Epigenetic Transcriptional Memory of *GAL* Genes Depends on Growth in Glucose and the Tup1 Transcription Factor in *Saccharomyces cerevisiae*

Varun Sood,[1] Ivelisse Cajigas,[1,2] Agustina D'Urso, William H. Light,[3] and Jason H. Brickner[4]
Department of Molecular Biosciences, Northwestern University, Evanston, Illinois 60208
ORCID ID: 0000-0001-8019-3743 (J.H.B.)

ABSTRACT Previously expressed inducible genes can remain poised for faster reactivation for multiple cell divisions, a conserved phenomenon called epigenetic transcriptional memory. The *GAL* genes in *Saccharomyces cerevisiae* show faster reactivation for up to seven generations after being repressed. During memory, previously produced Gal1 protein enhances the rate of reactivation of *GAL1*, *GAL10*, *GAL2*, and *GAL7*. These genes also interact with the nuclear pore complex (NPC) and localize to the nuclear periphery both when active and during memory. Peripheral localization of *GAL1* during memory requires the Gal1 protein, a memory-specific *cis*-acting element in the promoter, and the NPC protein Nup100. However, unlike other examples of transcriptional memory, the interaction with NPC is not required for faster *GAL* gene reactivation. Rather, downstream of Gal1, the Tup1 transcription factor and growth in glucose promote *GAL* transcriptional memory. Cells only show signs of memory and only benefit from memory when growing in glucose. Tup1 promotes memory-specific chromatin changes at the *GAL1* promoter: incorporation of histone variant H2A.Z and dimethylation of histone H3, lysine 4. Tup1 and H2A.Z function downstream of Gal1 to promote binding of a preinitiation form of RNA Polymerase II at the *GAL1* promoter, poising the gene for faster reactivation. This mechanism allows cells to integrate a previous experience (growth in galactose, reflected by Gal1 levels) with current conditions (growth in glucose, potentially through Tup1 function) to overcome repression and to poise critical *GAL* genes for future reactivation.

KEYWORDS transcriptional memory; *GAL* genes; gene positioning; epigenetic; RNA polymerase II; chromatin; nuclear pore complex

TRANSIENT stimuli can lead to changes in gene expression that are inherited for several cell divisions and play an important role in development and adaptation to the environment (Koornneef *et al.* 1994; Lee *et al.* 1994; Livingstone *et al.* 1995; Sung and Amasino 2004; Sung *et al.* 2006; Hansen *et al.* 2008; Seong *et al.* 2011; Nestorov *et al.* 2013). When a previous experience/stimulus leads to heritable changes in the transcriptional response/output, such phenomena are called "epigenetic memory" (Riggs 1996; Suganuma and Workman 2011; D'Urso and Brickner 2017). Epigenetic transcriptional memory enables certain inducible genes to respond much faster upon reexposure to the same stimulus in yeast, plants, and humans (Brickner *et al.* 2007; D'Urso and Brickner 2017). Although such genes are repressed between the two inductions, they are poised for faster reactivation. Transcriptional memory is inherited through multiple cell divisions, and serves as a model to study how cells can benefit from their previous experiences and how that information can be stored and inherited.

A well-characterized example of memory in budding yeast following inositol starvation leads to poising of the *INO1* gene for faster reactivation (Brickner *et al.* 2007; Light *et al.* 2010, 2013; D'Urso *et al.* 2016; D'Urso and Brickner 2017). *INO1* memory requires both a physical interaction of the promoter with the nuclear pore complex (NPC) as well as changes in chromatin structure to permit binding of a preinitiation form of RNA polymerase II (RNAPII; Light *et al.* 2010, 2013;

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[1]These authors contributed equally to this work.
[2]Present address: Department of Developmental Biology, Stanley Manne Children's Research Institute, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611.
[3]Present address: Cibus US LLC, 6455 Nancy Ridge, San Diego, CA 92121.
[4]Corresponding author: Department of Molecular Biosciences, Hogan 2100, 2205 Tech Dr., Northwestern University, Evanston, IL 60208. E-mail: j-brickner@northwestern.edu

D'Urso *et al.* 2016). Although *INO1* interacts with the NPC both when active and during memory, these interactions are independently regulated by distinct mechanisms (Brickner and Walter 2004; Brickner *et al.* 2007; Light *et al.* 2010). During memory, interaction with the NPC is mediated by the Sfl1 transcription factor binding to a *cis*-acting promoter element called the Memory Recruitment Sequence (MRS). Sfl1 is required for the interaction of *INO1* with the NPC protein Nup100, and this interaction is necessary and sufficient to induce increased incorporation of H2A.Z and dimethylation of histone 3, lysine 4 (H3K4) (Light *et al.* 2010; D'Urso *et al.* 2016). Both H3 dimethyl K4 (H3K4me2) and H2A.Z persist during memory at the *INO1* promoter and are themselves required for peripheral localization and binding of poised RNAPII. Thus, loss of Sfl1, Nup100, and H2A.Z, or mutations in the MRS, disrupts peripheral localization and binding of poised RNAPII, leading to slower reactivation of *INO1* (Light *et al.* 2010; Brickner *et al.* 2015; D'Urso *et al.* 2016).

Critical aspects of *INO1* transcriptional memory are conserved and widespread (Light *et al.* 2013). Hundreds of genes in HeLa cells exhibit interferon γ (IFN-γ)-induced transcriptional memory (Gialitakis *et al.* 2010; Light *et al.* 2013). IFN-γ memory requires the interaction of genes with the nuclear pore protein Nup98 (homologous to yeast Nup100), leading to H3K4 dimethylation and binding of poised RNAPII to the promoter (Gialitakis *et al.* 2010; Light *et al.* 2013). Likewise, yeast genes induced by oxidative stress show faster induction in cells previously exposed to high salt (Guan *et al.* 2012). This type of memory also leads to H3K4me2 and binding of poised RNAPII but requires Nup42 instead of Nup100 (Guan *et al.* 2012; D'Urso *et al.* 2016). This suggests that both gene-specific and general mechanisms promote epigenetic transcriptional memory.

Galactose-induced transcriptional memory leads to faster reactivation of yeast *GAL* genes (*GAL1*, *GAL10*, *GAL7*, and *GAL2*) for up to seven generations (~12 hr) after shifting from activating to repressing conditions (Brickner *et al.* 2007; Zacharioudakis *et al.* 2007; Kundu and Peterson 2010). However, *GAL* memory is more complex than *INO1* memory as it exhibits two distinct phases with different molecular requirements. During the first ~4 hr of repression, the NPC-associated protein Mlp1 facilitates looping between the 5′- and 3′- ends of the *GAL1* gene and this looping, combined with the SWItching deficient/Sucrose NonFermentable (SWI/SNF) chromatin remodeler, is required for faster reactivation (Kundu *et al.* 2007; Laine *et al.* 2009; Tan-Wong *et al.* 2009). Short-term *GAL* transcriptional memory is distinct from long-term *GAL* memory, which occurs between 4 and 12 hr of repression and is epigenetically inherited. Long-term memory requires the Gal1 protein, correlates with localization to the nuclear periphery and is independent of the SWI/SNF complex (Zacharioudakis *et al.* 2007; Kundu and Peterson 2010). Gal1 produced during activation acts as a coactivator by interfering with Gal80 repression during memory and is both necessary and sufficient to enhance the rate of reactivation (Bhat and Hopper 1992; Zacharioudakis *et al.* 2007).

Here, we have focused on understanding the molecular and cellular consequences of Gal1 expression during long-term, epigenetic *GAL* gene memory. Like *INO1*, *GAL1* and GAL2 localize at the nuclear periphery during memory (Brickner *et al.* 2007). We defined *cis*- and *trans*-acting factors that control *GAL* gene targeting to the nuclear periphery during epigenetic *GAL* memory. We find that Gal1 protein is necessary and sufficient to promote targeting of *GAL* genes to the nuclear periphery. A *cis*-acting DNA element (MRS_{GAL1}) in the *GAL1* promoter is necessary for targeting of the *GAL1* locus to the periphery during memory. Further, targeting by MRS_{GAL1} is both dependent on Nup100 and responsive to ectopic expression of Gal1. Although loss of Nup100 or mutations in the MRS_{GAL1} block peripheral localization, they do not affect *GAL1* transcription rates. Thus, although *GAL* gene transcriptional memory leads to interaction with the NPC, this interaction is not required to enhance transcriptional reactivation rates under these conditions.

GAL1 memory also leads to increased incorporation of histone variant H2A.Z, dimethylation of H3K4, and binding of poised RNAPII at the promoter. Furthermore, long-term *GAL* memory is most beneficial to cells that are currently growing in glucose and, in other carbon sources, memory is not observed. Both growth in glucose and the Tup1 transcription factor function upstream of peripheral localization and downstream of Gal1 during memory. Tup1 is necessary for incorporation of both H2A.Z and H3K4me2 at the *GAL1* promoter, leading to binding of poised RNAPII. Thus, future rates of *GAL* gene reactivation depend both on a previous experience (growth in galactose) and current conditions (growth in glucose).

Materials and Methods

Reagents

Unless noted otherwise, all chemicals were from Sigma ([Sigma Chemical], St. Louis, MO). Yeast media components were from Sunrise Science Products (San Diego, CA). Restriction enzymes were from New England Biolabs (Beverly, MA). Dynabeads, rabbit anti-GFP, goat anti-mouse-Alexafluor 594, and goat anti-rabbit Alexafluor 488 were from Invitrogen (Carlsbad, CA), mouse anti-Myc (9E10) was from Santa Cruz Biotechnology, mouse anti-RNAPII (8WG16) was from Covance, mouse anti-Nsp1 was from EnCor Biotechnology (Gainesville, FL), and rabbit anti-H2A.Z (4626) and rabbit anti-H3K4me2 (32356) were from Abcam. Rapamycin was from Millipore (Bedford, MA).

Plasmids, yeast strains, and molecular biology

Plasmids pAFS144 (Straight *et al.* 1996), p6LacO128-GAL1, and p6LacO128-GAL1-10prom have been described previously (Brickner and Walter 2004; Brickner *et al.* 2007, 2016). p6LacO128-GAL2 was created by amplifying the 3′ region of *GAL2* using PCR with the GAL2 3′ F and GAL2 3′ R primers. The PCR product was digested using *Not*I and *Bam*HI and

cloned into p6LacO128 (Brickner and Walter 2004). pRS304-ADH1-GAL1 was created by ligating P_{ADH1}-*GAL1*, excised from pGREG700, into *Sac*I- and *Kpn*I-digested pRS304 (Sikorski and Hieter 1989). pGREG700 in turn was generated from pGREG600 (Jansen *et al.* 2005) by swapping the *GAL1* promoter with the *ADH1* promoter using the *Sac*I and *Spe*I sites. Promoter fragments and MRS variants were integrated at *URA3*:*p6LacO128* using the pZIPKan plasmid (Egecioglu *et al.* 2014) or by cloning in p6LacO128 (Ahmed *et al.* 2010; Light *et al.* 2010). The plasmids were linearized by digestion and integrated at the desired locus.

Yeast strains used in this study appear in Supplemental Material, Table S1 in File S1. Except for cells containing Nup2–TAP, Nup100-TAP, and Gal1-GFP (Ghaemmaghami *et al.* 2003; Huh *et al.* 2003), all strains were constructed from CRY1 or CRY2 (Brickner and Fuller 1997), derived from the W303 background (*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*). Cells were grown in Synthetic Dextrose Complete (SDC), Synthetic Galactose Complete (SGC), or Synthetic Raffinose Complete (SRC) medium at 30° (Burke *et al.* 2000) for localization, RT- quantitative (q) PCR, and chromatin immunoprecipitation (ChIP) experiments. For flow cytometry of the Gal1-mCherry cells, cells were grown in either Yeast Peptone Dextrose (YPD) or Yeast Peptone Galactose (YPG).

A PCR-based system was used for deletion (Longtine *et al.* 1998) and C-terminally tagging genes with fluorophore or FRB tags. The mutant form of mrs_{GAL1} at the endogenous *GAL1* locus was generated by first replacing the promoter with the *Kan*^{r} marker and then transforming with the mutant promoter and selecting on galactose plates. Strains used for the chromatin localization assay using immunofluorescence (IF) were transformed with either pAFS144 (Straight *et al.* 1996) or pRS305-GFP-LacI for GFP-LacI expression, pRS304-Sec63myc for immunolabeling the nuclear envelope, and derivatives of the p6LacO128 plasmid to tag the locus of interest (Brickner *et al.* 2007). For live cell localization assays, the endoplasmic reticulum (ER)/nuclear envelope was visualized by tagging *PHO88* with *mCherry-His5*$^+$ cassette. For flow-cytometric study of *GAL1* expression, *GAL1* was C-terminally tagged with *mCherry-KanMx* cassette and P_{TDH}-*CFP-NATmx* cassette was inserted at the *HO* locus. For all Anchor-Away experiments the parent strain, HHY168, was adapted for live cell chromatin localization assay (Haruki *et al.* 2008). Cells were treated with 1 μg/μl rapamycin for depletion of FRB-tagged proteins for 1 hr before imaging.

Chromatin localization assay

Chromatin localization experiments using IF with fixed cells (Brickner *et al.* 2010) and with live cells (Egecioglu *et al.* 2014) were performed as described. Cells were imaged using SP5 Line Scanning Confocal Microscope (Leica Biosystems) at the Northwestern University Biological Imaging Facility. Gene localization was scored in stacks of images using LAS AF Lite software: in the *z*-slice with brightest and most focused LacO dot, if the center of the dot overlapped with the nuclear membrane the gene position was scored as peripheral. Localization was not scored in cells where the dot was either on the top or bottom of the nucleus. Error bars represent the SEM for three biological replicates of 30–50 cells each.

ChIP

Cells were fixed in 1% formaldehyde for 15 min at room temperature, 150 mM glycine was added to quench the formaldehyde reaction, and ChIP was performed as described previously (Brickner and Walter 2004; Ahmed *et al.* 2010; Light *et al.* 2010; Egecioglu *et al.* 2014). For Nup2 and Nup100 ChIP, cells were fixed at room temperature for 1 hr. RNAPII, H2A.Z, and H3K4me2 were recovered with respective antibodies coupled with either anti-pan-mouse (RNAPII) or sheep anti-rabbit IgG (H2A.Z and H3K4me2) Dynabeads, while Nup2 and Nup100 were recovered directly using anti-pan-mouse IgG Dynabeads. Recovery of the DNAs from *GAL1*, *BUD3*, and *PRM1* promoter by ChIP was quantified by qPCR as described previously (Brickner and Walter 2004) using primers listed in Table S2 in File S1. Error bars represent the SEM from three biological replicates.

RT-qPCR

For activation experiments, cells were grown in SDC to an OD_{600} 0.7–1. For reactivation experiments, cells were grown in SGC overnight, diluted to OD_{600} ~0.01 in SDC, and grown for 12 hr. After shifting from glucose to galactose medium, cells were harvested at various times, pelleted, and frozen in liquid nitrogen. RNA was isolated and RT-qPCR was performed as described previously (Brickner *et al.* 2007). *GAL1*, *GAL2*, and *GAL7* mRNA levels were quantified relative to *ACT1* levels using the *GAL1* coding sequence (CDS), *GAL2* CDS, and *GAL7* CDS primers, respectively (Table S2 in File S1). For experiments using the *gal1*Δ strain, cells were grown in SRC, shifted to SGC for 4 hr, and then shifted to SDC for 12 hr. Error bars represent the SEM of three biological replicates.

Flow cytometry

Cells with *GAL1-mCherry* were induced in YPG and maintained at $OD_{600} \leq 0.3$ throughout the induction. Next, 1 ml of culture was harvested at different times of induction, the cells were frozen in 10% glycerol, and stored at −80°. For flow cytometry, cells were thawed on ice and analyzed a BD LSRII flow-cytometer. mCherry and cyan fluorescent protein (CFP) were excited with 561 and 405 nm lasers, respectively. For detecting mCherry emission, a 600-nm long pass dichroic mirror and 610/20-nm band pass filter set was used, while for CFP emission a 505-nm long pass dichroic mirror and 525/50-band pass filter set was used. Roughly 5000 cells were analyzed to obtain the average intensity of Gal1-mCherry and CFP. The constitutively expressed CFP (P_{TDH}-*CFP*) served as a normalization control for Gal1-mCherry fluorescence; Gal1 expression levels were expressed as ratio of Gal1-mCherry to CFP fluorescence intensity.

Statistical analysis

To evaluate the significance of differences between peripheral localization scores or ChIP signals between strains or treatments with respect to the reference, an unpaired, two-tailed Student's *t*-test was performed.

Data availability

All the data necessary to support the conclusions of this study are presented within this article. Raw data are available upon request.

Results

Gal1 promotes targeting of GAL genes to the nuclear periphery during transcriptional memory

The Gal1 protein is necessary for faster reactivation of *GAL* genes during memory and ectopically expressed Gal1 is sufficient to promote faster *GAL* gene expression (Zacharioudakis *et al.* 2007; Kundu and Peterson 2010). Following 12 hr of repression in glucose, the rate of reactivation of *GAL2* was much faster than the initial activation and this effect is lost in cells lacking Gal1 (Figure 1A). Furthermore, ectopic expression of Gal1 (*ADH1* promoter driving Gal1, P_{ADH}-*GAL1*, integrated at the *TRP1* locus) leads to faster activation of *GAL7* mRNA (Figure 1B) or Gal1-mCherry protein (Figure 1C). Cells ectopically expressing mutant Gal1 lacking galactokinase activity (deletion of amino acids 171 and 172; gal1-ΔSA; Platt *et al.* 2000) also showed faster activation of Gal1-mCherry (Figure 1C). Thus, *GAL1* is necessary and sufficient to enhance the rate of *GAL* gene induction, suggesting that the production of Gal1 during activating conditions produces a *trans*-acting, cytoplasmically inherited factor that enhances reactivation rates (Zacharioudakis *et al.* 2007; Kundu and Peterson 2010).

To assess the effect of Gal1 on *GAL* gene positioning at the nuclear periphery during memory, *GAL1* and *GAL2* were tagged using an array of 128 Lac-repressor binding-sites (LacO array) in strains expressing the GFP-Lac repressor (Robinett *et al.* 1996; Brickner and Walter 2004). The fraction of the population in which the gene of interest colocalizes with the nuclear envelope can be determined either by immunofluorescence (IF) with fixed cells or directly in live cells using confocal microscopy (Brickner and Walter 2004; Brickner *et al.* 2010; Egecioglu *et al.* 2014). Genes that localize in the nucleoplasm colocalize with the nuclear envelope in ~30% of cells, corresponding to the baseline for this assay (shown as a blue hatched line throughout), whereas genes that interact with the NPC colocalize with the nuclear envelope in 50–65% of the population (Figure 1D; Brickner and Walter 2004; Casolari *et al.* 2004; Brickner *et al.* 2007). By IF, *GAL1* and *GAL2* localized at the nuclear periphery both when active and for up to 12 hr after repression, but not in glucose (Figure 1E; Brickner *et al.* 2007; Light *et al.* 2010). Consistent with previous studies, the fraction of the population that scored as colocalized with the nuclear periphery was lower for *GAL2* (~50%; Dieppois *et al.* 2006; Gard *et al.* 2009; Brickner *et al.* 2012) than for *GAL1* (~60%; Brickner *et al.* 2007). However, the increase in peripheral localization from repressing to either activating or memory conditions was clear and statistically significant ($P = 0.002$; two tailed *t*-test).

In the *gal1*Δ strain, the *GAL2* locus was targeted to the nuclear periphery under activating conditions, but not during memory (Figure 1E). Furthermore, P_{ADH}-*GAL1* caused both *GAL1* and *GAL2* to reposition to the nuclear periphery under repressing conditions (Figure 1E). Thus, Gal1 protein plays a critical role in controlling peripheral localization of *GAL* genes during memory.

GAL1 remained localized at the nuclear periphery for up to ~14 hr, or ~7.6 cell divisions, before returning to the nucleoplasm (Figure 1F). To approximate the concentration of Gal1 protein that is sufficient to promote peripheral localization, we quantified the steady-state amount of Gal1-GFP under activating conditions, as well as its rate of decay after repression. Using a standard curve of fluorescence intensity for 20 GFP-tagged proteins of known abundance (Newman *et al.* 2006), we estimated the abundance of Gal1 protein to be ~28,000 molecules per cell in cells grown overnight in galactose (Figure 1G). GFP fluorescence was measured over time after shifting the Gal1-GFP strain from galactose to glucose to measure the rate of Gal1 decay after repression (Figure 1H). The $t_{1/2}$ of Gal1-GFP fluorescence was ~130 min, somewhat longer than the cell division time in this experiment (~90 min). Because budding yeast cells divide asymmetrically, producing smaller daughters than mothers, this suggests that the rate of Gal1 decay reflects dilution by cell growth without any appreciable degradation. This may explain how *GAL* gene memory persists for so many generations. From these estimates, we calculate that ~300 Gal1 molecules per cell are sufficient to promote peripheral localization (Figure 1F) and faster reactivation of *GAL* genes (Figure S1 in File S1) after 14 hr of repression. This concentration is comparable to that of Gal80 under these conditions (~800 molecules per cell; Ghaemmaghami *et al.* 2003; Huh *et al.* 2003).

Peripheral localization of *GAL* genes during memory was observed using IF in which the ER/nuclear envelope was marked with the membrane protein Sec63-myc. However, in both live cells and fixed cells, *GAL* gene localization at the nuclear periphery was disrupted by overexpression of certain red fluorescent ER/nuclear membrane proteins (Figure S2C in File S1; Green *et al.* 2012). We do not yet understand the reason for this effect. Fortunately, we found that tagging the endogenous ER/nuclear envelope-resident protein Pho88 with mCherry did not disrupt peripheral localization during *GAL* memory (Figure S2D in File S1) or *INO1* memory (D'Urso *et al.* 2016). This system permitted both IF and live cell experiments to study the localization of *GAL* genes during memory.

Peripheral localization of GAL1 during transcriptional memory requires a cis-acting DNA element and Nup100

Localization of *INO1* to the nuclear periphery during memory requires a specific *cis*-acting element (the MRS) and the

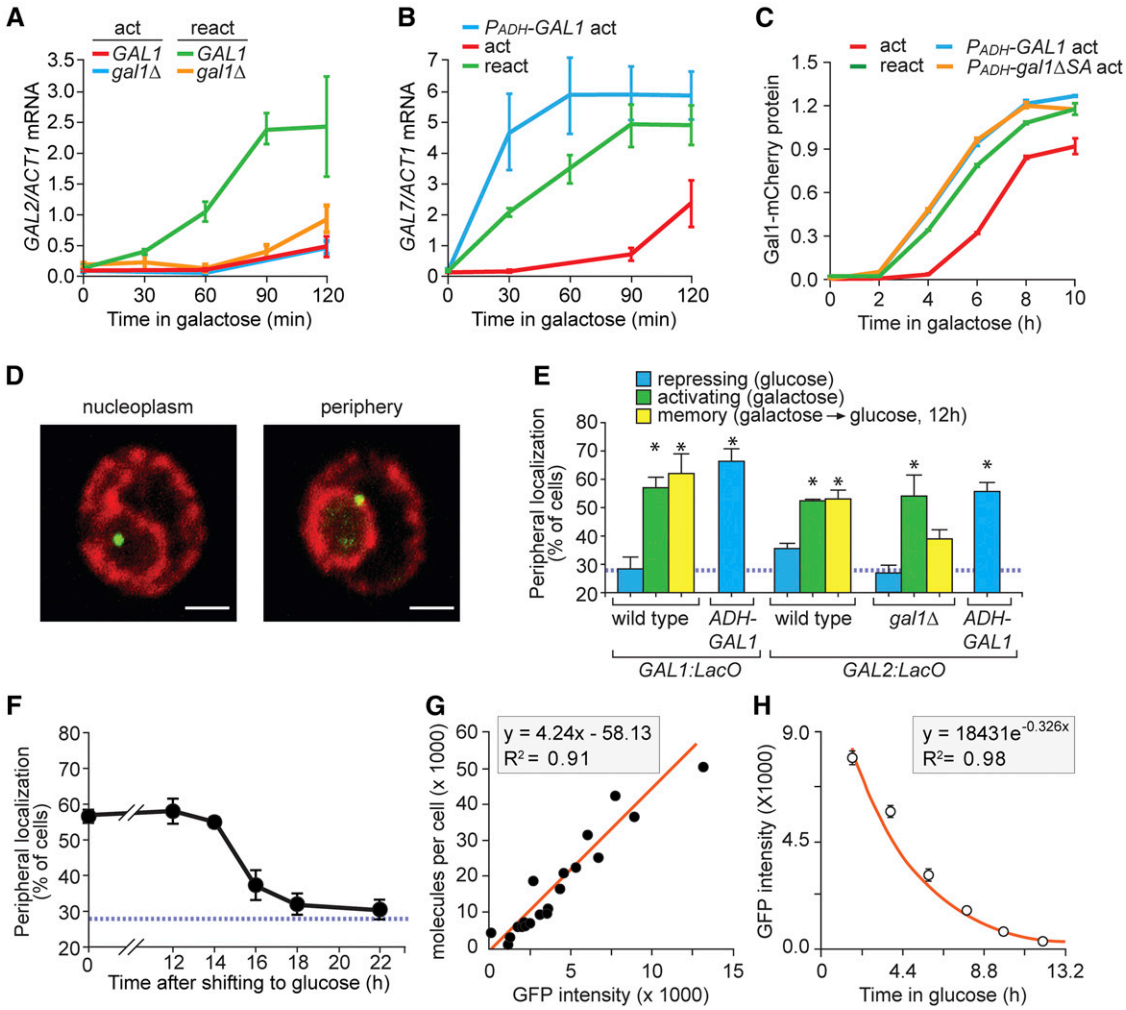


Figure 1 Gal1 promotes *GAL* gene localization at the nuclear periphery during memory. (A–C) Cells were shifted from glucose to galactose (act; activation) or grown overnight in galactose, shifted to glucose for 12 hr and then shifted to galactose (react; reactivation). Cells were harvested at the indicated times, RNA was prepared and mRNA levels were quantified relative to *ACT1* by reverse-transcriptase quantitative PCR (RT qPCR) (A and B) or fluorescence was quantified using flow cytometry (C). (A) *GAL2* activation and reactivation in wild-type and *gal1*Δ cells. (B) *GAL7* activation and reactivation or activation with P_{ADH}-*GAL1*. (C) Gal1-mCherry levels, normalized to constitutively expressed cyan fluorescent protein (CFP) (P_{TDH}-*CFP*) during activation, reactivation, and activation in cells with ectopically expressed wild-type *GAL1* (P_{ADH}-*GAL1*) or catalytically inactive mutant (P_{ADH}-*gal1-ΔSA*). (D) Immunofluorescence images of cells having the LacO array integrated downstream of the *GAL1* gene, stained for GFP-LacI (green) and Sec-63myc (red) and scored as either nucleoplasmic or peripheral. Bar, 1 μm. (E) Peripheral localization of *GAL1* and *GAL2* under repressing (glucose), activating (galactose), and memory (galactose → glucose, 12 hr) conditions in wild-type or *gal1*Δ cells and in the presence of P_{ADH}-*GAL1*. (F) Cells with the LacO array downstream of *GAL1* were shifted from galactose to glucose media for the indicated length of times and the percentage of cells in which *GAL1* colocalized with the nuclear envelope was plotted. The hatched blue line in (E and F) represents the baseline colocalization predicted by chance (Brickner and Walter 2004). (G) Plot of the fluorescence intensities of 20 GFP-tagged proteins (Ghaemmaghami *et al.* 2003; Huh *et al.* 2003), measured by flow cytometry, against protein copy number per cell (Newman *et al.* 2006). (H) Gal1-GFP fluorescence decay after shifting from galactose to glucose. Note: to avoid potential effects of continued translation and maturation of GFP, the initial point for curve fitting was 2 hr after repression. Error bars represent SEM for ≥ three biological replicates. Each replicate for localization (E and F) consisted of 30–50 cells and for fluorescence estimation using flow cytometer (C, G, and H) consisted of ≥ 5000 cells, respectively. * $P \leq 0.05$ (Student's *t*-test) relative to the repressing condition.

nuclear pore protein Nup100, neither of which are required for localization of active *INO1* to the nuclear periphery. This element functions as a DNA zip code that is sufficient to reposition an ectopic locus to the nuclear periphery (Light *et al.* 2010). We asked if targeting of *GAL1* to the nuclear periphery during memory also requires a specific *cis*-acting DNA zip code or Nup100. To identify DNA zip codes, we exploited the *URA3* locus, which normally localizes in the nucleoplasm (Figure 2A). Insertion of the full-length *GAL1* promoter at *URA3* (*URA3*:P_{GAL1}) causes *URA3* to localize at the nuclear periphery under both activating (Brickner *et al.* 2016) and memory (Figure 2A) conditions, supporting the hypothesis that this promoter possesses DNA zip code activity. Using this assay, we mapped a 63-bp MRS (MRS$_{GAL1}$; Figure S2A in File S1). The MRS$_{GAL1}$ did not overlap with two other zip codes in the *GAL1* promoter (GRS4 and GRS5; Brickner *et al.* 2016) that mediate peripheral localization of active *GAL1* (Figure S2A in File S1). Inserting the MRS$_{GAL1}$ alone at *URA3* led to

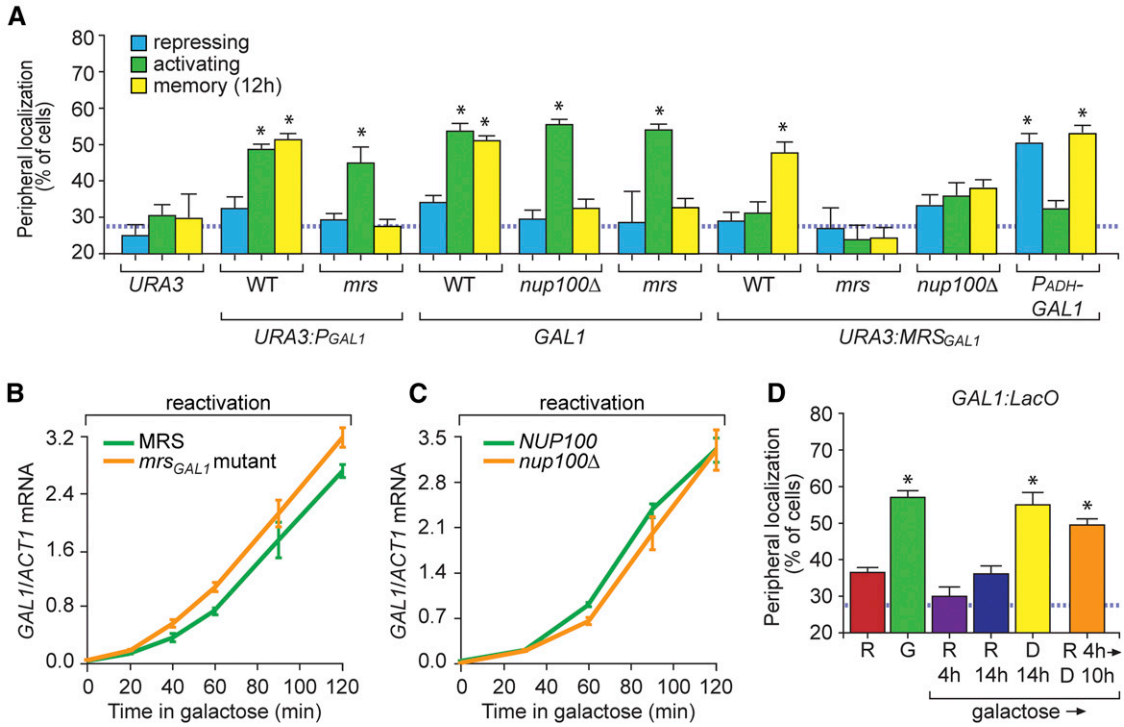


Figure 2 Memory Recruitment Sequence (MRS)$_{GAL1}$-dependent peripheral localization of *GAL1* during memory requires growth in glucose and Tup1. (A) Peripheral localization of *URA3*, *GAL1*, *URA3:P*$_{GAL1}$, or *URA3:MRS*$_{GAL1}$ was quantified under repressing (glucose), activating (galactose), and memory (galactose → glucose, 12 hr) conditions in wild-type (WT) or *nup100*Δ cells using immunofluorescence or live cell microscopy. The full-length *GAL1* promoter (*P*$_{GAL1}$, 667 bp) or the 63-bp MRS$_{GAL1}$ were inserted at *URA3* along with a LacO array as described (Egecioglu *et al.* 2014). The *mrs* mutation is shown in Figure S2B in File S1. (B and C) Cells were grown in galactose overnight, shifted to glucose for 12 hr, and then shifted to galactose (reactivation) to assay *GAL1* expression using RT-quantitative PCR in WT, mrs$_{GAL1}$ (B), and *nup100*Δ (C) mutant cells. (D) Peripheral localization of *GAL1* in cells grown in raffinose (R), galactose (G), and upon shift from galactose to: raffinose for 4 hr (R 4 hr), raffinose for 14 hr (R 14 hr), glucose for 14 hr (D 14 hr), or raffinose 4 hr followed by glucose 10 hr (R 4 hr → D 10 hr). The hatched line represents the level of colocalization with the nuclear envelope predicted by chance (A and D). Error bars represent SEM for at least three biological replicates. * $P \leq 0.05$ (Student's *t*-test) relative to the repressing condition.

peripheral localization specifically during memory (Figure 2A). Furthermore, mutations in this element (Figure S2B in File S1) disrupted targeting to the periphery of *URA3:MRS*$_{GAL1}$, *URA3:P*$_{GAL1}$, and the endogenous *GAL1* locus during memory (Figure 2A). Thus, the MRS$_{GAL1}$ is necessary and sufficient to control targeting to the nuclear periphery during *GAL* memory.

Loss of Nup100 also specifically disrupted *GAL1* peripheral localization during memory, but had no effect on *GAL1* peripheral localization during activating conditions (Figure 2A). Likewise, targeting of *URA3:MRS*$_{GAL1}$ to the nuclear periphery during memory required Nup100 (Figure 2A). ChIP against nuclear pore proteins Nup2 and Nup100 showed that, while Nup2 interacted with the *GAL1* promoter under both activating and memory conditions, Nup100 interacted with the *GAL1* promoter only during memory (Figure S3A in File S1). Finally, while inactivation of a conditional allele of Nup2 using the Anchor-Away technique (Haruki *et al.* 2008) led to rapid loss of peripheral localization under both activating and memory conditions, inactivation of Nup100 disrupted peripheral localization only during memory (Figure S3, B and C in File S1). Thus, while Nup2 plays a general role in *GAL1* peripheral localization, the molecular mechanism of *GAL1* targeting to the NPC during memory specifically requires the *cis*-acting MRS$_{GAL1}$ and the nuclear pore protein Nup100.

Although mutations in the MRS$_{GAL1}$ or loss of Nup100 blocked targeting of *GAL1* to the nuclear periphery during memory, these mutations did not alter the rate of reactivation of *GAL1* following 12 hr of repression (Figure 2, B and C). This suggests that targeting to the nuclear periphery is a product of *GAL* memory, but that the interaction with the NPC is not essential to promote faster *GAL* gene reactivation.

Targeting GAL1 to the nuclear periphery during memory requires both Gal1 protein and growth in glucose

Ectopic expression of Gal1 was sufficient to cause *URA3:MRS*$_{GAL1}$ localization to the nuclear periphery under repressing conditions (Figure 2A). Thus, like the native *GAL1*, MRS$_{GAL1}$-mediated targeting to the nuclear periphery is stimulated by expression of Gal1. Therefore, peripheral localization serves as a useful single-cell assay for long-term *GAL* transcriptional memory. Unexpectedly, ectopic expression of Gal1 did not lead to peripheral targeting of *URA3:MRS*$_{GAL1}$ in galactose medium (activating, Figure 2A). This suggested that MRS$_{GAL1}$-mediated peripheral localization during *GAL* transcriptional memory either required growth in glucose or is inhibited in galactose.

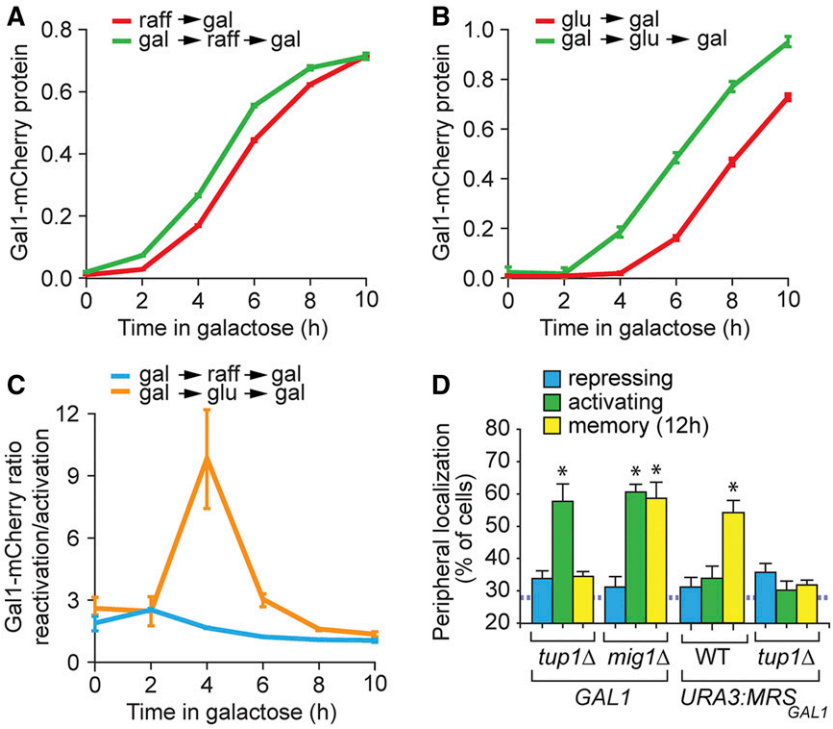


Figure 3 The adaptive value of memory in cells grown in nonrepressing and repressing carbon sources. (A and B) Gal1-mCherry expression during activation and reactivation, measured by flow cytometry. Activation: cells were shifted to galactose (gal) from either a nonrepressing carbon source, raffinose (raff) (A), or a repressing carbon source, glucose (glu) (B). Reactivation: cells were shifted from gal to either raff (A) or glu (B) for around seven cell divisions and then reactivated in galactose. (C) Gal1-mCherry reactivation:activation ratio at the indicated time points after shifting cells from raffinose to galactose or glucose to galactose. (D) Peripheral localization of *GAL1* or *URA3:MRS*$_{GAL1}$ in *tup1*Δ and *mig1*Δ mutant strains. The hatched line represents the level of colocalization with the nuclear envelope predicted by chance. WT, wild type. * $P \leq 0.05$ (Student's *t*-test) relative to the repressing condition. Error bars represent SEM for at least three biological replicates.

If glucose is necessary for the peripheral localization of *GAL1* and potentially other aspects of memory, we expected that recently-repressed *GAL1* would localize in the nucleoplasm in raffinose medium, a nonrepressing and nonactivating condition. Whereas induced *GAL1* in cells grown in galactose localized at the nuclear periphery, uninduced *GAL1* in cells grown in raffinose localized to the nucleoplasm (Figure 2D). This result conflicts with previous work showing that *GAL1* localizes at the nuclear periphery in cells growing in raffinose (Green *et al.* 2012). However, we find that expression of the ER/nuclear envelope marker used in that study, red fluorescent protein-HDEL (RFP-HDEL) is responsible for the discrepancy (data not shown).

Unlike *GAL1* in cells shifted from galactose to glucose, which remained at the periphery (D 14 hr, Figure 2D), *GAL1* in cells shifted from galactose to raffinose for either 4 or 14 hr localized in the nucleoplasm (R, Figure 2D). This was not due to lower Gal1 protein levels in cells shifted to raffinose; 4 hr after shifting from galactose to raffinose, Gal1-mCherry levels were slightly higher than in cells shifted from galactose to glucose for 4 hr (Figure S4 in File S1). Furthermore, cells shifted from galactose to raffinose retain the ability to target repressed *GAL1* to the nuclear periphery; in cells shifted from galactose to raffinose for 4 hr and then shifted to glucose for 10 hr, *GAL1* relocalized to nuclear periphery (R 4 hr → D 10 hr; Figure 2D). Therefore, Gal1 and glucose together promote targeting of *GAL* genes to the nuclear periphery during memory.

The rate of activation of *GAL* genes is much slower in cells shifted from glucose than in cells shifted from a nonrepressing carbon source like raffinose (Biggar and Crabtree 2001; Kundu and Peterson 2010). Cells shifted from galactose to glucose, upon returning to galactose, induce *GAL1* more rapidly than cells that have not previously grown in galactose. We hypothesized that memory is only evident in glucose because it only provides an adaptive advantage in cells growing in glucose. If so, then cells shifted from galactose to raffinose would, upon returning to galactose, induce *GAL1* with similar kinetics as naïve cells. We tested this idea by quantifying the effect of previous growth in galactose on the rate of induction of Gal1-mCherry when cells were shifted either from raffinose to galactose or from glucose to galactose (Figure 3). In cells shifted from raffinose to galactose, the rates of activation (raff → gal) and reactivation (gal → raff, seven divisions → gal) were similar (Figure 3A). In contrast, in cells shifted from glucose to galactose, the rate of activation (glu → gal) was significantly slower than the rate of reactivation (gal → glu, seven divisions → gal; Figure 3B). The difference between these two repressive sugars was also evident from the reactivation: activation ratio of Gal1-mCherry during induction (Figure 3C). This ratio was maximal (~11) in cells shifted from glucose back to galactose for 4 hr, illustrating the much greater impact of memory in cells grown in glucose.

In glucose, the Mig1 repressor and the corepressors Tup1 and Cyc8 bind to the *GAL* gene promoters to repress transcription (Santangelo 2006; Broach 2012). Therefore, we asked if these factors played a role in *GAL1* localization during transcriptional memory by scoring *GAL1* localization in *mig1*Δ and *tup1*Δ cells. The *cyc8*Δ mutant showed a severe growth defect, so it was not included in this analysis. While loss of Mig1 had no effect on *GAL1* localization, loss of Tup1 led to a specific defect in the targeting of *GAL1* to the nuclear periphery

during memory and disrupted peripheral localization of *URA3:MRS*$_{GAL1}$ (Figure 3D). Thus, Tup1 is required for MRS$_{GAL1}$-mediated peripheral localization of *GAL1* during memory.

Tup1 regulates binding of poised RNAPII to the GAL1 promoter and faster reactivation of GAL genes

Faster reactivation during memory in yeast and humans is associated with binding of preinitiation RNAPII to the promoter (Light *et al.* 2010, 2013; D'Urso *et al.* 2016). To test if *GAL1* transcriptional memory involves a similar mechanism, we used ChIP to monitor binding of RNAPII at the *GAL1* locus under repressing and activating conditions and at different times after repression. Recovery of both the *GAL1* promoter and the 5′-end of the *GAL1* CDS was quantified by qPCR (Figure 4A). RNAPII occupancy was low over both the *GAL1* promoter and CDS under repressing conditions and was high over both under activating conditions (Figure 4A). Shortly after shifting the cells from activating to repressing conditions (memory 20 min), RNAPII occupancy returned to background levels at both the promoter and the CDS (Figure 4A). However, between 2 and 4 hr of repression, RNAPII association with the promoter increased (Figure 4A). Binding of RNAPII during memory was unaffected by loss of Nup100 or mutations in the MRS$_{GAL1}$ (Figure 4D). However, loss of Tup1 specifically blocked RNAPII binding to the *GAL1* promoter during memory (Figure 4A). This suggests that long-term *GAL1* memory leads to binding of poised RNAPII to the promoter.

We next assessed the effects of Tup1 on *GAL1* activation and reactivation using RT-qPCR to measure mRNA levels (Figure 4B). In the wild-type strain, the rate of reactivation of *GAL1* was much faster than the rate of initial activation (Figure 4B, green *vs.* red). Consistent with a role in glucose repression, the rate of *GAL1* activation was slightly faster in absence of Tup1 (Figure 4B, cyan). However, following 12 hr of repression, the rate *GAL1* reactivation was significantly slower in the *tup1*Δ strain (Figure 4B, orange) and the rates of *GAL1* activation and reactivation were quite similar. This was not true under conditions of short-term *GAL1* memory; after 1 hr of repression in glucose, *tup1*Δ cells showed very rapid reactivation that was faster than the wild-type cells (Figure S5 in File S1). During osmotic stress, the Hog1 kinase converts the Tup1-Cyc8-Sko1 repressor complex into an activator (Rep *et al.* 2001; Proft and Struhl 2002). However, loss of Sko1 had no effect on *GAL* memory (Figure S6 in File S1). Thus, Tup1 plays a role in both glucose repression and in long-term *GAL* gene memory.

To establish the order of function of Tup1 and Gal1 in *GAL1* memory, we asked if loss of Tup1 is epistatic to ectopic expression of Gal1. Gal1-mCherry protein levels were measured using flow-cytometry in wild-type and *tup1*Δ cells in the presence and absence of P$_{ADH}$-*GAL1* (Figure 4C). In wild-type cells, P$_{ADH}$-*GAL1* led to a dramatic increase in the rate of activation of Gal1-mCherry (Figure 4C, green *vs.* red). As observed with mRNA quantification, activation of Gal1-mCherry was slightly faster in the *tup1*Δ strain (Figure 4C, cyan *vs.* red). However, loss of Tup1 blocked the effect of ectopic expression of Gal1 (Figure 4C, orange *vs.* cyan). This suggests that Tup1 functions downstream of Gal1 to promote faster *GAL* gene reactivation.

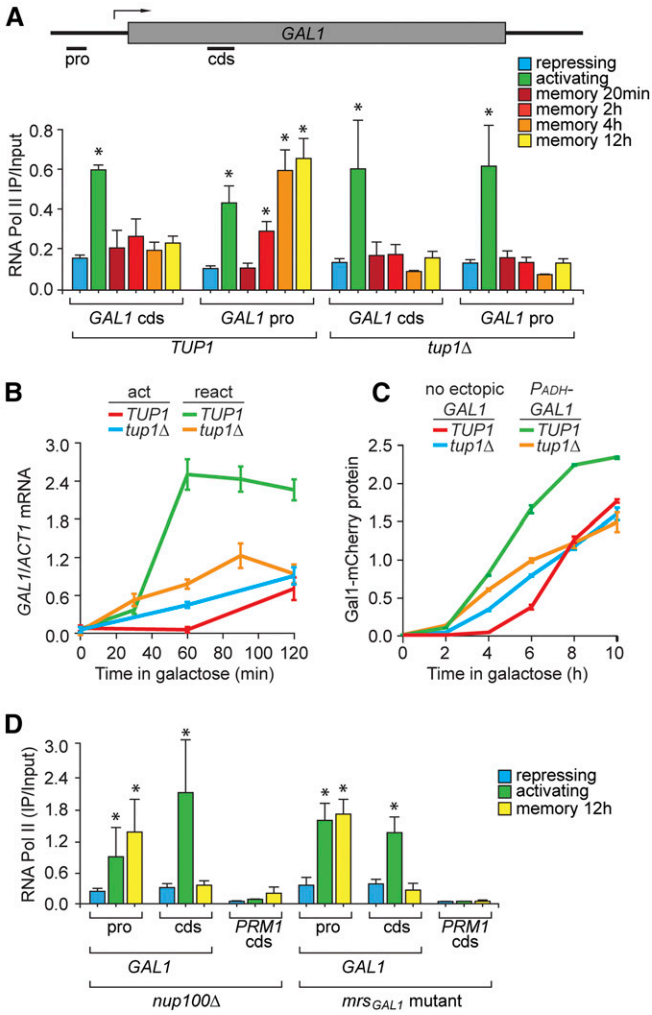


Figure 4 Tup1 functions downstream of Gal1 to promote binding of RNAPII to the promoter and faster reactivation of *GAL1* during memory. (A) RNAPII ChIP from wild-type and *tup1*Δ cells under repressing (glucose), activating (galactose), and at different times during memory (galactose → glucose, 20 min to 12 hr) conditions. Recovery of the *GAL1* promoter and cds was quantified relative to input by qPCR. (B) Time course of RT-qPCR for *GAL1* expression relative to *ACT1* during activation (act; glucose → galactose) and reactivation (react; galactose → glucose 12 hr → galactose) in WT and *tup1*Δ cells. (C) Gal1-mCherry expression during activation in wild-type and *tup1*Δ cells with or without P_{ADH}-*GAL1* integrated at the *TRP1* locus. (D) RNAPII ChIP under repressing (glucose), activating (galactose), and memory (galactose → glucose, 12 hr) conditions for *mrs*$_{GAL1}$ and *nup100*Δ mutant. Error bars represent SEM for at least three biological replicates. * $P \leq 0.05$ (Student's *t*-test) relative to the repressing condition. cds, coding sequence; ChIP, chromatin immunoprecipitation; pro, promoter; qPCR, quantitative PCR; RNAPII, RNA polymerase II; WT, wild type.

H2A.Z functions downstream of Gal1 to promote GAL memory

In addition to its role in glucose repression, Tup1 also promotes incorporation of H2A.Z into the *GAL1* promoter after

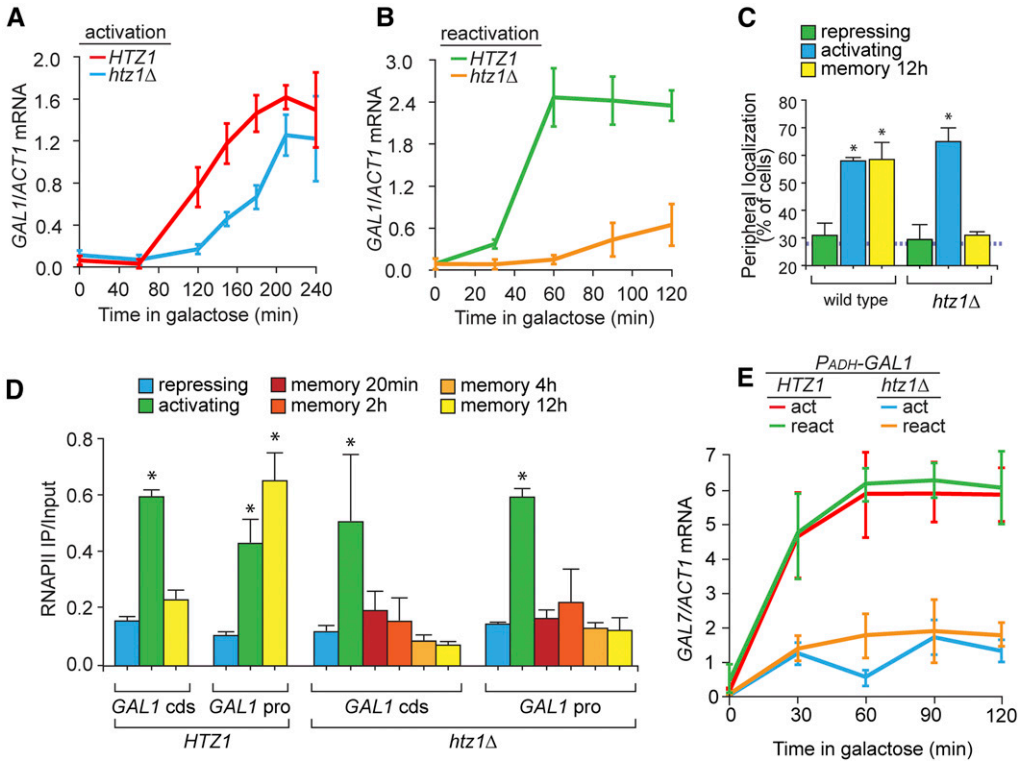


Figure 5 H2A.Z functions downstream of Gal1 to promote *GAL* transcriptional memory. (A and B) *GAL1* expression, relative to *ACT1*, measured by RT-qPCR over time in wild-type and *htz1*Δ cells during activation (A) and reactivation after 12 hr of repression (B). (C) Peripheral localization of *GAL1* under repressing (glucose), activating (galactose), and memory (galactose → glucose, 12 hr) conditions in wild-type and *htz1*Δ cells. The hatched line represents the level of colocalization with the nuclear envelope predicted by chance. (D) RNAPII ChIP from wild-type and *htz1*Δ cells under repressing, activating, and at different times during memory (galactose → glucose, 20 min to 12 hr) conditions. (E) *GAL7* expression, relative to *ACT1*, measured by RT-qPCR during activation or reactivation in wild-type and *htz1*Δ cells transformed with P_{ADH}-*GAL1*. Error bars represent SEM from at least three independent replicates. * $P \leq 0.05$ (Student's *t*-test) relative to the repressing condition. cds, coding sequence; ChIP, chromatin immunoprecipitation; pro, promoter; qPCR, quantitative PCR; RNAPII, RNA polymerase II.

repression (Gligoris *et al.* 2007). H2A.Z incorporation into the *INO1* promoter is essential for *INO1* transcriptional memory and loss of H2A.Z also leads to a strong, specific defect in the rate of *INO1* reactivation during memory (Brickner *et al.* 2007; Light *et al.* 2010). However, understanding the role of H2A.Z in *GAL* gene memory has been challenging because loss of H2A.Z leads to a defect in both activation and reactivation (Figure 5, A and B; Halley *et al.* 2010). To explore the role of H2A.Z in *GAL1* memory, we determined the effect of loss of H2A.Z using assays that are specific to memory: *GAL1* localization to the nuclear periphery and RNAPII binding after repression. Loss of H2A.Z disrupted both *GAL1* localization to the nuclear periphery (Figure 5C) and binding of poised RNAPII to the promoter during memory (Figure 5D), but did not affect *GAL1* localization to the nuclear periphery or RNAPII recruitment under activating conditions. Furthermore, loss of H2A.Z blocked the effect of ectopic expression of *GAL1* on the rate of induction of *GAL7* (Figure 5E). Thus, in addition to its role(s) in promoting *GAL* gene activation, H2A.Z plays an important role downstream of Gal1 in promoting *GAL* gene transcriptional memory.

Tup1 promotes incorporation of H2A.Z and H3K4me2 chromatin modification at the GAL1 promoter during memory

INO1 memory requires both persistent H2A.Z incorporation and H3K4me2 chromatin modification at the promoter (Light *et al.* 2010, 2013; D'Urso *et al.* 2016). Therefore, we tested if *GAL* gene transcriptional memory is associated with these chromatin alterations. The recovery of the CDS of the repressed *PRM1* gene served as a negative control for these ChIP experiments, and the recovery of the *BUD3* promoter served as a positive control for H2A.Z incorporation (Light *et al.* 2010; D'Urso *et al.* 2016). During memory, both H2A.Z occupancy and dimethylation of H3K4 increased significantly at the *GAL1* promoter, relative to the repressed condition (Figure 6, A and B). Likewise, expression of P_{ADH}-*GAL1* under repressing conditions also led to an increase in both H2A.Z occupancy and H3K4me2 (Figure 6, C and D). Thus, Gal1-mediated transcriptional memory leads to increased incorporation of H2A.Z and dimethylation of H3K4.

The increased H2A.Z incorporation and the dimethylation of H3K4me2 over the *GAL1-10* promoter associated with memory or ectopic expression of Gal1 was lost in strains lacking Tup1 (Figure 6). This effect was specific; loss of Tup1 had no effect on the H2A.Z incorporation into the *BUD3* promoter. Thus, Tup1 functions downstream of Gal1 to promote the changes in chromatin structure or modification associated with memory.

Discussion

The yeast *GAL* genes localize to the nuclear periphery and physically interact with the NPC during both activation and

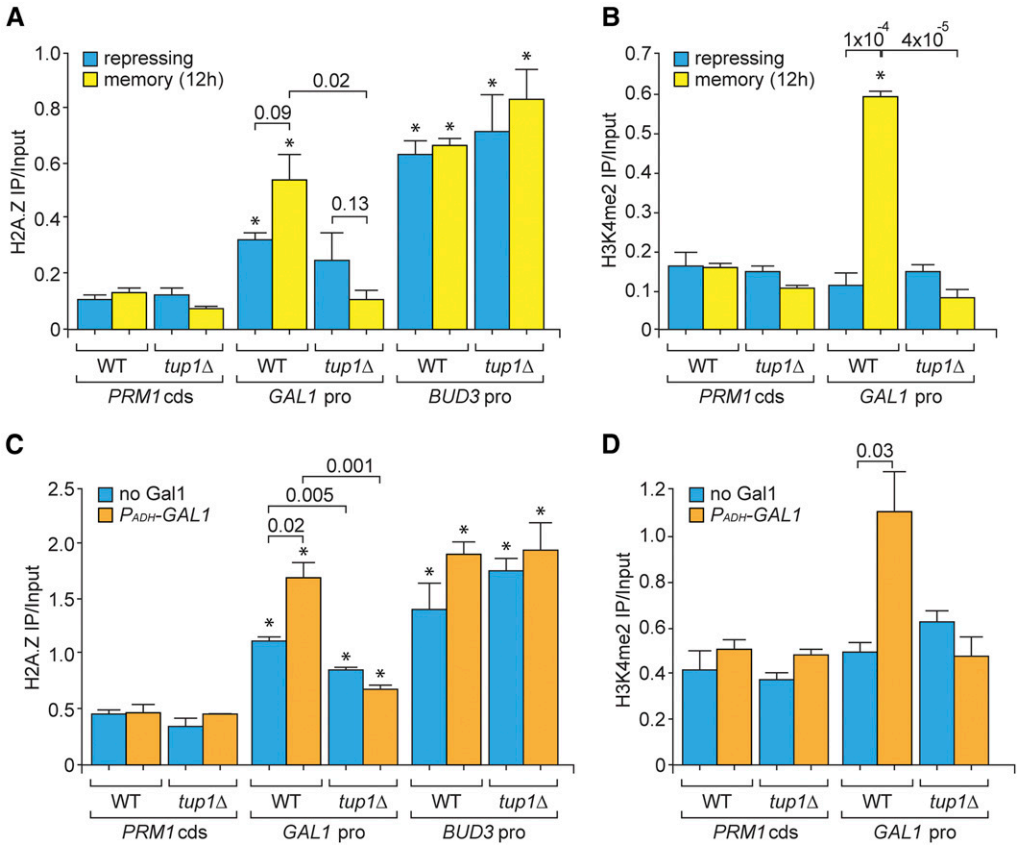


Figure 6 Tup1 promotes H2A.Z incorporation and H3K4me2 modification during *GAL* memory. (A and C) H2A.Z ChIP in WT and *tup1*Δ cells under repressing (glucose) and memory (galactose → glucose, 12 hr) conditions (A) or under repressing conditions with P_{ADH}-*GAL1* (C). The recovered DNA fragments in IP were analyzed for sequences arising from the *GAL1* promoter, *PRM1* coding sequence (negative control), and *BUD3* promoter (positive control) and plotted relative to input fraction. (B and D) H3K4me2 ChIP in WT and *tup1*Δ cells, performed as described in (A and C). Error bars represent SEM from at least three independent replicates. * $P \leq 0.05$ (Student's *t*-test) relative to the repressing condition. cds, coding sequence; ChIP, chromatin immunoprecipitation; H3K4me2, histone 3 dimethyl lysine 4; IP, immunoprecipitation; pro, promoter; qPCR, quantitative PCR; WT, wild type.

memory (Brickner *et al.* 2007). During activation, peripheral localization of *GAL1* requires the GRS4 and GRS5 DNA zip codes and is necessary for full expression (Brickner *et al.* 2016). We find that a different DNA zip code, the MRS_{GAL1}, controls the persistent localization to the nuclear periphery during *GAL1* memory. Targeting to the nuclear periphery is downstream of Gal1 protein; loss of Gal1 disrupts peripheral retention during memory and ectopic expression of Gal1 leads to MRS_{GAL1} zip code- dependent targeting of *GAL1* to the nuclear periphery even under repressing conditions. However, the association of *GAL* genes with the NPC is not necessary for faster reactivation, suggesting that it is a product, rather than a driver, of memory. Because localization to the nuclear periphery during memory required growth in glucose, this led us to uncover a critical role for the Tup1 transcription factor in *GAL* memory. Tup1 contributes to repression of *GAL* genes in the presence of glucose. However, during transcriptional memory, Tup1 functions downstream of Gal1 to promote changes in chromatin structure and binding of RNAPII to the *GAL1* promoter.

Among yeast genes that exhibit transcriptional memory, the *GAL* genes show the strongest increase in reactivation kinetics and the longest duration (~8 generations). The *GAL* genes remain associated with the nuclear periphery during this period. Although faster reactivation of *GAL1* does not require peripheral localization, peripheral localization requires all of the factors that are required for faster reactivation (Gal1, Tup1, and H2A.Z). Thus, the NPC association reflects the memory state and serves as a useful assay for this phenomenon. It is possible that, under conditions distinct from those that we have tested, interaction with the NPC contributes to the rate of *GAL* gene reactivation. Alternatively, interaction with the NPC might impact events that we have not assessed. Finally, interaction with the NPC may be functionally redundant with another pathway that promotes *GAL* gene reactivation, both of which are downstream of Gal1.

Exploring the conditions under which the MRS_{GAL1} leads to peripheral localization highlighted the role of glucose in *GAL* transcriptional memory. Peripheral localization mediated by MRS_{GAL1} requires growth in the presence of glucose, even in cells expressing ectopic Gal1. Furthermore, the benefit of previous growth in galactose is most apparent when cells are shifted from glucose to galactose, where memory provides a large adaptive benefit. Glucose regulates expression of *GAL* genes via the Mig1-Tup1-Cyc8 repressor complex (Treitel and Carlson 1995). Although Mig1 recruits the Tup1-Cyc8 corepressor to the *GAL1* promoter in glucose (Nehlin *et al.* 1991), Tup1 is also recruited to the active *GAL1* promoter in a Mig1-independent manner (Papamichos-Chronakis *et al.* 2004). This suggests that Tup1 has function(s) in addition to glucose repression. Consistent with this notion, loss of Mig1 had different effects than loss of Tup1. While loss of Mig1 did not affect *GAL1* localization and accelerated both activation and reactivation (Figure S7 in File S1), loss of Tup1 specifically disrupted *GAL1* peripheral localization during

memory, and led to slightly faster activation and significantly slower reactivation. This suggests that Tup1 plays distinct roles during activation and reactivation. Tup1-Cyc8 is mostly characterized as a corepressor (Smith and Johnson 2000) that masks activation domains (Wong and Struhl 2011), binds hypoacetylated histones (Davie *et al.* 2002), recruits histone deacetylases (Wu *et al.* 2001), interacts with mediator subunits (Lee *et al.* 2000; Papamichos-Chronakis *et al.* 2000), and repositions nucleosomes (Cooper *et al.* 1994). However, Tup1 can also function as a coactivator, facilitating recruitment of Spt-Ada-Gcn5 acetyltransferase SAGA or SWI/SNF to promote transcription (Zhang and Guarente 1994; Ozcan *et al.* 1997; Conlan *et al.* 1999; Papamichos-Chronakis *et al.* 2002; Hickman and Winston 2007). Thus, the different effects of Tup1 on active *GAL1* and recently-repressed *GAL1* may reflect different activities of Tup1 at the *GAL1* promoter during repression and memory.

Our current model for Tup1 function in memory is that this protein alters the chromatin of the promoter by promoting H2A.Z incorporation and H3K4me2 modification, allowing both peripheral localization and RNAPII binding. Tup1-Cyc8 promotes H2A.Z incorporation into the active *GAL1* promoter and SAGA recruitment (Papamichos-Chronakis *et al.* 2002; Gligoris *et al.* 2007). Loss of H2A.Z leads to a defect in the rate of activation and reactivation of *GAL1*, but leads to specific defects in RNAPII binding at the *GAL1* promoter and *GAL1* peripheral localization during memory (Brickner *et al.* 2007; Halley *et al.* 2010). Furthermore, H2A.Z is required for Gal1-mediated faster reactivation of *GAL7*. Thus, we propose that Tup1 promotes transcriptional memory through increasing H2A.Z incorporation and, potentially, enhancing dimethylation of H3K4.

Because only a few hundred Gal1 molecules are sufficient to induce *GAL* transcriptional memory, memory persists through at least seven cell divisions, providing a very long adaptive benefit to previous growth in galactose. However, memory is most adaptive when cells are switched from glucose and glucose is required for features of memory. Although we do not yet understand how growth in glucose impinges upon *GAL* memory, it is plausible that Tup1 function requires the presence of glucose. Because Gal1 requires Tup1 to mediate memory, these two factors may function to integrate prior growth in galactose with current growth in glucose to regulate memory. Such a mechanism would allow cells to induce memory only when it would be most beneficial.

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