CHAPTER II. THE REGULATORY ROLES OF THE GALACTOSE PERMEASE AND KINASE IN THE INDUCTION RESPONSE OF THE GAL NETWORK IN *SACCHAROMYCES CEREVISIAE*

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Abstract

The GAL genetic switch of *Saccharomyces cerevisiae* exhibits an ultrasensitive response to the inducer galactose as well as the 'all-or-none' behavior characteristic of many eukaryotic regulatory networks. We have constructed a strain which allows intermediate levels of gene expression from a tunable GAL1 promoter at both the population and single-cell level by altering the regulation of the galactose permease Gal2p. Analogous modifications to other feedback loops regulating the Gal80p repressor and the Gal3p signaling protein did not result in similarly tuned responses, indicating that the level of inducer transport is unique in its ability to control the switch response of the network. In addition, removal of the Gal1p galactokinase from the network resulted in a regimed response due to the dual role of this enzyme in galactose catabolism and transport. These two activities have competing effects on the response of the network to galactose such that transport effects of Gal1p are dominant at low galactose concentrations, whereas its catabolic effects are dominant at high galactose concentrations. In addition, flow cytometry analysis revealed the unexpected phenomenon of multiple populations in the *gal1*Δ strains which were not present in the isogenic *GAL1* background. This result indicates that Gal1p may play a previously undescribed role in the stability of the GAL network response.

2.1. Introduction

Saccharomyces cerevisiae inducible promoter systems have long been used for expression of heterologous proteins, gene function studies, and other areas of molecular genetics. Native inducible promoters such as GAL1[7], MET25[8], and CUP1[9, 10], although used successfully without modification, exhibit certain properties that are undesirable for many applications. One common feature of these systems is their autocatalytic or switch-like behavior, where addition of small amounts of inducer leads to large increases in gene expression. In prokaryotes and bacteriophages, this is generally due to cooperative interactions between transcription factors and promoter elements. In more complex eukaryotic networks, other elements such as feedback loops[11], zero-order sensitivity[11], multi-step signaling mechanisms[11], and nucleocytoplasmic transport of regulatory proteins[12] often contribute to nonlinear responses. In addition, native inducible promoter systems are often characterized by an all-or-none behavior, in which genes are either maximally expressed or virtually not expressed in individual cells[13]. In such cases the observed population-averaged response upon addition of inducer is due to an increase in the probability that a given cell will become fully induced. In contrast are systems that enable a homogenous cell population response and intermediate levels of gene expression in all cells proportional to the given stimulus[13]; however, examples of this are relatively rare in eukaryotic systems.

The widely used GAL promoter system is taken from an endogenous metabolic network regulating expression of a number of structural and regulatory genes required for efficient utilization of galactose as a primary carbon source (Fig. 2.1a). This complex and tightly-controlled network has served as a paradigm for gene regulatory circuits in

eukaryotic organisms. In noninducing-nonrepressing media, the Gal4p transcriptional activator binds as a dimer to recognition sites upstream of each galactose-regulated gene referred to as upstream activation sites (UASs). An inhibitory protein Gal80p dimerizes and binds to nuclear Gal4p in the absence of galactose, preventing recruitment of activator proteins by Gal4p and effectively repressing gene expression. In the presence of inducer, Gal3p becomes activated and gains affinity for Gal80p, thereby reducing the amount of Gal80p bound to Gal4p and permitting transcription from GAL promoter elements. Gal3p is an exclusively cytoplasmic protein, whereas Gal80p continuously shuttles between the nucleus and cytoplasm and becomes sequestered in the cytoplasm when bound to activated Gal3p[14]. In the presence of glucose, the same genes are rapidly and fully repressed by multiple mechanisms; the intracellular galactose concentration is reduced via transcriptional repression and catabolite inactivation of Gal2p[15], and the Mig1p repressor inhibits both the transcription and activity of Gal4p[16, 17]. The inducer molecule galactose is transported across the cell membrane by both a facilitated diffusion mechanism and a galactose permease protein Gal2p, which has both a high-affinity and low-affinity galactose transport mechanism[18]. Galactose is utilized as a sugar source by the cell through an initial conversion step catalyzed by a galactokinase Gal1p[19]. The levels of Gal2p, Gal3p, and Gal80p are regulated by GAL promoters, thereby forming three nested feedback control loops (Fig. 2.1b)[20]. A number of other structural and regulatory proteins are under the control of GAL promoters with either one or two UASs.

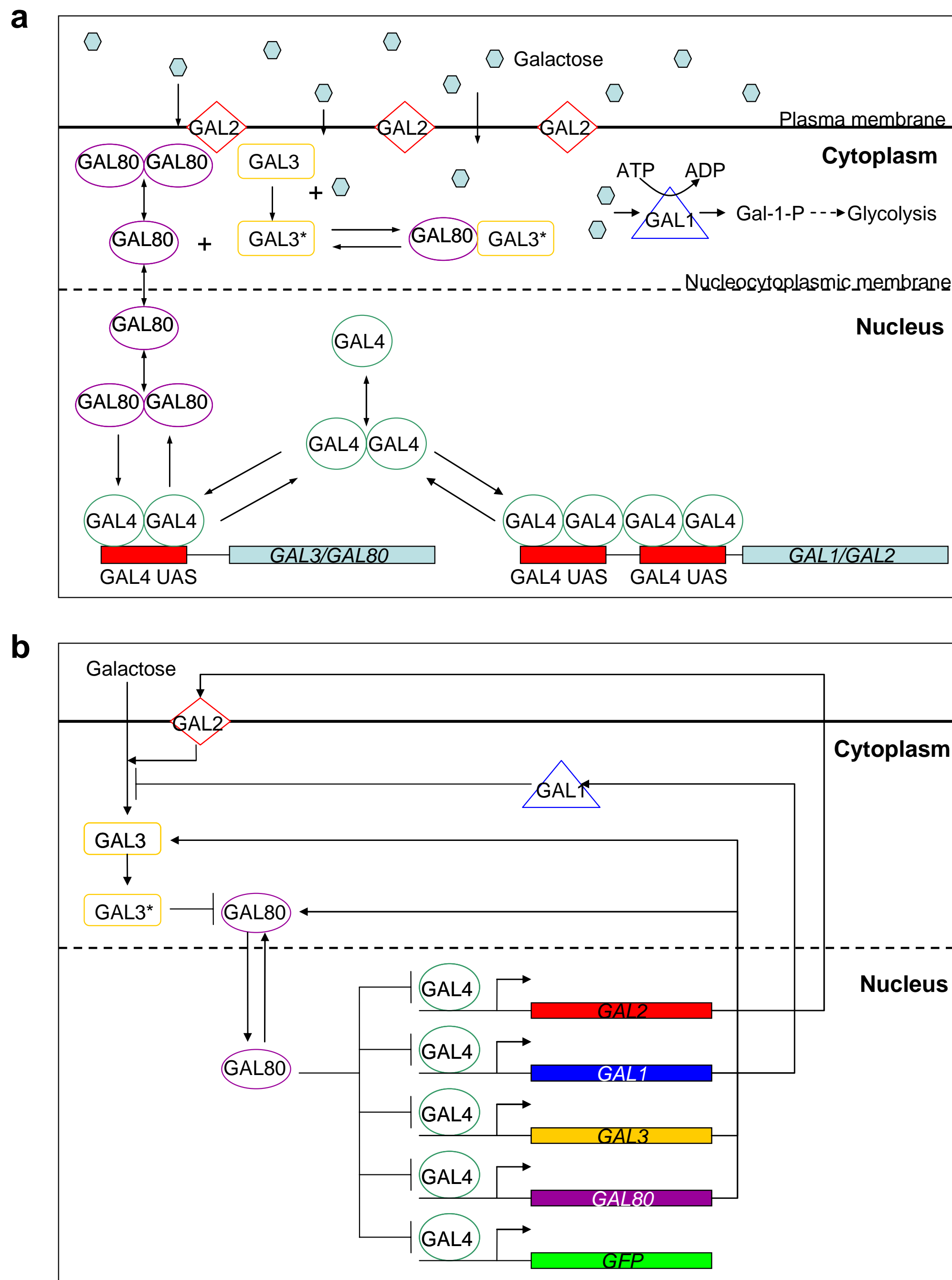

Fig. 2.1. Diagram of the native GAL network. **(a)** Schematic of the native GAL network in *S. cerevisiae*. **(b)** Schematic of the nested feedback control loops regulating the response of the GAL network in *S. cerevisiae*.

The nature of the autocatalytic response of the GAL genetic switch has been a topic of considerable research. Recent modeling work implicates the nucleocytoplasmic

shuttling of Gal80p and the feedback response of the regulatory proteins Gal3p and Gal80p as being critical to both the dynamic and steady-state performance of this system, and in particular the ultrasensitive response of the GAL induction curve[12, 21]. Modeling has also indicated that the switch is only functional if Gal80p and Gal3p are subject to the same regulation[12]. Prior work has demonstrated that the response properties of the system are highly sensitive to relative levels of Gal4p, Gal80p, and Gal3p[22]. The Gal2p galactose permease promoter region contains two UASs, whereas the promoter regions for Gal80p and Gal3p contain one UAS. Genes with multiple UASs are more tightly controlled by galactose, demonstrating lower basal expression and higher maximal induction; however, the effects of the permease feedback loop and transporter levels on the response of the network to varying galactose concentrations have not been examined.

Recent efforts have demonstrated that the response properties of inducible promoter systems can be altered by engineering interactions between components of the network. For example, several groups have altered the network connectivity of the arabinose metabolic network to exhibit a more tunable, homogenous response from arabinose-inducible promoter systems in *Escherichia coli* as opposed to its native, all-or-none response[23, 24]. This tunable promoter system was designed by altering the regulation of the arabinose transporter gene from autocatalytic control to constitutive or researcher-controlled systems that resulted in a more linear induction response[23]. In another recent study, the feedback loops of Gal3p and Gal80p were implicated in the memory response of the GAL network to growth history in *S. cerevisiae*[20]. Although numerous factors certainly play critical roles in regulating the response behavior of the galactose network as described above, it is possible that the genetic switch response of the network may be

altered by removing the positive feedback control loop regulating galactose transport mediated by the Gal2p permease.

This work demonstrates that removing the positive feedback control loop regulating Gal2p expression is sufficient to alter the autocatalytic nature of this network such that the GAL promoter responds in a more linear manner to changes in galactose levels. While complete removal of the permease enables a population-averaged linear response from the GAL promoter, constitutive expression of the permease largely maintains the linear response and increases the overall magnitude of the response at a particular galactose concentration. Identical modifications to the promoter regions of the regulatory proteins Gal3p and Gal80p did not have the same effect, indicating that the feedback loop around Gal2p is unique in its ability to affect this linear versus switch-like response. The Gal2p-modified network also alters the population distribution to a more homogenous and gradual response at the single-cell level. In addition, deletion of the galactokinase Gal1p from this network has varying effects dependent on strain background and galactose concentrations due to its dual roles in catabolism and transport. At low galactose concentrations transport effects dominate such that the network response is more linear compared to the wild-type, whereas at higher galactose concentrations catabolic effects dominate such that the network response is amplified. Finally, our studies indicate that Gal1p may play a role in network stability as its removal results in the formation of multiple steady-state populations independent of strain background.

2.2. Results

2.2.1. Galactose permease deletion results in a linear induction response

Galactose is transported into the cell through both an induced high-affinity and low-affinity transport mechanism and an uninduced facilitated diffusion mechanism. The response of the GAL network was determined when the outermost positive feedback loop controlling the autocatalytic expression of the galactose permease Gal2p was removed. Initial studies examined the response of the network in the absence of the induced transport response. A *GAL2* deletion strain was constructed by inserting a kanamycin resistance marker into the *GAL2* locus of the chromosome. This system enabled the examination of the network response under conditions where the transport of galactose is limiting. Transcriptional activation, or the level of Gal4p not bound by Gal80p, in both the *gal2*Δ and the wild-type strain was determined by measuring fluorescence levels in cells harboring yEGFP under the control of a GAL1 promoter, which harbors two UASs. The data from these studies indicate that the steady-state population-averaged induction response is linear with respect to galactose in the *gal2*Δ strain across a wide range of inducer concentrations, whereas the wild-type strain demonstrates the expected autocatalytic response curve. As illustrated in Fig. 2.2a, both strains exhibit similar induction levels of approximately 25-fold over uninduced cells at the highest concentration of three percent galactose.

2.2.2. Constitutive expression of the galactose permease results in a tunable linear response

The complete removal of the induced transport mechanism eliminates the switch-like response of the GAL network. The response of the network in the presence of the inducible high- and low-affinity transport mechanisms removed from their feedback

regulation scheme was also determined. A constitutive *GAL2* strain, *tetO$_2$:GAL2*, was constructed to allow for tunable levels of Gal2p while removing the native positive feedback control loop. A cassette was constructed to replace the GAL2 promoter with a tetracycline-repressible promoter[25]. This cassette, which also contains the tTA transactivator and a kanamycin resistance gene, was inserted into the GAL2 promoter region of the chromosome. Prior studies have indicated that in the absence of an appropriate tetracycline analog such as doxycycline, the expression levels from this promoter are approximately 10-20% of those observed from a fully induced GAL1 promoter[26, 27]. Similar steady-state assays of transcriptional activation in these strains were performed under varying concentrations of galactose and doxycycline. The former is expected to modulate the response of the GAL network in the presence of a constant level of galactose transporter, whereas the latter is expected to modulate the level of the galactose transporter. In the absence of doxycycline, permitting high Gal2p expression, the resulting induction curve is shifted upward compared to the *gal2*Δ strain but largely retains linearity (Fig. 2.2b). Addition of varying concentrations of doxycycline shifts the response curve to lower response levels, while at concentrations of 5 μg ml^{-1} Gal2p expression is fully repressed and demonstrates a response identical to that of the *gal2*Δ strain. In addition, the maximum induction level observed in the *tetO$_2$:GAL2* strain is significantly greater than that observed in the wild-type strain. It should be noted that at high Gal2p expression levels and low galactose concentrations the response of the system is slightly nonlinear.

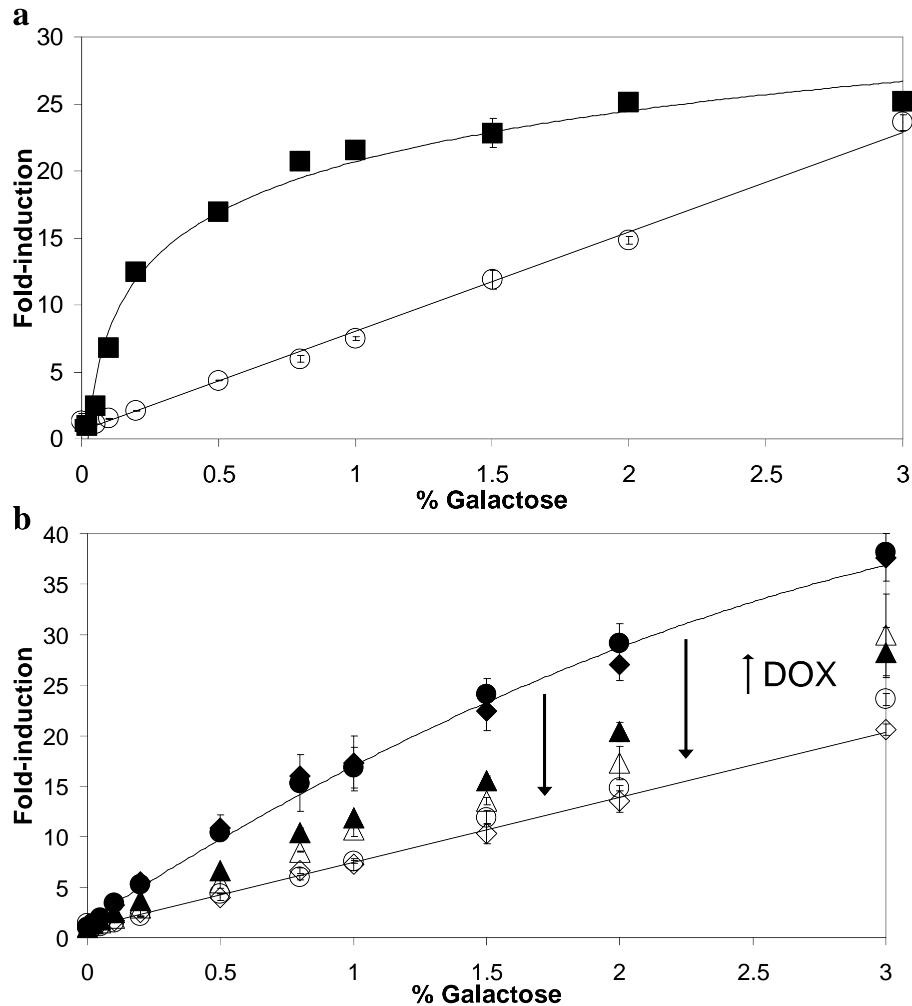

Fig. 2.2. Population-averaged response from strains with altered Gal2p regulation. **(a)** Population-averaged response of the Gal2p deletion strain (*gal2Δ*) (open circles) and the wild-type strain (filled squares). **(b)** Population-averaged response of the constitutive Gal2p strain (*tetO₂:GAL2*) at various concentrations of doxycycline (DOX). Levels of Gal2p decrease with increasing concentrations of doxycycline with full repression at concentrations over 1 mg ml^{-1}. Filled diamonds, no doxycycline; filled circles, 5 ng ml^{-1}; filled triangles, 25 ng ml^{-1}; open triangles, 50 ng ml^{-1}; open diamonds, 5 μg ml^{-1}; and open circles, *gal2Δ* strain.

2.2.3. Constitutive expression of regulatory proteins enhances the switch-like response of the network

The GAL network is regulated by three nested feedback control loops. The Gal2p loop is the exterior feedback loop and the presented data indicate that removal of this

loop is sufficient for modulating the sharp native response of this network to a linear response. The effects of the two interior nested loops regulating the expression of Gal80p and Gal3p on the steady-state population-averaged response of the GAL network were determined. Separate constitutive Gal80p and Gal3p strains were constructed by replacing the GAL80 and GAL3 promoters with previously described tetracycline-repressible promoter cassettes harboring the *his5*$^{+}$ and kanamycin selection markers, respectively. In addition, a combined constitutive Gal80p/Gal3p strain was constructed by sequential insertion of these cassettes into the wild-type strain. These systems enabled the examination of the response of the GAL network under conditions where the two internally nested control loops regulating the transcriptional repressor and activator were individually and combinatorially removed. Similar steady-state population-averaged assays of transcriptional activation in these strains were performed under varying concentrations of galactose and doxycycline.

Constitutive strains for either regulatory protein Gal3p or Gal80p did not produce the same linear response observed from the constitutive Gal2p strain. The $tetO_2$*:GAL3* strain exhibited a steeper response curve under nonrepressed conditions (Fig. 2.3a). In addition, the repressed response curve demonstrated a memory response such that when doxycycline and galactose were added at the same time point the response was similar to that under the nonrepressed conditions, whereas when cells were grown in the presence of doxycycline prior to galactose addition the overall response curve was much lower. The $tetO_2$*:GAL80* strain also exhibited a steeper response curve under nonrepressed conditions (Fig. 2.3b). However, the addition of doxycycline either prior to or at the same time as the addition of galactose did not significantly alter the induction response. In

addition, the induction response from the Gal3p/Gal80p constitutive strain was much lower than any of the other strains (Fig. 2.3c). In this strain a history-dependent response was also observed in the repressed response curve such that slightly higher induction levels were observed when doxycycline and galactose were added at the same time point versus when the cells were grown in doxycycline prior to induction.

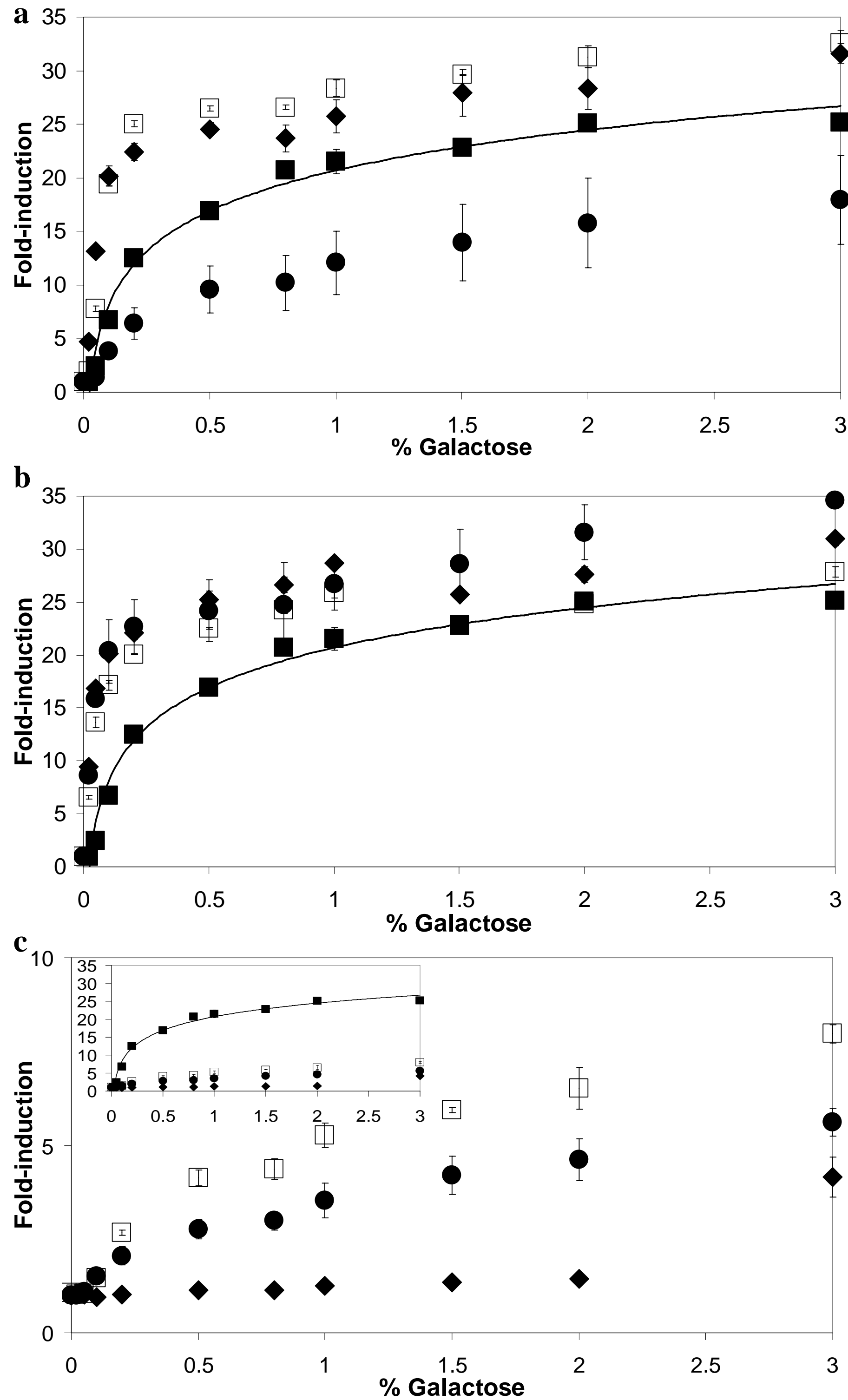

Fig. 2.3. Population-averaged response from strains with altered Gal3p and Gal80p regulation. (**a**) Population-averaged response of the wild-type strain (filled squares) and constitutive Gal3p strains (*tetO₂:GAL3*) at nonrepressed conditions (filled diamonds, 0 μg ml^{-1} doxycycline), fully repressed conditions (open squares, 5 μg ml^{-1} doxycycline), and

fully repressed conditions grown overnight in doxycycline (filled circles, 5 μg ml^{-1} doxycycline). **(b)** Population-averaged response of the wild-type strain (filled squares) and constitutive Gal80p strain (*tetO$_2$:GAL80*) at nonrepressed conditions (filled diamonds), repressed conditions (open squares), and repressed conditions grown overnight in doxycycline (filled circles). **(c)** Population-averaged response of the constitutive Gal3p, Gal80p strain (*tetO$_2$:GAL3 tetO$_2$:GAL80*) at nonrepressed conditions (filled diamonds), repressed conditions (open squares), and repressed conditions grown overnight in doxycyline (filled circles). The inset illustrates induction levels relative to the wild-type strain (filled squares).

2.2.4. Galactokinase deletion results in a regimed network response

The data support that the nested positive and negative feedback loops in the GAL network influence the observed steady-state induction response to varying levels of galactose. The galactokinase Gal1p is also anticipated to play a key regulatory role in the response of the network as a result of its two distinct activities. The immediate role of this enzyme is in converting galactose into an energy source for the cell. Therefore, it is anticipated that removal of this activity will increase the overall response of the network at a given galactose concentration as the intracellular levels of galactose available for activating Gal3p will be effectively higher. Prior work has demonstrated higher fully induced response levels in a Gal1p knockout strain[28]. However, the galactokinase also plays a key role in the high-affinity transport mechanisms associated with Gal2p[18]. To examine the role of the galactokinase on the response of the GAL network, Gal1p deletion strains were constructed in the three different Gal2p regulatory strains: wild-type, *gal2*Δ, and *tetO$_2$:GAL2*. These strains were constructed by inserting a *His3MX6* selection marker into the *GAL1* locus of the chromosome. These systems enable examination of the effects of the galactokinase in the response of the system under conditions where the normal Gal2p feedback control is present, Gal2p is present but the feedback control loop is removed, and in the absence of Gal2p. Similar steady-state

population-averaged assays of transcriptional activation in these strains were performed under varying concentrations of galactose.

The effects of deleting Gal1p were dependent on strain background and galactose concentration. For instance, in both the *gal1*Δ and the *gal1*Δ *tetO*$_2$*:GAL2* strains the induction response exhibited a more linear response in comparison to their respective *GAL1* isogenic strains (Fig. 2.4a,b). The response can be broken up into two different regimes: the low galactose regime, where the Gal1p deletion strains exhibit a lower induction response relative to their isogenic strains, and the high galactose regime, where the Gal1p deletion strains exhibit a higher induction response that increases linearly with galactose concentration relative to their isogenic strains. In the absence of Gal2p the deletion of Gal1p results in a different induction pattern (Fig. 2.4c). The response of the *gal1*Δ *gal2*Δ strain exhibits only one regime across all galactose concentrations, where the response curve maintains its linear response and is shifted upward from its isogenic strain across all galactose concentrations.

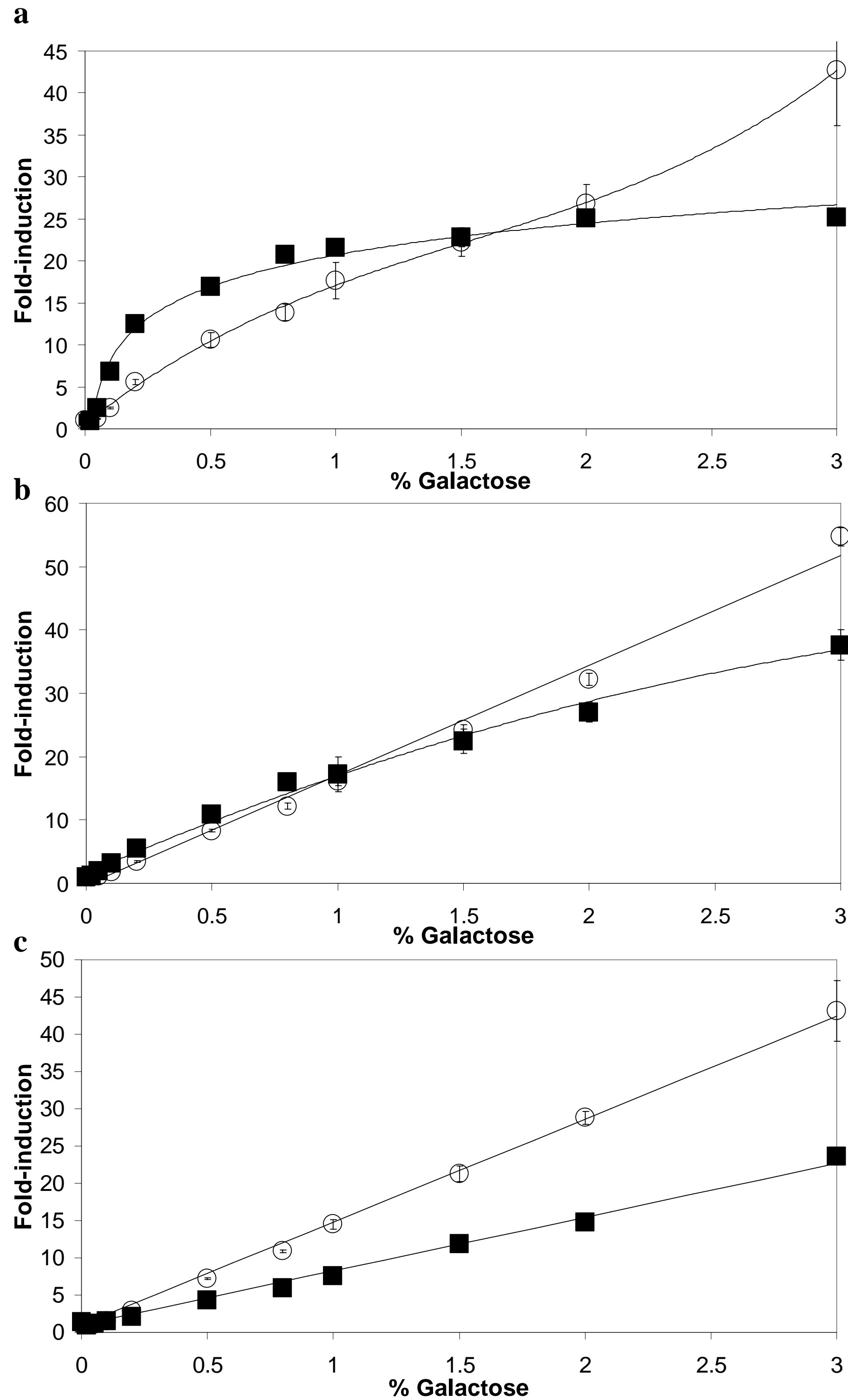

Fig. 2.4. Population-averaged response from strains with no Gal1p activity. **(a)** Population-averaged response of the Gal1p deletion strain (*gal1Δ*) (open circles) and the wild-type strain (filled squares). **(b)** Population-averaged response of the Gal1p deletion, constitutive Gal2p strain (*gal1Δ* $tetO_2$*:GAL2*) (open circles), and the corresponding

isogenic strain (*tetO₂:GAL2*) (filled squares). **(c)** Population-averaged response of the Gal1p, Gal2p deletion strain (*gal1Δ gal2Δ*) (open circles) and the corresponding isogenic strain (*gal2Δ*) (filled squares).

2.2.5. Population distributions in GAL2-modified strains exhibit graded responses

Alteration of the regulatory schemes at various control points modifies the steady-state population-averaged response of the GAL network. The effects of these targeted alterations on the population response of the network were determined. Flow cytometry was used to analyze the response of the cell population to alterations in Gal2p regulation. Wild-type, *gal2*Δ, and *tetO$_2$:GAL2* cells were cultured under the same conditions as the population-averaged studies prior to preparation for analysis. The wild-type strain exhibited two distinct populations of fully induced and uninduced cells (Fig. 2.5a). In accordance with the all-or-none effects observed in other inducible promoter systems[13], the percentage of fully induced cells increases with increasing galactose concentrations. While both *GAL2*-modified strains, *gal2*Δ and *tetO$_2$:GAL2*, exhibited a significant uninduced or negative population, they did not exhibit the all-or-none effect observed with the wild-type strain. Specifically, the average level of GFP expression from the induced population and the number of cells that were induced increased with galactose concentration (Fig. 2.5b,c). This graded response was most dramatic in the *gal2*Δ strain. The *tetO$_2$:GAL2* strain also demonstrated a slightly graded response to galactose with a higher mean fluorescence at all concentrations compared to the *gal2*Δ strain consistent with the population-averaged data (Fig. 2.5d). The three strains exhibited similar population distributions in the fully induced state, or at high galactose concentrations, with a majority of the population expressing the maximum level of GFP. Slight

differences in the negative populations between the *tetO₂:GAL2* strain and the wild-type account for the differences in maximum induction at the population-averaged level.

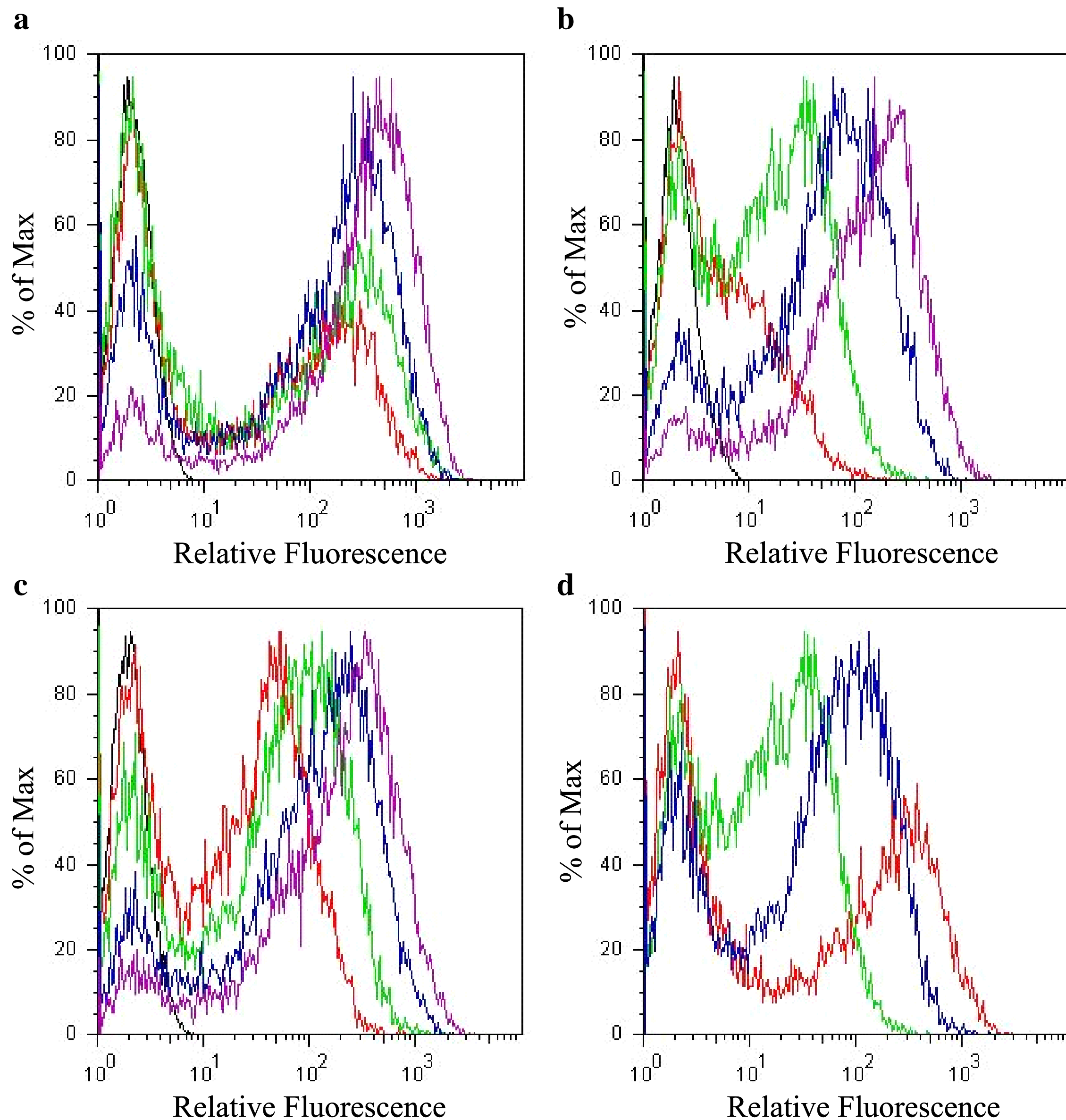

Fig. 2.5. Population response from strains with altered Gal2p regulation. For (a), (b), and (c), galactose concentration is indicated as: black (0%), red (0.2%), green (0.5%), blue (1%) and purple (3%). **(a)** Population distribution of cells with the native Gal2p positive feedback control loop (wild type) across various concentrations of galactose. **(b)** Population distribution of cells with constitutive Gal2p levels (*tetO*$_2$*:GAL2*) under nonrepressed conditions (0 μg ml^{-1} doxycycline) across various concentrations of galactose. **(c)** Population distribution of cells with no Gal2p (*gal2Δ*) across various concentrations of galactose. (d) Population distributions in 0.5% galactose of wild-type cells exhibiting feedback Gal2p control (red), *tetO*$_2$*:GAL2* cells exhibiting constitutive

Gal2p expression (blue), and *gal2Δ* cells in which Gal2p is absent from the network (green).

2.2.6. Deletion of the galactokinase results in multiple stable populations

Studies support the regimed effects of the galactokinase Gal1p on the steady-state population-averaged response of the GAL network as a result of its role in the high-affinity Gal2p transport mechanism. The effects of the removal of Gal1p in a variety of Gal2p regulatory backgrounds on the population response of the network were determined. Flow cytometry analysis was conducted to determine the population response in the absence of Gal1p. *Gal1*Δ, *gal1*Δ *gal2*Δ, and *gal1*Δ *tetO*$_2$*:GAL2* cells were cultured under the same conditions as the population-averaged studies prior to preparation for analysis. The population data matches the general trends observed in the population-averaged data across different concentrations of galactose (Fig. 2.6). Interestingly, all of the Gal1p deletion strains, regardless of background, exhibited multiple, distinct cell populations across all ranges of galactose concentration measured between 0.05 and 3%. In contrast to the all-or-none response of the wild-type strain, these populations allow intermediate levels of gene expression in all *gal1*Δ strains.

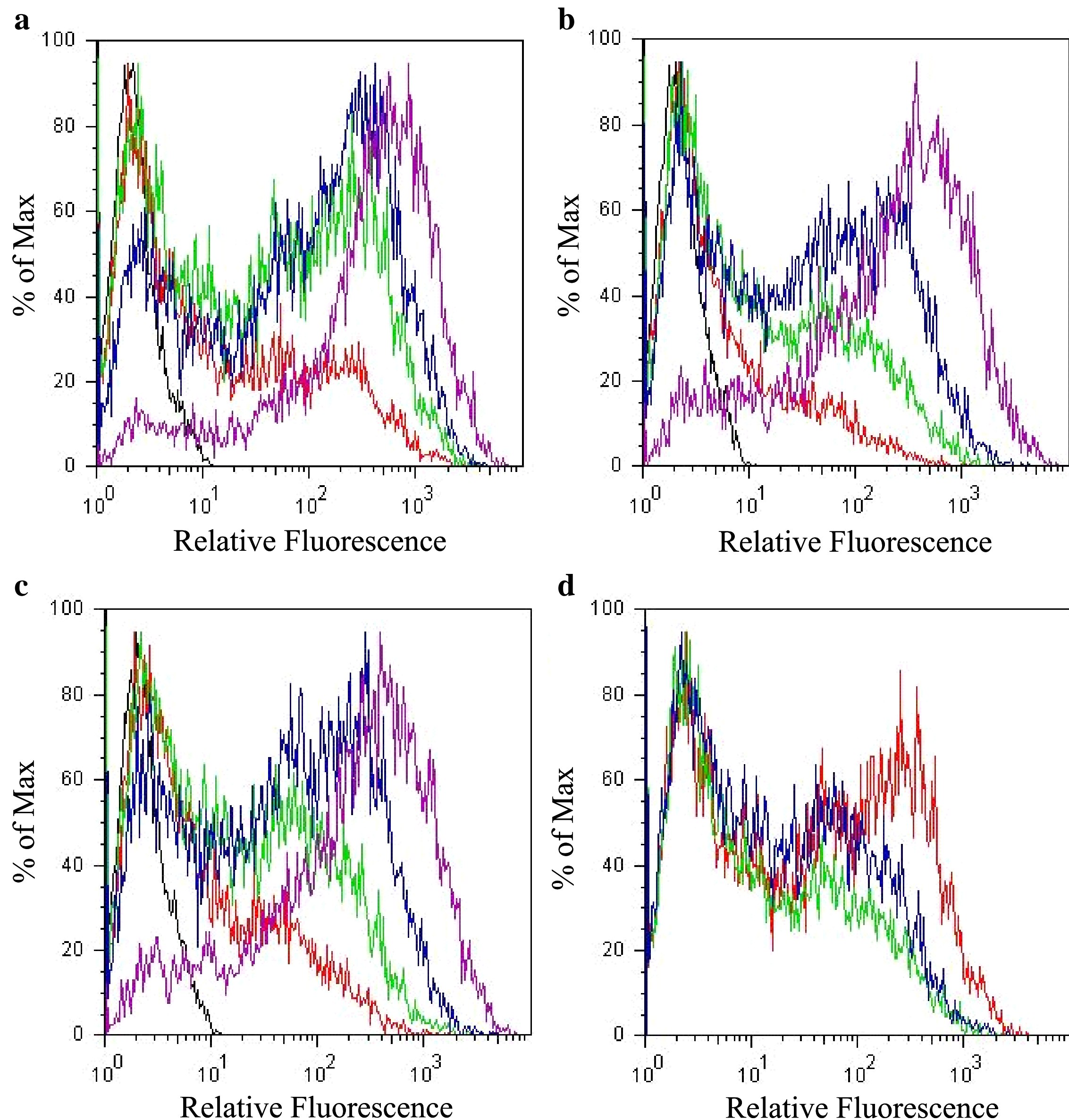

Fig. 2.6. Population response from strains with no Gal1p activity. For (a), (b), and (c), galactose concentration is indicated as: black (0%), red (0.2%), green (0.5%), blue (1%) and purple (3%). **(a)** Population distribution of cells with the native Gal2p positive feedback control loop and no Gal1p (*gal1Δ*) across various concentrations of galactose. **(b)** Population distribution of cells with constitutive Gal2p expression and no Gal1p (*gal1Δ tetO$_2$:GAL2*) under nonrepressed conditions (0 μg ml^{-1} doxycycline) across various concentrations of galactose. **(c)** Population distribution of cells with no Gal2p and no Gal1p (*gal1Δ gal2Δ*) across various concentrations of galactose. **(d)** Population distributions in 0.5% galactose of *gal1Δ* cells exhibiting feedback Gal2p control and no Gal1p (red), *gal1Δ tetO$_2$:GAL2* cells exhibiting constitutive Gal2p expression and no Gal1p (blue), and *gal1Δ gal2Δ* cells exhibiting neither Gal2p nor Gal1p (green).

2.3. Discussion

The data from the population-averaged transcriptional activation assays demonstrate that the positive feedback loop regulating the expression of Gal2p is necessary for the sharp, autocatalytic response of the system to galactose observed in the wild-type strain. In the *gal2*Δ strain, galactose and its nonphosphorylatable analogs are transported solely by an uninduced facilitated diffusion mechanism[18]. We propose that under these conditions transport effects limit the intracellular galactose concentration and the ensuing network response. Specifically, there will be fewer molecules of galactose to activate Gal3p such that more Gal80p remains bound to Gal4p and therefore a decrease in the transcriptional activation response is observed. This is in contrast to the wild-type environment, where the amount of galactose in the cells increases sharply over a narrow concentration range once galactose gets into the cells as a result of the positive feedback loop regulating Gal2p.

Furthermore, we demonstrate that modulating the levels of the galactose transporter in the absence of its positive feedback control loop is an effective way of tuning the linear response of the system. In the $tetO_2$*:GAL2* strain, galactose is transported by a constitutive high-affinity and low-affinity transport mechanism in addition to the facilitated diffusion mechanism. While removal of the positive feedback loop eliminates much of the switch-like response of the system, under conditions of high transporter levels (low doxycycline levels) and low galactose levels the system does exhibit a slightly nonlinear response. This data indicate that at low galactose concentrations the high-affinity transport mechanism is dominant and inducer transport is not a limiting

factor in GAL promoter activation. In addition, higher maximum induction levels are observed in the *tetO*$_2$*:GAL2* strain, most likely due to the removal of the negative feedback loop on the regulation of Gal2p from increased levels of Gal80p. Furthermore, under conditions of full tetracycline repression of Gal2p, the induction curve mimics that of the *gal2*Δ strain and supports that the observed shifts in the system response with doxycycline are due solely to a corresponding change in Gal2p levels.

The data from the flow cytometry assays demonstrate that alterations in Gal2p regulation also changed the population response of the GAL network. Specifically, the positive feedback control loop regulating the expression of the galactose permease is a necessary component of the observed all-or-none response of this network. This has been demonstrated in simpler bacterial networks such as the arabinose and lactose operons[23]. The results demonstrate a significant negative population irrespective of Gal2p regulation except at high galactose concentrations. The persistence of the negative population is likely due, in part, to the recently described cellular memory of this network[20], as cells were grown on noninducing-nonrepressing media prior to induction. Previous work has demonstrated that growing initial cell cultures in the presence of galactose will reduce, but not eliminate, this negative population[20].

Similar studies with the regulatory proteins indicate that the feedback loops regulating Gal3p and Gal80p levels are not necessary for the autocatalytic induction response. The tetO$_2$ promoter is weaker than the native GAL promoters, yet fully induced response levels comparable to the native promoter systems are still attained when Gal3p or Gal80p are individually controlled in this manner. Reducing the levels of the repressor protein Gal80p has the anticipated effect of a higher response and a lower galactose

requirement for full induction. However, the response of the network was similar under both repressed and nonrepressed conditions in this strain (*tetO*$_2$*:GAL80*), indicating that the relative levels over which the tetO$_2$ promoter can regulate Gal80p expression is not sufficient for tuning the network response. Reducing the levels of the activator protein Gal3p had the unanticipated effect of also increasing the response of the network and lowering the galactose level at which full induction is observed. The sharper response curve observed under constitutive Gal3p regulation versus feedback regulation may be explained by the higher concentrations of the activator protein potentially present at lower concentrations of galactose in the constitutive strain background. In addition, unlike the *tetO*$_2$*:GAL80* strain the response of the system in the *tetO*$_2$*:GAL3* strain was highly dependent on the concentration of Gal3p at the time of induction and indicated that the relative level over which the tetO$_2$ promoter can regulate Gal3p expression is sufficient for tuning the network response. This difference in observed tunability may be explained by differences in the relative levels of these two regulator proteins, as Gal3p has been estimated to be at a 5-fold higher concentration than Gal80p at full induction conditions[14]. Furthermore, unlike the constitutive *GAL2* strain, the behavior of the complete knockouts is not replicated under conditions of full repression indicating that low levels of Gal3p and Gal80p are sufficient to maintain switch functionality. In a Gal3p knockout strain the network is not inducible with the exception of long-term adaptation occurring after several days[29]. In a Gal80p knockout strain the Gal4p activation domain is not repressed and the population remains fully induced independent of galactose[30]. The data indicate that basal expression from the tetO$_2$ promoter produces sufficient Gal3p to

activate the switch even at low inducer concentrations and enough Gal80p to fully repress Gal4p in the absence of inducer.

The response of the network under constant and equal levels of both regulatory proteins was unexpected. Previous modeling work had predicted that the switch response of the network would be unaffected if Gal80p and Gal3p were not autoregulated[22]. The results from these studies indicate that under equal levels of the activator and repressor proteins expressed from the $tetO_2$ promoter under nonrepressing conditions the galactose network is not inducible. However, when levels of these regulatory proteins are both lowered under repressed conditions the network exhibits low levels of induction that depend on the concentrations of Gal3p and Gal80p at the time of induction. The low induction levels observed in this strain may indicate the sensitivity of this network to the ratio of Gal3p/Gal80p levels, and in particular lowering this ratio to one. Finally, the observed memory response in this strain supports the sensitivity of the system to starting levels of Gal3p, attaining higher induction levels when Gal3p is present at the time of galactose addition.

These studies indicate that the feedback loops controlling the levels of these two regulatory proteins may not be essential to the native switch-like response of the GAL network. In the case of Gal3p, it has been suggested that the feedback loop is a remnant of the evolution of this regulatory protein. This signal transduction molecule is the result of paralogous evolution from Gal1p[31] and effectively separates galactose sensing and metabolism as it has lost its galactokinase activity[32]. However, it is currently not clear why Gal80p evolved the same type of autoregulation mechanism if it is not necessary for

maintaining the response of the network, other than to prevent overexpression at high galactose concentrations.

The complex response properties observed in the *gal1*Δ strains are proposed to be a result of the competing roles of Gal1p in catabolism and transport in the GAL network. Removal of the Gal1p catabolic activity increases the effective concentration of galactose in the cell, which would be expected to increase the response of the network at all galactose concentrations. However, removal of the Gal1p transport activity, which effectively removes the high-affinity Gal2p transport mechanism, would be expected to decrease the response of the network particularly at low galactose concentrations, where this transport mechanism is essential to efficient galactose transport. This dual role model is supported by the data from the population-averaged transcriptional activation assays. In the absence of Gal2p the transport role of Gal1p is no longer a factor in the pathway and therefore the shifted response is solely due to the absence of galactose catabolism (*gal1*Δ *gal2*Δ versus *gal2*Δ). However, when Gal2p is present either at constitutive levels or under feedback regulated control both the transport and catabolic roles of Gal1p influence the response of the system. At low inducer concentrations the transport effects of Gal1p are dominant in the response of the system (observed as lower induction levels from the *gal1*Δ strains), whereas at high inducer concentrations the catabolic effects of Gal1p are dominant in the response of the system (observed as higher induction levels).

The data from the flow cytometry assays indicate that Gal1p also plays a role in effecting the population response of the GAL network. The data demonstrate the occurrence of multiple cell populations in all galactokinase deletion strains regardless of the regulation of the galactose permease or even its presence. This supports that the

occurrence of the multiple populations is due to the loss of the galactokinase function of Gal1p and not due to the loss of the high-affinity transport mechanism mediated by Gal2p. To our knowledge, the removal of a network kinase has not been demonstrated to result in the occurrence of multiple, steady-state cell populations in other networks. Multiple cell populations are often associated with different steady-state or stability regimes. It is possible that Gal1p, either through its galactokinase activity or some as yet unidentified activity, plays a role in stabilizing the population response. A recent structural study comparing Gal1p and Gal3p suggests that the galactokinase can function as a transcriptional activator[33], and the loss of this activity may potentially contribute to the emergence of multiple steady-states in the absence of Gal1p.

In summary, this work demonstrates that the removal of key regulatory loops alters the steady-state and population response of the galactose metabolic network in *S. cerevisiae*. The feedback loop regulating the Gal2p permease is critical to the observed autocatalytic induction response and all-or-none response of the system. The permease also presents a suitable control point at which titrating levels of this protein with available tools enables tuning of the network response with the *GAL2* deletion strain exhibiting a linear response between 0 and 3 % galactose. The feedback loops regulating the activator and repressor proteins are not necessary for the autocatalytic induction response and are not suitable control points for tuning the response of the system with the promoter system used in this work. In addition to further elucidating the roles of the various regulatory loops in the response of this network, this work also presents a number of engineered networks that will be useful as tunable, homogenous promoter systems in *S. cerevisiae*.

2.4. Materials and Methods

2.4.1. Yeast strain construction

The wild-type haploid yeast strain used in this study is W303α (MATα *his3-11,15 trp1-1 leu2-3 ura3-1 ade2-1*). All other strains were constructed by making modifications to the chromosome of this wild-type strain through standard homologous recombination procedures[34]. For each strain an insertion cassette was constructed with the appropriate insertion sequences and regions of homology to the desired targeted sites on the chromosome. A cassette harboring an *E. coli* kanamycin resistance gene and associated promoter and terminator elements with ends homologous to regions flanking *GAL2* on the chromosome was constructed by amplifying the appropriate segment from pFA6a-ZZ-TEV-S-kanMX6[35]. A second cassette harboring the $tetO_2$ response element and minimal CYC1 promoter, the tTA transactivator and associated promoter and terminator elements, and the kanamycin resistance gene and associated promoter and terminator elements with ends homologous to regions flanking the GAL2 promoter was constructed in two steps (Fig. 2.7a). In the first step the kanamycin resistance cassette was amplified from pFA6a-ZZ-TEV-S-kanMX6 and the tetracycline-regulatable promoter cassette was amplified from pCM188[27] separately. In a second round PCR step, these two cassettes were combined to form one cassette by overlap extension techniques[34]. A third cassette harboring a *Schizosaccharomyces pombe* histidine biosynthetic gene (*his5*$^+$) and associated promoter and terminator elements with ends homologous to regions flanking *GAL1* on the chromosome was constructed by amplifying the appropriate segment from pFA6-S-TEV-ZZ-HIS3MX6[35]. Analogous cassettes with regions flanking the GAL3 and GAL80 promoters were also constructed (Fig. 2.7b).

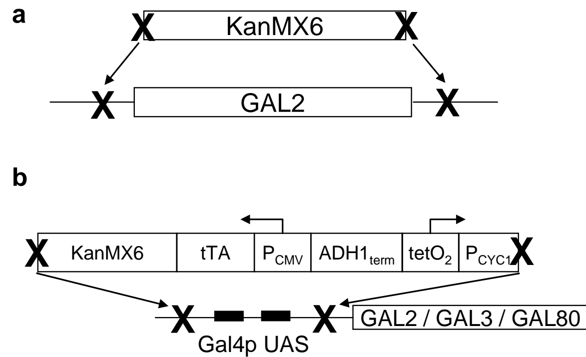

Fig. 2.7. Cassettes for chromosomal replacements. **(a)** Schematic of the *GAL2* knockout constructed by replacement of the entire coding region with the KanMX6 cassette. **(b)** General schematic of the constructs used to replace the native promoter of *GAL2*, *GAL3*, and *GAL80* with tetracycline-repressible promoters.

The individual fragments for the GAL2, GAL3, and GAL80 promoter substitution cassettes were amplified using the TripleMaster PCR System (Eppendorf). All other cassettes were constructed with standard PCR procedures in a Dyad PCR machine (MJ Research) with Taq DNA polymerase (Roche). Oligonucleotide primers were purchased from Integrated DNA Technologies, and primer sequences are available upon request. Cassettes were transformed into the appropriate strains using a standard lithium acetate procedure[36]. The *GAL2* knockout and *GAL2*, *GAL3*, and *GAL80* tetracycline-regulatable expression cassettes were inserted into the wild-type strain. The *GAL1* knockout cassette was inserted into the wild-type strain, the *GAL2* knockout strain, and the *GAL2* tetracycline-regulatable strain. Strains with inserted cassettes were selected by growth on synthetic complete media with the appropriate antibiotic selection and dropout media. Confirmation of cassette insertion into the correct chromosomal location was conducted by PCR amplification of the targeted region of the chromosome. A list of yeast strains and primer sequences used in this work are provided in Table 2.1 and Table 2.2.

Table 2.1. List of yeast strains.

Strain Number	Genotype	Plasmid
CSY3	MATα *his3-11,15 trp1-1 leu2-3 ura3-1 ade2-1*	
CSY22	*gal2Δ*	
CSY13	Gal2p::KanMX6-tTA-tetO_2	
CSY50	wild-type	pGAL-GFP
CSY52	*gal2Δ*	pGAL-GFP
CSY40	Gal2p::KanMX6-tTA-tetO_2	pGAL-GFP
CSY53	*gal1Δ*	pGAL-GFP
CSY54	*gal1Δ gal2Δ*	pGAL-GFP
CSY55	*gal1Δ* Gal2p::KanMX6-tTA-tetO_2	pGAL-GFP
CSY89	Gal3p::KanMX6-tTA-tetO2	pGAL-GFP
CSY90	Gal80p::His3MX6-tTA-tetO_2	pGAL-GFP
CSY91	Gal3p::KanMX6-tTA-tetO_2 Gal80p::His3MX6-tTA-tetO_2	pGAL-GFP

* All strains are derivatives of CSY3; only modifications to the wild-type background are indicated.

Table 2.2. List of primer sequences used in the construction of plasmids and yeast strains.

Name	Sequence	Description
GAL2kan.fwd	AGAATAGTAATAGTTAAGTAAACACAAGATTAACATAATAGGCAGATCCGCTAGGGATAA	Fwd primer for replacing GAL2 gene with KanMX
GAL2kan.rev	CATGAAAAATTAAGAGAGATGATGGAGCGTCTCACTTCAAGAATTCGAGCTCGTTTAAAC	Rev primer for replacing GAL2 gene with KanMX
tTA-tetO.fwd	CCTTGACTGCAATACGGCATCTACCCACCGTACTCGTCAA	Fwd primer for amplifying tTA and tetO_2 for cassette construction
GAL2tetO.rev	TTACTATTCTTGATGATAATTGAATAAGGTGCATAATGAACCCCCGAATTGATCCGGTAA	Rev primer for amplifying tTA and tetO_2 with 40bp homology to GAL2 promoter region
KanR.fwd	ATGCCGTATTGCAGTCAAGGGGCAGATCCGCTAGGGATAA	Fwd primer for KanMX portion of promoter replacement cassettes
GAL2p.kan.rev	ATATTCGAAAGGGGCGGTTGCCTCAGGAAGGCACCGGCGGGAATTCGAGCTCGTTTAAAC	Rev primer for KanMX portion of cassette with 40bp homology to GAL2 promoter region
GAL2p.screen.fwd	CATTAATTTTGCTTCCAAGACGACAGTAATATGTCTCCTA	Fwd primer for screening GAL2 promoter replacement
GAL2p.screen.rev	ATGGGAATCTTTACTGAGTGAAGAGATCACGTCTTC	Rev primer for screening GAL2 promoter replacement
GAL1HISko.fwd	GTGCGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAGGGCAGATCCGCTAGGGATAA	Fwd primer for amplifying HIS5 with 40bp homology to sequence upstream of GAL1
GAL1HISko.rev	CTACTCGTTATTATTGCGTATTTTGTGATGCTAAAGTTATGAATTCGAGCTCGTTTAAAC	Rev primer for amplifying HIS5 with 40bp homology to sequence downstream of GAL1
GAL1ko.screen.fwd	TGGAACTTTCAGTAATACGCTTAACTGCTC	Fwd primer for screening of GAL1 knockout
GAL1p.fwd	TCATGAATTCCCTTGAATTTTCAAAAATTCTTACTTTTTTTTTGG	Fwd primer for cloning of GAL1 promoter
GAL1p.rev	CATAGGATCCGTTTTTTCTCCTTGAC	Rev primer for cloning of GAL1 promoter
yEGFP.fwd	GCGGATCCATGTCTAAAGGTGAAGAATTATTCACTG	Fwd primer for cloning yEGFP-CLN2-PEST
yEGFP.rev	GCACGCGTTATATTACCCTGTTATCCCTAGCG	Rev primer for cloning yEGFP-CLN2-PEST
GAL3p.kan.rev	ATTAAACACAGTGGTTTCTTTGCATAAACACCATCAGCCTGAATTCGAGCTCGTTTAAAC	Rev primer for KanMX portion of cassette with 40bp homology to GAL3 promoter region
GAL3tetO.rev	CTCTGACCGGAGAACTGAATATTGGAACGTTTGTATTCATCCCCCGAATTGATCCGGTAA	Rev primer for amplifying tTA and tetO_2 with 40bp homology to GAL3 promoter region
GAL3p.screen.fwd	CTTATTAACCGCTTTTACTATTATCTTCTACGCTGACAGT	Fwd primer for screening GAL3 promoter replacement
GAL3p.screen.rev	TGAATGTGCCACATGTAGTCCGCATTTAAAGTAATTCGAC	Rev primer for screening GAL3 promoter replacement
GAL80p.his.fwd	TGACTGCCACTGGACCTGAAGACATGCAACAAAGTGCAAGGAATTCGAGCTCGTTTAAAC	Rev primer for His3MX portion of cassette with 40bp homology to GAL80 promoter region
GAL80tetO.rev	GAAAGAACGGGAAACCAACTATCGAGATTGTATACGCTGGCCCCCGAATTGATCCGGTAA	Rev primer for amplifying tTA and tetO_2 with 40bp homology to GAL80 promoter region
GAL80p.screen.fwd	GAACCTCCTCCAGATGGAATCCCTTCCATA	Fwd primer for screening GAL80 promoter replacement
GAL80p.screen.rev	ATCTAGTGAACATGCAAGGGCCCATTCTACGAAAAGATAC	Rev primer for screening GAL80 promoter replacement

2.4.2. Yeast expression plasmids

Standard molecular biology cloning techniques were used to construct the reporter plasmid used to assay Gal4p activation[34]. The plasmid was generated by cloning into the pCM190[27] shuttle plasmid. This plasmid contains an *E. coli* origin of replication (f1) and selection marker for ampicillin resistance, as well as a *S. cerevisiae* 2 μM high copy origin of replication and a selection marker for a uracil biosynthetic gene for plasmid maintenance in synthetic complete media supplemented with the appropriate amino acid

dropout solution. A yeast enhanced green fluorescent protein (yEGFP) gene with a degradation tag (CLN2-PEST)[37] and ADH1 terminator was inserted into the multi-cloning site of pCM190 behind the $tetO_7$ promoter between BamHI and MluI restriction sites. The GAL1 promoter was then cloned into this vector between EcoRI and BamHI restriction sites. The *yEGFP-CLN2-PEST* gene was amplified from pSVA15[37] using standard PCR procedures as previously described. The GAL1 promoter was amplified from pRS314-Gal[38]. This promoter contains two UASs and has been used in previous studies to measure Gal4p activity levels[39].

The reporter plasmid was constructed using restriction endonucleases and T4 DNA ligase from New England Biolabs. Plasmids were screened by transformation into an electrocompetent *E. coli* strain, DH10B (Invitrogen; F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G), using a Gene Pulser Xcell System (BioRAD) according to manufacturer's instructions. Subcloning was confirmed by restriction analysis. Confirmed plasmids were then transformed into the appropriate *S. cerevisiae* strains using a standard lithium acetate protocol[36]. *E. coli* cells were grown on Luria-Bertani media (DIFCO) with 100 μg ml^{-1} ampicillin (EMD Chemicals) for plasmid maintenance and *S. cerevisiae* cells were grown on synthetic complete media (DIFCO) supplemented with the appropriate dropout solution (Calbiochem) for plasmid maintenance. Plasmid isolation was conducted using Perfectprep Plasmid Isolation Kits (Eppendorf) according to manufacturer's instructions.

2.4.3. Fluorescence assays

Cell cultures were grown at 30°C in test tubes shaken at 200 rpm. Strains containing the reporter plasmid were grown in synthetic complete medium with the appropriate dropout solution (lacking uracil) and sugar source (2% raffinose, 1% sucrose). Overnight cultures were backdiluted 30-fold into fresh noninducing-nonrepressing media to an OD_{600} between 0.05 and 0.1. For assaying the network response, this fresh media contained appropriate concentrations of galactose (DIFCO), doxycycline (Sigma) for tetracycline-regulatable *GAL2*, *GAL3*, and *GAL80* strains, or water (negative control). Fluorescence and OD_{600} readings were measured using a Safire (TECAN) fluorescent plate reader after 8 h. Sample volumes of 200 μL were aliquoted into 96-well flat-bottom black plates (Greiner). The excitation and emission wavelengths were set to 485 nm and 515 nm, respectively, with a bandwidth of 12 nm. Fluorescence was measured from the bottom of the plate with a gain setting of 100. Fluorescence was normalized for cell number by dividing relative fluorescence units (RFUs) by the OD_{600} of the culture after subtracting the media background from each. All measurements were repeated at least in triplicate.

2.4.4. Flow cytometry assays

Yeast cells were grown according to methods detailed in fluorescence assays prior to preparation for flow cytometry analysis. After 7 h of induction, 5 ml of cells were harvested by centrifugation at 6000 rpm for 5 min, resuspended in 5 ml of phosphate buffered saline and incubated on ice for 30 min. This wash was repeated and the cell solution was subsequently filtered through a 40 μM cell strainer (Falcon). Cells were

analyzed on a FACSCalibur instrument (Becton Dickinson; San Jose, CA) using a 15 mW Argon laser with a 400 nm excitation wavelength and a 488 nm emission wavelength. For each sample approximately 10,000 cells were analyzed and each sample was repeated in duplicate. Data from the population fluorescence was analyzed using FlowJo software (Tree Star, Inc).