Different mechanisms confer gradual control and memory at nutrient and stress regulated genes in yeast

Running title: Dose sensitive gene regulation in yeast

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ABSTRACT

Cells respond to environmental stimuli by fine tuned regulation of gene expression. Here we investigate the dose dependent modulation of gene expression at high temporal resolution upon nutrient and stress signals in yeast. The $GAL1$ activity in cell populations is modulated in a well defined range of galactose concentrations, correlating with a dynamic change of histone remodeling and RNAPII association. This behavior is the result of a heterogeneous induction delay by decreasing inducer concentrations across the population. Chromatin remodeling appears to be the basis for the dynamic $GAL1$ expression because mutants with impaired histone dynamics show severely truncated dose response profiles. In contrast, the $GRE2$ promoter operates like a rapid off/on switch in response to increasing osmotic stress with almost constant expression rates and exclusively temporal regulation of histone remodeling and RNAPII occupancy. The Gal3 inducer and the Hog1 MAP kinase seem to determine the different dose response strategies at the two promoters. Accordingly, $GAL1$ becomes highly sensitive and dose independent if previously stimulated because of residual Gal3 levels, whereas $GRE2$ expression diminishes upon repeated stimulation due to acquired stress resistance. Our analysis reveals important differences in the way dynamic signals create dose sensitive gene expression outputs.
**Introduction**

Cells continuously adapt their protein composition to changing environmental conditions. The regulation of gene expression is one of the fundamental mechanisms to adjust the global protein repertoire of the cell in order to maintain cell function and integrity upon environmental challenges. Budding yeast is a powerful model to unravel the modes of transcriptional adaptation both at the level of specific genes or of the whole organism (1, 2). Additionally, the basic structure of the signaling cascades responding to environmental perturbations is conserved from yeast to man. It implies the alteration of core kinase activities, which modulate the expression of defense genes through a range of specific transcription factors. Extensive knowledge has accumulated which precisely describes the molecular machinery and its global impact on gene expression upon many types of stress (3-7). However, the vast majority of these studies are performed upon harsh environmental insults and therefore saturating stimulation. As a consequence, only very limited information or approaches are available to understand how cells adapt their gene expression programs to small or gradual changes in their environment.

It is assumed that cells have acquired mechanisms that assure a transcriptional response which is finely adjusted according to the strength of the stress or stimulation. However, the nature of the signaling molecules which confer gradual transcription outputs remains to be determined in most cases. Fine tuning of gene expression responses can occur with different purposes and the generation of a graded response can be achieved at different stages along the signal transduction path. For example, a linear response to mating pheromone has been described for the yeast mating MAP kinase cascade (8).
Additionally, specific transcriptional activators such as yeast Msn2 or Crz1 and mammalian NF-KB transmit linear signals to their cognate promoters by modulating their nuclear accumulation (9-12).

The same signal transduction pathway might have to distinguish related signals that originated from different stressors. This has been very recently described for yeast Msn2, a transcription factor responding to general stress and capable of filtering different stress inputs to generate graded gene expression outputs (13). Furthermore, among the often numerous activated genes upon a given stress the cell has to impose different sensitivities to guarantee an equilibrated adaptive response. Here, chromatin structure has been implied in modulating the threshold of gene activation in the yeast phosphate response (14) and different natural promoters and cis regulatory elements confer characteristic dose sensitive expression profiles upon osmotic and oxidative stress (15).

Here we investigate the mechanisms that confer a gradual and dose sensitive gene expression for two types of environmental cues: (i) the availability of a specific carbon and energy source and (ii) cytotoxic stress. We use two very well defined model genes, the nutrient regulated GAL1 and stress regulated GRE2.

The expression of the yeast GAL genes is specifically up-regulated by the presence of galactose in the growth medium via the transcriptional activator Gal4 (16). Gal4 is already bound at its target promoters under non-inducing conditions (-galactose), but its activation domain is inhibited by direct binding of the Gal80 repressor protein (17, 18). Upon growth in glucose limiting and galactose containing medium, GAL gene expression is induced with the help of the Gal3 inducer. Upon stimulation, Gal3 binds galactose and ATP, interrupts Gal80 inhibition of Gal4 and permits transcriptional
activation (19-21). Gal4 additionally recruits chromatin modifying complexes and mediator (22-27) in order to efficiently induce GAL gene expression.

GRE2 is a prototypical gene involved in the hyperosmotic and oxidative stress defense, which includes the stimulated gene expression encompassing hundreds of different cellular functions (5, 28). Its promoter is bound by the specific transcription factor Sko1 in a complex with the general corepressor Cyc8-Tup1 under normal non-inducing growth conditions (29). Upon hyperosmotic stress, transcriptional activation of GRE2 is rapidly achieved by the association of Sko1 with the stress activated MAP kinase Hog1, which switches Sko1 from repression to activation by multiple phosphorylation and the additional recruitment of chromatin modifiers and the mediator complex (30). As a result, GRE2 gene expression is very fast and transiently activated as commonly observed for transcriptional stress responses in yeast.

Additionally, the cell's history can modulate the transcriptional response at specific genes. Transcriptional memory has been described for several inducible yeast genes including GAL1. Here, a previous galactose induction facilitates the transcriptional response to the second galactose exposure. Different mechanisms have been proposed to establish transcriptional memory at the GAL genes including the tethering of actively transcribed GAL1 to the nuclear envelope via the histone variant Htz1, prolonged chromatin remodeling via Swi/Snf, or the inheritance of signaling compounds such as the Gal1 and Gal3 inducers (31-34).

The general architecture of the GAL1 and GRE2 regulons is very similar, which involves a switch of a promoter bound transcription factor from an inactive (or repressed) to an active state by the direct association with a specifically activated inducer. Here, we identify important differences in how both systems respond to gradual
or repeated stimulation. This was possible by the high time resolution and quantification of gene expression using destabilized luciferase reporters for the two types of genes.

This allowed us to accurately define the dynamic range of gene regulation and identify the molecules which modulate the characteristic dose-response pattern for GAL1 and GRE2 during nutrient sensing or acute osmotic stress adaptation.

**MATERIALS AND METHODS**

**Yeast strains**

*Saccharomyces cerevisiae* strains used in this study were: wild type BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and the mutant alleles *gal3::KanMX4; gcn5::KanMX4; snf2::KanMX4; gal11::KanMX4; htz1::KanMX4* (35). Yeast strains expressing chromosomally tagged TAP fusion proteins were: BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) with *GAL4-TAP-His3MX* and *GAL3-TAP-His3MX* (36).

Yeast strains expressing chromosomally tagged HA fusion proteins were: W303-1A (*MATα; leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; his3-11,15*) with *3xHA-HOG1, 3xHA-SKO1* (30) and *3xHA-RPB3* (37). Yeast strains expressing firefly luciferase were in MMY116-2C background (*MATa; leu2-3,112; trp1-1; can1-100; ura3-1; his3-11,15*) with *GAL1-FLuc* integrated in *URA3* locus (gift from A. Mazo-Vargas). DBY746 (*MATa; ura3-52; trp1-289; his3Δ1; leu2-3,112*) and its *ena1-4::LEU2* derivative were used to study the effect of the *ENA* gene dose on the GRE2 transcriptional memory.

**Plasmid constructions**

Single-copy reporter fusions with a destabilized luciferase gene (*lucCP*) were constructed as described previously (38). The upstream regulatory sequences of GRE2
(nucleotides -940 to -7), GAL1 (nucleotides -450 to -1) (38), CTTI (nucleotides -983 to -10), SOD2 (nucleotides -977 to -16) (15), ALD6 (nucleotides -785 to -2) and HOR2 (nucleotides -948 to -33) (this study). An integrative version of the GAL1-lucCP+ reporter fusion was constructed by insertion of the lucCP+ gene into the pAG306GAL1-ccdB Gateway destination vector (39), which was integrated into the URA3 locus of yeast wild type strain W303-1A. An integrative version of the GRE2-lucCP+ reporter fusion was constructed by insertion of the lucCP+ gene before the KAN MX marker in the pUG6 plasmid. The lucCP+ KAN MX containing cassette was PCR amplified and fused to the GRE2 promoter in the genome of yeast wild type strains BY4741 and W303-1A. Multi-copy integration plasmid pRS406-GAL1pr-Fluc was built by swapping MET25 with GAL1 promoter in pRS406-MET25-Fluc and integrating into strain MMY116-2C (40). Both the plasmid and integrated strains were gifts from A. Mazo-Vargas. For constitutive or induced overexpression of GAL3 under control of the TDH3 or GAL1 promoters, the entire GAL3 gene was inserted in the Gateway destination vectors pAG416GPD-ccdB and pAG416GAL1-ccdB (39). For constitutive overexpression of ENA1 under control of the PMA1 promoter, the plasmid pRS699-ENA1 was used (gift from J.M. Mulet, Valencia, Spain).

**Live cell luciferase assays**

Yeast strains containing the indicated luciferase fusion genes were grown at 28°C in synthetic dextrose or raffinose (SD or SRaff) medium lacking histidine (0.67% yeast nitrogen base, 2% glucose, 50mM succinic acid pH 5.5, 0.1g/liter leucine, 0.1g/liter methionine, 0.025g/liter uracil) to exponential growth phase. Culture aliquots were then incubated with 0.5mM luciferin (Sigma) on a roller at 28°C for 90min. The cells were then transferred in 100-μl aliquots in white 96-well plates (Nunc) with or without the indicated concentrations of NaCl, menadione or galactose supplied from appropriate
stock solutions. The light emission was then continuously recorded in a GloMax microplate luminometer (Promega) in three biological replicates. Data were processed with Microsoft Excel software. For representation of the relative light units of each reporter gene, we normalized the raw data for the number of cells in each assay. The maximal synthesis rate \( V_{\text{max}} \) and the maximal luciferase activity \( A_{\text{max}} \) were calculated as described previously (38).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was performed essentially as described previously (41). For the immunoprecipitation of HA fusion proteins, a mouse monoclonal anti-HA antibody (12CA5; Roche) was used in combination with Dynabeads protein A (Invitrogen). For the immunoprecipitation of TAP fusion proteins, Pan mouse IgG Dynabeads (Invitrogen) were used. For the immunoprecipitation of histone H3, a polyclonal anti-H3 antibody (ab1791, Abcam) was used in combination with Dynabeads protein A (Invitrogen). Quantitative PCR analyses at the indicated chromosomal loci were performed in real time using an Applied Biosystems 7500 sequence detector and Fast EvaGreen Mastermix for qPCR (Biotium). All occupancy data are presented as fold IP over the **POL1** coding sequence (+1796/+1996) internal control. Each ChIP was performed in triplicate with three different chromatin samples. All primer sequences used for ChIP are available upon request.

**Transcriptional memory experiments**

For memory experiments at **GAL1**, cells containing the **GAL1-lucCP** reporter gene were grown over night in synthetic raffinose (SRaff) medium lacking histidine to exponential growth phase. A first round of induction was then performed for 2 hours with 2% galactose, while naïve cells remained in SRaff medium. Both cell cultures were
then precipitated, washed once with water and then incubated in fresh SD medium for 1
hour. Finally cells were changed to SRaff medium containing 0.5mM luciferin for 90
min before starting the next induction with the indicated galactose concentrations and
continuous measurement of luciferase activity. For the memory experiment under
gradual Gal3 expression levels, the duration of the first round of galactose induction
was reduced to 30 min.

For memory experiments at GRE2, cells containing the GRE2-lucCP reporter gene
were grown over night in synthetic glucose (SD) medium lacking histidine to
exponential growth phase. A first salt shock was applied by adding 0.7M NaCl for 1
hour, while naive cells remained in SD medium. We confirmed that those salt stress
conditions did not cause any decrease in cell viability. Both cell cultures were then
precipitated, washed once with water and then incubated in fresh SD medium with
0.5mM luciferin for 90 min. The indicated NaCl doses were then applied to aliquots of
both cultures and the luciferase activity continuously measured.

**Single cell timelapse luminescence microscopy**

Cells with integrated GAL1-FLuc reporter gene were grown over night in synthetic
raffinose (SRaff) medium. We sonicated our yeast in a Diagenode Bioruptor UCD-200
sonicator for 30 seconds at medium intensity to obtain single-cell suspensions before
loading them onto microfluidic plates (CellAsic™). Bioluminescence imaging of yeast
cells was performed with a DV Elite microscope equipped with UltimateFocus™,
Evolve™ EMCCD camera and a 60X/1.25NA phase oil objective lens. Cells were
grown for 60 minutes at 30°C in SRaff medium at pH 3.8 with 200 μM of beetle D–
luciferin before switching to SRaff + galactose version of the same medium. We imaged
cells every 4 minutes and processed raw data using same protocols as before (40).
Briefly, cell segmentation was done in CellStat (MATLAB plug-in; (42)) and single-cell gene expression was fit to an exponential curve using the induction model described previously (40).

RESULTS

Comparison of transcriptional memory at the nutrient controlled \textit{GAL1} and stress induced \textit{GRE2} genes.

Transcriptional memory effects in yeast have been predominantly found at nutrient regulated genes. We therefore sought to compare how the dose dependent gene expression was modulated after a previous stimulation at nutrient- versus stress-regulated loci. We chose two prototypical yeast genes, which represent the two classes of regulation, \textit{GAL1} and \textit{GRE2}. To determine the dose response behavior of both genes we applied reporter fusions with destabilized luciferase, which allow monitoring gene expression in real time in the living yeast cell upon a wide range of stimulus concentrations. We first investigated how the dose sensitive induction of \textit{GAL1} was modulated by repeated galactose exposure. The expression profile from \textit{GAL1}-luciferase reporters in naïve yeast cells was compared with cells that previously experienced a galactose induction. As shown in Fig 1A, without pre-stimulus \textit{GAL1} responded with a gradual increase in reporter activity over the dynamic range of inducer concentrations. After previous stimulation, however, we observed a much faster response to galactose and an increased synthesis rate of the \textit{GAL1}-luciferase reporter. Importantly, in the second response to galactose even the lowest inducer concentration (0.01%) was able to activate \textit{GAL1} gene expression to the maximal synthesis rate. We concluded that the \textit{GAL1} dose response largely changed during the establishment of transcriptional memory and that the gene expression profile switched from a dose
sensitive graded mode to a dose insensitive but highly responsive mode. These dynamic
dynamic changes in dose responsive \textit{GAL1} stimulation are faithfully reproduced by centromeric
luciferase reporters used throughout this study when compared to chromosomally
integrated reporter genes (data not shown).

We next tested whether the dose response behavior of the stress activated \textit{GRE2} gene
changed upon repeated activation. Memory experiments were performed with the
\textit{GRE2}-luciferase real time reporter upon multiple NaCl induction. We observed in this
case (Fig 1B) that the induction of \textit{GRE2} expression was neither faster nor more
efficient nor more sensitive at low stress doses in the second round of stimulation.
Irrespective of previous gene induction, \textit{GRE2} expression always occurred at the same
time and with remarkably similar synthesis rates. The only difference we observed was
that cells, which responded to salt stress for the second time, aborted the induced \textit{GRE2}
expression a few minutes earlier than naïve cells. Thus, it seemed that \textit{GRE2} induction
is not further enhanced or sensitized by previous stress treatment, and instead reduced
the amplitude of the transcriptional burst at \textit{GRE2} in the second round of activation.

Next we wanted to gain insights into the mechanisms which sensitized \textit{GAL1} gene
expression to dose independent maximal induction rates after previous induction. It has
been previously reported that the transcriptional memory at \textit{GAL1} was dominantly
regulated by signaling molecules such as the Gal3 inducer (33). Thus, we manipulated
the Gal3 levels and tested its impact on the dose dependent induction profile of \textit{GAL1}.
As depicted in Fig 1C, constitutive overexpression of \textit{GAL3} led to a highly efficient
\textit{GAL1}-luciferase expression independently of the galactose inducer concentration.
Therefore high \textit{GAL3} levels can mimic the enhanced sensitivity of \textit{GAL1} expression
acquired during repeated galactose induction. The Gal3 inducer level is therefore a key
element in changing the dose response behavior at the \textit{GAL1} gene during memory.
GRE2 expression was modulated by repeated stimulation in an opposite manner to GAL1, as the second wave of transient gene expression is shorter than the initial one in this case. We hypothesized that the accumulation of defense proteins in the first round of stimulation could prepare the cells for the second salt shock and thereby permit an efficient adaptation with a diminished transcriptional response. We considered two physiological adaptations as most relevant for the tolerance to salt stress: the accumulation of the osmolyte glycerol and the enhanced extrusion of Na\(^+\). Both processes can be blocked by single deletions in key structural genes, such as the gpd1 or ena1-4 mutants, respectively. We repeated the memory experiments in those specific mutant strains. The gpd1 cells were indistinguishable from wild type (data not shown), but we detected important differences for the ena1-4 mutant, which lacks all copies of the Ena Na\(^+\) extrusion ATPase. As shown in Fig. 1D, in this mutant the GRE2-lucCP\(^+\) reporter responded almost equally during the first and second exposure to NaCl stress. Finally, we altered the ENA1 expression levels by the use of an additional copy of the gene under control of the constitutive PMA1 promoter. The effect of increasing ENA1 expression was a subsequent decrease of the GRE2 expression peak (Fig. 1E), thus providing additional evidence that the gradual GRE2 expression depended on the amount of the Ena1 Na\(^+\) pump. Taken together, these experiments show that important differences exist in the response of differentially regulated genes to previous exposure. GAL1 gene expression is highly sensitized to previous exposure, which leads to a dose independent activation likely driven by the Gal3 inducer. The transient GRE2 expression is not sensitized to previous exposure and seems to be modulated principally by the physiology of the cell, which dictates the amplitude of the transcriptional response at this gene.
Dose dependent expression of \textit{GAL1} in cell populations corresponds with gradual histone remodeling and RNAPII association.

The pattern of the gradual response of \textit{GAL1} to increasing galactose concentrations was recorded with real time luciferase reporters, which were expressed from centromeric plasmids or integrated into the yeast genome. The \textit{GAL1}-luciferase expression was stimulated to a detectable level with a minimal galactose concentration of approximately 0.02\% for plasmid or 0.01\% for genomic expression. Increasing stimulus concentrations provoked a continuous increase of the reporter activity until reaching a threshold concentration of 0.5\% (Fig. 2A). Greater galactose concentrations did not further increase the reporter activity, however, they slightly decreased the lag time between stimulation and response. Since \textit{GAL1} transcript levels are actively repressed in the absence of galactose, we interpreted the \textit{GAL1}-luciferase expression data as the actual mRNA synthesis rates which are dynamically modulated in a stimulus dependent manner. Galactose concentrations from 0.02\% - 0.5\% result in a gradual activation of \textit{GAL1} promoter activity. We next addressed whether this dynamic behavior was attributable to a galactose dependent regulation of RNA polymerase II (RNAPII) association at \textit{GAL1}. We performed in vivo ChIP experiments to quantify the association of the RNAPII subunit Rpb3 with the \textit{GAL1} promoter within the range of galactose concentrations, which apparently cause graded transcription outputs. As shown in Fig. 2B, RNAPII recruitment is slow and inefficient at low threshold concentrations (0.03\% galactose) and is continuously faster and more efficient until an upper threshold concentration of 0.5\% galactose. As a result, we can correlate the dynamic behavior observed with the \textit{GAL1p} driven luciferase expression system with the gradual association of the transcription machinery at the \textit{GAL1} promoter.
We then wanted to know whether the gradual increase of *GAL1* promoter activity was accompanied by graded chromatin remodeling. Therefore we determined the histone H3 density at *GAL1* by ChIP over the same range of galactose concentrations. As depicted in Fig. 2C, we found that the speed and efficiency of nucleosome remodeling at *GAL1* is gradually increased in the dynamic range of galactose concentrations. Thus, at the nutrient regulated *GAL1* gene an ample range of inducer concentrations is transduced to a graded remodeling of its nucleosomal promoter structure and the dynamic entry of the transcription machinery.

Dose dependent expression of *GRE2* relies on the temporal regulation of histone remodeling and RNAPII association at constant synthesis rates.

We next extended our analysis of graded gene regulation to the transiently activated *GRE2* gene. We first determined the complete dose response profile upon NaCl stress in vivo by the use of plasmid born or integrated *GRE2*-luciferase reporters. As shown in Figure 2D, *GRE2* responded with characteristic and transient activation profiles in a NaCl concentration range from 0.1M to 1M. Moderate salt concentrations (0.15-0.4M) induced *GRE2* always at the same time and with almost identical synthesis rates. However, in the same dynamic range of salt concentrations, a gradual increase of the stimulus (NaCl) provoked gradually increasing maximal reporter activities. This apparently was achieved by continuously prolonging the time during which *GRE2*-lucCP+ remained actively expressed at maximal synthesis rates. In summary, the *GRE2*-luciferase reporter, as opposed to the gradual regulation of synthesis rates seen in the case of *GAL1*, was dynamically regulated temporally while maintaining constant gene expression rates.
We then wanted to prove whether this particular dose response profile correlated with transcriptional events. Thus, we directly measured the association of RNAPII and histone H3 by ChIP at GRE2 at NaCl concentrations, which cause dynamic changes in the maximal expression. We found that RNAPII association with the GRE2 promoter occurred very rapidly at low salt concentrations (0.1-0.3M) (Fig. 2E). Clearly, increasing salt concentrations did not stimulate the absolute RNAPII levels but continuously increased the time during which the RNA polymerase remained associated with GRE2. These data correlated well with the rapid loss of histone H3 from the GRE2 promoter region, which was continuously prolonged, but not more efficient, in response to stimulation by increasing NaCl shock (Fig. 2F). Taken together, we find that the differential expression of GRE2 caused by increased stress doses is mainly achieved by regulating the time during which the promoter remains actively transcribed with practically constant synthesis rates, RNAPII occupancy and histone eviction.

We then wanted to prove whether this temporal pattern of dose response was general for stress responsive genes. Therefore we quantified the dose sensitive expression pattern of two more osmostress inducible natural promoters, HOR2 and ALD6. The respective fusions with destabilized luciferase were suitable to determine the entire dose response profiles for both genes upon NaCl stress (Fig. 3A). The comparison of the stimulus dependent modulation of, both the maximal expression and the synthesis rates, revealed an almost identical pattern for GRE2, ALD6 and HOR2 (Fig. 3B). At all three genes the maximal synthesis rate was reached with low stress doses (0.2M NaCl), while the maximal expression further increased until 0.4M NaCl due to prolonged activation of the respective fusion genes. Finally we tested the dose response behavior of two additional genes, SOD2 and CTT1, upon a different type of environmental cue such as oxidative stress (Fig. 3C). As depicted in Figure 3D, both genes showed a very similar
pattern of maximal gene expression and synthesis rates upon increasing stimulation with menadione as compared to the previous patterns obtained for salt stress. Thus the temporal modulation of gene activity in response to increasing stress doses might be a general feature for stress responsive genes in yeast.

**Increasing galactose stimuli gradually decrease the lag phase and cell to cell variability to engage in active GAL1 gene expression.** We investigated the dose-sensitive response of GAL1 at the level of single cells. GAL1 gene expression has been reported to occur in a bimodal fashion especially at lower galactose concentrations (43-46). Therefore we wanted to test to what degree bimodality was the source of the gradual GAL1 regulation. We performed time elapsed induction studies and recorded the traces of GAL1-luciferase expressing single cells upon stimulation with different galactose concentrations. As shown in Figure 4, a high galactose stimulus (0.5%) leads to a fast and homogeneous induction throughout the cell population, while lower galactose concentrations increase the lag phase and the heterogeneity of gene induction. However, even very low inducer concentrations (0.02%) activated GAL1 expression in most of the cells over time and the slope of GAL1 induction was largely unaffected by the inducer concentration (Figure 4 D and E). Thus, the gradual decrease of GAL1 expression in a cell population is mostly the result of a heterogeneous induction delay caused by suboptimal inducer concentrations.

**Mutants in SAGA, SWI/SNF or mediator cause severely reduced dose responses of GAL1.**

The expression of the GAL1 gene is finely tuned dependent of the galactose availability and we have shown above that this regulation involves the graded modulation of promoter activity and nucleosome eviction in a cell population. We next wanted to
know how impaired nucleosome remodeling affected the dynamic adaptation of *GAL1* promoter activity to changing inducer concentrations. Therefore we determined the induction profile of the *GAL1*-luciferase reporter gene upon a wide range of galactose concentrations in mutants with defects in various coactivator complexes. We included in this study the *gcn5* (SAGA histone acetyltransferase), *snf2* (SWI/SNF chromatin remodeling complex) and *gal11* (Mediator complex) mutants previously identified as important for full *GAL1* transcriptional activation via Gal4 (22-27), and additionally the *htz1* mutant in the histone variant H2AZ. The comparison of the dose response profiles obtained for all mutant strains (Fig. 5A) revealed that loss of SAGA or SWI/SNF function significantly reduced the dynamic range of luciferase synthesis rates driven by the *GAL1* promoter (Fig 5B). While wild type cells continuously increase the expression rate until galactose concentrations of 0.5%, *gcn5* and *snf2* mutants have truncated dose responses. Both mutants reach a maximal synthesis rate at very low inducer concentrations, which cannot be further increased. Mediator mutants *gal11* and *htz1* revealed an even stronger reduction in the dynamic gene expression at *GAL1*. To attribute the observed loss of dynamic *GAL1* promoter activity in response to gradual increment of inducer to impaired chromatin remodeling, we next compared the changes in histone H3 occupancy among the different mutant strains. As shown in Fig 5C, loss of SAGA, SWI/SNF or mediator function impaired the efficient and dose dependent histone eviction from the *GAL1* promoter. Thus, a correlation exists between the efficient nucleosome removal and the gradual adaptation of *GAL1* promoter activity, which relies on the activity of the here investigated coactivator complexes. In the absence of histone variant H2AZ, we still observed efficient histone H3 remodeling at *GAL1* (Fig 5B). Therefore the defect of *htz1* mutants for the galactose dependent
modulation of \textit{GAL1} gene expression is likely caused by other effects than impaired nucleosome eviction.

We next applied the same exhaustive analysis of dose response profiling at the stress regulated \textit{GRE2} gene. A NaCl gradient was applied to the same set of mutants and their dose dependent \textit{GRE2} expression profiles were determined (Fig 6A). As the \textit{GRE2} gene expression is dynamically regulated in response to increasing salt stress via modulation of its maximal expression level, we chose this parameter to identify alterations in the dose dependent behavior of this gene. As shown in Fig 6B, the loss of SWI/SNF activity did not affect the dose response profile of \textit{GRE2}. Mutations in SAGA or H2AZ caused a general reduction in the maximal \textit{GRE2}-luciferase activities, however, a continuous increase in reporter activity was still observed in the dynamic inducer range (0.1-0.5M NaCl). Loss of mediator function caused a very poor expression of \textit{GRE2} at any salt concentration. Importantly and different to the \textit{GAL1} gene, SWI/SNF activity is dispensable for the efficient adaptation of \textit{GRE2} activity to increased stimulation by salt. Also, in the absence of SAGA, the absolute expression levels of \textit{GRE2} decreased, however, the gradual increase in the maximal expression following the NaCl gradient was maintained. In conclusion, chromatin modifiers such as SWI/SNF or SAGA have distinct roles in the establishment of specific dose responses exemplified here for the \textit{GAL1} and \textit{GRE2} genes.

\textbf{Gradual association of Gal3 and temporally regulated recruitment of Hog1 recapitulate the different dose response behavior of \textit{GAL1} and \textit{GRE2}.}

The regulation of the dose dependent expression of \textit{GAL1} and \textit{GRE2} depends on different mechanisms. We next investigated the signaling compounds which were responsible to establish a specific dose response pattern at the two genes. We first
focused at the specific transcription factors Gal4 and Sko1, which bind directly to the 
*GAL1* or *GRE2* promoter regions and confer galactose or salt induced transcriptional 
activation. We found that Gal4 binding to *GAL1* was generally stimulated by galactose 
but independently of the concentration tested (Fig 7A). Sko1 binding to *GRE2* was 
slightly increased by low salt doses (Fig 7B), but did not correlate with the increasing 
*GRE2* promoter activity observed before in this range of salt stimuli. Therefore the 
differential binding of the direct transcriptional activators Gal4 or Sko1 was not a 
mechanism to establish the dynamic dose responses at the *GAL1* or *GRE2* genes. We 
them then determined the association of a second class of regulators, the Gal3 inducer and the 
Hog1 MAP kinase. Both signaling molecules are imported into the nucleus upon 
stimulation, associate with the promoter regions via Gal4 or Sko1 and are required to 
trigger the transcriptional switch from repression to activation. As shown in Fig 7A, 
Gal3 association with the *GAL1* promoter increases gradually with growing galactose 
concentrations. The Gal3 inducer bound slowly and less efficiently with low galactose 
concentrations and faster and more efficiently with higher galactose stimulation. These 
data correlated with the dynamic nucleosome remodeling, entry of RNAPII and the 
modulation of promoter activity in the same range of galactose concentrations. Of note, 
Gal3 association with *GAL1* upon high galactose concentrations was transient although 
extression of *GAL1* occurs for longer times. However, our observation is in agreement 
with previous findings that report transient Gal3 association with *GAL* genes only in the 
early phase of galactose induction (47).

The recruitment over time of the Hog1 MAP kinase was finally determined for 
increasing salt stress at *GRE2*. As shown in Fig 7B, Hog1 association was 
indistinguishable at early time points for the different NaCl doses tested, however, Hog1 
remained bound for longer time along with increasing stimulus. These data correlated
with the temporal regulation of gene expression, nucleosome remodeling and RNAP II association at \textit{GRE2} observed before. In conclusion, the different patterns of dose dependent gene expression activity at \textit{GAL1} and \textit{GRE2} are recapitulated by the specific association pattern of transcriptional inducer molecules such as Gal3 or Hog1.

We finally wanted to prove whether the Gal3 inducer level was the decisive factor for the sensitivity and efficiency of \textit{GAL} gene expression. We therefore placed the \textit{GAL3} gene under control of the \textit{GAL1} promoter and monitored the effect of a gradual activation of \textit{GAL3} by limiting galactose concentrations on the second round of \textit{GAL1}-luciferase expression. As shown in Figure 7C, the gradual increase of \textit{GAL3} preactivation mimicked the transition from slow and unsensitive to fast and highly sensitive \textit{GAL1} gene activation. These data indicate that the Gal3 inducer level is an important determinant of regulating the dose sensitive \textit{GAL} gene expression.

\textbf{Discussion}

Cells execute transcriptional programs upon many different environmental stimuli and threats. A single cell such as yeast has acquired a multitude of gene expression responses triggered by external stimuli, which is well documented by extensive literature over the past decades. Traditionally these environmental stress responses were investigated upon severe insults, which activate the signaling pathways to a maximal level. However, the adaptation to subtle changes in the cells environment might be of more physiological importance and generally we expect that cells are able to adapt their transcriptional responses gradually to the severity of the stress. It is largely unknown how gene regulatory systems adapt to these “suboptimal” signals, mostly because it is experimentally challenging to quantify the dynamic gene expression upon gradually changing stimulation. Here, the recent application of destabilized luciferase reporters for
continuous live cell measurements in yeast turned out to be especially useful (15, 38). This real time survey of gene expression activity reveals the dynamic range of differentially regulated groups of genes. In the case of the two genes studied in detail here in cell populations, we find a graded dose-response in concentration ranges well below the normally used stimuli for these types of genes: GAL1 expression gradually adapts from 0.01 to 0.5% of galactose, while GRE2 expression amplitude continuously increases from 100 to 400mM NaCl. These specific stimulus concentrations that provoke gradual outputs might reflect evolutionary adaptation to the naturally occurring environmental changes. It is important to note that the sensitivity of gene expression to a common signal, such as a nutrient or a stress, can be different for specific responsive genes. Here, the chromatin structure and the combination of different cis regulatory elements in promoters have been implied in creating characteristic dose sensitivities of yeast genes (14, 15). In our present study we reveal different strategies that ensure an appropriate transcriptional activation corresponding to subtle environmental changes.

Galactose induction at the GAL1 gene is slow and inefficient at threshold concentrations. This might be due to the repressive chromatin structure at the GAL1 promoter region, which has to be overcome by the activated Gal3 inducer. The initial repressed levels of Gal3 in combination with low galactose concentrations delay the transition of GAL1 to the on state. Consequently, the time point of active gene expression becomes much more variable for individual cells at low galactose doses, however, it is important to note that even at the lowest inducer concentrations finally all cells actively express GAL1 with comparable induction kinetics. Therefore the key determinants, which explain a gradual galactose response, are the signaling events that permit the first round of transcription (Figure 7D). It is likely that with few Gal3 molecules present in a cell that has not metabolized galactose over a longer time, the
rate limiting step for efficient GAL gene transcription is a threshold concentration of active Gal3 bound at the Gal4 transcriptional activator. In this model, the grade of active Gal3 counteracting the Gal80 mediated repression and additional recruitment of SAGA and Swi/Snf coactivators would increasingly favor GAL1 transcriptional initiation along with growing galactose concentrations. On the other hand, the fact that the expression of GAL3 itself is activated by galactose makes the GAL system especially modulatable. An inducible sensor such as Gal3 allows to adapt the sensitivity of the GAL gene activation to environmental needs in a way that for example yeast cells which frequently encounter galactose as an energy source would respond more readily in the following round of stimulation (33, 34). Of note, transcriptional memory in yeast has been predominantly identified at genes responsive to nutritional stimuli (31, 48). Thus, gradually regulated promoter activity over a range of metabolite concentrations with the ability to modulate the sensitivity by specific inducible signal transducers might be a general scheme of nutrient stimulated gene expression in yeast.

The adaptation of gene expression to different grades of cytotoxic stress seems to follow a different principle. Intuitively one might think that a gene product that functions in the detoxification of an acute stress has to be produced as soon as possible and, at least in the beginning of the stress defense, irrespectively of the strength of the insult. Such an “emergency” response is identified here in the case of the prototypical stress defense gene GRE2. At this gene, maximal levels of nucleosome eviction and preinitiation complex formation are observed almost immediately (experimentally at 2 min) after salt stress exposure. Importantly, and opposed to GAL1, low stress doses provoke maximal induction at GRE2. Our data suggest that activation of the HOG signaling pathway in the range of mild salt stress always triggers the same signal to its target promoters, which in all cases leads to full transcriptional activation in the first instances of
adaptation. Only at salt concentrations above 0.4M NaCl, a progressive delay in gene expression can be observed, which can be explained by general inhibition of the transcription process and a slow down of signal transduction at high osmolarity (49-51). Our results also indicate that the switch like behavior is a general feature for stress responsive promoters and not restricted to GRE2 activation by salt stress. We therefore speculate that genes of acute stress responses might generally switch to active transcription easily and independently of the stress dose and that mainly the duration of the on-state would be dictated by the strength of the stress. This regulatory mode can provide the cell the most efficient protection, as the absolute production of defense gene mRNAs continuously increases from very low stress levels to stress levels that actually start to inhibit gene expression in general (Figure 3B). This also implies that the dynamic adaptation of gene expression to stress does not result from gradual activation but from the timely shutdown of transcription, a process, whose molecular basis is greatly unknown and therefore of special interest for future studies on stress regulation.

In general, the osmotic and oxidative stress responses might be optimized to execute very rapid transcriptional activation, which cannot be further enhanced during transcriptional memory. In line with this assumption the most notable effect of memory on the GRE2 expression is a reduction in the amplitude during repeated salt stress. This reduction is produced by the accumulation of defense proteins such as the cation exporter Ena1 in the case of NaCl stress. Therefore stress-induced genes might be predominantly modulated by the cellular defense capacity, which determines the time needed to maintain maximal gene expression. It is worth noting that a positive memory effect has been reported recently for the yeast response to oxidative stress, when the cells were previously treated with a mild dose of salt (52). Future work might therefore
reveal the importance of acquired resistance versus transcriptional memory for different stress types and doses.

The nuclear expression of both GAL1 and GRE2 is modulated by signals, which originate in the cytoplasm and are then sent to the chromosomal genes via signaling proteins Gal3 or Hog1 respectively. Galactose bound Gal3 and phosphorylated Hog1 physically interact with their target genes through DNA bound transcription factors.

Here we show that the dynamics of Gal3 or Hog1 association during gradual stimulation faithfully reflects the grade of chromatin remodeling, RNAPII density and transcriptional output of the regulated genes. Therefore Gal3 and Hog1 are very likely to be responsible for the specific dose responses observed at their target genes.

Importantly, Gal3 protein levels in the uninduced state are very low which explains the need for high inducer concentrations to efficiently switch on transcription of GAL genes. On the contrary, Hog1 protein levels are constitutively high (approximately 10 fold more abundant than uninduced Gal3 (36)) independently on the stress condition thereby assuring maximal signaling rates at low stress doses. Of note, the increasingly longer, but not more efficient, association of Hog1 with its GRE2 target promoter reported here is in agreement with gradually longer phosphorylation of the MAP kinase upon increasing salt stimulation (53). An additional layer of regulation might impose the chromatin structure at stress- versus nutrient-regulated genes. In the case of GAL1, nucleosome remodeling seems to be more important to achieve efficient transcription and accordingly we find that the Swi/Snf and SAGA chromatin modifiers are crucial for the dynamic increase of GAL1 activity. In the case of GRE2, nucleosome remodeling might occur either in a much easier fashion or might be less important for activated transcription. This notion is supported by our finding that Swi/Snf is completely dispensable for the dose dependent GRE2 regulation and even in the absence of SAGA
the transcriptional output is gradually stimulated by increasing salt stress. Both coactivator complexes, however, have been shown to be recruited to the GRE2 promoter upon salt shock (30). Taken together, gradual stimulation of inducible yeast genes can be conferred by different principles, modulation of the time in the “on” state in the case of stress genes or gradual modulation of the transition to the “on” state in the case of nutrient regulated genes. The efficiency of signal transduction is a key determinant for the type of response and its reinforcement during memory provides a way to switch from one mode to another.

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References


Figure legends

FIG 1 Comparison of transcriptional memory at the GAL1 and GRE2 genes. (A) GAL1 expression is efficiently sensitized upon subsequent galactose induction. A GAL1-lucCP\(^+\) reporter gene was used in live cell luciferase assays to determine the expression rates in raffinose containing minimal medium after supplementing with the indicated concentrations of galactose. Naïve cells (- memory) were stimulated just once with galactose, while transcriptional memory (+ memory) was achieved by a previous galactose induction as explained in the methods section. (B) The stress induced GRE2 expression decreases and is not sensitized by repeated stimulation. A GRE2-lucCP\(^+\) reporter gene was used in live cell luciferase assays to determine the expression rates in glucose containing minimal medium after supplementing with NaCl. Cells were pretreated with 0.7M NaCl (+ memory) or not (- memory) before the induction with the
indicated NaCl doses. (C) Effect of Gal3 levels on the dose response of GAL1. A
GAL1-lucCP+ reporter gene was used in live cell luciferase assays to determine the
expression rates in raffinose containing minimal medium after supplementing with the
indicated concentrations of galactose. Cells were grown in synthetic glucose medium
before induction was started in synthetic raffinose medium containing the indicated
galactose concentrations. Δgal3 mutants containing plasmid encoded GAL3 under
constitutive control (GPD-GAL3) or the empty vector (Δgal3) were compared with wild
type cells containing the empty vector (GAL3). Constitutive overexpression of GAL3
leads to GAL1 induction by raffinose, therefore an additional control in glucose
containing SD medium is included in the last panel. (D) The decrease of GRE2
expression upon repeated NaCl induction depends on a functional ENA gene cluster.
The indicated strains (DBY746 background) were compared for GRE2-lucCP+
expression upon the identical conditions as in (B). (E) Transient activation of GRE2
depends on the Ena1 levels. Yeast wild type cells (BY4741) were assayed for GRE2-
lucCP+ expression in the presence of constitutive ENA1 overexpression (PMA1p-ENA1)
or the empty plasmid (wt) upon exposure to 0.4M NaCl. Cells were pretreated (+
memory) or not (- memory) with 0.7M NaCl. For all panels, the mean values of three
independent biological replicas are shown for each galactose or NaCl concentration, SD
< 15%.

FIG 2 Comparison of the gradual gene expression, RNAPII occupancy and histone
remodeling at the GAL1 and GRE2 genes. (A) The expression of a GAL1-lucCP+
reporter gene is dynamically modulated at the level of the synthesis rate. A live cell
luciferase assay was used to determine the expression rates in raffinose containing
minimal medium after supplementing with the indicated concentrations of galactose.
GAL1-luciferase fusions were expressed from centromeric plasmids (upper panel) or
after integration in the genome (lower panel). Data of the upper panel are taken from (38). The mean values of three independent biological replicas are shown for each galactose concentration, SD < 15%. (B) Gradual association of RNAPII with the \textit{GAL1} promoter modulated by the galactose inducer concentration. ChIP of Rpb3-HA expressing cells was used to determine the RNAPII density at the \textit{GAL1} promoter in synthetic raffinose medium before and after the induction with the indicated galactose concentrations. The mean values of three independent biological replicas are shown with the corresponding SD. (C) Nucleosome remodeling is gradually stimulated by increasing galactose inducer concentrations. ChIP of histone H3 was used to determine the nucleosome occupancy at the \textit{GAL1} promoter in synthetic raffinose medium before and after the induction with the indicated galactose concentrations. The mean values of three independent biological replicas are shown with the corresponding SD. (D) The dynamic dose response profile of a GRE2-lucCP\textsuperscript{+} reporter gene upon NaCl stress. A live cell luciferase assay was used to determine the expression rates in glucose containing minimal medium after supplementing with the indicated concentrations of NaCl. GRE2-luciferase fusions were expressed from centromeric plasmids (upper panel) or after integration in the genome (lower panel). The mean values of three independent biological replicas are shown for each salt concentration, SD < 15%. (E) Modulation of RNAPII recruitment at \textit{GRE2} over the dynamic range of NaCl concentrations. ChIP of Rpb3-HA expressing cells was used to determine the RNAPII density at the \textit{GRE2} promoter in synthetic glucose medium before and after the induction with the indicated NaCl concentrations. The mean values of three independent biological replicas are shown with the corresponding SD. (F) The duration of transient nucleosome remodeling at \textit{GRE2} is stimulated by increasing NaCl concentrations. ChIP of histone H3 was used to determine the nucleosome occupancy at the \textit{GRE2} promoter.
in synthetic glucose medium before and after the induction with the indicated NaCl concentrations. The mean values of three independent biological replicas are shown with the corresponding SD.

FIG 3 The dose sensitive modulation of salt and oxidative stress regulated yeast genes. (A) Live cell reporter fusions with destabilized luciferase were used to determine the dose response profiles of the HOR2 and ALD6 genes in response to NaCl stress. The mean values of three independent biological replicas are shown for each salt concentration, SD < 15%. (B) The maximal expression levels (A_max) and synthesis rates (V_max) upon NaCl stress of the ALD6, HOR2 and GRE2 genes. Error bars are SD. For each gene, the highest value for A_max or V_max was adjusted to 100. (C) Live cell reporter fusions with destabilized luciferase were used to determine the dose response profiles of the SOD2 and CTT1 genes in response to menadione stress. The mean values of three independent biological replicas are shown for each menadione concentration, SD < 15%. (D) The maximal expression levels (A_max) and synthesis rates (V_max) upon menadione stress of the SOD2 and CTT1 genes. Error bars are SD. For each gene, the highest value for A_max or V_max was adjusted to 100.

FIG 4 Graded dose-response of GAL1 expression results from heterogeneous induction delay across the population. We used timelapse luminescence microscopy to measure gene expression in single cells after induction (dashed, vertical line) in medium with (a) 0.02%, (b) 0.06%, and (c) 0.5% galactose. Average gene expression is shown as thick, black curve. We plot the distribution of the (d) delay and (e) slope of induction across different numbers of cells (n=90,136,97 respectively). Statistical analysis confirms that delay becomes longer whereas the slope is not significantly different across all galactose concentrations (student’s t-test, ***:p<0.001).
FIG 5 The efficiency of dose sensitive regulation at GAL1 depends on coactivator complexes and histone remodeling. (A) The GAL1-lucCP reporter gene was used in live cell luciferase assays in the indicated yeast strains to determine the expression rates in raffinose containing minimal medium after supplementing with the indicated concentrations of galactose. The mean values of three independent biological replicas are shown for each galactose concentration, SD < 15%. (B) Comparison of the galactose dependent modulation of GAL1 synthesis rates. Data shown represent the mean values for the maximal synthesis rate for each galactose concentration determined in three independent biological replicates for the indicated yeast strains. Error bars are SD. (C) Comparison of nucleosome remodeling at GAL1 upon increasing galactose inducer concentrations. ChIP of histone H3 was used to determine the nucleosome occupancy at the GAL1 promoter in YP raffinose medium before and after the induction with the indicated galactose concentrations. The mean values of two independent biological replicas are shown with the corresponding SD.

FIG 6 The function of coactivator complexes and histone H2AZ in the dose sensitive regulation of GRE2. (A) The GRE2-lucCP reporter gene was used in live cell luciferase assays in the indicated mutant strains to determine the expression rates in glucose containing minimal medium after supplementing with the indicated concentrations of NaCl. The mean values of three independent biological replicas are shown for each salt concentration, SD < 15%. (B) Comparison of the NaCl dependent modulation of GRE2 gene expression. Data shown represent the mean values for the maximal expression rate for each salt concentration determined in three independent biological replicates. Error bars are SD.

FIG 7 The Gal3 inducer and the Hog1 MAP kinase show different patterns of dose dependent recruitment at target promoters. (A) Gal3 association with the GAL1
promoter is gradually increased with galactose concentration. ChIP of Gal4-TAP or
Gal3-TAP expressing yeast cells was used to determine the association of both factors
with the GAL1 promoter in synthetic raffinose medium after the indicated time of
induction with different galactose concentrations. Left panel: Gal4 occupancy before
and after 20 min of galactose induction; right panel: Gal3 occupancy before and after
the indicated galactose induction. The mean values of three independent biological
replicas are shown with the corresponding SD. (B) Hog1 occupancy at the GRE2
promoter is temporally regulated upon increasing NaCl stimuli. ChIP of Sko1-HA or
Hog1-HA expressing yeast cells was used to determine the association of both factors
with the GRE2 promoter in synthetic glucose medium after induction with the indicated
concentrations of NaCl. Left panel: Sko1 binding to GRE2, right panel: Hog1
recruitment at the GRE2 promoter upon increasing salt stress. The mean values of three
independent biological replicas are shown with the corresponding SD. (C) Gradual
induction of Gal3 confers increasing sensitivity of GAL1 expression. The memory
experiment of Figure 1A was modified by a brief first induction with limiting galactose
concentrations (depicted above the graphs). A gal3 mutant was used either transformed
with the empty vector (left panel) or transformed with single copy GAL1prom-GAL3
(rest of the panels). The galactose concentrations used for the second induction are
given at the right. (D) Model of the dose dependent regulation of GAL1 expression: low
galactose concentrations provoke slow and heterogeneous promoter activation at the
level of individual cells. The time needed for engagement of most of the cells in active
transcription is long, while high galactose concentrations (or a low galactose stimulus
after previous induction) provoke a fast and homogeneous transition to the on state. This
regulation leads to a gradual reduction of GAL1 expression levels in a cell population
(depicted at the right). Green (Red) lines show single cell $GAL1$ expression upon high (low) galactose stimulation with the dotted lines indicating the population average.
Figure 3

A. 

\[\text{Relative Light Units} \times \text{time [min]}\]

B. 

\[\frac{A_{\text{max}}}{\text{NaCl [M]}}\]

C. 

\[\text{Relative Light Units} \times \text{time [min]}\]

D. 

\[\frac{A_{\text{max}}}{\text{Menadione [µM]}}\]
Figure 5

A

Relative Light Units

\[ \text{wt} \]

Relative Light Units

\[ \Delta \text{snf2} \]

Relative Light Units

\[ \Delta \text{gc} \text{n}5 \]

Relative Light Units

\[ \Delta \text{gal}11 \]

Relative Light Units

\[ \Delta \text{htz}1 \]

time [min]

B

\[ V_{\text{max}} \]

wt
gcn5
snf2
gal11
htz1

% galactose

C

H3 occupancy

\[ \text{wt} \]

H3 occupancy

\[ \Delta \text{gc} \text{n}5 \]

H3 occupancy

\[ \Delta \text{snf}2 \]

H3 occupancy

\[ \Delta \text{gal}11 \]

H3 occupancy

\[ \Delta \text{htz}1 \]

% galactose
Figure 6

A

\[ \text{Relative Light Units} \]
\[ \text{Time [min]} \]

\( \Delta \text{gcn5} \)

\( \Delta \text{snf2} \)

\( \Delta \text{htz1} \)

\( \Delta \text{gal11} \)

B

\[ A_{\max} \]
\[ \text{NaCl [M]} \]

\( \text{wt} \)

\( \text{gcn5} \)

\( \text{snf2} \)

\( \text{gal11} \)

\( \text{htz1} \)
Figure 7

A. Gal4 occupancy over time for different Gal3 concentrations (0.06%, 0.13%, 0.25%, 0.50%). The bars show the mean occupancy with error bars indicating standard deviation. The graphs show the occupancy over time for 0 min, 20 min.

B. Sko1 occupancy over time for different Hog1 concentrations (0.2 M, 0.3 M, 0.4 M). The bars show the mean occupancy with error bars indicating standard deviation. The graphs show the occupancy over time for 0 min, 5 min, 10 min.

C. GAL1-GAL3 expression levels under different conditions and concentrations of Gal3. The graphs show the relative light units over time for different time points.

D. Proposed model for Gal3 active and memory effects on GAL1 expression. The model shows the transition from galactose to active [Gal3] and the effect on GAL1 expression over time.