*te11 through *Mu*cin / *Sh*o1 pathway.

We sought to determine if cells that lack *SMuSh* mechanosensing pathway components are intrinsically unstable when mechanically compressed. To address this question, we developed a new microfluidic device that allowed us to exert an instantaneous mechanical compressive stress (Fig. 3a). In this system, cells were loaded into the chamber and then the loading valve was sealed. Subsequently, pressure was induced in two alternative ways: Either the confined cells were allowed to divide to build-up growth-induced pressure over several hours (Fig. 3b and Fig. S5), or a thin membrane "micro-piston" at the base of the chamber was distorted to instantaneously compress the cell population (Fig. 3c). When cells progressively built up pressure through growth and division there was a large increase in cell death in the *ste11*Δ background compared to wild-type cells (Fig. 3d). However, when cells were instantaneously compressed to a comparable pressure, there was no cell death in either strain (Fig. 3e). These results demonstrate that loss of *SMuSh* components does not cause intrinsic mechanical instability. Rather, the cell death phenotype that we observe in mutants for the mechanosensing pathway only occurs when cells grow and/or divide under pressure.

In the wild-type situation, the build-up of compressive, growth-induced mechanical stress was accompanied by an increase in cells delayed in the G1 phase of the cell cycle (Fig. 4a). We observed that this cell cycle delay was lost in *ste11*Δ cells (Fig. 4b), suggesting that cell cycle arrest could be key to cell survival. In agreement with this hypothesis, all *ste11*Δ cells that we could observe dying ($N \geq 10$) had a cytosolic Whi5 signal, indicating that these cells had progressed beyond START to enter the cell division cycle (Fig. S6).

The activation of the MAPKKK Ste11 mediated by Msb2 / Sho1 has been reported to activate two main pathways: the osmotic response pathway, through its MAPK Hog1 and the filamentous growth pathway, through its MAPK Kss1 [17]. In addition, Ste11 has recently been shown to signal to the cell wall integrity pathway and its MAPK Slt2 [16, 18–21](Fig. 4c). We deleted the MAPK for each of these three pathways, and found that, even though cell death doubled in *kss1*Δ and *hog1*Δ strains, deletion of *SLT2* had the most dramatic effect, increasing cell death six-fold to levels similar to those observed when *SMuSh* components were deleted. Importantly, we also observed that the pressure-induced cell cycle delay in G1 depended on the presence of Slt2p: no G1 delay was observed in the *slt2*Δ background (Fig. 4b). Several recent studies have reported interactions between the *MSB2/SHO1/STE11* pathway and the cell wall integrity pathway at the level of, or upstream of the MAPKKK Bck1 [18]. Consistent with these studies, we observed a dramatic cell death phenotype in a *bck1*Δ background (Fig. S7). Thus, cell cycle arrest and cell survival require activation of the cell wall integrity pathway by *SMuSh* (Fig. 4c, orange).

In contrast to osmotic stress, which is mainly integrated through Hog1, compressive mechanical stress appears to mainly activate downstream elements of the Cell Wall Integrity pathway, and affects cell survival through Slt2. Our results also indicated that compressive stress induces cell cycle arrest *via* a distinct mechanism. The Hog1-dependent cell cycle delay

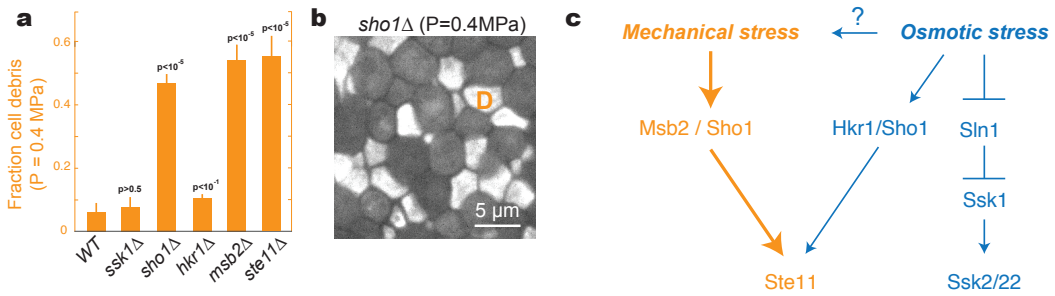

Figure 2: **The SMuSh pathway is essential for survival under compressive mechanical stress. a**. Fraction of cell debris under a mechanical pressure of 0.4 MPa in different genetic backgrounds. p-values were calculated by a T-test on more than 5 replicates for each genetic background compared to wild-type (*WT*). **b**. Fluorescent picture of a *sho1*Δ background under mechanical stress, displaying the accumulation of cell debris. **c**. Pathway diagrams for putative osmosensors in *S. cerevisiae*. Our results suggest that the Msb2/Sho1 module is a mechanosensor (orange) while Sln1 and Hkr1/Sho1 modules are osmosensors (blue).

that occurs under osmotic stress depends on the kinase inhibitor Sic1 [22]. We found that *HOG1* and *SIC1* were not required for G1 arrest under mechanical stress (Fig. S8). Rather, we found that this cell cycle arrest is mediated by the MAPK Slt2. Recent work demonstrated that Slt2p can arrest cells by inhibiting DNA replication through Cdc6 degradation [23].

One salient difference between osmotic stress and compressive mechanical stress is the anisotropic nature of the latter, which results in strong cell deformation (Fig. 1b). The *SLN1* branch has long been hypothesized to be a true osmosensor [11, 24, 25]. We found that SLN1 is dispensable for cell survival under compressive mechanical stress, reinforcing this notion. We can still only speculate about the SLN1 activation mechanism: One possibility might be that the isotropic volume reduction imposed through an osmotic stress may crowd the Sln1 molecules, which are uniformly distributed on the plasma membrane [12].

In contrast, Sho1p is localized asymmetrically at the site of polarized growth [26]. Moreover, Sho1p is required for proper septation of *S. cerevisiae* cells, by recruiting the HICS complex, consisting of Hof1p, Inn1p and Cyk3p, that may link the cell membrane to the acto-myosin ring during cytokinesis [27]. Thus, we speculate that Sho1 may be tuned to sense anisotropic cell deformation. The HICS complex also recruits Slt2p and is thought to activate the Cell Wall Integrity pathway. Deletion of Sho1p leads to cell wall defects, including fragility of the bud neck. Thus, *SMuSh* pathway mutants may mechanically fail at sites of septation, which would explain the increase in cell death under mechanical stress only when cells progress beyond START. *MSB2*, but not *HKR1*, has recently been shown to directly interact with the actin cytoskeleton [15]. This coupling raises the possibility that Msb2 / Sho1 could sense mechanical stress through deformation of the cortex or changes in plasma membrane tension, which would also naturally occur during cell division. The cell cortex is also reorganized and membrane tension is altered when cells are exposed to osmotic stress [28], perhaps explaining how the Msb2 / Sho1 complex also acts as an osmosensor.

Our results identified the *SMuSh* pathway, the first pathway required for cell survival in mechanical compressive stress. The identification of the Msb2 / Sho1 module as the key sensor in this mechanosensing paradigm opens new avenues to understand the physical details of compressive mechanosensing. Transmembrane mucins are also important in human physiology and are frequently overexpressed in cancer, another context where compressive stresses arise [29, 30]. This raises the intriguing possibility that mechanosensing through mucins may be widely conserved in eukaryotes.

Acknowledgements

The authors would like to thank J. Hartung for his help with the finite element simulations, as well as the Thorner and Boeke labs for some of the strains used in this study. Research reported in this publication was supported by the National Institute Of General Medical Sciences of the National Institutes of Health under Award Number R01GM115851, by a Simons Investigator award from the Simons Foundation (O.H.) and by the German Research Foundation (DFG) in the framework of the SFB 937/A15. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

[1] G. Helmlinger, P. A. Netti, H. C. Lichtenbeld, R. J. Melder, and R. K. Jain, “Solid stress inhibits the growth

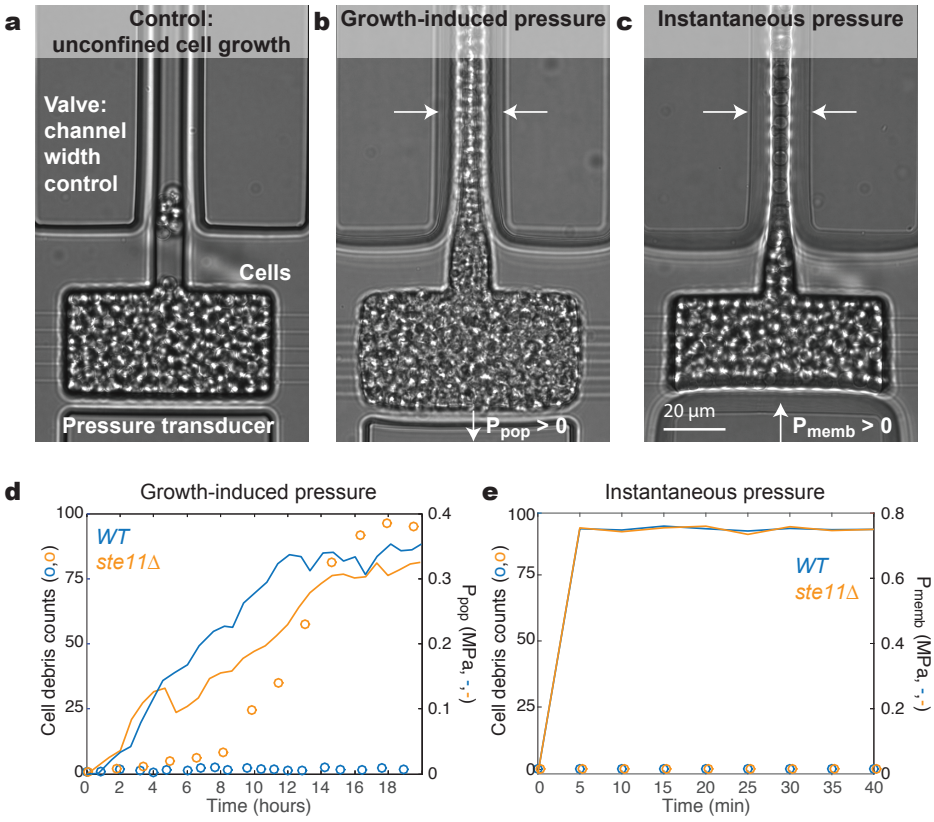

Figure 3: **Cell death only accumulates through confined cell proliferation in a *ste11*Δ background, not through instantaneous compression.** When the valve channel is opened (**a**), the cells flow out of the chamber and do not build-up a mechanical stress, in contrast to a situation where the valve is closed (**b**). A water-driven pressure can be applied on the pressure transducer in order to instantaneously compress the cell population (**c**). We observe accumulation of cell debris under the build-up of pressure in a *ste11*Δ background but not in the wild-type (**d**). An instantaneous compression does not increase cell death in both cases, suggesting that the cells are intrinsically mechanically stable.

of multicellular tumor spheroids," *Nature biotechnology*, vol. 15, no. 8, pp. 778–783, 1997.

[2] K. Alessandri, B. R. Sarangi, V. V. Gurchenkov, B. Sinha, T. R. Kießling, L. Fetler, F. Rico, S. Scheuring, C. Lamaze, A. Simon, *et al.*, "Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression in vitro," *Proceedings of the National Academy of Sciences*, vol. 110, no. 37, pp. 14843–14848, 2013.

[3] T. Stylianopoulos, J. D. Martin, V. P. Chauhan, S. R. Jain, B. Diop-Frimpong, N. Bardeesy, B. L. Smith, C. R. Ferrone, F. J. Hornicek, Y. Boucher, *et al.*, "Causes, consequences, and remedies for growth-induced solid stress in murine and human tumors," *Proceedings of the National Academy of Sciences*, vol. 109, no. 38, pp. 15101–15108, 2012.

[4] M. Delarue, J. Hartung, C. Schreck, P. Gniewek, L. Hu, S. Herminghaus, and O. Hallatschek, "Self-driven jamming in growing microbial populations," *Nature Physics*, 2016.

[5] S. Huang and D. E. Ingber, "Cell tension, matrix mechanics, and cancer development," *Cancer cell*, vol. 8, no. 3, pp. 175–176, 2005.

[6] M. A. Schwartz, "Integrins and extracellular matrix in mechanotransduction," *Cold Spring Harbor perspectives in biology*, vol. 2, no. 12, p. a005066, 2010.

[7] R. O. Hynes, "The extracellular matrix: not just pretty fibrils," *Science*, vol. 326, no. 5957, pp. 1216–1219, 2009.

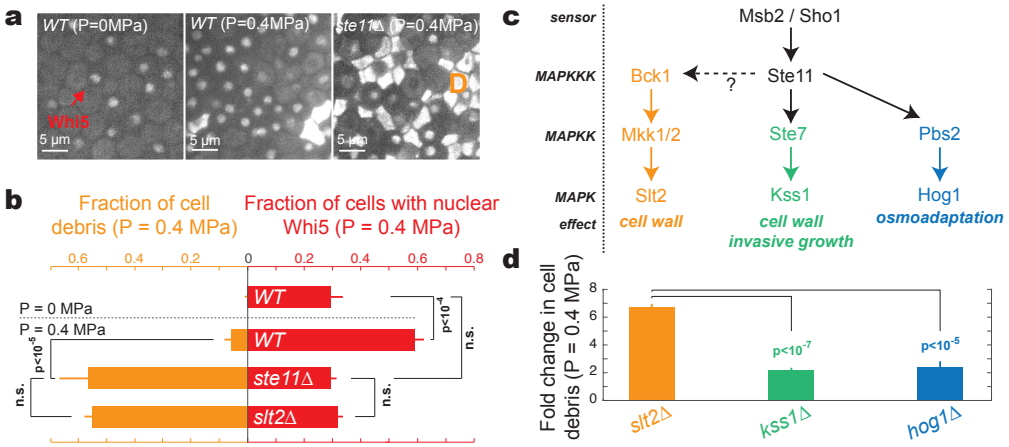

Figure 4: **Slt2-dependent cell cycle arrest prevents cell death under mechanical pressure a**. Pictures of the *WHI5-mCherry* construct in the wild-type situation and a *ste11*Δ background. **b**. Quantification of the fraction of cell debris and cells displaying nuclear Whi5 under a compressive mechanical stress. **c**. Ste11, through Msb2/Sho1, can activate three major MAPK pathways, through the MAPKs Slt2, Kss1 and Hog1. **d**. Fold change in cell debris as compared to the wild-type for different genetic backgrounds, showing that the downstream response of Slt2p is essential for cell survival.

[8] D. E. Discher, P. Janmey, and Y.-l. Wang, "Tissue cells feel and respond to the stiffness of their substrate," *Science*, vol. 310, no. 5751, pp. 1139–1143, 2005.

[9] M. H. Zaman, L. M. Trapani, A. L. Sieminski, D. MacKellar, H. Gong, R. D. Kamm, A. Wells, D. A. Lauffenburger, and P. Matsudaira, "Migration of tumor cells in 3d matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis," *Proceedings of the National Academy of Sciences*, vol. 103, no. 29, pp. 10889–10894, 2006.

[10] A. J. Engler, S. Sen, H. L. Sweeney, and D. E. Discher, "Matrix elasticity directs stem cell lineage specification," *Cell*, vol. 126, no. 4, pp. 677–689, 2006.

[11] S. M. O'Rourke, I. Herskowitz, and E. K. O'Shea, "Yeast go the whole hog for the hyperosmotic response," *TRENDS in Genetics*, vol. 18, no. 8, pp. 405–412, 2002.

[12] S. Hohmann, "Osmotic stress signaling and osmoadaptation in yeasts," *Microbiology and molecular biology reviews*, vol. 66, no. 2, pp. 300–372, 2002.

[13] M. Costanzo, J. L. Nishikawa, X. Tang, J. S. Millman, O. Schub, K. Breitkreuz, D. Dewar, I. Rupes, B. Andrews, and M. Tyers, "Cdk activity antagonizes whi5, an inhibitor of g1/s transcription in yeast," *Cell*, vol. 117, no. 7, pp. 899–913, 2004.

[14] K. Tatebayashi, K. Tanaka, H.-Y. Yang, K. Yamamoto, Y. Matsushita, T. Tomida, M. Imai, and H. Saito, "Transmembrane mucins hkr1 and msb2 are putative osmosensors in the sho1 branch of yeast hog pathway," *The EMBO journal*, vol. 26, no. 15, pp. 3521–3533, 2007.

[15] K. Tanaka, K. Tatebayashi, A. Nishimura, K. Yamamoto, H.-Y. Yang, and H. Saito, "Yeast osmosensors hkr1 and msb2 activate the hog1 mapk cascade by different mechanisms," *Sci. Signal.*, vol. 7, no. 314, pp. ra21–ra21, 2014.

[16] J. M. Rodríguez-Peña, S. Díez-Muñiz, C. Bermejo, C. Nombela, and J. Arroyo, "Activation of the yeast cell wall integrity mapk pathway by zymolyase depends on protease and glucanase activities and requires the mucin-like protein hkr1 but not msb2," *FEBS letters*, vol. 587, no. 22, pp. 3675–3680, 2013.

[17] R. E. Chen and J. Thorner, "Function and regulation in mapk signaling pathways: lessons learned from the yeast saccharomyces cerevisiae," *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, vol. 1773, no. 8, pp. 1311–1340, 2007.

[18] C. Bermejo, E. Rodríguez, R. García, J. M. Rodríguez-Peña, M. L. R. de la Concepción, C. Rivas, P. Arias, C. Nombela, F. Posas, and J. Arroyo, "The sequential activation of the yeast hog and slt2 pathways is required for cell survival to cell wall stress," *Molecular biology of the cell*, vol. 19, no. 3, pp. 1113–1124, 2008.

[19] G. Leng and K. Song, "Direct interaction of ste11 and mkk1/2 through nst1 integrates high-osmolarity glycerol and pheromone pathways to the cell wall integrity mapk

pathway," *FEBS letters*, vol. 590, no. 1, pp. 148–160, 2016.

[20] B. B. Fuchs and E. Mylonakis, "Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways," *Eukaryotic cell*, vol. 8, no. 11, pp. 1616–1625, 2009.

[21] J. M. Rodríguez-Peña, R. García, C. Nombela, and J. Arroyo, "The high-osmolarity glycerol (hog) and cell wall integrity (cwi) signalling pathways interplay: a yeast dialogue between mapk routes," *Yeast*, vol. 27, no. 8, pp. 495–502, 2010.

[22] X. Escoté, M. Zapater, J. Clotet, and F. Posas, "Hog1 mediates cell-cycle arrest in g1 phase by the dual targeting of sic1," *Nature Cell Biology*, vol. 6, no. 10, pp. 997–1002, 2004.

[23] K. Kono, A. Al-Zain, L. Schroeder, M. Nakanishi, and A. E. Ikui, "Plasma membrane/cell wall perturbation activates a novel cell cycle checkpoint during g1 in saccharomyces cerevisiae," *Proceedings of the National Academy of Sciences*, vol. 113, no. 25, pp. 6910–6915, 2016.

[24] W. Tao, R. J. Deschenes, and J. S. Fassler, "Intracellular glycerol levels modulate the activity of sln1p, a saccharomyces cerevisiae two-component regulator," *Journal of Biological Chemistry*, vol. 274, no. 1, pp. 360–367, 1999.

[25] D. B. Ostrander and J. A. Gorman, "The extracellular domain of the saccharomyces cerevisiae sln1p membrane osmolarity sensor is necessary for kinase activity," *Journal of bacteriology*, vol. 181, no. 8, pp. 2527–2534, 1999.

[26] D. C. Raitt, F. Posas, and H. Saito, "Yeast cdc42 gtpase and ste20 pak-like kinase regulate sho1-dependent activation of the hog1 mapk pathway," *The EMBO journal*, vol. 19, no. 17, pp. 4623–4631, 2000.

[27] K. Labedzka, C. Tian, U. Nussbaumer, S. Timmermann, P. Walther, J. Müller, and N. Johnsson, "Sho1p connects the plasma membrane with proteins of the cytokinesis network through multiple isomeric interaction states," *J Cell Sci*, vol. 125, no. 17, pp. 4103–4113, 2012.

[28] I. Slaninová, S. Šesták, A. Svoboda, and V. Farkaš, "Cell wall and cytoskeleton reorganization as the response to hyperosmotic shock in saccharomyces cerevisiae," *Archives of microbiology*, vol. 173, no. 4, pp. 245–252, 2000.

[29] J. Taylor-Papadimitriou, J. Burchell, D. Miles, and M. Dalziel, "Muc1 and cancer," *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, vol. 1455, no. 2, pp. 301–313, 1999.

[30] B. Macao, D. G. Johansson, G. C. Hansson, and T. Härd, "Autoproteolysis coupled to protein folding in the sea domain of the membrane-bound muc1 mucin," *Nature structural & molecular biology*, vol. 13, no. 1, pp. 71–76, 2006.