Molecular Systems Biology 5; Article number 281; doi:10.1038/msb.2009.36
Citation: ***Molecular Systems Biology*** **5:281**

www.molecularsystemsbiology.com

molecular systems biology

REPORT

Robustness and fragility in the yeast high osmolarity glycerol (HOG) signal-transduction pathway

Marcus Krantz[1,2,*], Doryaneh Ahmadpour[1,6], Lars-Göran Ottosson[1,6], Jonas Warringer[1], Christian Waltermann[3], Bodil Nordlander[1], Edda Klipp[3], Anders Blomberg[1], Stefan Hohmann[1] and Hiroaki Kitano[2,4,5]

[1] Department of Cell and Molecular Biology, University of Gothenburg, Göteborg, Sweden, [2] The Systems Biology Institute, Tokyo, Japan, [3] Theoretical Biophysics, Institute of Biology, Humboldt University, Berlin, Germany, [4] Sony Computer Science Laboratories, Tokyo, Japan and [5] Okinawa Institute of Science and Technology, Okinawa, Japan
[6] These authors contributed equally to this work
* Corresponding author. Cell and Molecular Biology, University of Gothenburg, Box 462, SE-40530 Gothenburg, Sweden. Tel.: +467 364 547 67; Fax: +463 178 625 99; E-mail: marcus.krantz@cmb.gu.se

Received 28.5.08; accepted 13.5.09

Cellular signalling networks integrate environmental stimuli with the information on cellular status. These networks must be robust against stochastic fluctuations in stimuli as well as in the amounts of signalling components. Here, we challenge the yeast HOG signal-transduction pathway with systematic perturbations in components' expression levels under various external conditions in search for nodes of fragility. We observe a substantially higher frequency of fragile nodes in this signal-transduction pathway than that has been observed for other cellular processes. These fragilities disperse without any clear pattern over biochemical functions or location in pathway topology and they are largely independent of pathway activation by external stimuli. However, the strongest toxicities are caused by pathway hyperactivation. *In silico* analysis highlights the impact of model structure on *in silico* robustness, and suggests complex formation and scaffolding as important contributors to the observed fragility patterns. Thus, *in vivo* robustness data can be used to discriminate and improve mathematical models.
Molecular Systems Biology **5**: 281; published online 16 June 2009; doi:10.1038/msb.2009.36
Subject Categories: metabolic & regulatory networks; signal transduction
Keywords: gTow; HOG; robustness; signal transduction; systems biology

Introduction

Robustness is an intrinsic feature of life as all cellular systems have to maintain functionality in the face of naturally occurring external and internal fluctuations. The resilience of cellular genetic networks lets the cell tolerate a certain level of environmental or mutational perturbations. This robustness can be achieved either by maintaining the cellular status stable against various fluctuations, or by adapting to external changes by triggering a series of cellular responses (Kitano, 2004; Stelling *et al*, 2004). The decision to respond and adapt is relayed via signal transduction systems, which, upon activation by specific stimuli, produce distinct regulatory signals in the form of changes in levels of activated signal-pathway components. A critical aspect of such processes is distinguishing a genuine signal from stochastic fluctuations in protein levels and activity, as misinterpretation of these has potentially disastrous fitness consequences. Thus, the robustness of cells to maintain such a function despite variations in dosage of the components is of primary importance for survival. Despite their importance for viability and fitness, little is known about how signalling systems distinguish between signals and natural fluctuations, or to what extent such fluctuations are tolerated.

Here, we approach this issue through a system-wide robustness study of the HOG pathway of *Saccharomyces cerevisiae*, which is one of the most extensively studied eukaryotic signal-transduction cascades. It is activated by high osmolarity and is essential under this condition. The signalling pathway, which is depicted in Figure 1, consists of a MAP kinase (MAPK) core module, upon which two independent upstream branches converge. The first of these consists of

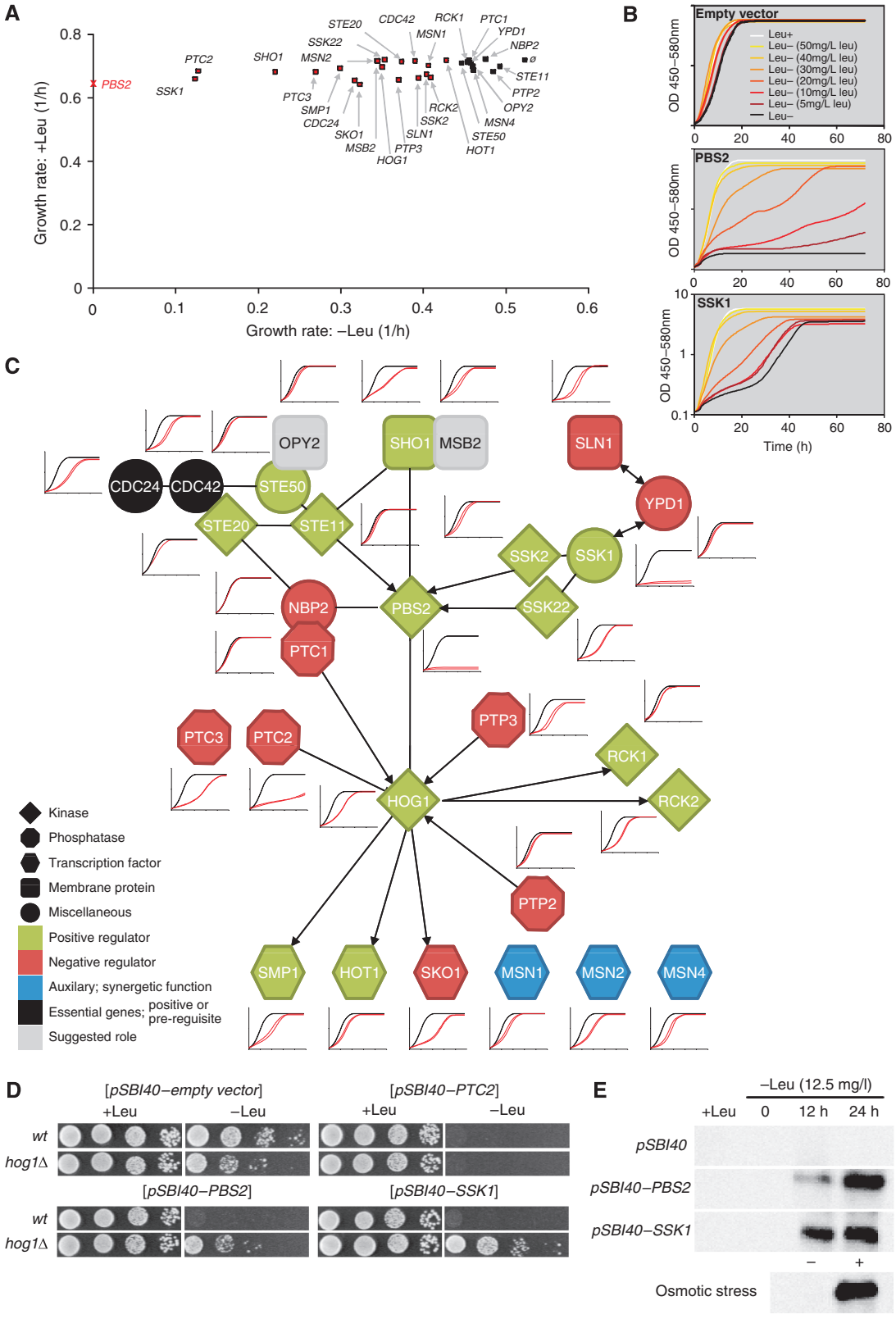

Figure 1 **(A)** gToW growth phenotypes occur in the absence of leucine, and red squares indicate a significant growth-rate defect as compared with the empty plasmid control (Ø). *PBS2*'s growth rate could only be determined in the presence of leucine. **(B)** The severity of the growth defect increases with the level of leucine starvation and **(C)** they spread over different pathway functions. Graphs in (C) indicate growth with (black) or without (red) leucine. **(D)** Phenotypes caused by *PBS2* and *SSK1*, unlike *PTC2*, are partially suppressed by the deletion of *HOG1*. **(E)** Overexpression of Pbs2p and Ssk1p causes dual phosphorylation of Hog1p, which after leucine limitation (12.5 mg/l), reaches levels comparable to those caused by osmotic stress (+) within 24 h. The empty plasmid control (pSBI40) remains similar to unstressed cells (−).

a phosphotransfer module, including the histidine kinase and presumed osmosensor Sln1p, the phosphotransfer protein Ypd1p and the response regulator Ssk1p. When active, this module keeps Ssk1p phosphorylated and inactive. When the module is inactive, dephosphorylated Ssk1p binds to and activates the MAPK kinase kinases Ssk2p and Ssk22p (Saito and Tatebayashi, 2004). Two mucin-like proteins Msb2p and Hkr1p were recently suggested as putative osmosensors of the second input branch (Tatebayashi *et al*, 2007). On activation, the transmembrane protein Sho1p is believed to receive signals from these sensors and convey these signals to the interior of the cell. Sho1p also assembles the MAPK kinase Pbs2p and the MAPK kinase kinase Ste11p through its cytoplasmic domain. The Sho branch also requires Cdc42p and Ste20p for the transmission of the signal. Once active, Ste11p, Ssk2p and Ssk22p are each able to phosphorylate Pbs2p, which also acts as a scaffold for the MAPK module. Pbs2p in turn phosphorylates and activates the MAPK Hog1p, which has numerous targets, including the cytoplasmic kinase Rck2p and several transcription factors such as Hot1p, Sko1p and Smp1p. Active Hog1p accumulates in the nucleus and partakes in transcription (Hohmann, 2002).

The methodology used here links the expression of each target gene of interest to that of a defective allele of a metabolic gene. This allele, *leu2-d*, has a defective promoter and needs to be present in a high copy number to support high Leu2p levels and thus unperturbed growth in the absence of leucine (Schneider and Guarente, 1991). By placing a target gene of interested on the same episomal plasmid as *leu2-d*, the copy number and thus the expression level of this target gene can be controlled via the leucine concentration in the media. The gene in question is still under the control of its normal promoter, allowing expression that is regulated but increases proportionally with the increase in copy number (Moriya *et al*, 2006; Torres *et al*, 2007). If the increase in target gene product interferes with cellular function, a negative pressure on plasmid copy number will balance the positive pressure conferred by the metabolic gene, resulting in a genetic tug-of-war (gToW; Supplementary Figure S1). Such a compromise will result in a decrease in cellular fitness, which can be measured precisely using high-resolution microcultivation (Warringer *et al*, 2003).

Results and discussion

The HOG pathway shows a low level of robustness particularly during the adaptation phase

On applying the gToW methodology to investigate the HOG-pathway robustness, we found a high prevalence of negative impacts from gene dosage perturbations within the HOG pathway (Figure 1A). We used three physiological 'windows' to assess the robustness; growth rate, growth adaptation time and growth efficiency. Growth rate was considered the primary readout because of its strong correlation to plasmid copy number (Moriya *et al*, 2006). Adaptation defects (prolonged growth lag) turned out to be similar to growth-rate defects, although even more pronounced and frequent (Supplementary Figure S2a). There was almost no effect on growth efficiency (Supplementary Figure S2b). Altogether, overexpression of 22 out of 29 HOG-pathway components caused a significant defect in at least one of these three growth variables ($P<0.001$, see Supplementary information). These phenotypes were strongly linked to leucine starvation and thus to selective pressure for high plasmid copy numbers (Figure 1B). They were also observed for all protein classes, and for both positive and negative regulators of the pathway (Figure 1C). The negative effects from increases in gene dosage were emphasized by a complete lack of positive fitness effects mediated by any of the gToW constructs. The highest frequency and strength of defects were observed during adaptation, which may reflect the delicate balance of signal transmission in initiating proliferation.

The high frequency (76%) of HOG-pathway gToW-imposed defects stands in stark contrast to the cell-cycle system, for which only 25–30% of the gToW constructs caused a clear growth retardation (Moriya *et al*, 2006), and to a global *GAL1* promoter-driven overexpression study using galactose induction in which a mere 15% of the targets conferred detectable growth defects (Sopko *et al*, 2006). Interestingly, little correlation in terms of cellular toxicity was found between the gToW- and galactose-driven overexpressions of the same, but GST tagged proteins, even on galactose (Supplementary Figure S3). The sole exception was Ssk1p that scored as highly toxic with both methods. The lack of correlation may be explained by the varying absolute levels of overexpressions (Supplementary Figure S3e) in the different screens and by the influence of the GST tag on protein function.

The high prevalence of nodes of fragility within the HOG pathway may be partly explained by the very nature of signal transduction, as overexpression defects are enriched among components that transduce adaptation signals, that is kinases, phosphatases and transcription factors (Sopko *et al*, 2006). However, the high fraction of such components in the HOG pathway cannot be the sole reason for the high frequency of fitness defects, as the system is also sensitive to overexpression of more than half of the components, which do not partake in (de)phosphorylation or transcription. In fact, each such component that does not impair normal growth when overexpressed is either known or presumed to be a targeting or activating partner of catalytic components within the pathway, that is Nbp2p for Ptc1p, Ste50p for Ste11p and Opy2p for Ste50p/Ste11p (Posas *et al*, 1998; Mapes and Ota, 2004; Wu *et al*, 2006). Overexpression of their catalytic partners, Ste11p and Ptc1p, also failed to cause significant growth defects ($P>0.001$; see Supplementary information). Overall, these paired components are more robust against overexpression ($P=0.022$, Fisher's exact test).

Robustness analysis at the genetic extremes

The gToW targets' phenotypes vary much more than the corresponding deletion mutants' phenotypes. Four of the genes included here are essential (*CDC24*, *CDC42*, *SLN1* and *YPD1*), but the twenty-five viable deletion strains show at the most mild growth defects during normal conditions. Only in the presence of NaCl stress does deletion of those genes cause strong but viable phenotypes. However, these deletion phenotypes have little in common with those caused by

overexpression (Supplementary Figure S4). The strongest gToW phenotype is caused by Pbs2p, which is well known to be highly important for growth on salt as well as severely toxic when overexpressed. Hog1p is likewise important for osmotic tolerance but, unlike Pbs2p, not toxic when overexpressed. In addition, Ssk1p is severely toxic when overexpressed, but dispensable for osmotic tolerance. Although their roles in the osmotic stress response are well known, the toxicity mechanisms of Ssk1p and Pbs2p overexpression remain to be mechanistically resolved. However, both activate Hog1p constitutively and are suppressed by the deletion of *HOG1*, indicating that most, if not all, of the toxicity stems from the downstream pathway hyperactivation (Figure 1D and E).

Understanding toxicity mechanisms

In order to gain more insight into the mechanisms of toxicity of the two main nodes of fragility, Ssk1p and Pbs2p, we compared the observed *in vivo* sensitivity profiles with the *in silico* sensitivities with respect to nuclear, dually phosphorylated Hog1p predicted by the Hog model by Klipp *et al* (2005) (Figure 2A). As the *in vivo* differences in fragility between Ssk1p and Ssk2p and the fragility of the Pbs2p node cannot be captured using the original model, we studied seven variants with alternative motifs of regulation involving Ssk1p and Pbs2p (Supplementary Figure S5) and scored the relative improvements of each in the light of our data on overexpression (Figure 2C). We found that the *in silico* sensitivity

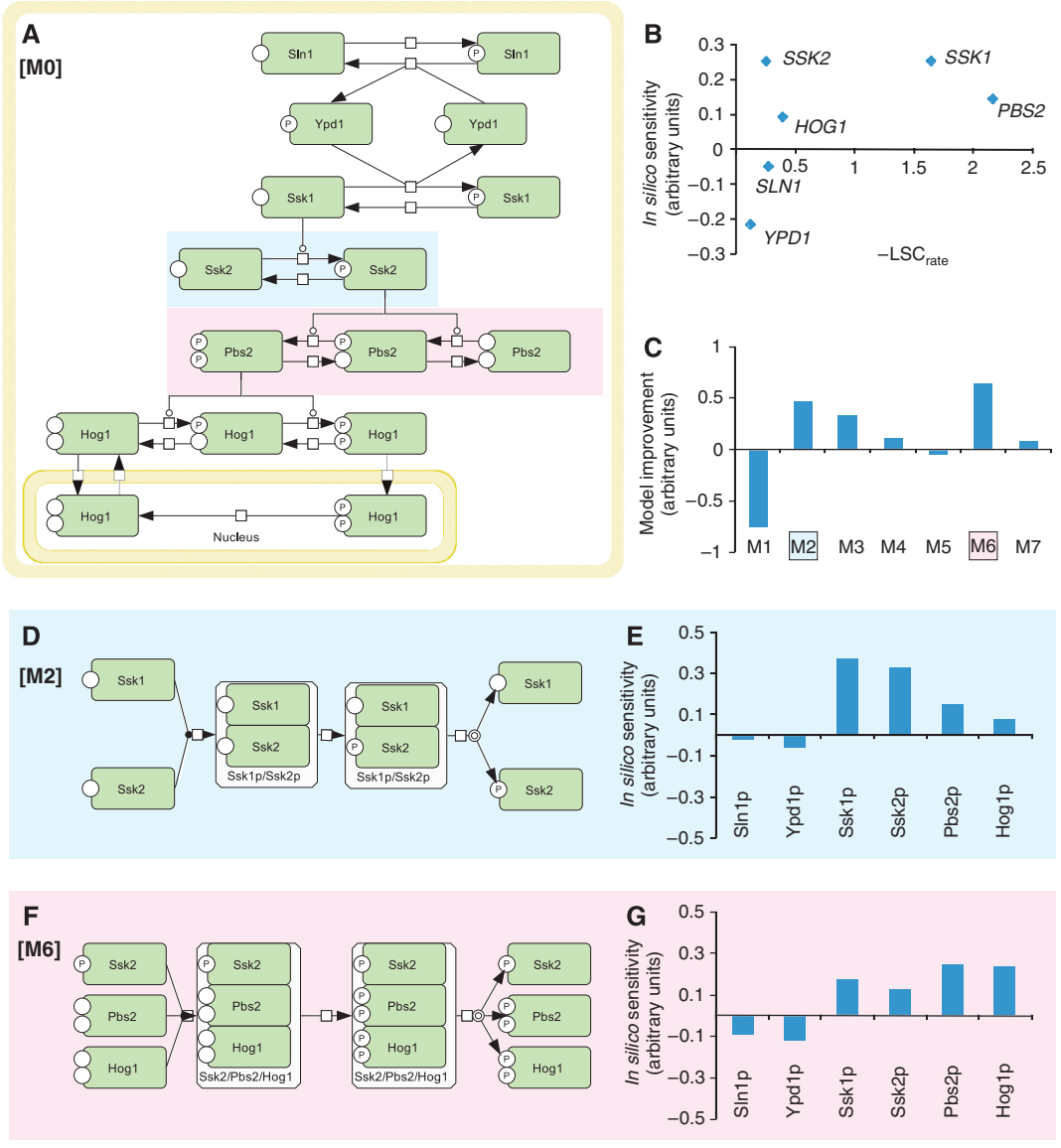

Figure 2 (**A**) The Hog part of the mathematical osmoregulatory model by Klipp *et al* (2005) (**B**) *In vivo* growth-rate defects in leucine-free medium are compared with *in silico* increases in basal levels of nuclear, dually phosphorylated Hog1p as a result of gene overexpression. The model does not capture the fragility of the Pbs2p node or distinguish the sensitivities of *SSK1* and *SSK2*. (**C**) The relative improvement in model performance by the inclusion of regulatory motifs around Ssk1p (M1–M3) and Pbs2p (M4–M7) (Supplementary Figure S5). (**D**) Requirement of dimerization before the phosphorylation of Ssk2p yielded the best improvements for Ssk1p (**E**; the corresponding sensitivity profile). (**F**) Explicit modelling of the scaffold function yields the best improvements for Pbs2p (**G**; the corresponding sensitivity profile). The LSC scores the growth difference compared with wild type, with a negative value, indicating a growth defect.

of Ssk1p is enhanced most when the dimerization of Ssk1p with Ssk2p is required for the phosphorylation and activation of Ssk2p (Figure 2D and E). In addition, explicit modelling of Pbs2p's function as a scaffold best improves its performance regarding the fragility of the Pbs2p node (Figure 2F and G). However, we observed no improvement of the *in silico* robustness through the implementation of the known dimerization of Ssk1p alone (Supplementary Figure S5; M1), suggesting that it is unlikely to contribute to the robustness pattern.

If *in vivo* toxicity stemmed from the indiscriminate interaction between protein pairs, we would expect the effect of overexpression to be roughly symmetrical for transient interactions or biased towards the component with lower expression levels in case of sustained interactions. Here, we see neither. Ssk1p has both a much stronger phenotype and higher basal expression level than Ssk2p (Supplementary Figure S6a). Ssk22p is even less abundant and the effect of Ssk1p overexpression is suppressed in *ssk2*Δ (Supplementary Figure S6d). As the phosphorylated, inactive state of Ssk1p has been reported to be stabilized by Ypd1p (Janiak-Spens *et al*, 2000), and the gToW overexpression brings Ssk1p into parity with Ypd1p levels (Supplementary Figure S6b), it may be the depletion of the stabilizing Ypd1p that leads to an accumulation of dephosphorylated and active Ssk1p (Supplementary Figure S7). Consistently, deletion of either *SLN1* or *YPD1* is lethal owing to the resulting constitutive activity of Ssk1p/Ssk2p and the HOG pathway. Pbs2p likewise stands out as being much more sensitive than its neighbours. Although this toxicity may stem from a disrupted balance with negative regulators, such as the Nbp2p–Ptc1p phosphatase complex, the high basal abundance of Pbs2p argues against the depletion of Nbp2–Ptc1 as the sole source of toxicity (Supplementary Figure S6a). However, the toxicity stems from the amplification of an existing residual signal, as it can be suppressed by deletion of the upstream kinase Ssk2p (Supplementary Figure S6d). As for Ssk1p, it leads to hyperactivation of Hog1p (Figure 1E) and this is the source of its toxicity (Figure 1D). In contrast to Ssk1p and Pbs2p, the phenotype caused by the phosphatase Ptc2p is not mediated

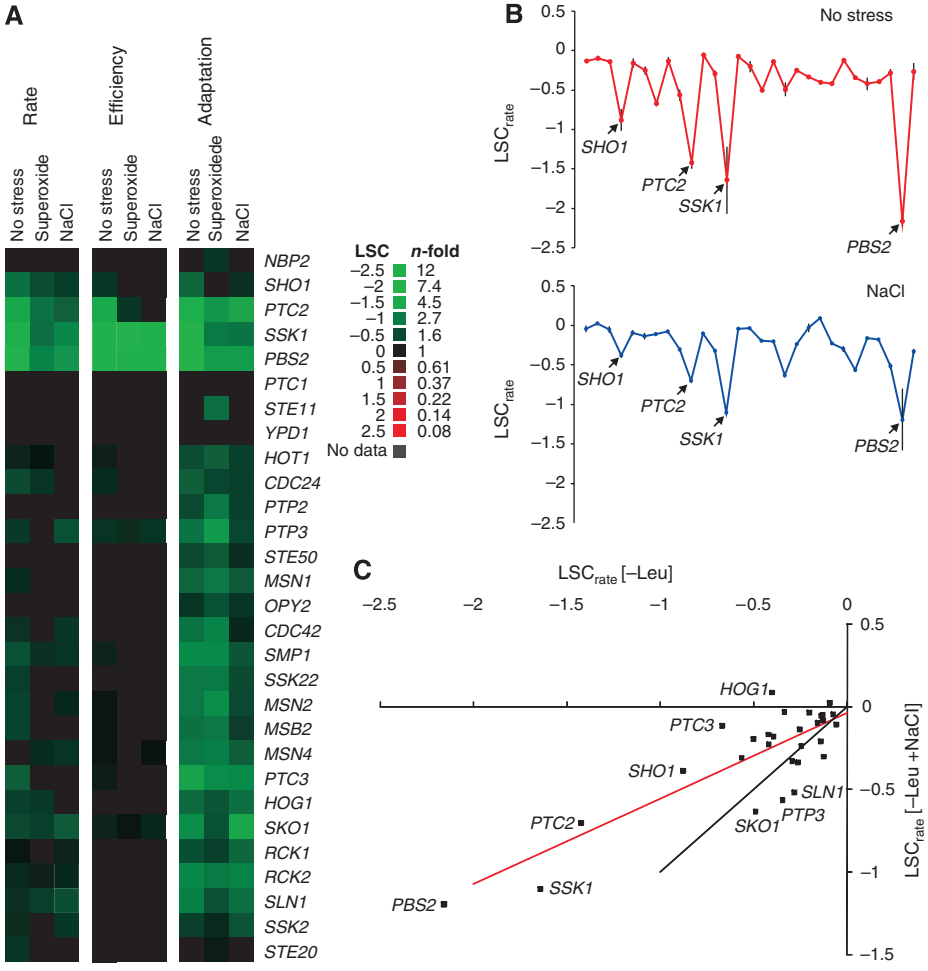

Figure 3 (**A**) Hierarchical clustering of the growth-phenotype profiles in the presence or absence of environmental perturbations. The phenotypic effect is indicated by colour. (**B**) Experimentally measured growth-rate-toxicity profiles in the presence or absence of an external pathway activator (LSC_{rate} ± s.d., n=2; gene order as in Supplementary Table SI). (**C**) There is a strong correlation between the phenotypic effect under the different conditions, although the relative gToW effect is milder under adverse growth conditions—NaCl (r^2=0.57, k=0.5 (red line; the black line indicates 1:1 correlation)).

through the activation of the HOG pathway and cannot be suppressed by the deletion of *HOG1* (Figure 1D), as would be expected because its overexpression phenotype is stronger than the deletion phenotype of Hog1p (Supplementary Figure S4b). Instead, the mechanism of its toxicity should be found outside the context of the HOG pathway.

The robustness of the HOG pathway is partly dependent on the environmental stress

To determine whether the HOG-pathway robustness is dependent on pathway activation status, we probed the HOG-pathway robustness during NaCl stress, which is known to activate the pathway, and superoxide stress (paraquat addition), which does not activate the pathway. Both stresses were applied in doses causing a similar (40–50%) reduction in reference strain growth rate. Interestingly, the relative gToW sensitivity patterns during these two stresses were very similar to that observed during unstressed conditions, indicating that the nodes of fragility remain regardless of pathway activation. The phenotypic correlations between these growth conditions ranged from 0.57 to 0.81 (Figure 3; r^2 for LSC rate). The impact of the HOG gToW perturbations were significantly stronger under normal growth conditions than under either NaCl or paraquat stress (P=0.005 and 7.4×10^{-5}, respectively, paired t-test of LSC rate). Apart from this general dampening effect, which is observed under different stress conditions, robustness is largely independent of pathway activation by environmental perturbations. However, we see an indication of interaction between the genetic and environmental perturbations. Although paraquat and NaCl stress give similar trends in the dampening of the phenotypes, the variance around this trend seems higher under NaCl (Supplementary Figure S8). An appealing interpretation would be conditional alleviation or aggravation, which would be expected if the effect of the genetic and environmental perturbation cancel out or act synergistically, respectively. We find it interesting that the targets furthest from the trend line under NaCl stress are *SLN1*, *SKO1* and *PTP3*, all known negative regulators of the osmotic stress response, on the negative side and *HOG1* and *PTC3* on the positive side. *PTC3* is equally and surprisingly alleviated by both paraquat and NaCl stress.

In summary, we used the gToW method to qualitatively capture nodes of fragility from overexpression within the HOG pathway. The quantitative correlation to the level of overexpression is more difficult to assess due to additional levels of gene, mRNA and protein regulation. However, previous results by Moriya *et al* show a correlation between growth phenotype, plasmid copy number and relative protein overexpression. Here, we report that expression changes have very strong impact on signalling. The system robustness against overexpression is heavily dependent on the target component and neighbouring nodes show very different fragility. In the HOG pathway, overexpression of Pbs2p and Ssk1p yield the strongest effects, whereas none of their neighbours; Ssk2p, Ste11p or Hog1p, are similarly sensitive. The *in silico* analysis of model variants clearly shows that model structure has a strong impact on the fragility of different nodes. Our results suggest that the stable formation of an Ssk1p–Ssk2p dimer and Pbs2p's scaffold function contribute to the fragility of their respective nodes. Although robustness information alone cannot be used to reject model structures, it provides information complementary to dynamic data that can be used to discriminate models, and should prove a valuable tool in any modelling endeavour.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

Acknowledgements

The authors would like to thank Hisao Moriya and Yuki Yoshida for their advice in creating the initial gToW set for this study, and Raul Garcia Salcedo for advice with the immuno-qPCR. MK was supported by JSPS as a postdoctoral fellow, and is currently financed by a repatriation grant from SSF. CW is supported by the IRTG 'Genomics and Systems Biology of Molecular Networks' from DFG. SH is supported by the Swedish Research Council, the 'Quantitative Biology' platform at GU and by the EC-funded 'QUASI' project. The collaboration between SH and HK is supported jointly by Vinnova and JST. AB is supported by the Swedish Research Council.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* **66:** 300–372

Janiak-Spens F, Sparling DP, West AH (2000) Novel role for an HPt domain in stabilizing the phosphorylated state of a response regulator domain. *J Bacteriol* **182:** 6673–6678

Kitano H (2004) Biological robustness. *Nature Reviews* **5:** 826–837

Klipp E, Nordlander B, Kruger R, Gennemark P, Hohmann S (2005) Integrative model of the response of yeast to osmotic shock. *Nat Biotechnol* **23:** 975–982

Mapes J, Ota IM (2004) Nbp2 targets the Ptc1-type 2C Ser/Thr phosphatase to the HOG MAPK pathway. *EMBO J* **23:** 302–311

Moriya H, Shimizu-Yoshida Y, Kitano H (2006) *In vivo* robustness analysis of cell division cycle genes in Saccharomyces cerevisiae. *PLoS Genet* **2:** e111

Posas F, Witten EA, Saito H (1998) Requirement of STE50 for osmostress-induced activation of the STE11 mitogen-activated protein kinase kinase kinase in the high-osmolarity glycerol response pathway. *Mol Cell Biol* **18:** 5788–5796

Saito H, Tatebayashi K (2004) Regulation of the osmoregulatory HOG MAPK cascade in yeast. *J Biochem* **136:** 267–272

Schneider JC, Guarente L (1991) Vectors for expression of cloned genes in yeast: regulation, overproduction, and underproduction. *Methods Enzymol* **194:** 373–388

Sopko R, Huang D, Preston N, Chua G, Papp B, Kafadar K, Snyder M, Oliver SG, Cyert M, Hughes TR, Boone C, Andrews B (2006) Mapping pathways and phenotypes by systematic gene overexpression. *Mol Cell* **21:** 319–330

Stelling J, Sauer U, Szallasi Z, Doyle III FJ, Doyle J (2004) Robustness of cellular functions. *Cell* **118:** 675–685

Tatebayashi K, Tanaka K, Yang HY, Yamamoto K, Matsushita Y, Tomida T, Imai M, Saito H (2007) Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. *EMBO J* **26:** 3521–3533

Torres EM, Sokolsky T, Tucker CM, Chan LY, Boselli M, Dunham MJ, Amon A (2007) Effects of aneuploidy on cellular physiology and cell division in haploid yeast. *Science* **317:** 916–924

Warringer J, Ericson E, Fernandez L, Nerman O, Blomberg A (2003) High-resolution yeast phenomics resolves different physiological features in the saline response. *Proc Natl Acad Sci USA* **100:** 15724–15729

Wu C, Jansen G, Zhang J, Thomas DY, Whiteway M (2006) Adaptor protein Ste50p links the Ste11p MEKK to the HOG pathway through plasma membrane association. *Genes Dev* **20:** 734–746