Molecular Microbiology (2014) 94(1), 202–217 ■
doi:10.1111/mmi.12757
First published online 1 September 2014

Perturbation of the interaction between Gal4p and Gal80p of the *Saccharomyces cerevisiae* GAL switch results in altered responses to galactose and glucose

Akshay Kumar Das Adhikari, Mohd. Tanvir Qureshi,† Rajesh Kumar Kar and Paike Jayadeva Bhat*
Laboratory of Molecular Genetics, Department of Bioscience and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India.

Summary

In *S. cerevisiae*, following the Whole Genome Duplication (WGD), *GAL1*-encoded galactokinase retained its signal transduction function but lost basal expression. On the other hand, its paralogue *GAL3*, lost kinase activity but retained its signalling function and basal expression, thus making it indispensable for the rapid induction of the *S. cerevisiae* GAL switch. However, a *gal3*Δ strain exhibits delayed growth kinetics due to the redundant signalling function of *GAL1*. The subfunctionalization between the paralogues *GAL1* and *GAL3* is due to expression divergence and is proposed to be due to the alteration in the Upstream Activating Sequences (UAS_G). We demonstrate that the GAL switch becomes independent of *GAL3* by altering the interaction between Gal4p and Gal80p without altering the configuration of UAS_G. In addition to the above, the altered switch of *S. cerevisiae* loses ultrasensitivity and stringent glucose repression. These changes caused an increase in fitness in the disaccharide melibiose at the expense of a decrease in fitness in galactose. The above altered features of the *Sc*GAL switch are similar to the features of the GAL switch of *K. lactis* that diverged from *S. cerevisiae* before the WGD.

Introduction

The discovery of a striking similarity between the homologous proteins of humans and chimpanzees led to the proposal that evolution is mainly driven by changes in the mechanism of gene expression rather than by the divergence of protein function (King and Wilson, 1975). Since then, the accumulated evidence has suggested that alteration in the transcriptional regulatory network (TRN) by 'tinkering' with the existing regulatory structure plays a crucial role in the overall scheme of evolution of gene expression (Jacob, 1977). TRN can evolve by altering the *cis*-regulatory sequences (DNA elements) or trans-acting factors (Coding sequences) or both (Wittkopp *et al.*, 2004). Adaptive evolution of morphological features is thought to occur mainly due to alteration in the *cis*-regulatory changes (Carroll, 2005; 2008) and is demonstrated in humans (McLean *et al.*, 2011), flies (Gompel *et al.*, 2005; McGregor *et al.*, 2007; Frankel *et al.*, 2011) and fish (Shapiro *et al.*, 2004; Chan *et al.*, 2010). In contrast, it is suggested that the alteration in the trans-acting factors of TRN is unlikely to contribute to adaptive evolution, primarily because of the deleterious pleiotropic effects of epistasis (Carroll, 2005; 2008; Stern and Orgogozo, 2009; Streisfeld and Rausher, 2011). Notwithstanding the above, recent evidence indicates that epistasis also contributes to the evolution of TRN, but the mechanism of escape from negative pleiotropy is not understood (Hoekstra and Coyne, 2007; Li and Johnson, 2010). Gene duplication is thought to circumvent the deleterious pleiotropic effects arising out of epistasis by providing a period of redundancy for novel or altered protein-protein interactions to emerge (Lynch and Wagner, 2008; Li and Johnson, 2010). However, how gene duplication provides an opportunity to incorporate regulatory features to fine tune the gene expression pattern and its impact on fitness under different environmental condition is not clear. We have used GAL switch of *S. cerevisiae* as a paradigm to unearth the possible changes that could result in the fine tuning of the gene expression following genome duplication.

Saccharomyces diverged from *Kluyveromyces* 100mya and underwent whole genome duplication (Wolfe and Shields, 1997) and rewired its metabolic lifestyle (Piskur *et al.*, 2006). During this transition, it evolved the ability to grow rapidly by fermenting glucose, even in the presence of oxygen and a tightly regulated GAL switch with exquisite sensitivity to glucose. This seems to have contributed towards its ability to utilize large quantities of glucose (Weirauch and Hughes, 2010). In contrast is the GAL

Accepted 13 August, 2014. *For correspondence. E-mail jayadeva@iitb.ac.in; Tel. (+91) 22 2576 7772; Fax (+91) 22 2576 3480. †Present address: Department of Chemistry, The Pennsylvania State University, 104 Chemistry Bldg., University Park, PA 16802, USA.

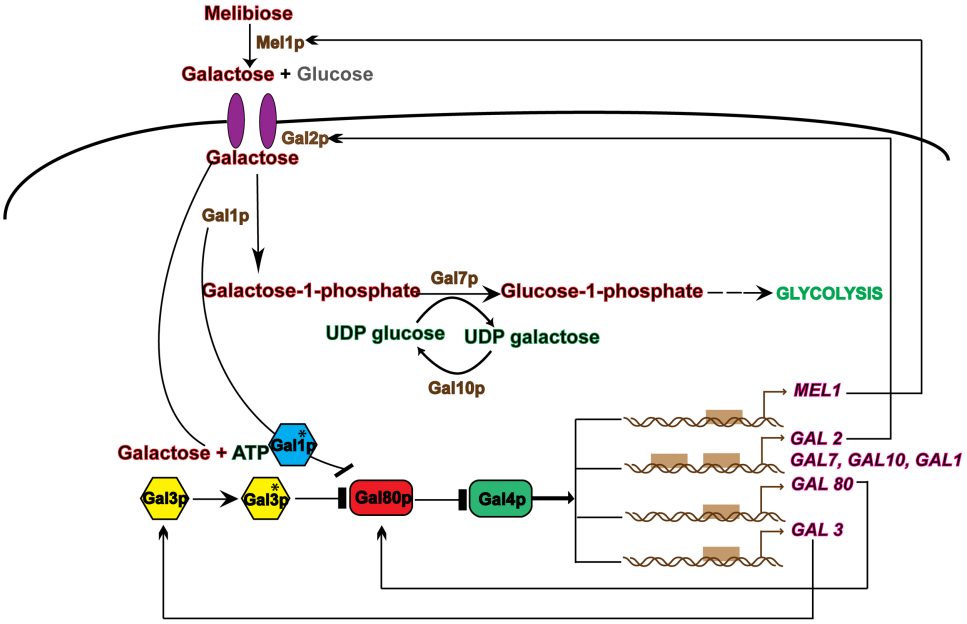

Fig. 1. GAL genetic circuit and pathway of galactose utilization in *S. cerevisiae*. The transcriptional activator Gal4p activates a set of enzymatic and regulatory genes by binding to the Upstream Activating Sequences (UAS_G) in their promoter (indicated by grey boxes). In the absence of galactose, Gal4p activity is repressed by the transcriptional repressor Gal80p. The permease Gal2p facilitates uptake of galactose into the cell. In presence of galactose and ATP, the signal transducer Gal3p becomes activated (indicated by asterisk) and inhibits Gal80p leading to the induction of *GAL* genes. Galactose is metabolized by a series of enzymatic reactions through the activities of the galactokinase (Gal1p), transferase (Gal7p), and epimerase (Gal10p) and enters glycolytic pathway. Apart from these, *MEL1* is also regulated by similar mechanism. *MEL1* although not a core member of galactose utilization pathway, encodes extracellular enzyme α-galactosidase that cleaves melibiose in to glucose and galactose. The regulatory genes *GAL3*, *GAL80* and *GAL2* are also activated in a similar fashion. Gal1p, a paralogue of Gal3p, is a bi-functional protein which also inhibits Gal80p in the presence of galactose and ATP albeit with lower affinity. *K. lactis* GAL genetic switch functions similarly except the absence of dedicated signal transducer like Gal3p and positioning of UAS_G in the promoter.

genetic switch of *K. lactis*, an aerobic yeast which is more adapted for the utilization of lactose than galactose (Rubio-Texeira, 2005). Accordingly, the *Kl*GAL switch is less sensitive to glucose repression. Therefore, the molecular basis of the above differential features of *Sc*GAL switch vis-à-vis *Kl*GAL switch and their possible impact on their lifestyle is not clearly understood.

The transcription of *GAL* genes is activated by Gal4p, a transcriptional activator that dimerizes and remains bound to the upstream activating sequences (UAS_G) (Keegan *et al.*, 1986; Ma and Ptashne, 1987a). However, in the absence of galactose, Gal4p is unable to activate the transcription due to its association with Gal80p. In the presence of galactose, this negative repression of Gal80p is removed by either the signal transducer Gal3p or Gal1p (Fig. 1). In *K. lactis, GAL1* (*KlGAL1*) encodes the bi-functional protein which serves both as a galactokinase (required for the conversion of galactose to galactose-1-phosphate, the first reaction of the catabolic pathway) as well as signal transducer which sequesters Gal80p in response to galactose. In contrast, in *S. cerevisiae*, *GAL1* codes for the bi-functional protein and its paralogue *GAL3* codes for the dedicated signal transducer (Bhat and Murthy, 2001; Rubio-Texeira, 2005). During this evolutionary transition, UAS_G present as '*trans*' (on the opposite faces of the DNA helix) in *KlGAL1*, got altered to '*cis*' (on the same face of the DNA helix) as observed in *ScGAL1* (Hittinger and Carroll, 2007). This altered configuration of UAS_G in *ScGAL1*, has conferred increased fold expression upon induction and low basal expression in the absence of galactose (Hittinger and Carroll, 2007). This change in the expression pattern is attributed to the cooperative binding of Gal4p dimer to the adjacently placed UAS_G elements which is stabilized by the dimer–dimer interaction between Gal80p bound to Gal4p (Melcher and Xu, 2001; Hittinger and Carroll, 2007). In contrast, the *GAL3* promoter with a single UAS_G has a basal expression and a moderate fold induction. The alteration in the expression pattern caused due to the change in the helical phasing of UAS_G elements contributed marginally towards the adaptive evolution of *Sc*GAL switch (Hittinger and Carroll, 2007).

In addition to the dimerization, cooperativity of Gal4p binding to DNA and protein sequestration, the GAL genetic switch also consists of Gal2p and Gal3p/Gal1p dependent positive feedback loops and Gal80p dependent negative feedback loop. These features determine the

dynamics of the GAL genetic switch. For example, disabling the Gal80p and Gal3p feedback loops increased both heterogeneity (Ramsey *et al*., 2006) and cellular memory (Acar *et al*., 2005) population wide. Recent studies have demonstrated the robust bimodal expression of *ScGAL* genes is due to bistability which is dependent on an ultrasensitive response. Disrupting the Gal1p and Gal3p feedback loops abolished the bimodality by disrupting the underlying ultrasensitive response. Based on modelling results, it has been suggested that the competitive sequestration of Gal80p by Gal1p and Gal3p is the source of ultrasensitivity which is crucial for the bistable response of the *Sc*GAL switch (Venturelli *et al*., 2012).

Our aim was to unravel a long standing paradox as to how *GAL3* induces the GAL switch when super-repressor $GAL80^{S\text{-}1}$ is in conjunction with constitutive allele $GAL4^{C}$ (here $GAL4^{C}$ is epistatic over $GAL80^{S\text{-}1}$) but not with the wild type *GAL4* (here $GAL80^{S\text{-}1}$ is epistatic over *GAL4*) (Nogi *et al*., 1977). In other words, what is the molecular basis of the epistatic effect of *GAL3* over $GAL80^{S\text{-}1}$ only when the latter is in conjunction with $GAL4^{C}$ but not with *GAL4* allele? We discovered that the epistatic effect of *GAL3* over $GAL80^{S\text{-}1}$ is due to an increase in basal expression of *GAL3*, which occurs in a strain with $GAL80^{S\text{-}1}GAL4^{C}$ but not with $GAL80^{S\text{-}1}GAL4$ alleles. Thus, the epistatic interaction between $GAL80^{S\text{-}1}$ and $GAL4^{C}$ (Nogi *et al*., 1977) is inseparable from the epistatic effect of *GAL3* over $GAL80^{S\text{-}1}$ due to the increase in basal expression, as observed in this study. During this analysis, we also observed that the ultrasensitive response and sensitive glucose repression that is otherwise present in the wild type GAL switch is severely impaired in the $GAL80^{S\text{-}1}GAL4^{C}$ switch. We discuss the possible implications of our results in light of the differences in the behaviour of *Sc*GAL and *Kl*GAL switch and in terms of evolutionary impact.

Results

How did we arrive at the idea that altering the interaction between Gal80p and Gal4p plays a significant role in imparting unique features to *S. cerevisiae* GAL genetic switch? Previous experimental results indicated that $Gal80^{S\text{-}1}$p (a super-repressor) is incapable of interacting with signal transducer Gal3p, and accordingly a strain bearing such an allele is not inducible (Yano and Fukasawa, 1997; Diep *et al*., 2008). A *S. cerevisiae* strain with a $GAL80^{S\text{-}1}$ genetic background regains inducibility upon substituting the wild type *GAL4* allele with a $GAL4^{C}$ allele [a mutant T859I allele that confers constitutive expression of *GAL* genes in an otherwise wild type genetic background (Salmeron Jr *et al*., 1990)]. That is, a strain bearing $GAL80^{S\text{-}1}GAL4$ is un-inducible while $GAL80^{S\text{-}1}GAL4^{C}$ is inducible similar to a wild type strain (Nogi *et al*., 1977; Salmeron Jr *et al*., 1990). The explanation that Gal3p cannot interact with $Gal80^{S\text{-}1}$p in un-inducible strain $GAL80^{S\text{-}1}GAL4$ does not explain the inducible nature $GAL80^{S\text{-}1}GAL4^{C}$ strain. This is because, for induction, Gal3p needs to interact with $Gal80^{S\text{-}1}$p. Thus, it is not understood as to how the Gal3p interaction with $Gal80^{S\text{-}1}$p is dependent on the allelic status of *GAL4* (summarized in Table S1). In contrast to the above, in *K. lactis*, corresponding $Gal80^{S\text{-}1}$p interacts with its signal transducer and the strain is inducible even in presence of the wild type *GAL4* (Zenke *et al*., 1999). Thus, the molecular basis of the inducible nature of $GAL80^{S\text{-}1}GAL4^{C}$ strain remained a paradox. Contrary to the available experimental evidences (Yano and Fukasawa, 1997; Diep *et al*., 2008), we reasoned that, Gal3p has to interact with $Gal80^{S\text{-}1}$p. Here, we first demonstrate that in fact Gal3p functionally and physically interacts with $Gal80^{S\text{-}1}$p. This breakthrough enabled us to look at the role of epistasis in the adaptive evolution of *Sc*GAL genetic switch in a new perspective.

Wild type Gal3p functionally and physically interacts with $Gal80^{S\text{-}1}$p

In *S. cerevisiae*, a dominant super-repressor allele $GAL80^{S\text{-}1}$ confers a non-inducible phenotype to an otherwise wild type strain (Nogi *et al*., 1977). A *gal80*Δ strain with wild type *GAL4* does not respond to galactose if both Gal3p and $Gal80^{S\text{-}1}$p are overexpressed (Yano and Fukasawa, 1997). It was further demonstrated that Gal3p cannot interact with $Gal80^{S\text{-}1}$p in response to galactose (Yano and Fukasawa, 1997). We reasoned that, upon overexpression of both, $Gal80^{S\text{-}1}$p was in far excess as compared to Gal4p, causing a reduced productive interaction between Gal3p and $Gal80^{S\text{-}1}$p. If this is true, then a $GAL80^{S\text{-}1}$ *GAL4* strain should respond to galactose upon overexpression of only Gal3p. As expected, we observed that $GAL80^{S\text{-}1}GAL4$ strain transformed with multiple copy plasmid wherein the expression of Gal3p is driven from *CYC1* promoter (pYJM-3) but not from endogenous promoter (pT1-3B) grows on galactose (Fig. 2A and B). As expected, transformants of wild type strain but not that of strains bearing $GAL80^{S\text{-}0}$ and $GAL80^{S\text{-}2}$ alleles grew on galactose (Yano and Fukasawa, 1997). These results indicate that Gal3p functionally interacts with $Gal80^{S\text{-}1}$p but not $Gal80^{S\text{-}0}$p and $Gal80^{S\text{-}2}$p, in response to galactose.

In order to confirm the above, we determined the activation of the GAL switch, by monitoring the α-galactosidase expression in wild type and strains with $GAL80^{S\text{-}0}$ or $GAL80^{S\text{-}1}$ or $GAL80^{S\text{-}2}$ (Fig. S1) allele transformed with pYJM-3 and pT1-3B. As expected, α-galactosidase induction in $GAL80^{S\text{-}1}$ strain transformed with pYJM-3 but not pT1-3B was of the order of 30% (Fig. 2C) of what is normally observed in the wild type strain induced by galactose (Fig. S1A and C). Under the above conditions, we

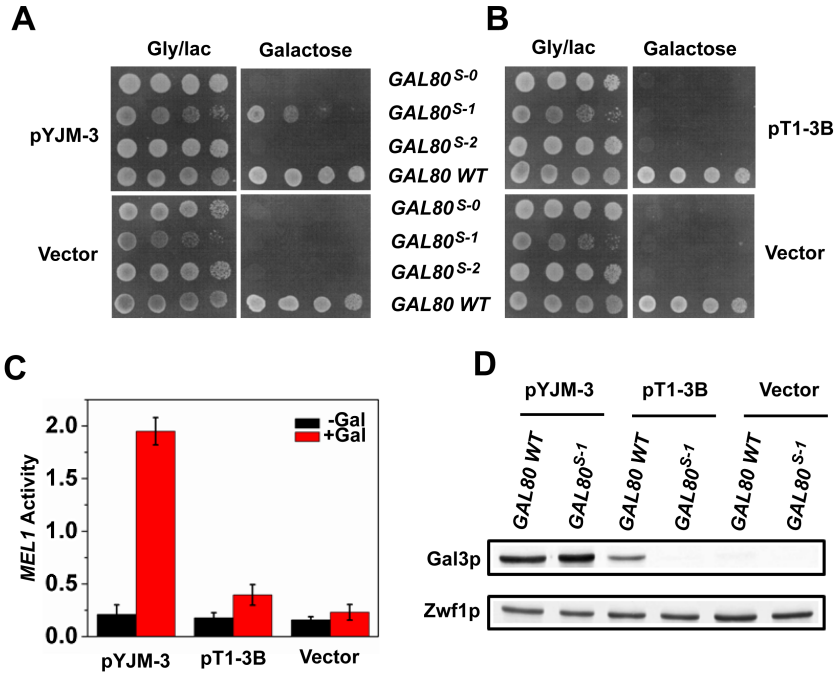

Fig. 2. Overexpression of Gal3p suppresses the non-inducible phenotype of a strain with *GAL80*$^{S-1}$ super-repressor allele.
A and B. Strains with indicated *GAL80* alleles (indicated between A and B) transformed with multicopy plasmid, in which *GAL3* expression is driven by either *CYC1* promoter (pYJM-3, A) or from its own promoter (pT1-3B, B). The respective vector controls are as indicated. Cells were pregrown in medium containing glycerol/lactate (gly/lac) and spotted on to media containing gly/lac or galactose.
C. α-Galactosidase activity was determined in strain with *GAL80*$^{S-1}$ super-repressor allele transformed with pYJM-3, pT1-3B or vector alone. Triplicate samples were taken for analysis and results represent average of three independent experiments with standard deviation.
D. Western blot for Gal3p in wild type or the strain with *GAL80*$^{S-1}$ allele bearing plasmids as indicated.

observed that Gal3p is overexpressed only in the *GAL80*$^{S-1}$ strain transformed with pYJM-3 but not pT1-3B (Fig. 2D). The lack of expression of Gal3p from endogenous promoter in a *GAL80*$^{S-1}$ background is probably because of the strong negative auto-regulatory influence of the *GAL80*$^{S-1}$ allele. As expected, Gal3p expression was detected in extracts obtained from a wild type strain transformed with either plasmid (Fig. 2D).

To determine whether Gal80^{S-1}p physically interacts with wild type Gal3p, His tagged Gal3 protein was immobilized on to Ni-NTA agarose column and allowed to interact with the cell-free extract containing Gal80p, Gal80^{S-0}p, Gal80^{S-1}p and Gal80^{S-2}p separately. The bound proteins were pulled down and analysed on a Western blot using Gal80p antisera. Gal3p interacted with Gal80p and Gal80^{S-1}p (Fig. 3A) but not Gal80^{S-0}p and Gal80^{S-2}p in a dose dependent fashion (Fig. S2, Supporting Text, section S2). Quantification of the relative intensities of the bound protein indicated that Gal3p binds Gal80p at least 5 times more strongly than Gal80^{S-1}p (Fig. S3A–C, Supporting Text, section S2). Unlike the interaction of Gal3p with Gal80p, galactose and ATP were indispensable for the interaction between Gal3p and Gal80^{S-1}p (Fig. 3B).

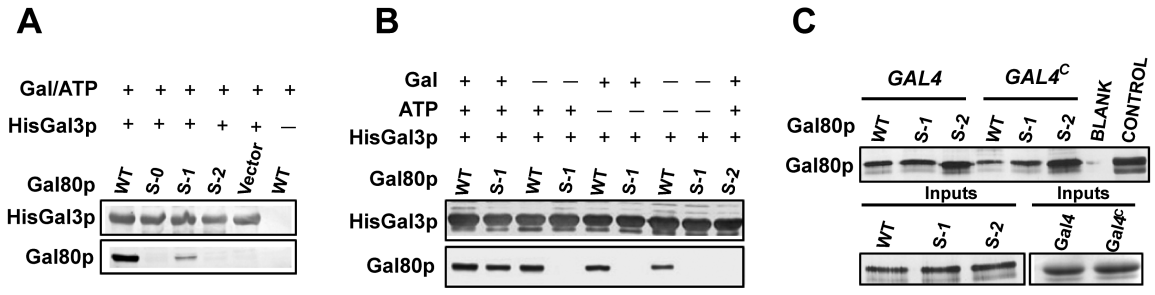

Fig. 3. Interaction of wild type and Gal80 super-repressor proteins with Gal3p, Gal4p and Gal4^{C}p.
A. Cell-free extract (equivalent to 1000 μg of protein) containing His-tagged Gal3p was immobilized on to Ni-NTA beads. Cell-free extract (equivalent to 1500 μg of protein) containing wild type and Gal80 super-repressor proteins were allowed to interact with the immobilized Gal3p, in presence of galactose and ATP. Gal3p–Gal80p complex was analysed by Western blot.
B. Interaction of Gal80p and Gal80^{S-1}p with Gal3p in presence or absence of galactose and ATP as indicated. The protocol used for this was same as in section A, except cell-free extract containing HisGal3p was twice. As a control, binding analysis with cell-free extract containing Gal80^{S-2}p was carried out.
C. Cell-free extract (equivalent to 1000 μg of protein) containing GST tagged C-terminal AD of Gal4p and Gal4^{C}p were immobilized on to glutathione agarose beads. Purified His tagged Gal80, Gal80^{S-1} and Gal80^{S-2} proteins (20 μg each) were allowed to interact with this immobilized Gal4p and Gal4^{C}p. Gal4p–Gal80p complex was analyzed by Western blot. Purified Gal80p was used as the control. As a control, binding analysis with cell-free extract containing Gal80p and Gal80^{S-2}p was carried out.

It was previously demonstrated that the D368V variant of Gal3p interacts with wild type Gal80p in the absence of galactose (Diep *et al*., 2008). It was also demonstrated that D368V variant interacted with Gal80^{S-1}p only in presence of galactose (Diep *et al*., 2008). Our study demonstrates that wild type Gal3p behaves similar to the D368V variant when excess Gal3p is used for binding experiments. Based on these observations, we suggest that a structural alteration brought about by D368V is not necessarily a prerequisite for Gal3p to interact with Gal80^{S-1}p. Overall these data suggest that Gal80^{S-1}p has reduced affinity towards wild type Gal3p which can be compensated either by introducing D368V or by overexpressing wild type *GAL3*. Thus, we suggest that the previous observation (Diep *et al*., 2008) that only the Gal3p variant D368V is capable of interacting with Gal80^{S-1}p and the prediction that G323R substitution in Gal80^{S-1}p prevents its interaction with wild type Gal3p (Lavy *et al*., 2012) needs to be revisited in the light of our results.

It was known that substituting the wild type with constitutive *GAL4^{C}* allele in a strain otherwise non-inducible because of the *GAL80^{S-1}* allele converts the switch to an inducible one (Nogi *et al*., 1977; Salmeron Jr *et al*., 1990). This phenotype is surprising in the context of our observation that affinity between Gal3p and Gal80^{S-1}p was reduced. If so, how does Gal3p induce the *GAL80^{S-1}GAL4^{C}*, but not a *GAL80^{S-1} GAL4* switch? A likely possibility is that the reduced interaction between Gal3p and Gal80^{S-1}p is probably compensated by a reduced Gal80^{S-1}p and Gal4^{C}p interaction. We demonstrate that the Gal80^{S-1}p interacts weakly with the C-terminal Activation Domain (AD) (Ma and Ptashne, 1987b) of Gal4^{C}p as compared to that of Gal4p (Fig. 3C; Fig. S3D, Supporting Text, section S3).

The response of the epistatically altered GAL switch to Gal3p is not as efficient as the wild type switch

Unlike in *S. cerevisiae*, in *K. lactis*, *GAL80^{S-1}GAL4* strain is inducible, albeit weakly (Zenke *et al*., 1999). This observation points out that the epistatic nature of Gal80p and Gal4p interaction dictates the responsiveness of the switch. To obtain further insight, we introduced the *GAL4^{C}* allele (T859I) in WT and *GAL80^{S-1}* background. The former was constitutive while the latter was inducible, which is in agreement with the previous report (Douglas and Hawthorne, 1972; Nogi *et al*., 1977; Salmeron Jr *et al*., 1990) (Fig. S4A and B). The epistatically altered strain has elevated basal expression of *GAL3* along with *MEL1* as compared to the wild type strain (Fig. S4B and C). Thus, the GAL switch of *GAL80^{S-1}GAL4^{C}* alleles is akin to the *GAL80GAL4* or *GAL80^{S-1}GAL4* switch of *K. lactis*. We then determined the consequence of overexpression of Gal3p in epistatically altered strain. As expected, in the wild type strain, the GAL switch was constitutive upon overexpression (Bhat and Hopper, 1992). In contrast, the epistatically altered strain did not exhibit constitutive expression of *GAL* genes upon overexpression of Gal3p (Fig. 4A and B). This is despite the fact that the amount of Gal3p produced is in the transformants of the epistatically altered strain is comparable to that of wild type (Fig. 4C). This difference is explained easily based on the low affinity of Gal3p and the Gal80^{S-1}p.

Epistatically altered S. cerevisiae *GAL switch resembles* K. lactis *GAL switch*

The induction kinetics of the *GAL1* promoter driven β-galactosidase expression in response to galactose by the epistatically altered switch resembles that of *K. lactis* switch rather than the wild type GAL switch (Fig. S5A). More importantly, in the strain with the epistatically altered switch, *GAL1* promoter driven activity was found to be leaky, indicating that the altered interaction is inefficient in implementing a strong repression in the absence of galactose. To determine whether the basal expression of the *GAL1* in the altered strain has biological significance, we disrupted *GAL3* and monitored the induction kinetics. We surmised that because Gal1p has signalling activity and has a basal expression in the epistatically altered strain, the delayed induction kinetics observed in a *gal3*Δ (Torchia and Hopper, 1986; Bhat and Murthy, 2001) in an otherwise wild type background should disappear. The growth kinetics, enzyme induction and the time taken for first doubling clearly demonstrate that the altered switch can function independent of Gal3p (Fig. 5A–C). To determine whether this property is intrinsic to the *GAL80^{S-1}GAL4^{C}* interaction and not due to the genetic background of our laboratory strain, we introduced *GAL80^{S-1}GAL4^{C}* alleles into BY strain back ground by crossing and segregation. Five independent segregants with *GAL80^{S-1}GAL4^{C}* configuration showed Gal3p independent activation, indicating that the above property is solely due to the altered epistatic interaction (Fig. S5B). This result suggests that following gene duplication, the recruitment of Gal3 as a dedicated signal transducer in *S. cerevisiae* entailed epistasis. Thus, the epistatically altered GAL switch resembles the *Kl*GAL switch.

The *Kl*GAL switch is less sensitive to glucose repression (Salmeron Jr *et al*., 1989; Zenke *et al*., 1993). The stringent glucose repression in *S. cerevisiae* evolved following genome duplication (Piskur and Langkjaer, 2004). Moreover, previous studies have implicated *GAL80* as a determinant of the glucose repression of GAL switch (Johnston *et al*., 1994; Biggar and Crabtree, 2001). Therefore, we wanted to test the sensitivity of the epistatically altered switch to glucose repression. We determined the *GAL1* promoter driven β-galactosidase expression in response to

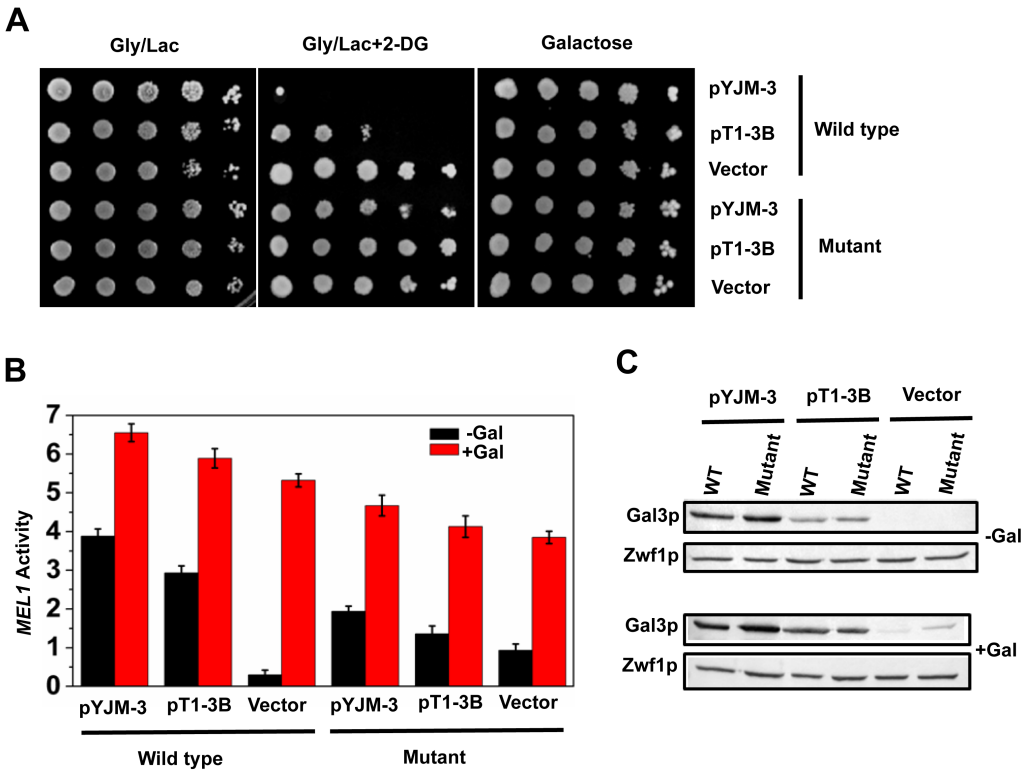

Fig. 4. Response of wild type and epistatically altered strain upon overexpression of *GAL3*.
A. Growth of wild type and epistatically altered (*GAL80*$^{S\text{-}1}$*GAL4*^{C}) strain (indicated as mutant) transformed with pYJM-3 or pT1-3B. pYJM (*URA3* multicopy plasmid) was taken as vector control.
B. α-Galactosidase activity of wild type and epistatically altered strains transformed with pYJM-3 or pT1-3B or vector alone. Triplicate samples were taken for analysis and results represent average of three independent experiments with standard deviation.
C. Western blot for Gal3p in wild type and epistatically altered strain bearing plasmids as indicated.

glucose under two different experimental regimes. In the first case, cells were grown in a neutral medium and then transferred to a medium containing a constant amount of galactose but increasing concentration of glucose. *GAL1* promoter driven β-galactosidase was determined after a growth period of 4 h (Fig. 6A). In the second regime, cells pre-grown in galactose were inoculated into a medium containing 1% galactose and allowed to grow for 1 h, at the end of which 0.5% glucose was added. β-Galactosidase was monitored as a function of time (Fig. 6B). Results of both the above experiments clearly indicate that the sensitivity of the altered switch towards glucose repression is more like that of the *Kl*GAL switch.

Given that the pattern of glucose repression in epistatically altered switch resembles that of *Kl*GAL switch, it was of interest to look at the growth profiles of wild type and the epistatically altered strains in galactose and melibiose. We consistently observed that the growth of the epistatically altered strain was not as robust as the wild type strain at low galactose concentration. However, at higher galactose concentration the stationary phase cell density of the mutant was higher than that of wild type (Fig. S6B). In contrast, the epistatically altered strain grew better at all the melibiose concentrations tested as compared to the wild type (Fig. S6C). To assess whether the above difference has any adaptive value, we determined the fitness in competition experiments. The wild type strain has a fitness advantage on galactose as compared to the epistatically altered strain (Fig. 6C). However, in melibiose the epistatically altered strain has a fitness advantage (Fig. 6C).

The epistatically altered GAL switch is not ultrasensitive

Our study demonstrates that without altering the topology of the switch, it is possible to reinstate the features of an ancestral GAL switch, as represented by *Kl*GAL switch in *S. cerevisiae* by altering the strength of sequestration of Gal4 by Gal80. Molecular sequestration is a prevalent mechanism of implementing ultrasensitive response in transcriptional regulation (Buchler and Louis, 2008). This is probably because, the binding surface between the interacting partners is flexible and more easily evolvable than modifying cooperativity through oligomerization or multiple binding of transcription factors (Venturelli *et al.*,

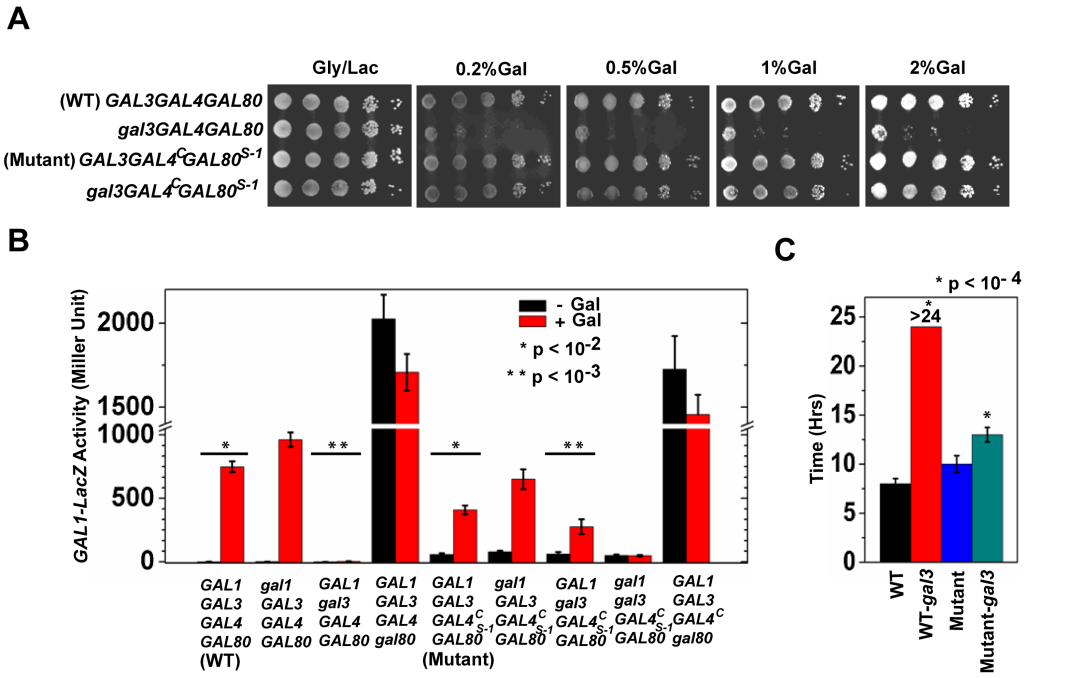

Fig. 5. Epistatically altered strain deleted for *GAL3 does not show long*-term adaptation due to the increased basal expression of *GAL1*.
A. Growth of wild type and epistatically altered strains with corresponding *gal3*Δ strains in different concentrations of galactose as indicated.
B. *GAL1-LacZ* reporter cassette was integrated in to the wild type and epistatically altered strains. β-Galactosidase activity in reporter strains with the genetic background as indicated. Triplicate samples were taken for analysis and results represent average of three independent experiments with standard deviation.
C. Time for first doubling was calculated after inoculating stationary gly/lac cultures of wild type and epistatically altered strains in to 2% galactose and monitoring OD_{600} at 1 h intervals ($n = 4$). Similar experiments are also carried out in corresponding *GAL1* and *GAL80* deletion strains as a control.

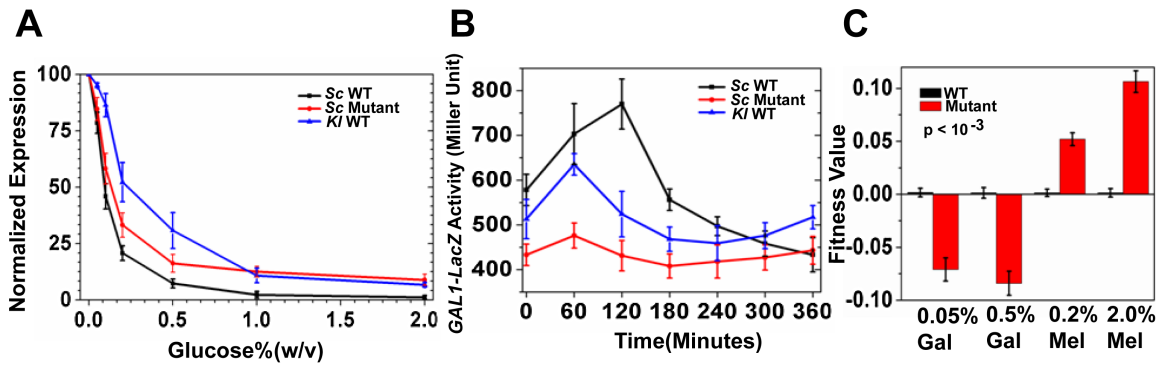

Fig. 6. Glucose repression and fitness analysis.
A. Reporter strains were pre-grown in gly/lac to an OD_{600} of 0.3 and cell cultures were divided into different tubes. Each culture was allowed to grow for additional 4 h after adding 1% galactose and different concentration of glucose as indicated. β-Galactosidase activity was determined and presented as normalized expression for each strain. *K. lactis* wild type strain was also subjected to similar analysis.
B. Reporter strains were pre-grown in galactose to stationary phase. Cells were freshly inoculated in to a medium containing 1% galactose. After 1 h, cultures were divided in to two parts. 0.5% glucose was added to one part and water was added to the other as control. β-Galactosidase was monitored as function of time in both cases. The activities in the control samples are not presented for clarity. *K. lactis* wild type strain was also subjected to similar analysis.
C. Fitness of wild type and epistatically altered strains in galactose and melibiose as indicated was determined by competition assay (see methods for details). Triplicate samples were taken for analysis and results represent average of three independent experiments with standard deviation.

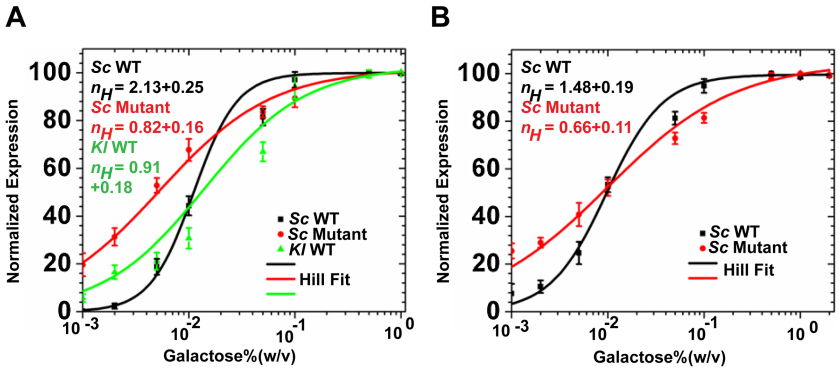

Fig. 7. Epistatically altered strain has lost ultrasensitive with respect to galactose concentration.
A. Reporter strains were pregrown in gly/lac to an OD_{600} of 0.3 and the cell cultures were equally distributed into different tubes. Galactose was added to the final concentration as indicated. After 4 h, β-galactosidase activity was determined. *K. lactis* wild type strain was also subjected to similar analysis.
B. Similar analysis was performed to determine the response from a single Gal4 binding site by determining α-galactosidase activity. Enzyme activities were normalized for each strain. Triplicate samples were taken for analysis and results represent average of five (A) and three (B) independent experiments with standard deviation. Each point represents the data obtained with standard deviation. Lines represent fitted Hill function.

2012). Given that the wild type *Sc*GAL switch is ultrasensitive (Verma *et al*., 2003), we determined the response of the wild type and the altered switch to varying concentrations of galactose. The Hill coefficient of *S. cerevisiae* wild type switch is 2.14 while the epistatically altered GAL switch is 0.82 and that of *K. lactis* is 0.92 (Fig. 7A). To determine whether change in galactose concentration as a function of growth affected the ultrasensitive response, both wild type and the epistatically altered *S. cerevisiae* strains were deleted for galactokinase and same analysis was performed. Under these conditions the Hill coefficient marginally decreased but the difference between the wild type and the epistatically altered switch remained the same (Fig. S7A). We also determined the response of the switch with respect to α-galactosidase, whose expression is driven by single Gal4p binding site. The wild type and the epistatically altered switch again showed a difference in the Hill coefficient (Figs 7B and S7B). These results indicate that without altering the configuration of the promoter elements, altering only epistatic interaction between Gal80p and Gal4p results in the loss of ultrasensitive response. The absence of ultrasensitivity in the epistatically altered strain and *K. lactis* is consistent with the observation that the basal expression is more than what is observed in *S. cerevisiae.*

Discussion

Our initial purpose was to unravel how wild type Gal3p that cannot induce the GAL switch of a strain with *GAL80*$^{S\text{-}1}$*GAL4* genetic constitution can in fact induce the GAL switch with *GAL80*$^{S\text{-}1}$*GAL4*^{C} genetic constitution. That is, we investigated how the interaction of wild type Gal3p with Gal80$^{S\text{-}1}$p is dependent on the allelic status of *GAL4*, with which Gal3p does not have any direct physical interaction. Based on our observations, we suggest that the difference in the ability of Gal3p to interact with Gal80$^{S\text{-}1}$p is a function of Gal3p expression status, which in turn is indirectly a function of the structural status of the GAL switch itself. That is, in *GAL80*$^{S\text{-}1}$ *GAL4*^{C} background, the Gal3p expression is more than what is normally observed in the wild type strain and can compensate for the otherwise low affinity with Gal80$^{S\text{-}1}$p. This is coupled with the fact that the affinity of Gal80$^{S\text{-}1}$p with Gal4^{C}p is reduced as compared with Gal4p. This fundamental observation made us wonder whether tweaking one specific parameter of the network has any other functional outcomes at the phenotypic and fitness level. We observed that the *S. cerevisiae* strain with *GAL80*$^{S\text{-}1}$*GAL4*^{C} background behaves like the *K. lactis* strain. Most of these consequences seem to arise due to the alteration of ultrasensitivity, a parameter that is known to impact network dynamics.

The *K. lactis* GAL switch superficially resembles *S. cerevisiae* GAL switch at the structural level but minor differences abound. For example, the *Kl*GAL switch only has the *GAL1* dependent signalling pathway. It lacks the adjacent UAS_G elements, thus preventing the co-operative interaction of Gal4p. However, it has a positive autoregulatory feedback loop for Gal4p which is absent in *Sc*GAL switch. Therefore, it was unexpected that by only altering the interaction between Gal80p and Gal4p in *S. cerevisiae*, the switch behaves more like *K. lactis* switch, not only in making the *Sc*GAL switch independent of *GAL3* and increasing the basal level of expression of *GAL* genes but also at the level of carbon source preferences.

Ultrasensitivity of a signalling network can normally arise due to cooperativity, dimerization, feedback loops

and protein sequestration. It is generally suggested that since protein sequestration can be altered through mutation, this mechanism of changing the ultrasensitivity is more evolvable than other possibilities (Buchler and Louis, 2008; Venturelli *et al*., 2012). We (the present study) and others (Venturelli *et al*., 2012) have demonstrated that the *GAL* gene promoter with single UAS_G responds in an ultrasensitive manner. This suggests that the adjacently placed UAS_G elements may not have a direct role in implementing ultrasensitivity, as inferred earlier (Griggs and Johnston, 1991; Verma *et al*., 2003). Although the ultrasensitivity of the GAL system was robust, it was recently demonstrated that abolishing the dual feedback loops of *GAL1* and *GAL3* resulted in the loss of ultrasensitivity and bistability (Venturelli *et al*., 2012). Based on modelling, these authors suggested that the competitive sequestration of Gal80p by both Gal1p and Gal3p is essential but not cooperatively. However, we observed that, in a *GAL1* deletion strain ultrasensitivity was not completely abolished but the magnitude was reduced as compared to the wild type. This suggests that sequestration of Gal80p by Gal3p alone is sufficient to introduce ultrasensitivity. If cooperativity of Gal4p binding is not required for ultrasensitivity, then how do we explain the loss of ultrasensitivity upon changing the affinity between Gal3p–Gal80p and Gal80p–Gal4p only on the basis of Gal80p sequestration by Gal1p/Gal3p? Considering that Gal4p concentration in *S. cerevisiae* does not increase through auto-regulation as observed in *K. lactis* (Czyz *et al*., 1993), we propose that the role of adjacently placed UAS_G elements is limited to the recruitment of the low abundant Gal4p to the promoters, through cooperativity. The Gal4p dimer–dimer interaction subsequently stabilized by the Gal80p dimer–dimer interaction ensures that the limited amount of Gal4p is productively engaged in interacting with DNA and Gal80p. Thus, the affinity of Gal80 to Gal4p is precisely calibrated within narrow limits, so as to increase the productive interaction between Gal3p and Gal80p in to confer an ultrasensitive response. That is, if the affinity of Gal80p to Gal4p is decreased, then Gal3p cannot efficiently transduce the signal to the Gal80p–Gal4p complex, as there will be sufficient free Gal80p to sequester Gal3p, resulting in unproductive interaction. We suggest that in the $GAL4^{C}GAL80^{S\text{-}1}$ strain, because of the lower affinity between $Gal80^{S\text{-}1}$p and $Gal4^{C}$p, Gal3p is inefficient in transducing the signal leading to the loss of ultrasensitivity. Therefore, the notion of 'productive interaction' between Gal3p, Gal80p, Gal4p explains (a) our observation that over expression of Gal3p alone but not the overexpression of both $Gal80^{S\text{-}1}$p and Gal3p, as observed by others (Yano and Fukasawa, 1997), activates the switch in a $GAL80^{S\text{-}1}GAL4$ background (b) the observation that the increased basal expression of Gal3p in $GAL80^{S\text{-}1}GAL4^{C}$ strain compensates the low affinity between Gal3p and $Gal80^{S\text{-}1}$p, resulting in the inducible phenotype (c) lack of ultrasensitivity in *Kl*GAL switch because of the elevated basal expression of the regulatory proteins leading to the loss of productive interaction.

In vitro evolution approach yielded three independent *S. cerevisiae* strains with single mutations that have a higher specific growth rate in galactose, indicating that even single mutation is sufficient to alter the metabolic potential at a given environmental condition (Hong *et al*., 2011). Again using *in vitro* evolution experiments, it has been suggested that single mutation can optimize multiple phenotypes simultaneously (Chou *et al*., 2014). Recent theoretical analysis undertaken to address whether GAL regulatory network is capable of steering the metabolic flux at varying external conditions indicated that by adjusting kinetic parameters, the GAL regulatory network is capable of responding to the demands of metabolic network to the expected level (Berkhout *et al*., 2013). Of the 54 parameters tested using unidimensional sensitivity analysis, only 19 parameters affected the optimized metabolic flux. Interestingly, of the 19 parameters, an increase or decrease by two fold Gal80p–Gal4p–DNA interaction parameters impacts the gene expression (Berkhout *et al*., 2013), which is consistent with our experimental results. According to the authors, this indicates that the gene regulatory network is more complex than required for achieving the galactose metabolic flux alone. The authors suggest that this complex nature of the gene regulatory network may allow the optimization of different objective functions, such as the ability to use different carbon sources by mutations which change only a given parameter (Berkhout *et al*., 2013). In contrast to this analysis, in our study a decrease in Gal80p–Gal4p interaction by twofold impacted the Gal3p–Gal80p interaction by fivefold. This further supports the idea that it is difficult to predict the outcomes of the network response subjecting it to mutational perturbation.

We observe that the altered strain is more fit in melibiose than galactose. The efficient utilization of melibiose could also be due to the reduced glucose repression of the altered *Sc*GAL switch as demonstrated in this study. Based on the above, we speculate that the ancestral GAL switch, functionally similar to the epistatically altered GAL switch, is optimal for the simultaneous utilization of glucose and galactose, products of hydrolysis of disaccharides such as lactose or melibiose. It is important to note that *K. lactis*, an aerobic yeast which lacks ultrasensitive response to galactose, is adept in utilizing lactose and accordingly *Kl*GAL switch is not severely repressed by glucose (Rubio-Texeira, 2005). Thus, the features observed in *S. cerevisiae*, such as facultative anaerobic life style, sever glucose repression and ultrasensitive response to galactose is more compatible with the efficient and hierarchical

utilization of glucose and galactose. To the best of our knowledge, the only other known example where in *S. cerevisiae* demonstrates ultrasensitivity as compared to *K. lactis* is in MAP kinase pathway (Coyle *et al*., 2013). We speculate that, evolution of ultrasensitivity in *S. cerevisiae* following genome duplication might be more pervasive than currently understood.

Interestingly, following genome duplication, many members of *sensu stricto* species have become facultative anaerobes (Merico *et al*., 2007) and lost the ability to utilize melibiose (Naumova *et al*., 2003), suggesting that they may be in the path of evolutionary transition. Moreover, it is intriguing to note that all the members of the *sensu stricto* group exhibit similar disposition of UAS_G elements in the promoter of the *GAL* genes (Kellis *et al*., 2003) but show variation in the gene copies of *GAL4, GAL1/3* and *GAL80* (Hittinger *et al*., 2004; Rokas and Hittinger, 2007). For example, *S. castellii* has retained two copies of *GAL3*, *GAL80* and *GAL4* while *S. bayanus* has retained two copies of only *GAL80* (Hittinger *et al*., 2004; Rokas and Hittinger, 2007). However, unlike *S. kudriavzevii* which has retained only *GAL1* but not *GAL3* (Hittinger *et al*., 2010), *S. bayanus* Gal3p has retained galactokinase activity (Hittinger *et al*., 2004). A detailed comparison of sugar utilization and kinetics of induction in these strains would provide a more detailed perspective of the role of ultrasensitivity in adaptive evolution. Thus, our study highlights, how epistasis can emerge following gene duplication and impinge on the regulation of gene expression by altering network dynamics which can alter not just one but many closely related traits to adapt to changing circumstances.

Experimental procedures

Media and growth conditions

Yeast cells were grown either in YPD or synthetic complete medium or synthetic medium lacking the appropriate amino acid (drop out) supplemented with 3% (v/v) glycerol plus 2% (v/v) potassium lactate or 2% (w/v) galactose or glucose (Amberg *et al*., 2005). 2-Deoxygalactose (2-DG) was added to a final concentration of 0.1% with glycerol plus lactate to detect constitutive expression of *GAL* genes (Platt, 1984). Wherever growth on galactose as a sole carbon source was scored, ethidium bromide (EtBr) was included in the medium at a concentration of 20 μg ml^{-1} (Matsumoto *et al*., 1978). G418 and Hygromycin B were used in YPD medium to a final concentration of 200 μg ml^{-1} (Wach *et al*., 1994; Goldstein and McCusker, 1999).

Plasmids

Plasmid pYJM-3 is a *URA3* based multicopy plasmid containing *GAL3* under the control of *CYC1* promoter and pYJM is the corresponding parent plasmid (Murthy and Jayadeva, 2000). pT1-3B (Torchia and Hopper, 1986) is *URA3* based multicopy plasmid containing *GAL3* under its own promoter and the corresponding vectors plasmids is YEp24 (New England Biolab). pT80-3 is a multicopy plasmid bearing *GAL80* under its own promoter (Torchia and Hopper, 1986). pHS71, pHS141 and pHS222 contain 4.1 kb genomic fragment corresponding to $GAL80^{S-0}$, $GAL80^{S-1}$ and $GAL80^{S-2}$ alleles in YEp24 (Nogi *et al*., 1984). pAP45 is a multicopy plasmid derived from pYEX-BX (Clontech) bearing His tagged *GAL3* under Cu^{++} inducible *CUP1* promoter (Platt and Reece, 1998). pL754-4 contain 2.7 Kb BamHI–HindDIII genomic fragment corresponding to complete ORF of *GAL4* and 26 bases of 5′ untranslated and 239 bases of 3′ untranslated region, cloned in to pL754 (Promega). *GAL4 T859I* substitution (hence forth will be called as $GAL4^C$) allele was introduced by site-directed mutagenesis in pL754-4 and was performed using 'Quickchange site-directed mutagenesis protocol' originally developed and further modified by Stratagene. Mutation was confirmed by sequencing. The resultant plasmid containing $GAL4^C$ allele was named pAKD04. pGPDHIS80 (Murthy, 2000) constructed by cloning His tagged *GAL80* amplified from genomic DNA at BamHI, HinDIII sites of PGPD426 (Mumberg *et al*., 1995). His tagged $GAL80^{S-1}$ and $GAL80^{S-2}$ were cloned by amplifying $GAL80^{S-1}$ and $GAL80^{S-2}$ from pHS141 and pHS222 respectively at BamHI, HinDIII in PGPD426. C-terminal Activation Domain (amino acids 768–881) of *GAL4* and $GAL4^C$ were amplified from plasmid pL754-4 and pAKD04 respectively and cloned in to pGEX4T1 (GE Healthcare) at EcoRI site. The resultant plasmids express GST tagged *GAL4* or $GAL4^C$.

Strains

Saccharomyces cerevisiae used in this study are isogenic and listed in Table 1. *K. lactis* wild type strain JA6 (*MATα trp1-11 ura3-12 ade1-600 adeT-600*) used in this study was a generous gift from Dr K. D. Breunig (Breunig and Kuger, 1987). Genetic manipulation was carried out by lithium acetate mediated transformation (Gietz and Woods, 2002). Sc285 (Torchia and Hopper, 1986) expresses *GAL* genes constitutively due to a 0.595 kb deletion internal to *GAL80* open reading frame extending between two BglII sites. For integrating the mutant alleles of *GAL80*, Sc285 was co-transformed by 2.1 kb AccI and HinDIII fragment from pHS71, pHS141 and pHS222 containing the mutant versions of the *GAL80* with marker plasmid. The transformants containing the integrated *GAL80* alleles were then screened for the lack of constitutive expression of *GAL* genes by observing the colonies that are able to grow on glycerol plus lactate medium in presence of 2-DG. These putative integrants were then tested for the integration event by PCR amplification using the *GAL80* specific flanking primers. $GAL4^C$ allele (from pAKD04) was introduced to the strain containing wild type or super repressors of *GAL80* by integrating as BamHI and HinDIII fragment (Supporting Text, section S1). Gene disruption using *KanMX4* (Wach *et al*., 1994) was carried out by amplifying *KanMX4* cassette from the genomic DNA of corresponding gene deletion strain obtained from EUROSCARF. *HygMX4* cassette used for gene disruption was amplified from plasmid pAG32 (Goldstein and McCusker, 1999) (EUROSCARF).

Table 1. List of strains.

Strain	Genotype	Source/Reference
Sc 644(WT)	*MATa MEL1ade1 ile trp2-112 leu2-3,112 ura3-52*	J.E. Hopper (Johnston and Hopper, 1982)
Sc 252(WT)	*MATa MEL1ade1 ile leu2-3,112 ura3-52*	J.E. Hopper (Johnston and Hopper, 1982)
Sc 414	*MATa MEL1 ade1 ile ura3-52 gal4Δ gal80Δ*	J.E. Hopper (Mylin *et al.*, 1989)
Sc 285	*MATa MEL1 ade1 ile ura3-52 leu2-3,112 gal80Δ*	J.E. Hopper (Torchia *et al.*, 1984)
Sc 385	*MATα MEL1 ade1 ile ura3-52 leu2-3,112 gal3::LEU2*	J.E. Hopper (Torchia and Hopper, 1986)
BY4742*gal1Δ*	BY4742; *Mat α; his3Δ; leu2Δ; lys2Δ; ura3Δ; YBR020W::KanMX4*	EUROSCARF
BY4742*gal3Δ*	BY4742; *Mat α; his3Δ; leu2Δ; lys2Δ; ura3Δ; YDR009W::KanMX4*	EUROSCARF
BY4742*gal80Δ*	BY4742; *Mat α; his3Δ; leu2Δ; lys2Δ; ura3Δ; YML051W::KanMX4*	EUROSCARF
Sc MTQ 01	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}0}$ (G301R)GAL4*	This study
Sc MTQ 02	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ (G323R)GAL4*	This study
Sc MTQ 03	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}2}$ (E351K)GAL4*	This study
Sc AKD 45	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80 GAL4^{C}(T859I)*	This study
Sc AKD 06	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}0}$ GAL4^{C}*	This study
Sc AKD 07	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C}*	This study
Sc AKD 08	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}2}$ GAL4^{C}*	This study
Sc AKD 13	*MATa MEL1ade1 ile trp2-112 leu2-3,112 ura3-52 GAL1::KanMX4*	This study
Sc AKD 14	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ (G323R)GAL4 GAL1::KanMX4*	This study
Sc AKD 15	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C} GAL1::KanMX4*	This study
Sc AKD 17	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C} GAL3::KanMX4*	This study
Sc AKD 32	*MATa MEL1ade1 ile leu2-3,112 ura3-52 URA3::GAL1-LacZ*	This study
Sc AKD 23	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C} URA3::GAL1-LacZ*	This study
Sc AKD 46	*MATa MEL1ade1 ile leu2-3,112 ura3-52 URA3::GAL1-LacZ GAL1::KanMX4*	This study
Sc AKD 25	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C} URA3::GAL1-LacZ GAL1::KanMX4*	This study
Sc AKD 47	*MATa MEL1ade1 ile leu2-3,112 ura3-52 URA3::GAL1-LacZ GAL3::KanMX4*	This study
Sc AKD 27	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C} URA3::GAL1-LacZ GAL3::KanMX4*	This study
Sc AKD 31	*MATa MEL1 ade1 ile ura3-52 leu2-3,112 gal80Δ URA3::GAL1-LacZ*	This study
Sc AKD 29	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C} URA3::GAL1-LacZ GAL80::KanMX4*	This study
Sc AKD 30	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C} URA3::GAL1-LacZ GAL1::HygMX4 GAL3::KanMX4*	This study
Sc AKD 41	*MATa MEL1ade1 ile leu2-3,112 ura3-52 LYS2::KanMX4*	This study
Sc AKD 42	*MATa MEL1ade1 ile leu2-3,112 ura3-52LYS2::HygMX4*	This study
Sc AKD 44	*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C} LYS2::HygMX4*	This study

GAL1-LacZ reporter cassette was introduced at *URA3* locus by linearizing plasmid pGAL1-LacZ (Li *et al.*, 2002) by digesting with StuI followed by transformation and selection on ura drop out plate.

Genetic cross and tetrad analysis

Our laboratory strain bearing *GAL80$^{S\text{-}1}$GAL4^{C}* alleles (*MATa MEL1 ade1 ile leu2-3,112 ura3-52 GAL80$^{S\text{-}1}$ GAL4^{C}*) was crossed with BY4742 *gal80Δ* strain (BY4742; *Mat α*; *his3Δ*; *leu2Δ*; *lys2Δ*; *ura3Δ*; *YML051W::KanMX4*) obtained from EUROSCARF. Diploid was selected according to the markers and plated on to sporulation medium containing 0.5% potassium acetate (Amberg *et al.*, 2005). Spores were dissected and five independent segregants bearing *GAL80$^{S\text{-}1}$GAL4^{C}* alleles were selected on the basis of their growth on galactose, susceptibility to G418 and ability to grow on glycerol plus lactate medium in presence of 2-DG.

Spotting assay

Growth phenotype was scored by spotting assay. Strains or transformants were pre-grown in synthetic complete or drop out glycerol plus lactate medium (gly/lac) to stationary phase. Cells were harvested, washed with sterile water and 10-fold serial dilution was done according to their normalized cell densities. A fixed volume of diluted samples were spotted on to the culture plates containing assay media as indicated. Growth phenotype was analysed after incubation depending upon the experiment.

Growth analysis

Wild type and epistatically altered (Mutant) strains were pre-grown in synthetic complete gly/lac medium to stationary phase. Approximately equal amount of cells from each strain were taken by normalizing their cell densities and freshly inoculated into media containing different concentrations of galactose or melibiose as indicated. Growth of each strain in different medium was monitored by measuring the cell densities at regular intervals. Growth profile was generated by plotting OD_{600} versus time. Wild type and epistatically altered (Mutant) strains were pre-grown on synthetic complete gly/lac medium to stationary phase. Cell cultures were inoculated in synthetic complete media containing 2% galactose to an initial OD_{600} of 0.1. And OD_{600} was measured at 1 h intervals

to determine the time to first doubling (Hittinger *et al.*, 2010). Each experiment was done in triplicates and results represent average of four independent experiment with standard deviation.

Western blotting

Yeast strains or transformants carrying the appropriate plasmids were grown in synthetic complete or drop out medium containing gly/lac as the carbon source up to an OD_{600} of 0.3. Galactose to a final concentration of 1% was added whenever required. After 4 h of galactose addition, cells were harvested by centrifugation at 5000 rpm for 5 min and then washed once with cold autoclaved double distilled water and re-suspended in Y-PER Yeast Protein Extraction Reagent (Thermo Scientific). Protease inhibitor cocktail and PMSF were added to the final concentration of 2.5 mM and 1 mM respectively and kept at room temperature for 40 min in a vertical cell mixer. Cell-free extract was obtained by centrifuging at 10 000 rpm for 20 min at 4°C. Protein content was estimated using BCA protein estimation kit (Thermo Scientific). Cell-free extract containing known amount of protein was boiled with SDS buffer for 10 min and resolved in SDS-PAGE, transferred to PVDF membrane. After transfer to PVDF membrane, blot was treated with blocking buffer (PBS pH8.0 containing 1% w/v milk powder). The blot was then probed with primary antibodies/antisera and incubated for 1 h. Then the membrane was washed thrice with PBS and Tween 20 (0.3% v/v). The blot was then probed with the secondary antibody (Sigma) conjugated with alkaline phosphatase. Membrane was washed three times with PBS and Tween 20 (0.3% v/v). Finally, blot was developed with developing buffer containing NBT and BCIP. Every experiment was repeated at least three times. Gal3p antibody (Lakshminarasimhan and Bhat, 2005) and Gal80p antisera (Diep *et al.*, 2008) used in this study have been described earlier. Strains used Gal3p Western blot were deleted for *GAL1*, the paralogue of *GAL3*, to eliminate the background signal. His antibody used in this study was obtained from Santacruz Biotech. Zwf1p antibody (Sigma) has been used as loading control in separate blot when ever required.

α-Galactosidase assay (MEL1 activity)

To determine the expression level of the *MEL1*, α-galactosidase assay was carried out. Yeast strains or transformants carrying the appropriate plasmids were grown in synthetic complete or drop out medium containing gly/lac as the carbon source up to an OD_{600} of 0.3. Galactose to a final concentration of 1% was added and both uninduced and the induced cultures were incubated for an additional 4 h. One millilitre of each culture was centrifuged and α-galactosidase activity was determined in the supernatant. One hundred and twenty microlitres of the supernatant was mixed with 360 µl of assay buffer [2 volumes of 0.5 m sodium acetate, pH 4.5, and 1 volume of 100 mm p-nitrophenyl α-D-galactopyranoside (Sigma)]. The reaction was incubated at 30°C for 5 h and terminated by adding 520 µl of stop buffer (1 M sodium carbonate), and the absorbance at 410 nm was measured (Diep *et al.*, 2006). The selective media served as the blank. *MEL1* activity is presented as OD_{410} per unit OD_{600}. Triplicate samples were taken for the analysis and results represent average of at least three independent experiment with standard deviation.

β-Galactosidase assay

GAL1-LacZ reporter activity was measured using a cell permeabilization method. Yeast strains or transformants carrying the appropriate plasmids were grown in synthetic complete or drop out medium containing gly/lac as the carbon source up to an OD_{600} of 0.3. Galactose to a final concentration of 1% was added and both uninduced and induced cultures were incubated for an additional 4 h. One millilitre of each culture was pelleted down, washed with Z buffer (100 mM Sodium phosphate buffer pH 7.0, 10 mM KCl, 1 mM $MgSO_4$ without BME) and resuspended in 150 µl of Z buffer with 0.3% BME. Twenty microlitres of 0.1% SDS and 50 µl chloroform were added. Samples were mixed vigorously for 15 s, 700 µl of pre-warmed Z buffer containing 0.3 % β-mercaptoethanol and 1 mg ml^{-1} ONPG was added and reactions were allowed at 30°C till pale yellow colour appeared. Reactions were stopped by addition of 500 µl 1 M $NaCO_3$ and time was noted. Reaction tubes were centrifuged at 13 000 rpm for 10 min and the OD at 420 nm of the supernatant was measured. β-Galactosidase activity was calculated as: Miller unit = $[1000 \times OD_{420}]/[OD_{600} \times$ Vol (1 ml) $\times$ Reaction time (min)] (Muratani *et al.*, 2005). Triplicate samples were taken for the analysis and results represent average of at least three independent experiment with standard deviation.

Gal3p and Gal80p interaction assays

pAP45 bearing His tagged *GAL3* was expressed as described (Platt and Reece, 1998) in a strain lacking *GAL80* (Sc285). Cells were harvested by centrifugation at 5000 rpm for 5 min and then washed once with cold autoclaved double distilled water and re-suspended in Y-PER Yeast Protein Extraction Reagent (Thermo Scientific). Protease inhibitor cocktail and PMSF were added to the final concentration of 2.5 mM and 1 mM respectively and kept at room temperature for 40 min in a vertical cell mixer. Cell-free extract was obtained by centrifuging at 10 000 rpm for 20 min at 4°C. Protein content was estimated using BCA protein estimation kit (Thermo Scientific). Cell-free extract with known amount of protein (500 or 1000 or 2000 µg as described) was immobilized on to Ni-NTA agarose (Qiagen) in Eppendorf tube at 4°C. HisGal3p was purified by removing non-specific proteins by washing with buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole-pH8.0). *GAL80, GAL80^{S-0}, GAL80^{S-1}* and *GAL80^{S-2}* were expressed from plasmids pT80-3, pHS71, pHS141 and pHS222, respectively, in a strain lacking *GAL4* and *GAL80* (Sc414; Mylin *et al.*, 1989). Empty vector YEp24 was taken as control. Cell-free extract preparation and protein estimation was done by the above described method. Cell-free extract containing approximately 1500 µg of protein was added to the immobilized HisGal3p. Galactose and ATP was added to final concentration of 50 mM and 2 mm respectively and placed in a vertical cell mixer at 4°C. After 2 h,

unbound proteins were washed off by adding wash buffer supplemented with 50 mM galactose and 2 mM ATP. HisGal3-Gal80 complex were then boiled for 10 min in 40 µl of 1× SDS buffer containing imidazole to a final concentration 250 mM. The samples were analysed by Western blotting separately with His antibody and Gal80p antisera. All experiments were done at least three times.

Expression of GST tagged Gal4p and Gal4^{C}p activation domain (AD)

Plasmids bearing GST tagged C-terminal of *GAL4* and *GAL4^{C}* correspond to Activation Domain were separately expressed in *E. coli* BL21. Overnight cultures in LB were freshly inoculated in flasks containing LB supplemented with 0.5% glucose and allowed to grow at 37°C to an OD_{600} of 1. IPTG was added to a final concentration of 1 mM and cultures were grown at 25°C for additional 8–10 h. Cells were harvested by centrifugation at 2000 rpm for 5 min and then washed once with cold Phosphate buffered saline (PBS), 10 mM phosphate buffer, pH 7.4, 150 mM NaCl and stored in –80°C for further analysis.

Purification of His tagged Gal80p wild type and super-repressors

His tagged *GAL80* and super-repressor alleles were expressed in a yeast strain lacking *GAL4* and *GAL80* (Sc414). Plasmids bearing Transformants bearing His tagged *GAL80* and super-repressor alleles were grown in uracil drop out glucose medium to an OD_{600} of 0.5. Cells were harvested by centrifugation at 5000 rpm for 5 min and then washed once with cold autoclaved double distilled water and re-suspended in Y-PER Yeast Protein Extraction Reagent (Thermo Scientific). Protease inhibitor cocktail and PMSF were added to the final concentration of 2.5 mM and 1 mM respectively and kept at room temperature for 40 min in a vertical cell mixer. Cell-free extract was obtained by centrifuging at 10 000 rpm for 20 min at 4°C. Cell-free extracts from each was immobilized on to with Ni-NTA agarose (Qiagen) in Eppendorf tube at 4°C. HisGal80p was purified by removing non-specific proteins by washing with buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole-pH8.0). Purified proteins were eluted by adding elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 200 mM imidazole-pH8.0) and stored in –80°C. Protein content was estimated using BCA protein estimation kit (Thermo Scientific).

Gal4p (AD) Gal80p interaction assays

E. coli (BL21) cells induced for GST tagged AD of Gal4p or Gal4^{C}p, stored at –80°C (see previous section) were thawed and re-suspended in BugBuster HT Protein Extraction Reagent (Novagen). Protease inhibitor cocktail and PMSF were added to the final concentration of 2.5 mM and 1 mM respectively and kept at room temperature for 20 min in a vertical cell mixer. Cell-free extract was obtained by centrifuging at 10 000 rpm for 20 min at 4°C. Protein content was estimated using BCA protein estimation kit (Thermo Scientific). Cell-free extract containing equal amount of GST tagged AD of Gal4 or Gal4^{C} protein was immobilized on to Glutathione Agarose (Sigma) in Eppendorf tube at 4°C and unbound proteins were removed by washing with PBS-T (1% triton-100). To this immobilized GST tagged AD of Gal4 and Gal4^{C}, purified His tagged Gal80, Gal80$^{S\text{-}1}$ and Gal80$^{S\text{-}2}$ (20 µg each) proteins were added. Binding was allowed for 2 h. Unbound Gal80 proteins were removed by washing with washing buffer. Gal4p–Gal80p complex were then boiled for 10 min in 40 µl of 1× SDS buffer containing reduced glutathione to a final concentration 10 mM. The samples were analysed by Western blotting separately with Gal80p antisera. All experiments were done at least three times. Purified Gal80p was used as the control. Gal80p input was determined by Western blot while Gal4p input was determined by Coomassie staining.

Quantification of relative binding affinity

Intensity of bands obtained by Western blot was quantified by using ImageJ software. The intensities were in linear range. Relative affinity was determined by factoring in the dilutions of sample used in Western blot.

Statistics

Data were analysed in Origin and *P*-value was calculated using one-way ANOVA when ever required.

Competition assays

The fitness of epistatically altered strain relative to the wild type was assayed by conducting competition assay. Both strains were tagged with different antibiotic markers. Competitions were performed at 30°C in the synthetic medium containing galactose or melibiose as indicated. Before each fitness assay, the two competitors were grown separately in neutral medium (gly/lac) up to saturation. Approximately equal number of cells from each competitor was inoculated in the same medium to a final cell density of 2×10^5. Immediately sample was taken, diluted and plated for determining cell count at zero time point. After 24 h, the cultures were diluted and plated for determining the final cell count. The fitness of the epistatically altered strain relative to the wild type was calculated as ln[EA(f)/EA(0)]/ln[WT(f)/WT(0)], where EA(0) and WT(0) are the initial densities of the epistatically altered and wild type strain, respectively, and EA(f) and WT(f) represent the corresponding densities at the end of the competition (Gagneux *et al.*, 2006). For control, wild type strain was competed with wild type by tagging two different antibiotics marker. The relative fitness calculated by the above formula was deducted from 1 and is presented as fitness value for each strain. Cells were diluted in such a way that approximately 200 colonies per plate were obtained to ensure that the counting error is minimized. For each sample at least three plates were used. Each experiment was done in triplicates and results represent average of three independent experiments with standard deviation.

Acknowledgements

We would like to thank DST, CSIR and BRNS for financial support to P.J.B. Research fellowships from UGC to A.K.D., CSIR to M.T.Q. and R.K.K. are greatly acknowledged.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceived and designed the experiments: A.K.D., P.J.B. Performed the experiments: A.K.D., M.T.Q., R.K.K. Analysed data: A.K.D., P.J.B. Wrote the paper: A.K.D., P.J.B.

References

Acar, M., Becskei, A., and van Oudenaarden, A. (2005) Enhancement of cellular memory by reducing stochastic transitions. *Nature* **435:** 228–232.

Amberg, D.C., Burke, D.J., and Strathern, J.N. (2005) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*. New York: Cold Spring Harbor Laboratory Press.

Berkhout, J., Teusink, B., and Bruggeman, F.J. (2013) Gene network requirements for regulation of metabolic gene expression to a desired state. *Sci Rep* **3:** 1417.

Bhat, P.J., and Hopper, J.E. (1992) Overproduction of the GAL1 or GAL3 protein causes galactose-independent activation of the GAL4 protein: evidence for a new model of induction for the yeast GAL/MEL regulon. *Mol Cell Biol* **12:** 2701–2707.

Bhat, P.J., and Murthy, T.V. (2001) Transcriptional control of the GAL/MEL regulon of yeast *Saccharomyces cerevisiae*: mechanism of galactose-mediated signal transduction. *Mol Microbiol* **40:** 1059–1066.

Biggar, S.R., and Crabtree, G.R. (2001) Cell signaling can direct either binary or graded transcriptional responses. *EMBO J* **20:** 3167–3176.

Breunig, K.D., and Kuger, P. (1987) Functional homology between the yeast regulatory proteins GAL4 and LAC9: LAC9-mediated transcriptional activation in *Kluyveromyces lactis* involves protein binding to a regulatory sequence homologous to the GAL4 protein-binding site. *Mol Cell Biol* **7:** 4400–4406.

Buchler, N.E., and Louis, M. (2008) Molecular titration and ultrasensitivity in regulatory networks. *J Mol Biol* **384:** 1106–1119.

Carroll, S.B. (2005) Evolution at two levels: on genes and form. *PLoS Biol* **3:** e245.

Carroll, S.B. (2008) Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. *Cell* **134:** 25–36.

Chan, Y.F., Marks, M.E., Jones, F.C., Villarreal, G., Jr, Shapiro, M.D., Brady, S.D., *et al*. (2010) Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a Pitx1 enhancer. *Science* **327:** 302–305.

Chou, H.H., Delaney, N.F., Draghi, J.A., and Marx, C.J. (2014) Mapping the fitness landscape of gene expression uncovers the cause of antagonism and sign epistasis between adaptive mutations. *PLoS Genet* **10:** e1004149.

Coyle, S.M., Flores, J., and Lim, W.A. (2013) Exploitation of latent allostery enables the evolution of new modes of MAP kinase regulation. *Cell* **154:** 875–887.

Czyz, M., Nagiec, M.M., and Dickson, R.C. (1993) Autoregulation of GAL4 transcription is essential for rapid growth of *Kluyveromyces lactis* on lactose and galactose. *Nucleic Acids Res* **21:** 4378–4382.

Diep, C.Q., Peng, G., Bewley, M., Pilauri, V., Ropson, I., and Hopper, J.E. (2006) Intragenic suppression of Gal3C interaction with Gal80 in the *Saccharomyces cerevisiae* GAL gene switch. *Genetics* **172:** 77–87.

Diep, C.Q., Tao, X., Pilauri, V., Losiewicz, M., Blank, T.E., and Hopper, J.E. (2008) Genetic evidence for sites of interaction between the Gal3 and Gal80 proteins of the *Saccharomyces cerevisiae* GAL gene switch. *Genetics* **178:** 725–736.

Douglas, H.C., and Hawthorne, C.D. (1972) Uninducible mutants in the gal i locus of *Saccharomyces cerevisiae*. *J Bacteriol* **109:** 1139–1143.

Frankel, N., Erezyilmaz, D.F., McGregor, A.P., Wang, S., Payre, F., and Stern, D.L. (2011) Morphological evolution caused by many subtle-effect substitutions in regulatory DNA. *Nature* **474:** 598–603.

Gagneux, S., Long, C.D., Small, P.M., Van, T., Schoolnik, G.K., and Bohannan, B.J. (2006) The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science* **312:** 1944–1946.

Gietz, R.D., and Woods, R.A. (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* **350:** 87–96.

Goldstein, A.L., and McCusker, J.H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15:** 1541–1553.

Gompel, N., Prud'homme, B., Wittkopp, P.J., Kassner, V.A., and Carroll, S.B. (2005) Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in Drosophila. *Nature* **433:** 481–487.

Griggs, D.W., and Johnston, M. (1991) Regulated expression of the GAL4 activator gene in yeast provides a sensitive genetic switch for glucose repression. *Proc Natl Acad Sci USA* **88:** 8597–8601.

Hittinger, C.T., and Carroll, S.B. (2007) Gene duplication and the adaptive evolution of a classic genetic switch. *Nature* **449:** 677–681.

Hittinger, C.T., Rokas, A., and Carroll, S.B. (2004) Parallel inactivation of multiple GAL pathway genes and ecological diversification in yeasts. *Proc Natl Acad Sci USA* **101:** 14144–14149.

Hittinger, C.T., Goncalves, P., Sampaio, J.P., Dover, J., Johnston, M., and Rokas, A. (2010) Remarkably ancient balanced polymorphisms in a multi-locus gene network. *Nature* **464:** 54–58.

Hoekstra, H.E., and Coyne, J.A. (2007) The locus of evolution: *evo devo* and the genetics of adaptation. *Evolution* **61:** 995–1016.

Hong, K.K., Vongsangnak, W., Vemuri, G.N., and Nielsen, J. (2011) Unravelling evolutionary strategies of yeast for improving galactose utilization through integrated systems

level analysis. *Proc Natl Acad Sci USA* **108:** 12179–12184.
Jacob, F. (1977) Evolution and tinkering. *Science* **196:** 1161–1166.
Johnston, M., Flick, J.S., and Pexton, T. (1994) Multiple mechanisms provide rapid and stringent glucose repression of GAL gene expression in *Saccharomyces cerevisiae*. *Mol Cell Biol* **14:** 3834–3841.
Johnston, S.A., and Hopper, J.E. (1982) Isolation of the yeast regulatory gene GAL4 and analysis of its dosage effects on the galactose/melibiose regulon. *Proc Natl Acad Sci USA* **79:** 6971–6975.
Keegan, L., Gill, G., and Ptashne, M. (1986) Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science* **231:** 699–704.
Kellis, M., Patterson, N., Endrizzi, M., Birren, B., and Lander, E.S. (2003) Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* **423:** 241–254.
King, M.C., and Wilson, A.C. (1975) Evolution at two levels in humans and chimpanzees. *Science* **188:** 107–116.
Lakshminarasimhan, A., and Bhat, P.J. (2005) Replacement of a conserved tyrosine by tryptophan in Gal3p of *Saccharomyces cerevisiae* reduces constitutive activity: implications for signal transduction in the GAL regulon. *Mol Genet Genomics* **274:** 384–393.
Lavy, T., Kumar, P.R., He, H., and Joshua-Tor, L. (2012) The Gal3p transducer of the GAL regulon interacts with the Gal80p repressor in its ligand-induced closed conformation. *Genes Dev* **26:** 294–303.
Li, H., and Johnson, A.D. (2010) Evolution of transcription networks–lessons from yeasts. *Curr Biol* **20:** R746–R753.
Li, X.Y., Bhaumik, S.R., Zhu, X., Li, L., Shen, W.C., Dixit, B.L., and Green, M.R. (2002) Selective recruitment of TAFs by yeast upstream activating sequences. Implications for eukaryotic promoter structure. *Curr Biol* **12:** 1240–1244.
Lynch, V.J., and Wagner, G.P. (2008) Resurrecting the role of transcription factor change in developmental evolution. *Evolution* **62:** 2131–2154.
Ma, J., and Ptashne, M. (1987a) A new class of yeast transcriptional activators. *Cell* **51:** 113–119.
Ma, J., and Ptashne, M. (1987b) Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell* **48:** 847–853.
McGregor, A.P., Orgogozo, V., Delon, I., Zanet, J., Srinivasan, D.G., Payre, F., and Stern, D.L. (2007) Morphological evolution through multiple cis-regulatory mutations at a single gene. *Nature* **448:** 587–590.
McLean, C.Y., Reno, P.L., Pollen, A.A., Bassan, A.I., Capellini, T.D., Guenther, C., *et al*. (2011) Human-specific loss of regulatory DNA and the evolution of human-specific traits. *Nature* **471:** 216–219.
Matsumoto, K., Toh-e, A., and Oshima, Y. (1978) Genetic control of galactokinase synthesis in *Saccharomyces cerevisiae*: evidence for constitutive expression of the positive regulatory gene gal4. *J Bacteriol* **134:** 446–457.
Melcher, K., and Xu, H.E. (2001) Gal80-Gal80 interaction on adjacent Gal4p binding sites is required for complete GAL gene repression. *EMBO J* **20:** 841–851.
Merico, A., Sulo, P., Piskur, J., and Compagno, C. (2007) Fermentative lifestyle in yeasts belonging to the Saccharomyces complex. *FEBS J* **274:** 976–989.
Mumberg, D., Muller, R., and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156:** 119–122.
Muratani, M., Kung, C., Shokat, K.M., and Tansey, W.P. (2005) The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing. *Cell* **120:** 887–899.
Murthy, T.V., and Jayadeva, B.P. (2000) Disruption of galactokinase signature sequence in gal3p of *Saccharomyces cerevisiae* does not lead to loss of signal transduction function. *Biochem Biophys Res Commun* **273:** 824–828.
Murthy, T.V.S. (2000) Structure Function Correlation of Gal3 Protein of *Saccharomyces cerevisiae*. Theses submitted to IIT Bombay. IIT Bombay.
Mylin, L.M., Bhat, J.P., and Hopper, J.E. (1989) Regulated phosphorylation and dephosphorylation of GAL4, a transcriptional activator. *Genes Dev* **3:** 1157–1165.
Naumova, E.S., Korshunova, I.V., and Naumov, G.I. (2003) [Molecular analysis of alpha-galactosidase MEL genes from *Saccharomyces sensu stricto*]. *Mol Biol (Mosk)* **37:** 825–833.
Nogi, Y., Matsumoto, K., Toh-e, A., and Oshima, Y. (1977) Interaction of super-repressible and dominant constitutive mutations for the synthesis of galactose pathway enzymes in *Saccharomyces cerevisiae*. *Mol Gen Genet* **152:** 137–144.
Nogi, Y., Shimada, H., Matsuzaki, Y., Hashimoto, H., and Fukasawa, T. (1984) Regulation of expression of the galactose gene cluster in *Saccharomyces cerevisiae*. II. The isolation and dosage effect of the regulatory gene GAL80. *Mol Gen Genet* **195:** 29–34.
Piskur, J., and Langkjaer, R.B. (2004) Yeast genome sequencing: the power of comparative genomics. *Mol Microbiol* **53:** 381–389.
Piskur, J., Rozpedowska, E., Polakova, S., Merico, A., and Compagno, C. (2006) How did Saccharomyces evolve to become a good brewer? *Trends Genet* **22:** 183–186.
Platt, A., and Reece, R.J. (1998) The yeast galactose genetic switch is mediated by the formation of a Gal4p–Gal80p–Gal3p complex. *EMBO J* **17:** 4086–4091.
Platt, T. (1984) Toxicity of 2-deoxygalactose to *Saccharomyces cerevisiae* cells constitutively synthesizing galactose-metabolizing enzymes. *Mol Cell Biol* **4:** 994–996.
Ramsey, S.A., Smith, J.J., Orrell, D., Marelli, M., Petersen, T.W., de Atauri, P., *et al*. (2006) Dual feedback loops in the GAL regulon suppress cellular heterogeneity in yeast. *Nat Genet* **38:** 1082–1087.
Rokas, A., and Hittinger, C.T. (2007) Transcriptional rewiring: the proof is in the eating. *Curr Biol* **17:** R626–R628.
Rubio-Texeira, M. (2005) A comparative analysis of the GAL genetic switch between not-so-distant cousins: *Saccharomyces cerevisiae* versus *Kluyveromyces lactis*. *FEMS Yeast Res* **5:** 1115–1128.
Salmeron, J.M., Jr, Langdon, S.D., and Johnston, S.A. (1989) Interaction between transcriptional activator protein LAC9 and negative regulatory protein GAL80. *Mol Cell Biol* **9:** 2950–2956.

Salmeron, J.M., Jr, Leuther, K.K., and Johnston, S.A. (1990) GAL4 mutations that separate the transcriptional activation and GAL80-interactive functions of the yeast GAL4 protein. *Genetics* **125:** 21–27.

Shapiro, M.D., Marks, M.E., Peichel, C.L., Blackman, B.K., Nereng, K.S., Jonsson, B., *et al.* (2004) Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* **428:** 717–723.

Stern, D.L., and Orgogozo, V. (2009) Is genetic evolution predictable? *Science* **323:** 746–751.

Streisfeld, M.A., and Rausher, M.D. (2011) Population genetics, pleiotropy, and the preferential fixation of mutations during adaptive evolution. *Evolution* **65:** 629–642.

Torchia, T.E., and Hopper, J.E. (1986) Genetic and molecular analysis of the GAL3 gene in the expression of the galactose/melibiose regulon of *Saccharomyces cerevisiae*. *Genetics* **113:** 229–246.

Torchia, T.E., Hamilton, R.W., Cano, C.L., and Hopper, J.E. (1984) Disruption of regulatory gene GAL80 in *Saccharomyces cerevisiae*: effects on carbon-controlled regulation of the galactose/melibiose pathway genes. *Mol Cell Biol* **4:** 1521–1527.

Venturelli, O.S., El-Samad, H., and Murray, R.M. (2012) Synergistic dual positive feedback loops established by molecular sequestration generate robust bimodal response. *Proc Natl Acad Sci USA* **109:** E3324–E3333.

Verma, M., Bhat, P.J., and Venkatesh, K.V. (2003) Quantitative analysis of GAL genetic switch of *Saccharomyces cerevisiae* reveals that nucleocytoplasmic shuttling of Gal80p results in a highly sensitive response to galactose. *J Biol Chem* **278:** 48764–48769.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10:** 1793–1808.

Weirauch, M.T., and Hughes, T.R. (2010) Conserved expression without conserved regulatory sequence: the more things change, the more they stay the same. *Trends Genet* **26:** 66–74.

Wittkopp, P.J., Haerum, B.K., and Clark, A.G. (2004) Evolutionary changes *in cis* and *trans* gene regulation. *Nature* **430:** 85–88.

Wolfe, K.H., and Shields, D.C. (1997) Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **387:** 708–713.

Yano, K., and Fukasawa, T. (1997) Galactose-dependent reversible interaction of Gal3p with Gal80p in the induction pathway of Gal4p-activated genes of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **94:** 1721–1726.

Zenke, F.T., Zachariae, W., Lunkes, A., and Breunig, K.D. (1993) Gal80 proteins of *Kluyveromyces lactis* and *Saccharomyces cerevisiae* are highly conserved but contribute differently to glucose repression of the galactose regulon. *Mol Cell Biol* **13:** 7566–7576.

Zenke, F.T., Kapp, L., and Breunig, K.D. (1999) Regulated phosphorylation of the Gal4p inhibitor Gal80p of *Kluyveromyces lactis* revealed by mutational analysis. *Biol Chem* **380:** 419–430.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.