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Report

# A Yeast Catabolic Enzyme Controls Transcriptional Memory

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## Summary

**It has been postulated that chromatin modifications can persist through mitosis and meiosis, thereby securing memory of transcriptional states [1–4]. Whether these chromatin marks can self-propagate in progeny independently of relevant *trans*-acting factors is an important question in phenomena related to epigenesis. "Adaptive cellular memory" displayed by yeast cells offers a convenient system to address this question. The yeast *GAL* genes are slowly activated by Gal4 when cells are first exposed to galactose, but their progeny, grown in glucose media, exhibit a fast activation mode upon re-exposure to this sugar [5]. This "galactose memory" persists for several generations and was recently proposed to involve chromatin modifications and perinuclear topology of the *GAL* genes cluster [5, 6]. Here, we perform a heterokaryon assay demonstrating that this memory does not have a chromatin basis but is maintained by cytoplasmic factor(s) produced upon previous galactose induction. We show that Gal3, the cytoplasmic rate-limiting factor that releases the Gal4 activator, is dispensable for preserving galactose memory. Instead, the important memory determinant is a close Gal3 homolog, the highly expressed Gal1 galactokinase, the residual activity of which preserves memory in progeny cells by rapidly turning on the Gal4 activator upon cells' re-exposure to galactose.**

## Results and Discussion

### Analysis of Transcriptional Memory at a Single-Cell Level

The activation kinetics of *GAL* genes transcription has been previously studied by measuring their average mRNA levels in populations of yeast cells. In this report, we use yeast strains expressing Gal1-GFP or Gal7-GFP hybrid proteins and FACS analysis, which allows for the quantitative measurements of transcriptional kinetics and provides additional information on the structure of a yeast population at a single-cell resolution [7, 8]. We first show that this method can be used to confirm the previously described "galactose memory" phenomenon [6]. Yeast cultures pregrown in glucose responded to galactose by initially activating transcription of *GAL1* (Figures 1A and 1C) and *GAL7* (Figures S1A and S1C in the Supplemental Data available online) slowly and with a clear binary (either on or off) fashion over the time required to reach a fully activated uniform pattern. When the progeny cells of such a fully activated culture were consecutively grown back in glucose for 6–7 generations (12 hr) and then rechallenged with galactose, they reactivated *GAL1* and *GAL7* transcription rapidly and in a graded (uniform) pattern as opposed to the binary one displayed by their progenitors (Figures 1B and 1C; Figures S1B and S1C). This graded fashion of the second response indicates that every single cell in the yeast population acquires galactose memory characterized by the accelerated transcriptional activation rate of *GAL* genes. We should mention that when pregrown in no glucose media, such as in raffinose media, yeast cells respond to galactose for the first time with a graded and very rapid kinetics, masking the accelerated second response after consequent growth in glucose (data not shown).

### Transcriptional Memory Is Based on Cytoplasmic Determinants

It has been recently proposed that modified chromatin, such as that characterized by the deposition of H2A.Z histone variant [6] or that resulting from the function of the Swi/Snf chromatin remodeler [5], is required for the rapid reactivation of the *GAL1* promoter in cells that were previously exposed to galactose and consequently grown for some generations in glucose. This idea for a chromatin, or more generally nuclear, basis of memory was investigated by using a heterokaryon approach; normally, conjugation of haploid yeast cells of opposite mating types is followed by plasma membrane fusion and immediate nuclear fusion resulting in the formation of a diploid zygote. Mutations of genes required specifically for efficient nuclear fusion, such as the *kar1-1* mutation [9], allow plasma membarane fusion to occur, resulting in the formation of a heterokaryon cell, in which the two nuclei are kept separately in a common cytoplasmic environment. We exploited this nuclear fusion defect conferred by the *kar1-1* mutation and generated heterokarya derived from differently treated cells: glucose-grown (naive) *KAR1* cells carrying the *GAL1-GFP* allele were crossed with *kar1-1* mutants with no GFP-tagged *GAL1* that had been pregrown in galactose and successively cultured in glucose. Galactose induction of the crossed mixed cell population revealed rapid activation of the *GAL1-GFP* promoter specifically in heterokarya cells, as opposed to *GAL1-GFP*-carrying haploid cells (Figure 2A). Importantly, a much slower response was observed in heterokarya derived when crossing naive *GAL1-GFP* cells with *kar1-1* mutants continuously grown in glucose (Figure 2B). Thus, otherwise naive nuclei allow rapid transcriptional activation once they are found in a cytoplasmic environment that had been previously

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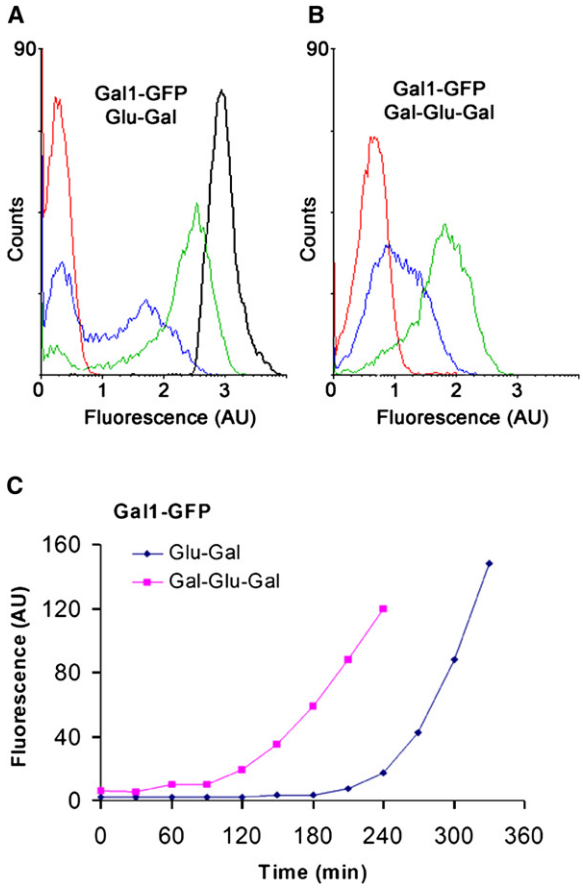


Figure 1. Galactose Activation and Reactivation in the Single-Cell Level

(A) Single-cell pattern of *GAL1-GFP* activation of glucose-pregrown cells. Histograms of FACS analysis at representative time points showing GFP fluorescence measurements taken from at least 10,000 yeast cells growing in glucose (red) and subsequently growing in galactose for 270 min (blue), 360 min (green), or 12 hr (black). The Gal1-GFP hybrid protein contains the entire Gal1 amino acid sequence and is expressed from the native GAL1-10 promoter and chromosomal position. Cells were cultured for at least 24 hr in rich YP-2% glucose medium, and then they were washed and transferred to YP-2% galactose medium, while kept at low cell density (OD < 0.2) throughout the experiment.

(B) *GAL1-GFP* reactivation of cells previously exposed to galactose. Histograms representing GFP distributions taken at 0 min (red), 120 min (blue), and 210 min (green) after galactose reinduction. Cells were grown for 24 hr in YP-2% galactose medium, washed, and transferred in YP-2% glucose for 12 hr before they are reinduced in galactose and used for FACS analysis as in (A). Optical density measurements and colony formation ability indicated that yeast cells have undergone 6 to 7 cell divisions during 12 hr culture in glucose.

(C) Activation kinetics of the *GAL1-GFP* reporter expressed in cells treated as described in (A) and (B), (blue and pink curves, respectively). Mean GFP values at indicated time points (30 min intervals) were estimated by the WinMdi software and represent average of at least three independent experiments. Day-by-day experimental variation did not exceed that of 15% of the average values.

exposed to galactose. This observation strongly suggests that transcriptional memory is not dependent on prior transcriptional activation and relevant book-marking of chromosomal regions in *cis*, but it rather relies on factors acting in *trans*.

## The Signal Transducer Gal3 Is Dispensable for Memory Persistence

Transcription of *GAL* genes strictly depends on the Gal4 transcriptional activator, which, although it is expressed and binds DNA even in glucose-grown cells [10, 13], is inactive because of its interaction with the negative regulator Gal80 [11, 12]. Exposure to galactose leads to the sequestration of Gal80 to the cytoplasm by a complex formed between galactose, ATP, and the Gal3 regulator. As a result, active Gal4 initiates transcriptional activation of the *GAL* catabolic pathway and further activates *GAL3* transcription, thereby setting up a Gal3-Gal4 positive-feedback loop [14–17]. Although Gal3 is expressed at low levels compared to the other *GAL* genes (e.g., *GAL1*), its key role in activating Gal4 makes it a candidate for being the cytoplasmic factor preserving galactose memory in progeny cells. This hypothesis can be examined by using cells lacking *GAL3* and tested for their ability to exhibit galactose memory. However, transcriptional activation of *GAL* genes and cell growth upon first exposure to galactose is prevented in *gal3*Δ cells. To circumvent this problem, we applied a standard gene-disruption protocol to delete the *GAL3* gene from wild-type cells growing continuously in galactose media. By selecting and purifying *GAL3* gene-disrupted cells in growth media containing galactose as a sole carbon source, we had been able to generate *gal3*Δ mutants that fully express Gal1-GFP (Figure 3A). This confirms that Gal3 is not required for the maintenance of the *GAL* gene activation state and is consistent with the previously described "long-term adaptation" property of *gal3*Δ cells, which ultimately succeed to activate the *GAL* pathway after being cultured in galactose for days [18]. Unexpectedly, when the *gal3*Δ mutants expressing *GAL1-GFP* are consequently cultured in glucose for 12 hr and then rechallenged with galactose, they retain the ability to rapidly and uniformly reactivate *GAL1-GFP* transcription (Figures 3A and 3C). This clearly indicates that Gal3 neither is the cytoplasmic factor responsible for galactose memory nor is it required for the initiation of the "memorized" rapid transcriptional response. The reactivation ability of the *gal3*Δ cells is permanently lost after longer, memory-erasing, time (30 hr) of growth in glucose (Figures 3B and 3C); by that time point, *GAL1* transcription can not be induced by galactose, as is typically observed in *gal3*Δ mutants [18].

## The Gal1 Galactokinase Is Responsible for Transcriptional Memory

It has been previously shown that the Gal1 galactokinase, which is a close homolog of Gal3 (70% identity, 90% similarity [19]), interacts with Gal80 in vivo, albeit with lower affinity compared to that of Gal3, and that its constitutive expression suppresses the *gal3*Δ mutation [20–22]. On the other hand, our analysis indicates that reduction of the intracellular Gal1 levels in galactose-pregrown cells after cell divisions in glucose media correlates with galactose memory loss (see Figure S3). These results suggest that residual Gal1 can perform a Gal3-like function in sequestering Gal80, thereby allowing Gal4 to reactivate transcription in progeny cells, so we investigated whether Gal1 determines galactose memory. Indeed, heterokarya between *KAR1*, *GAL1-GFP* naive cells and *kar1-1,gal1*Δ double mutants pregrown in

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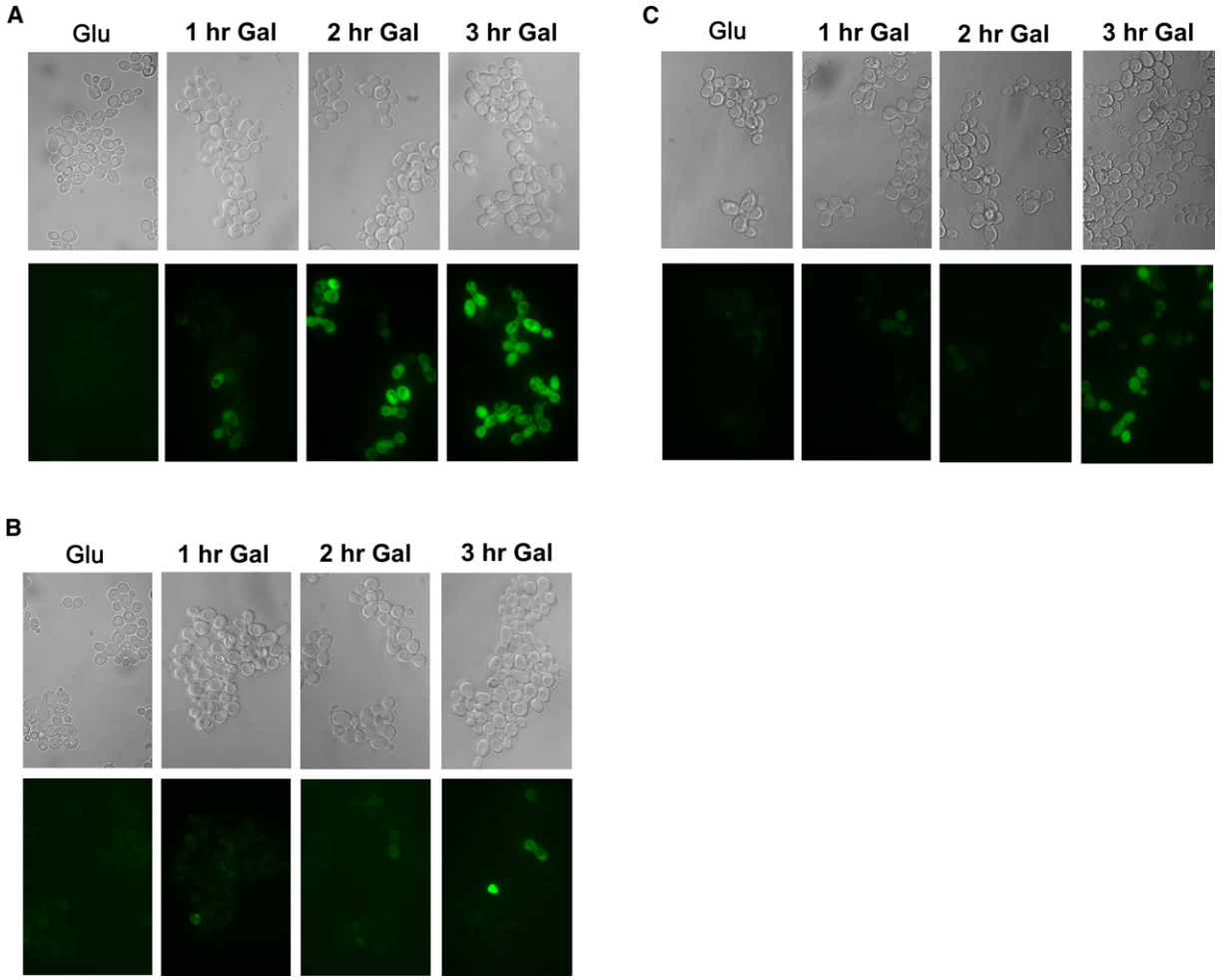


Figure 2. Heterokaryon Assay for Identifying the Base of Galactose Memory

(A) Light and GFP images taken at indicated time points of galactose induction of naive *GAL1-GFP*-carrying cells crossed with galactose-pregrown *kar1-1* mutants. Wild-type *GAL1-GFP* cells grown for 24 hr in YP-2% glucose continuously kept at low density (<0.2) were mixed with an equal number ($\sim 10^7$) of opposite mating type *kar1-1* mutant cells that had been grown for 12 hr in YP-2% galactose and consecutively for 6 hr in YP-2% glucose. For mating to take place, the mixture of haploid cells was spotted and further incubated for additional 5 hr in YP-2% glucose agar plate. By that time, the mating efficiency was roughly estimated to exceed 60%. After this mating period, cells were diluted in YP-2% galactose medium and samples taken at indicated time points were subjected to UV-epifluoroscence microscopy. Heterokarya display a characteristic elongated morphology with a bud often emerging from central position and two distinct nuclei, as indicated by DAPI staining shown in Figure S2.

(B) Light and GFP images at indicated time points of galactose induction of naive *GAL1-GFP*-carrying cells crossed with *kar1-1* mutants continuously grown in glucose. Heterokarya were formed, challenged, and examined for galactose response as in (A), with the exception that *kar1-1* haploid mating partners was continuously (18 hr) grown in glucose.

(C) Light and GFP images at indicated time points of galactose induction of heterokarya derived by crossing *kar1-1, gal1*Δ double mutants with glucose-grown *GAL1-GFP*-carrying wild-type cells. *kar1-1, gal1*Δ cells were grown in YP-2% galactose-2% raffinose media, transferred to YP-2% glucose, crossed with glucose-grown *GAL1-GFP* wild-type cells as in (A), and challenged for reactivation in YP-2% galactose-2% raffinose.

galactose fail to respond rapidly to galactose induction (Figure 2C). In order to quantify the Gal1 involvement in galactose memory, we analyzed the reactivation kinetics of a *gal1*Δ strain expressing the Gal7-GFP hybrid protein. By contrast to *gal3*Δ or wild-type cells, the *gal1*Δ mutants pregrown to galactose and consecutively cultured for 12 hr in glucose media fail to reactivate rapidly the *GAL7* gene after a second exposure to galactose (Figure 4A). In fact, the activation kinetics of these cells is identical to those that have never experienced galactose before (Figure 4A). Identical results were obtained with *gal1*Δ cells expressing the GFP protein from the native GAL1 promoter, with a gene reporter (*GAL1pr-GFP*) that lacks the *GAL1* coding sequence (Figure S4A). In order to exclude the possibility that compromised galactose catabolism in *gal1*Δ cells accounts for memory loss, we also demonstrate that cells expressing *GAL1* by the constitutive ADH1 promoter activate rapidly and uniformly transcription of the Gal7-GFP hybrid with the same kinetics either after a first exposure or after a re-exposure to galactose (Figures 4B–4D). In fact, the rate of activation of Gal7-GFP hybrid in these cells displays a more rapid pattern than the one acquired by the wild-type cells preexposed to galactose (Figure 4D). Identical results were obtained when the *GAL1-GFP* reporter was assayed (Figure S4B). In order to support that it is the Gal3-like activity

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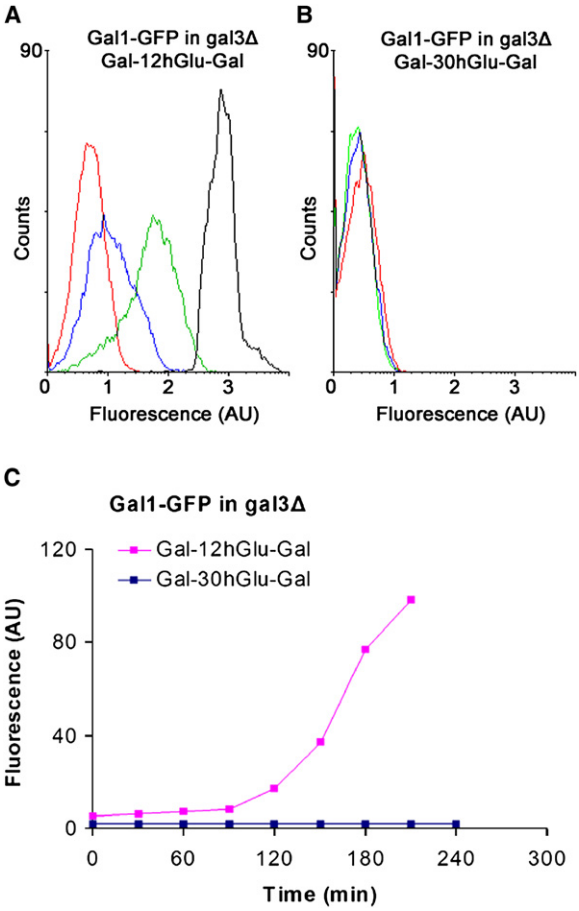


Figure 3. Role of Gal3 in Galactose Memory Persistence

(A) *GAL1-GFP* reactivation in galactose-experienced *gal3*Δ mutant cells. Histograms of FACS analysis at representative time points showing GFP fluorescence measurements taken from *gal3*Δ cells grown continuously in galactose (black), subsequently cultured in glucose media for 12 hr (red), and reinduced by galactose for 120 min (blue) or 180 min (green). *gal3*Δ yeast colonies were obtained by transforming wild-type *GAL1-GFP*-carrying cells grown o/n in YP-2% galactose medium with *GAL3* disruption linear DNA fragment. All subsequent colony-purification process was done in the presence of galactose as the sole carbon source. *gal3*Δ colonies were confirmed by PCR and cultured in liquid YP-galactose medium, and a sample was subjected to FACS analysis (black). After consecutive growth for 12 hr in glucose (GFP reaching close to background levels, red curve), cells were challenged again by galactose and analyzed by FACS.

(B) Activation defect of *gal3*Δ mutants. Galactose-pregrown cultures of *gal3*Δ cells bearing *GAL1-GFP* fusion were transferred in YP-glucose media and cultured for 30 hr, instead of 12 hr described in (A), before galactose reinduction. Representative histograms display GFP measurements taken at 0 min (red), 210 min (blue), and 240 min (green) after galactose reinduction.

(C) Graphs of GFP mean values over time obtained by the FACS analysis of (A) and (B) (blue and pink graphs, respectively) taken in 30 min intervals.

of Gal1 that is responsible for preserving galactose memory, we expressed Gal3 itself from the GAL1 promoter (GAL1pr-Gal3) in a strain lacking the *GAL1* gene (*gal1*Δ), and we investigated the reactivation kinetics of the *GAL7-GFP* reporter. As indicated in Figure S5, galactose-pregrown cells carrying *GAL1pr-GAL3* can reinduce *GAL7-GFP* transcription after consequent growth in glucose, as opposed to *gal1*Δ cells expressing the native *GAL3* gene. The above analysis strongly suggests that Gal1 is absolutely required for preserving galactose memory and argues against a parallel operation of additional redundant mechanism(s) that may contribute to this phenomemon. Conversely, it seems that Gal1 is also a sufficient source of "galactose information" for a naive culture. We suggest that the Gal3-like function of the highly expressed Gal1 in galactose pre-exposed cells is responsible for rapid reactivation.

Exposure of yeast cells to a galactose environment has a strong impact on the general physiology of the organism [23], and therefore memory of this environment may confer a beneficial adaptation to subsequent generations. Our results point out that this memory resides on the accumulated Gal1 galactokinase that is transmitted via the cytoplasm to successive generations. After a second exposure to galactose, Gal1 sets up a Gal1-Gal4 positive-feedback loop that rapidly releases the activation potential of the Gal4 activator. Our model proposes that this cellular memory expires as Gal1 concentration reaches a critically low level in progeny cells grown in glucose by being gradually diluted after each mitotic event.

Our results suggest that rapid reactivation of GAL genes transcription clearly depends on the Gal3-like function provided by the Gal1 protein and does not require previous galactose metabolism per se: when Gal1 was expressed by the ADH promoter in glucose-growing cells, *GAL* genes were induced rapidly, resembling the pattern displayed by galactose-pregrown cells, and when Gal3 was expressed by the GAL1 promoter in *gal1*Δ cells, which are unable to catabolize galactose, cells' memory was restored. We have to mention that the key role of the Gal3 activity in determining both the rate and the mode (binary versus graded) of *GAL* gene activation has been recognized previously; rapid *GAL* activation occurs in glucose-pregrown cells that ectopically express Gal3, and similar rates of induction were observed in cells pregrown in glycerol or raffinose media, in which Gal3 expression is derepressed [7, 8] (data not shown).

Our study on this yeast "adaptive cell memory" paradigm strongly argues against a mechanism involving self-propagating chromatin bookmarks that are acquired upon prior transcriptional activation. In concert, histone tail modifications (acetylation and methylation) that were measured at activated yeast promoters, including those of *GAL* genes, were removed upon deactivation and hence can not be stably inherited through cell divisions [24, 25]. On the other hand, a recent report [6] has proposed that histone H2A.Z-dependent translocation of the *GAL* cluster to the nuclear periphery upon transcriptional activation, and its persistence after growth in glucose is the basis for galactose memory. Because no evidence directly supporting this hypothesis was presented, it is conceivable that the persistence of altered topology of previously activated genes might be just the consequence of chromosomal relocation dynamics. In addition, this proposal is inconsistent with the uniform cell pattern of *GAL* reactivation revealed by our FACS analysis, because the persistent perinuclear localization of *GAL* genes was observed for only a fraction (~60%) of the population [6]. In another study [5],

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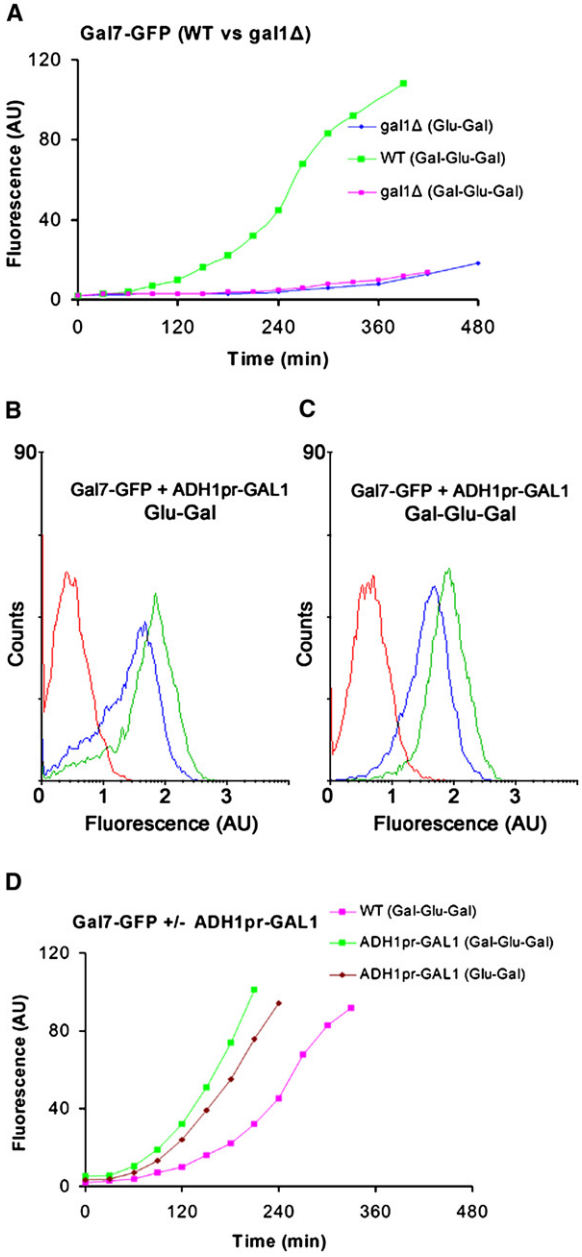


Figure 4. Role of Gal1 in Galactose Memory

(A) Reactivation kinetics of the *GAL7-GFP* reporter in galactose-experienced *gal1*Δ mutants. Graphs represent mean GFP values over time taken at 30 min intervals after galactose reactivation of *gal1*Δ mutants (pink) and wild-type (green) cells. The activation kinetics of glucose-grown *gal1*Δ mutants after galactose induction is shown for comparison (blue). *GAL7-GFP*-expressing *gal1*Δ mutants were grown in YP media containing 2% galactose plus 2% raffinose, transferred in glucose, reinduced in galactose (plus reffinose) media, and subjected to FACS analysis.

(B) Single-cell pattern of *GAL7-GFP* activation in glucose-pregrown cells expressing Gal1 constitutively. Histograms of FACS analysis at representative time points showing GFP fluorescence measurements taken from cells growing in glucose (red) and subsequent growth in galactose for 150 min (blue) and 210 min (green). The Gal7-GFP hybrid protein contains the entire Gal7 amino acid sequence and is expressed from the native promoter and chromosomal position. Cells containing a plasmid expressing Gal1 by the ADH1 promoter were cultured for 24 hr in 2% glucose, washed, and transferred in 2% galactose medium.

Swi/Snf function has been proposed to be required for rapid reactivation of the *GAL1* promoter in cells that have been previously exposed to galactose. This was shown to be evident for transcriptional memory after only one generation, and it might simply reflect a requirement of this coactivator for the rapid transcriptional reactivation set up by the Gal1-Gal4-positive loop. Our results demonstrate that this system of transcriptional memory has a cellular basis that targets the transcriptional activator. In agreement, recent proposals implicate DNA-binding transcriptional regulators rather than chromatin modifications as the basis of epigenesis in higher organisms (see for review [26]).

## Experimental Procedures

### Yeast Strains and Reporters

Yeast strains were derived from the wild-type strain FT5 (S288c, *GAL*$^{+}$) [27]. *GAL1-GFP* was constructed by inserting GFP at the 3′ end of the *GAL1* coding sequence of FT5 by standard homologous recombination with a yEGFP-KanMX cassette amplified from pFA6a-link-yEGFP-KAN [28] with synthetic primers (1A and 1B, listed in Table S1), bearing regions homologous to 3′ end of *GAL1* ORF. Same methodology was used for generating the *GAL7-GFP* carrying strain by tagging the 3′ end of *GAL7* ORF gene with yEGFP with primers 3A and 3B (Table S1). GAL1 promoter-driven GFP strains were constructed by homologous recombination with linear yEGFP-KanMX DNA amplified from FA6a-link-yEGFP-KAN with primers (4A and 1B, Table S1) designed so as to replace *GAL1* ORF with the yEGFP sequence, thus creating a *gal1*Δ null mutation. *GAL3* gene was disrupted from wild-type strain by standard one-step homologous recombination techniques, with linear KITRP1 DNA amplified from PYM6 [29] with primers (2A and 2B) harboring homologous 5′ ends to the respective gene. To generate *gal3*Δ cells fully expressing the *GAL1-GFP* reporter, wild-type cells were grown in YP-2% galactose media and *GAL3* disruptants were purified in selective media with 2% galactose as a carbon source. Gene deletion was confirmed by both PCR and phenotypic analysis. *gal1*Δ strains were constructed by disrupting the *GAL1* gene by a linear DNA fragment containing the URA3 marker flanked by 5′ and 3′ *GAL1* sequences and confirmed by PCR and phenotypic analysis. The ADH1-driven *GAL1* plasmid was constructed by in vivo three-piece recombination process: a linear DNA fragment containing the ADH1 promoter and a second one containing the entire *GAL1* ORF and 3′ UTR were amplified from the yeast genome by PCR with primers (5A-5B and 6A-6B, respectively) designed to allow recombination with each other and with the pRS314 plasmid vector. PCR products along with single cut pRS314 plasmid recombined in vivo giving rise to an ADH1 promoter-driven *GAL1* gene cloned in pRS314. Recombinant plasmid was rescued in *E. coli* cells and confirmed by sequencing analysis.

### Growth Conditions and Flow Cytometry

Glucose-to-galactose induction experiments were performed with cells inoculated in YP-2% glucose medium and cultured at 30°C kept at low optical density (OD < 0.2) by continuous dilutions. After

(C) Single-cell pattern of *GAL7-GFP* reactivation in galactose-pregrown cells expressing Gal1 constitutively. Histograms representing GFP distributions taken at 0 min (red), 150 min (blue), and 210 min (green) after galactose induction. Cells carrying *GAL7-GFP*- and ADH-driven Gal1 were grown for 24 hr in galactose medium, transferred in glucose for 12 hr and back to galactose, followed by FACS analysis as in (B).

(D) Graphs of mean GFP values showing activation and reactivation kinetics of *GAL7-GFP* transcription in cells constitutively expressing *GAL1*. Mean values (at 30 min intervals) of FACS analysis presented in (B) and (C) were plotted in brown (glucose-pregrown) or green (galactose-pregrown) curves. The reactivation kinetics of *GAL7-GFP* in wild-type strain (pink curve, taken from Figure S1) are shown for comparison.

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24 hr of growth, cells were washed twice with an equal volume of water and transfered to prewarmed YP medium containing 2% galactose. For galactose to glucose and back to galactose (galactose memory testing) experiments, cells were inoculated and cultured in YP-2% galactose medium for 24 hr at 30°C, transferred to YP-2% glucose, and cultured for 12 hr (keeping optical density OD < 0.2) and then washed and transferred to YP-2% galactose, as above. For FACS analysis, 250 μl of yeast culture (OD ~0.2) in indicated media and time points were harvested, washed, and resuspended in PBS. FACS analysis was performed with Calibour instrument (Becton Dickinson) in excitation and emission wavelength at 488 nm and 530/30 nm, respectively. Statistical analysis was performed with the WinMDI application.

### Supplemental Data

Five figures and one table are available at http://www.current-biology.com/cgi/content/full/17/23/■■■/DC1/.

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