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The Mechanism, Adaptive Fitness, and Evolution of Galactose Induced Transcriptional Memory in *Saccharomyces cerevisiae*

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Abstract

Certain inducible genes show faster reactivation if they were recently expressed. This epigenetic phenomenon is called transcriptional memory and is inherited for several generations after the first round of induction. During this phase, genes show several conserved molecular features that are essential for faster reactivation: peripheral localization of the gene, binding of poised RNA polymerase II, H2A.Z incorporation, and H3K4me2 modification at the promoter. However, it is unclear how regulatory systems of different genes are modified by transcriptional memory to mount faster reactivation. Furthermore, it is unknown how transcriptional memory evolved and whether it has any adaptive value. To address these questions, I have investigated the mechanism of *GAL* gene transcriptional memory in yeast. *GAL* genes show a strong upregulation of expression kinetics during memory that persists for seven cell divisions, making it an excellent model. I found that during memory, *GAL* genes localize to the nuclear periphery and exhibit the conserved chromatin changes, as seen during transcriptional memory of *INO1*. However, unlike *INO1* memory, peripheral localization is dispensable for faster reactivation of *GAL* genes. Using both a candidate based approach and a genetic screen, I found that faster reactivation is regulated by factors both upstream and downstream of Gal4 transcription factor and by a domain within Gal4. A Gal1 co-activator, produced during initial induction, acts upstream of Gal4 by neutralizing the Gal80 inhibitor. This leads to the faster uni-modal expression of *GAL* gene. The faster co-activation by Gal1 is dependent on the interaction of Gal4 central domain with its activation domain. This interaction is necessary for high levels of expression from Gal4. Downstream of Gal4, Tup1 transcription factor together with H2A.Z promote binding of a pre-initiation form of RNA

polymerase II at the *GAL1* promoter, poising the *GAL* genes for faster reactivation. The faster expression of *GAL* gene during memory confers a huge fitness advantage in *S. cerevisiae* by decreasing the growth lag upon shift to galactose. However, a related yeast species, *S. uvarum*, does not show similar benefit from memory. Rather, it shows a constitutive memory-like response due to leaky expression of *GAL1*. The absence of such constitutive memory in *S. cerevisiae* represents a trade-off for better fitness in mixed sugars. Thus, *GAL* memory is a recently evolved phenomenon that allows cells to integrate a previous experience (growth in galactose, reflected by Gal1 levels) with current conditions (growth in glucose, potentially through Tup1 function). These inputs modulate both the levels of expression and fraction of cells that expresses *GAL* genes in a population. The resulting faster expression promotes rapid adaptation to changes in carbon source during memory.

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Dedication

I would like to dedicate this dissertation to my parents, Ms. Usha Sood and Mr. Vijay Kumar Sood. All my achievements are a result of their sacrifices. They faced a lot of hardships but always ensured I get the best upbringing. The values they instilled in me have guided me through testing times. I love them, respect them, and hope to make them proud.

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Chapter I: Introduction

1.A Epigenetic memory

Cells with identical genomes can exhibit different gene expression or phenotypic states. When such states persist for several generations after the initiating stimuli have been removed, they are referred as epigenetic states [1-4]. Such epigenetically inherited states regulate cellular identity and response to environmental changes [5]. Epigenetic mechanisms thus provide an additional layer ("*epi*") of heritable gene regulation, without changing the DNA sequence, and play physiologic and adaptive roles. The epigenetic states can either be invariant (telomere silencing and X-inactivation) or dynamically inherited for short periods of time. When the epigenetic states are dynamic, they are referred as epigenetic memory: a heritable change in gene expression or behavior that is established by previous stimuli [6]. There are at least three types of memory: cellular, transgenerational and transcriptional. Out of these, transcriptional memory is the focus of this thesis. The three kinds of memory differ on the duration of inheritance but utilize similar mechanisms [6]. These mechanisms fall into two broad categories. First, *trans*-acting mechanisms that involve a positive feedback loop of a diffusible factor. Such a factor propagates the epigenetic state as well as its own renewal. Second, *cis*-activating mechanisms that involve either inheritance of DNA methylation or histone modifications that impact transcription. Here, I will review the different types of epigenetic memory, the mechanisms of epigenetic inheritance and its impact on transcription. Using *INO1* as a model I will review the known mechanisms of transcriptional memory and then present what is known about *GAL* transcriptional memory.

Cellular memory

The cellular memory refers to epigenetic states that are heritable over mitosis [6]. A paradigm example of this phenomenon is the inheritance of homeotic gene expression patterns in *Drosophila*. Different sets of homeotic genes are activated during development depending upon the position of cells along the embryo axis [7]. These expression patterns are re-established after mitosis by Trithorax and Polycomb group of proteins, through many cell divisions [3, 8-10]. The Trithorax proteins maintain the active chromatin mark, (methylation of Lysine 4 on Histone 3, H3K4me) at homeotic genes marked for activation. The Polycomb proteins, on the other hand, maintain a repressive chromatin mark (methylation of Lysine 27 on Histone 3, H3K27me) over the silent homeotic genes [3, 8-10]. These chromatin modifications are thought to act as "bookmarks" that transmit the epigenetic states across DNA replication. Cellular memory can also be established by environmental stimuli. In *Arabidopsis*, cold stress leads to repression of an inhibitor of flowering, *FLC*, through *VRN2* [11, 12]. VRN2 represses *FLC* by introducing the repressive H3K27me mark, during winters. VRN2 maintains this mark during spring and promotes flowering, even in absence of cold stress [11-14]. Both VRN2 and Polycomb complex act through a positive feedback loop: both are recruited by H3K27me and also introduce the same modification [9, 14]. Thus, mechanisms of cellular memory in response to both developmental and environmental stimuli involve heritable chromatin modification.

Transgenerational Memory

Despite global changes in chromatin structure and expression during gametogenesis and embryogenesis, memory of a previous signal can be passed to the next generation [15]. Such epigenetic states that are inherited over meiosis are referred as transgenerational memory [6]. A well-studied example of this phenomenon is genomic imprinting [16]. In humans, a small set of genes is expressed only from the paternal or the maternal allele i.e. imprinted. For example, *IGF2* gene is expressed only from the paternal allele [16-18]. During gametogenesis in the females, the *IGF2* locus is marked for silencing through DNA methylation [15]. *IGF2* locus in males remains rather un-methylated. The DNA methylation and repression of the maternal allele is inherited in the zygote and maintained throughout the adult life [15]. Transgenerational memory can also be induced by environmental stimuli. For example, upon heat shock the *Drosophila* repressors ATF-2 gets phosphorylated and losses binding to heterochromatin [19]. This leads a to loss of repressive H3K9me mark and increased transcription from heterochromatin [19]. After repeated heat stress over successive generations, ATF-2 biding is lost for several generations even in the absence of heat stress [20]. Increased transcription from heterochromatin is thought to improve tolerance to variable challenging environments [20]. Thus, transgenerational memory plays both physiologic and adaptive roles.

Transcriptional memory

In response to previous experiences, certain inducible genes show a mitotically heritable increase in the rate of transcription [6, 21]. During this epigenetic phase, the genes are

repressed but remain competent for faster induction. This phenomenon, referred as transcriptional memory, is observed in yeast, *Drosophila* and humans. In yeast, the *INO1* shows a much faster induction upon inositol starvation if the cells have been recently starved for inositol [6, 22, 23]. This *INO1* transcriptional memory lasts for 2-3 cell divisions. Similarly, prior induction with galactose primes *GAL* genes for faster reactivation upon second induction with galactose. The *GAL* gene memory lasts for up to seven cell divisions [24-26]. Cross priming for faster transcription can also occur. A brief salt stress primes hundreds of yeast genes for faster induction with H_2O_2.[27] In *Drosophila*, several ecdysone induced genes exhibit transcriptional memory that lasts for 20 h [28]. In HeLa cells, hundreds of interferon gamma induced genes show transcriptional memory that lasts up to seven cell divisions [23, 29]. Thus, transcriptional memory is widespread and affects genes with diverse function and regulatory systems. Despite this diversity, certain aspects of the mechanism for transcriptional memory are conserved. During transcriptional memory, the genes associate with the nuclear pore complex (NPC) and exhibit heritable changes in the chromatin structure [23, 30]. These changes together lead to binding of a form of RNA polymerase II (RNAPII) that is poised for transcription initiation, which by-passes the rate limiting step in RNAPII recruitment [23, 30].

1.B. Role of nuclear pore complex in gene expression and transcriptional memory

In addition to their role in nucleo-cytoplasmic transport, the NPCs play an important role in genome organization, gene regulation and transcriptional memory [31-33]. The NPC has a conserved modular structure that penetrates the nuclear envelope [34, 35]. A set of scaffolding nuclear pore proteins (Nups) constitutes the core of the concentric ring around the central

transport channel [34, 35]. This structure serves as docking site for several phenylalanine-glycine Nups (FG-Nups) [34, 35]. In addition, peripheral structures of the NPC project both into the nucleus (nuclear basket) and the cytoplasm (cytoplasmic filaments). While FG-Nups create a selective barrier for transport through the channel, components of the nuclear basket and some FG-Nups interact with transcriptional regulators, mRNA export factors and chromatin [33-35]. Thus, the modular structure allows NPCs to perform multiple biological functions.

NPC interacts with both active and repressed parts of the genome. Interaction with components of the nuclear basket, Nup2, Nup60, Mlp1, and Mlp2 is strongly correlated with active genes involved in glycolysis and protein biosynthesis [36]. Furthermore, recruitment of several genes to the NPC occurs upon activation with diverse stimuli: nutrient shift, osmotic stress, heat shock, and exposure to pheromone [37-44]. However, interaction with NPC is not always associated with active genes. For example, interaction with Nsp1, Nup84, Nup145 and Nup100 is not correlated with expression [36, 39]. Furthermore, some Nups are essential for repression: Nup170 is required for silencing of several ribosomal and subtelomeric genes through cooperation with silencing protein Sir4 [45]. Several mechanisms have been proposed on how NPC regulate transcription. Physical associations of NPC with transcriptional co-activator[46], histone acetylase complex SAGA [47, 48], and multiprotein mRNA export complex TREX-2[49, 50] are thought to promote transcription. Interactions with NPC, in some cases, increase the fraction of cells responding to these inducing signals [46, 51]. Several Nups can also act as insulators that restrict the spread of both active and repressed chromatin. Thus, NPCs play both activating and repressing roles in transcription [52, 53]. Although such

functions are conserved from yeast to humans [54], the mechanisms underlying these divergent functions are not clear. It has been proposed that these different roles might arise from different composition of a subset of NPCs. For example, Mlp1 and Mlp2, associated with active genes, are present in some but not all NPCs within the nucleus [55].

NPCs also play an essential role in transcriptional memory. In yeast, a nuclear basket protein, Mlp1, promotes transcriptional memory of galactose-induced genes, *HXK1* and *GAL1* [56, 57]. A chromatin loop between 5' and 3' of these genes, required for faster reactivation, is maintained by Mlp1 [56, 57]. A different Nup, Nup42, promotes salt-stress induced transcriptional memory of hundreds of yeast genes [27]. Transcriptional memory of *INO1* gene requires interaction with Nup100 at the nuclear periphery [22, 30]. This interaction is necessary for promoting chromatin modifications (H2A.Z incorporation and H3K4 modification) and binding of poised RNAPII [22, 30]. A homolog of Nup100, Nup98, plays a similar role during the transcriptional memory of interferon-gamma induced genes in HeLa cells [23]. However, unlike Nup100, Nup98 interacts with these genes in the nucleoplasm [23]. Nup98 is also required for transcriptional memory of ecdysone-induced gene in *Drosophila* embryo [28]. Nup98 promotes faster reactivation through promoter-enhancer looping and possibly through interaction with histone acetyltransferase CBP-p300 [28, 58]. Thus, although the involvement of NPC is conserved, it promotes transcriptional memory through diverse mechanisms: gene looping, promoter chromatin modifications, and enhancer-promoter interaction.

1.C. Mechanisms of epigenetic inheritance and gene regulation

Cis-acting epigenetic mechanisms

The heritable DNA and histone modifications that impact gene expression constitute the *cis*-acting epigenetic mechanisms. DNA modification occurs mainly at CpG islands by *de novo* DNA methylase, DNMT3A and 3B, during development [5, 59-62]. The DNA methylations are inherited by a semi-conservative mechanism: DNMT1 copies the methylation pattern from the parental strand on to the newly synthesized strand [63, 64]. The DNA methylation plays a repressive role [60, 61, 65-67]. Repression occurs either by inhibiting the binding of transcription activators or by recruiting methyl-binding proteins that have a repressor function [65-67]. As a result, DNA methylation stably perpetuates silencing during imprinting, X-inactivation, and retroviral silencing [15, 68-71].

Unlike DNA methylation, which does not exist in yeast, histone modifications exist in all eukaryotes [71, 72]. The four histones, H3, H4, H2A, and H2B, form a core particle that wraps around 147 base pairs of DNA [73]. This complex, called nucleosome, constitutes the basic repeating unit of chromatin. The histone tails protrude out of the nucleosome and undergo extensive post-translational modifications [74]. These modifications can occur at 60 histone residues, which can be acetylated, methylated, phosphorylated, sumoylated and ubiquitinylated [75, 76]. Some of these modifications affect nucleosomal interaction [77]. A

modification that decreases nucleosomal interaction (acetylation) destabilizes the nucleosomes and promotes expression. Other modifications act as docking sites for non-histone proteins that modify chromatin and affect transcription, DNA repair, and replication [76, 78]. To describe the effects on the transcription, "histone code" hypothesis asserts that certain sets of modifications are associated with either repressed or expressed genes. For example, expressed genes are associated with acetylation and tri-methylation at H3K4, K3K36, and K3K69, whereas repressed genes are associated with methylation at H3K9, H3K27, and H3K40 [79, 80]. The associations with transcription states are context dependent: active genes have H3K36me3 mark in the coding region, but same mark at the promoter region is associated with repressed genes [81, 82]. Furthermore, cross talk between histone modifications can be cooperative or antagonistic [83, 84]. Thus, the diverse histone modification reflects a spectrum of transcriptional states. However, it is unclear if histone modifications directly affects or rather they are an outcome of the transcriptional states [85]. Evidence supporting either scenario exists, implying a likely positive feedback between the histone modifications and transcriptional states [85].

Current models for inheritance of histone modifications propose a semi-conservative mechanism [78, 81, 86-91]. During S-phase, parental histones are randomly distributed between the replicated DNA [88, 92-94]. The epigenetic modifications on the parental histones can guide similar modifications on the newly incorporated histones [88, 92-94]. The epigenetic inheritance of such modification requires that the histone modifiers also bind to the histone modification they introduce, leading to a positive feedback loop [95]. For example, Sir complex

is recruited to partially de-acetylated telomere and catalyze de-acetylation of the surrounding regions [96-100]. This leads to a stable perpetuation of silent de-acetylated telomeres. Similar mechanism of inheritance occurs at homeotic genes. The Polycomb proteins bind H3K27me at the silent homeotic genes and introduce H3K27me to the surrounding chromatin [3, 8-10]. However, most histone modifications are not inherited and the extent to which they are heritable is not clear. For example, tethering Clr4 in *S. pombe* introduces the repressive H3K9me3 modification, which is also bound by a domain in Clr4 [101]. However, in the absence of tethered Clr4, H3K9me3 modification is removed by an Epe1 demethylase and therefore not inherited. But, if Epe1 is deleted, H3K9me3 is inherited for at least 50 generations after removing the tethered Clr4 [101]. Thus, although positive feedback loop for chromatin modification promotes epigenetic inheritance, it is not widespread probably due to presence histone modifiers with antagonistic activity.

Trans-acting epigenetic mechanisms

The inheritance of diffusible factors that propagate epigenetic states constitutes the *trans*-acting epigenetic mechanisms. Such a factor could be a master transcription regulator that establishes the expression state as well as promotes its own renewal. Wor1 in *Candida albicans* is one such transcription factor. Wor1 regulates the white-opaque phenotype switching. The white and opaque cells differ in morphology, gene expression and mating behavior. These states are stably inherited for several generations and switch at low frequency. Once expressed, Wor1 establishes the opaque epigenetic state as well as a positive feedback loop for its own expression [102]. Another *trans*-acting mechanism occurs through self-templating

conformation change by yeast prion proteins. Prions are unusual extended conformations of otherwise well-folded cellular proteins [103]. Prions propagate by templating other molecules of the same protein to a prion form, which coalesce and form insoluble aggregates [104]. The division of protein aggregates during cytokinesis ensures the epigenetic inheritance, through both mitosis and meiosis. The phenotype of the epigenetic state is determined by the loss of function associated with the prion protein. In yeast, prions form under stress and play an adaptive role [103, 105]. For example, Sup35, a well-characterized prion protein, is a translation terminator. Its prion form leads to stop codon read-through [105]. This leads to diverse phenotypic effects, including change in cell-adhesion, nutrient use, and resistance to toxins. Some of these phenotypes are adaptive under stress [103, 105]. Thus, *trans* epigenetic mechanisms involve a self-renewing diffusible factor that regulates the switch between epigenetic states.

1.D. *INO1* transcriptional memory

The mechanism of *INO1* transcriptional memory has been extensively studied (Figure 1.1) [22, 23, 30]. Following repression, *INO1* localizes to the nuclear periphery and exhibits heritable chromatin changes. These changes lead to binding of a poised RNAPII and faster reactivation for 2-3 generations (Figure 1.1; memory phase) [22, 23, 30]. During this period, Sfl1 transcription factor binds to the promoter DNA element, Memory Recruitment Sequence (MRS), and initiates all known aspects of *INO1* memory: peripheral localization, interaction with nuclear pore protein 100 (Nup100), di-methylation of histone 3 lysine 4 (H3K4me2),

incorporation of H2A.Z, and binding of poised RNAPII [30]. Disrupting any of these changes leads to a loss of rapid reactivation.

Epigenetic regulation of peripheral localization is critical for both *INO1* activation and transcriptional memory. During activation, two promoter DNA elements, GRSI and GRSII, guide peripheral localization and interaction with Nup2 [37]. GRSI and GRSII are bound by Put3 and Cbf1 transcription factors, respectively [41]. Mutating the GRS or deleting *NUP2* or *PUT3* leads to loss of peripheral localization during *INO1* activation. During the memory phase, Sfl1 binds the MRS and guides peripheral localization and interaction with Nup100 [30]. Nup100 is essential for H2A.Z incorporation, H3K4me2 modification, RNAPII binding, and faster reactivation [22]. Although, how Nup100 promotes these chromatin changes and faster reactivation is not clear [23].

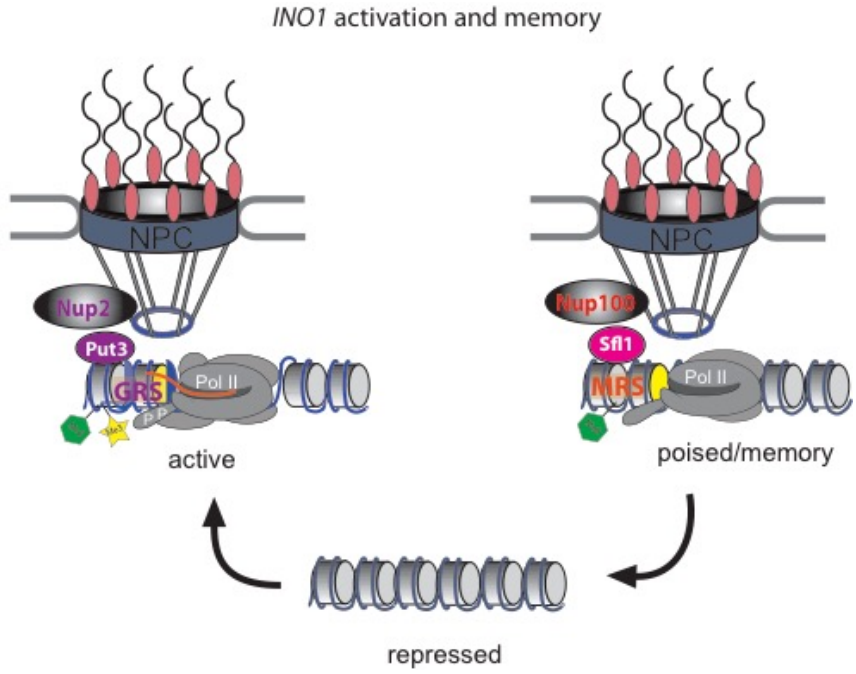

Figure 1.1: Model for epigenetic changes during *INO1* memory: Upon activation, Put3 binds the GRS and causes peripheral localization of *INO1* and its interaction with Nup2. The *INO1* promoter also shows chromatin changes: H3K4me3, H3K4me2, and H2A.Z incorporation (yellow). Upon repression, Sfl1 binds the MRS and causes peripheral localization of *INO1* and its

interaction with Nup10. The *INO1* promoter also shows a poised RNA polII bound at the promoter and inheritance of chromatin changes: H3K4me2 and H2A.Z incorporation (yellow).

Recent studies have shed light on the mechanism of inheritance and role of H3K4me2 modification. During *INO1* memory, both SET1/COMPASS and Mediator are repurposed to promote H3K4me2 modification and binding of poised RNAPII [30]. Set1, catalytic subunit of COMPASS H3K4 methyltransferase, deposits a tri-methylation mark on H3K4 [41, 106-109]. The H3K4me3 mark is associated with active genes [110, 111]. During *INO1* memory, however, a remodeled SET1/COMPASS lacking Spp1 introduces H3K4me2 modification instead [112-115]. Unlike H3K4me3, H3K4me2 is associated with poised promoters [29, 116-118], inactive genes, and repression of non-promoter cryptic transcripts [119-121]. The H3K4me2 mark is bound by the Set3, which is a part of SET3C histone deacetylase [121]. Set3 binding is necessary for maintaining H3K4me2 during *INO1* memory [30]. Pre-initiation complex (PIC) is also remodeled during *INO1* memory. This PIC lacks the Kin28 kinase subunit and has Cdk8+ form of mediator [30]. The Kin28 kinase is required for RNAPII escape from the promoter [22, 122, 123]. The Cdk8+ form of mediator is specifically found at promoters of genes with poised RNAPII [124]. Thus, PIC subunits are reorganized during *INO1* memory to maintain a poised RNAPII binding that does not escape the promoter. Conditional depletion of factors required for H3K4me2 leads to a loss of poised RNAPII, while depletion of some but not all PIC components leads to a loss of H3K4me2 retention [30]. Thus, H3K4me2 provides a platform for binding of poised RNAPII (unpublished results, Agustina), which in turn promote retention of H3K4me2.

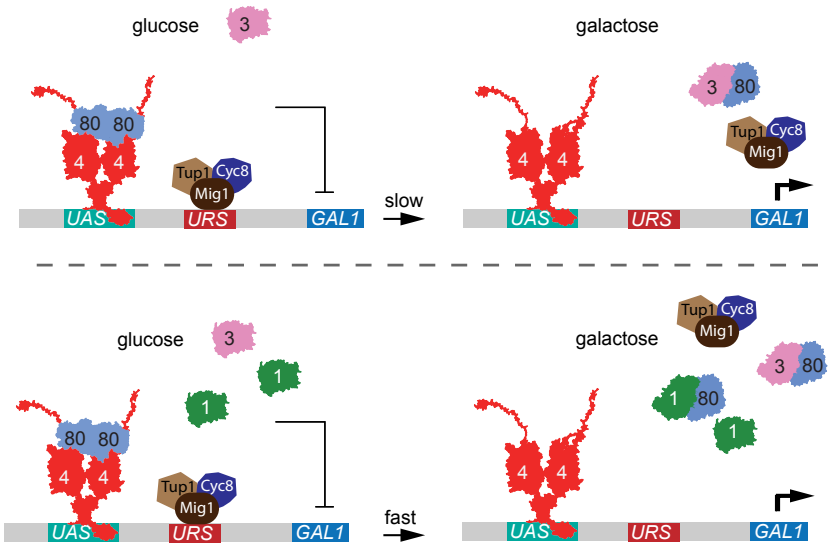

Figure 1.2: Schematic for *GAL1* gene regulation and transcriptional memory: Upper panel: during activation in galactose, Gal3 sequesters the Gal80 repressor from the Gal4 activator while Mig1 repressor along with the co-repressors, Tup1 and Cyc8, is exported out of the nucleus in the absence of glucose, leading to *GAL1* gene expression. Lower panel: during reactivation, residual Gal1 augments Gal3 co-activation, leading to faster expression kinetics.

1.E. *GAL* gene regulation and transcriptional memory

In yeast, *GAL* genes (*GAL1, GAL2, GAL10,* and *GAL7*) code for enzymes that converts galactose to glucose (Leloir pathway) [125]. Expression of *GAL* genes is tightly repressed in glucose and strongly induced in galactose by three regulators (*GAL3, GAL4,* and *GAL80*) [126-133]. In the absence of galactose, the Gal4 transcription factor binds upstream of *GAL* genes but the Gal80 repressor inhibits its productive interaction with SAGA and mediator complex [134-141] (Figure 1.2). During growth in glucose, another repressor, Mig1, recruits Tup1-Cyc8 co-repressors to further inhibit *GAL* gene transcription (Figure 1.2; top panel) [142]. Upon shift from glucose to galactose, Gal3 co-activator neutralizes Gal80 repression. The absence of glucose triggers Mig1's phosphorylation and its export out of the nucleus (Figure 1.2; top panel)

[143]. The cumulative de-repression of both Mig1 and Gal80, and upregulated levels of Gal4 in galactose media leads to a 1000-fold increase in the expression of *GAL* genes [129].

Cells that have not seen galactose for a long time (naïve cells) show a slow rate of *GAL* gene expression [24-26, 144]. This stems from the higher levels of Gal80 repressor relative to Gal3 co-activator [145, 146]. Consequently, only cells with lower levels of Gal80 express *GAL* genes initially, but eventually all cells follow suit [147]. This leads to a mixed population of expresser and non-expressers (bi-modal distribution) during early induction, which slows down the average rate of expression [147]. Since *GAL* genes are essential for growth in galactose, the slow rate of activation poses a fitness challenge. *GAL* gene transcriptional memory overcomes this limitation by increasing induction rates through the inheritance of a *trans*-factor, Gal1 (Figure 1.2; bottom panel) [25, 26]. Gal1 is both a galactokinase and a co-activator that is similar to Gal3 [148, 149]. Unlike Gal3, Gal1 is strongly induced in galactose and gets diluted with each cell division. As a result, Gal1 is present at higher stoichiometric levels over Gal80 during memory and accelerates *GAL* gene expression [25, 26, 150]. This transcriptional memory of *GAL* genes lasts for at least seven cell divisions (14h)[25, 26]. Like *INO1*, *GAL* genes show localization to the nuclear periphery during memory [24]. However, whether *GAL* genes exhibit other aspects of *INO1* memory and whether they show a causal relationship to rapid expression are not known.

1.F. Structure-function relationship of Gal4

Gal4 is an 881-amino-acid long, zinc-binuclear cluster transcription factor with a N-terminal DNA binding domain (DBD), a major C-terminal acidic activation domains (AD), and a big intervening central domain (CD; Gal4-238-767) [151] (Figure 1.3). Gal4 also has a minor activation domain next to the DBD [151]. Gal4 shows cooperative binding [152] to the 17mer biding-site, 5'-CGG-N11-CGG-3', in the UAS_{GAL}. The two zinc-finger domains in the Gal4 dimer directly bind to the major groove containing CGG elements [153]. The linker and dimerization domain next to DBD interact with the phosphate background of spacer residue in the 17mer binding site. The stretch of 19 amino acids in the Gal4 linker dictates different spacer lengths of binding site across this family of transcription factors [154]. The major activation domain at the C-terminal is bound by a Gal80 dimer [151, 155]. The DNA binding domain of Gal4 can be physically separated from its activation domain; these domains have been used in two-hybrid assay for protein-protein interactions [156].

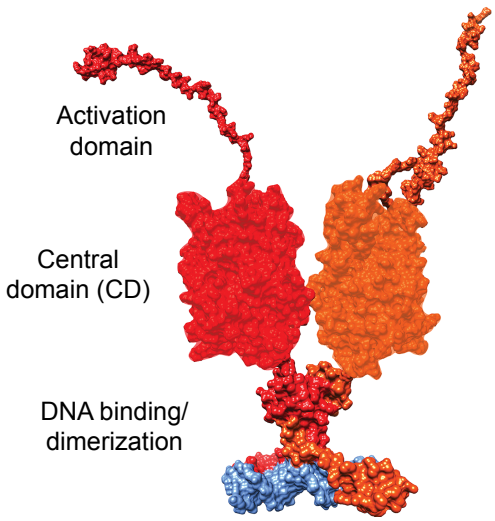

Figure 1.3. Predicted structure of Gal4 transcription factor: Gal4 has a N-terminal DNA-binding domain (1-238), a large central domain (239-767) and C-terminal activation domain (768-881).

Unlike the AD and DBD, role of the central domain of Gal4 is unclear. Phosphorylation of this domain at Serine 699 in galactose is essential for de-repression in *GAL80* cells but dispensable in *gal80Δ* mutant [157, 158]. This suggests that central domain interacts with Gal80, but the interaction has not been confirmed *in vitro* [140, 159-162]. Phosphorylation of Serine 699 by SRB10/CDK8 kinase creates a docking site for F-box protein, Dsg1 [158, 163]. A turnover of this form of Gal4 was shown to be important for *GAL* gene expression. However, in a different strain background, Serine 699 phosphorylation is dispensable [164]. Thus, there is still ambiguity regarding the role of Gal4 central domain. Although, it is likely that Gal4 central domain plays a regulatory role, similar to the central domain in related transcription factors [165-170].

Chapter 2. *GAL* gene epigenetic transcriptional memory in *Saccharomyces cerevisiae* depends on growth in glucose and the Tup1 transcription factor.

2.A. Introduction

Galactose-induced transcriptional memory leads to faster reactivation of yeast *GAL* genes (*GAL1*, *GAL10*, *GAL7,* and *GAL2*) for up to seven generations (~12h) after shifting from activating to repressing conditions [24, 26, 144]. However, *GAL* memory is more complex than *INO1* memory, as it exhibits two distinct phases with different molecular requirements. During the first ~4h 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 SWI/SNF chromatin remodeler, is required for faster reactivation [56, 57, 144]. Short-term *GAL* transcriptional memory is distinct from long-term *GAL* memory, which occurs between 4-12h of repression and is epigenetically inherited. Long-term memory requires the Gal1 protein and is independent of the SWI/SNF complex [26, 144]. Thus, it has been proposed that Gal1 produced during activation acts as a co-activator by interfering with Gal80 repression during memory and is both necessary and sufficient to enhance the rate of reactivation [26, 171]. In order to understand how gene-specific regulatory apparatuses are adopted for transcriptional memory we have focused on understanding the molecular and cellular consequences of Gal1 expression during long-term, epigenetic *GAL* gene memory.

2.B. 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 [26, 144]. Following 12h 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 2.1A). 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 2.1B) or Gal1-mCherry protein (Figure 2.1C). Cells ectopically expressing mutant Gal1 lacking galactokinase activity (deletion of amino acids 171 & 172; gal1-ΔSA;[172]) also showed faster activation of Gal1-mCherry (Figure 2.1C). 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 [26, 144].

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 GFP-Lac repressor [38, 173]. 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 [38, 174, 175]. 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 2.1D;[24, 36, 38]).

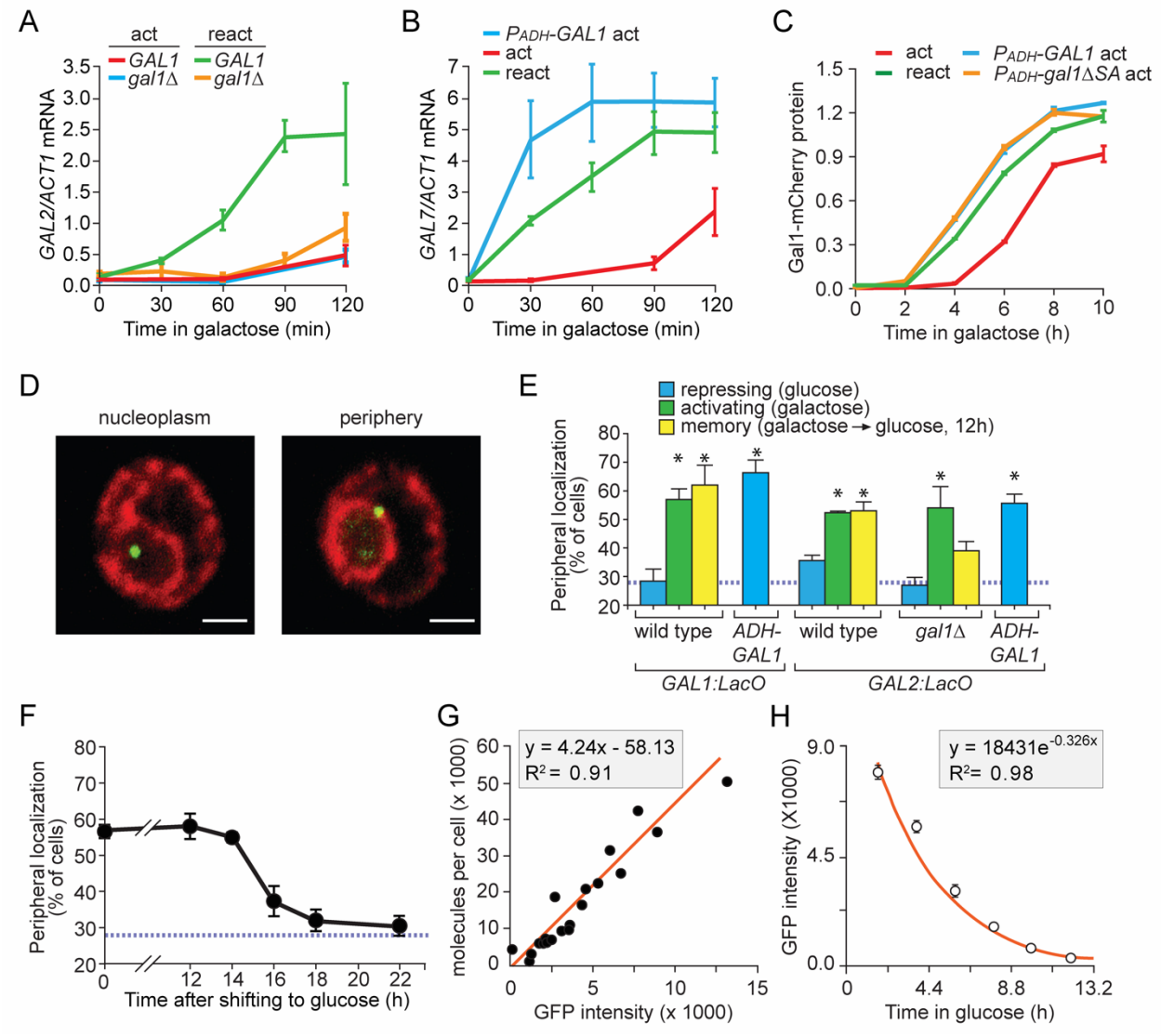

Figure 2.1. Gal1 promotes *GAL* gene localization at the nuclear periphery during memory. A, B and C. Cells were shifted from glucose to galactose (act; activation) or grown overnight in galactose, shifted to glucose for 12h and then shifted to galactose (react; reactivation). Cells were harvested at the indicated times, RNA was prepared and mRNA levels were quantified relative to *ACT1* by RT-qPCR (A and B) or fluorescence was quantified using flow cytometry (C). A. *GAL2* activation and reactivation in wild-type and *gal1Δ* cells. B. *GAL7* activation and reactivation or activation with P_{ADH}-*GAL1*. C. Gal1-mCherry levels, normalized to the constitutively expressed 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. Immunoflurescence images of cells having the LacO array integrated downstream of *GAL1* gene, stained for GFP-LacI (green) and Sec-63myc (red) and scored as either nucleoplasmic or peripheral. Scale bar = 1 µm. E. Peripheral localization of *GAL1* and *GAL2* under repressing (glucose), activating (galactose) and memory (galactose → glucose, 12h) conditions in wild-type or *gal1Δ* cells and in presence of P_{ADH}-*GAL1*. F. Cells with the LacO array

downstream of *GAL1* were shifted from galactose to glucose media for indicated length of times and percentage of cells in which *GAL1* co-localized with the nuclear envelope was plotted. The hatched blue line in panels E and F represents the baseline colocalization predicted by chance [38]. G. Plot of the fluorescence intensities of 20 GFP tagged proteins [146, 176], measured by flow cytometry, against protein copy number per cell [145]. 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 2h after repression. Error bars represent SEM for ≥ 3 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 ≥ 5,000 cells, respectively. * $p \leq 0.05$ (Student's t-test) relative to the repressing condition.

By IF, *GAL1* and *GAL2* localized at the nuclear periphery both when active and for up to 12h after repression, but not in glucose (Figure 2.1E;[22, 24]). Consistent with previous studies, the fraction of the population that scored as colocalized with the nuclear periphery was lower for *GAL2* (~50%;[40, 177]) than for *GAL1* (~60%;[24]). 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 *gal1Δ* strain, the *GAL2* locus was targeted to the nuclear periphery under activating conditions, but not during memory (Figure 2.1E). Furthermore, P_{ADH}-*GAL1* caused both *GAL1* and *GAL2* to reposition to the nuclear periphery under repressing conditions (Figure 2.1E). 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 ~14h, or ~7.6 cell divisions, before returning to the nucleoplasm (Figure 2.1F). 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 twenty GFP-tagged proteins of known

abundance [145], we estimated the abundance of Gal1 protein to be ~ 28,000 molecules per cell in cells grown overnight in galactose (Figure 2.1G). 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 2.1H). The $t_{1/2}$ of Gal1-GFP fluorescence was ~130min, somewhat longer than the cell division time in this experiment (~90min). 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 ~300 Gal1 molecules per cell are sufficient to promote peripheral localization (Figure 2.1F) after 14h of repression. This concentration is comparable to that of Gal80 under these conditions (~800 molecules per cell;[146, 176]).

2.C. 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 Memory Recruitment Sequence) and the 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 [22]. We asked if targeting of *GAL1* to the nuclear periphery during memory also requires a specific *cis*-acting DNA zip code or Nup100.

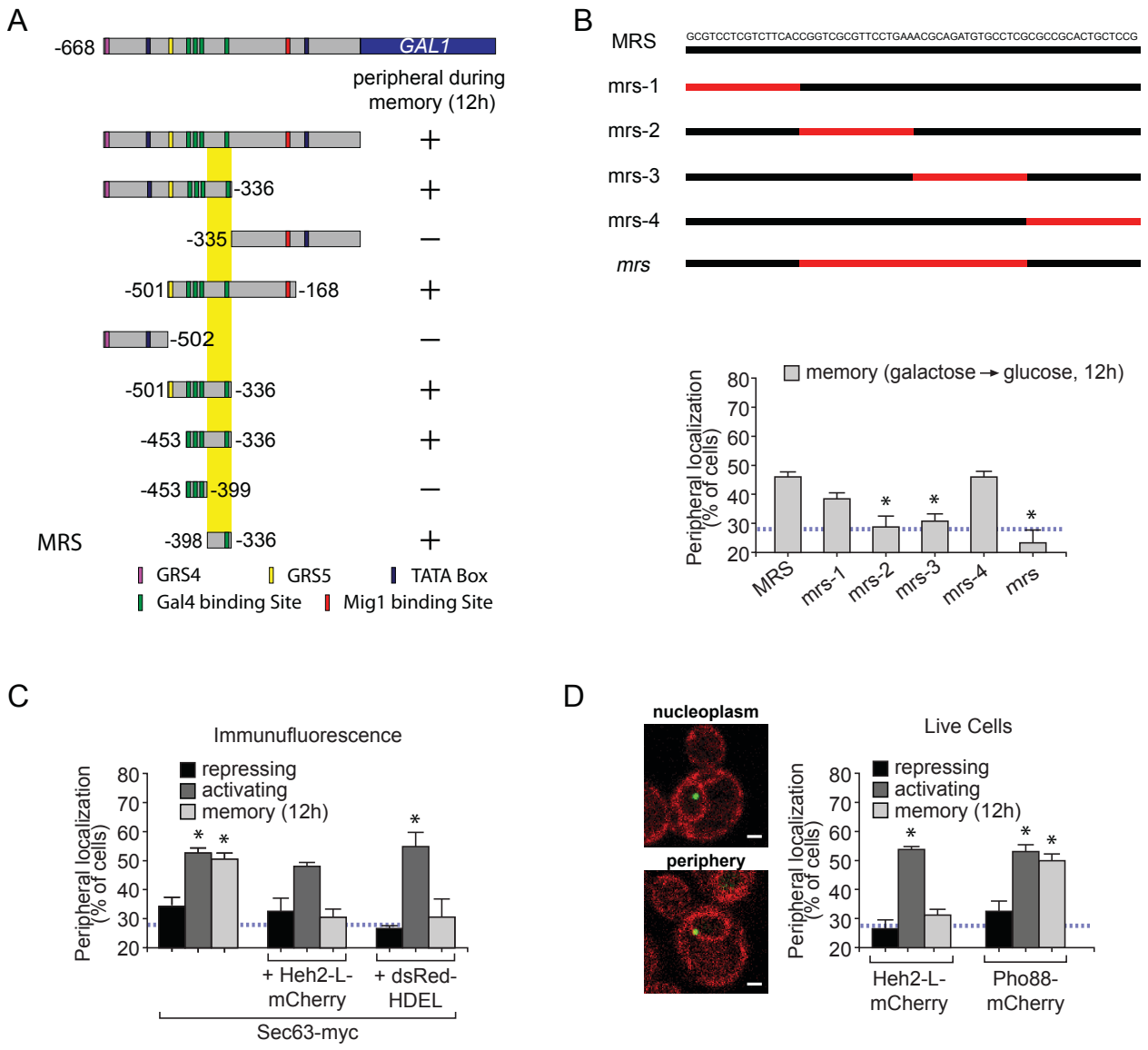

Figure 2.2. Memory Recruitment Sequence (MRS_{GAL1}) regulated *GAL1* peripheral localization during memory is sensitive to the fluorescent marker for nuclear envelope. A. Schematic of *GAL1* promoter fragments inserted next to the *URA3:LacO*. The + and - signs indicate fragments that did or did not lead to statistically significant peripheral localization under memory conditions (galactose → glucose, 12h). The MRS_{GAL1} (-336 to -398 within the *GAL1* promoter) is sufficient to target *URA3* to nuclear periphery during memory. Colored boxes indicate the relative positions of the annotated *cis*-regulatory elements [51]. B. The red bars in the schematic represent the segments of the MRS_{GAL1} in which transversion mutations were introduced at every alternate base. Below: localization of wild-type and transversion mutants of MRS_{GAL1} inserted at *URA3:LacO* scored for peripheral localization under memory conditions. *GAL1* peripheral localization either in fixed cells using immunofluorescence (C) or in live cells (D) grown under repressing (glucose), activating (galactose) and memory (galactose → glucose, 12h) conditions with and without overexpressed red fluorescent protein directed to either ER

membrane (Heh2-L-mCherry) or ER lumen (dsRed-HDEL)[175, 178]. D. Left: Representative images of cells having LacO array integrated downstream of *GAL1* gene, expressing GFP-LacI (green) and Pho88-mCherry (red) and scored as localized to nucleoplasm or periphery. The hatched line represents the level of co-localization with the nuclear envelope predicted by chance and error bars represent SEM from at least 3 independent replicates of 30-50 cells. Scale bar = 1 μm. * $p \leq 0.05$ (Student's t-test) relative to repressing condition.

Peripheral localization of *GAL* genes or promoter of *GAL* genes inserted at *URA3* was observed during memory 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 2.2C;[178]). 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 2.2D) or *INO1* memory [30]. This system permitted both IF and live cell experiments to study the localization of *GAL* genes during memory.

To identify DNA zip codes, we exploited the *URA3* locus, which normally localizes in the nucleoplasm (Figure 2.3A). Insertion of the full-length *GAL1* promoter at *URA3* (*URA3:*P_{GAL1}) causes *URA3* to localize at the nuclear periphery under both activating [51] and memory (Figure 2.3A) conditions, supporting the hypothesis that this promoter possesses DNA zip code activity. Using this assay, we mapped a 63 bp Memory Recruitment Sequence (MRS_{GAL1}; Figure 2.2A). The MRS_{GAL1} did not overlap with two other zip codes in the *GAL1* promoter (GRS4 and GRS5;[51]) that mediate peripheral localization of active *GAL1* (Figure 2.2A). Inserting the 2-3MRS_{GAL1} alone at *URA3* led to peripheral localization specifically during memory (Figure 2.3A). Furthermore, mutations in this element (Figure 2.2B) disrupted targeting to the periphery of

URA3:MRS$_{GAL1}$, *URA3:P$_{GAL1}$* and the endogenous *GAL1* locus during memory (Figure 2.3A). Thus, the MRS$_{GAL1}$ is necessary and sufficient to control targeting to the nuclear periphery during *GAL* memory.

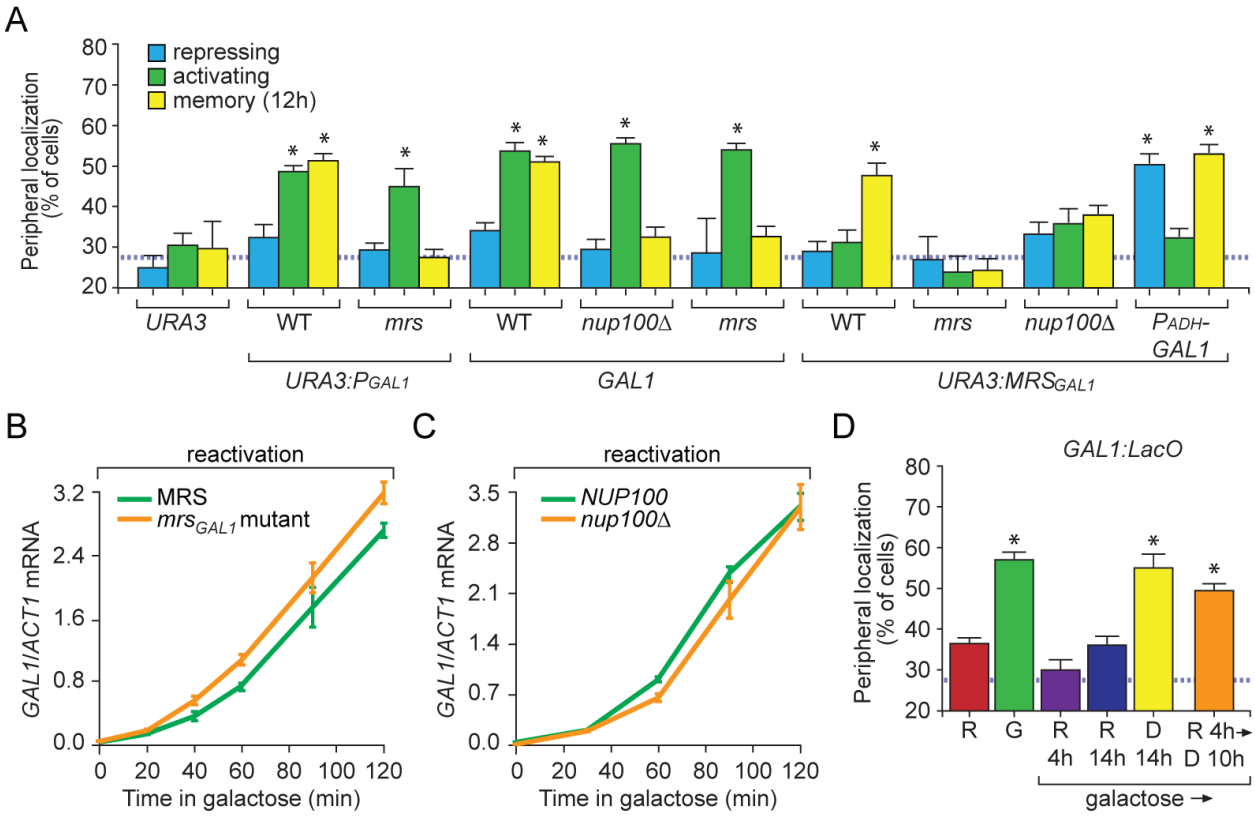

Figure 2.3. MRS$_{GAL1}$-dependent peripheral localization of *GAL1* during memory requires growth in glucose and Tup1. A. Peripheral localization of *URA3*, *GAL1*, *URA3:P$_{GAL1}$* or *URA3:MRS$_{GAL1}$* was quantified under repressing (glucose), activating (galactose) and memory (galactose → glucose, 12 h) conditions in wild-type or *nup100Δ* cells using immunofluorescence or live cell microscopy. The full-length *GAL1* promoter (*P$_{GAL1}$*, 667bp) or the 63bp MRS$_{GAL1}$ were inserted at *URA3* along with a LacO array as described [175]. The *mrs* mutation is shown in Figure S2B. B and C. Cells were grown in galactose overnight, shifted to glucose for 12h and then shifted to galactose (reactivation) to assay *GAL1* expression using RT-qPCR in wild type, mrs$_{GAL1}$ (B) and *nup100Δ* (C) mutant cells. D. Peripheral localization of *GAL1* in cells grown in Raffinose (R), Galactose (G) and upon shift from galactose to raffinose for 4h (R 4h), raffinose for 14hr (R 14h), to glucose for 14h (D 14h) and raffinose 4h followed by glucose 10h (R 4h → D 10h). The hatched line represents the level of co-localization with the nuclear envelope predicted by chance (A and D). Error bars represent SEM for ≥ 3 biological replicates. * $p \leq 0.05$ (Student's t-test) relative to the repressing condition.

Loss of Nup100 also specifically disrupted *GAL1* peripheral localization during memory, but had no effect on *GAL1* peripheral localization during activating conditions (Figure 2.3A). Likewise, targeting of *URA3:MRS*$_{GAL1}$ to the nuclear periphery during memory required Nup100 (Figure 2.3A). Chromatin Immunoprecipitation (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 2.4A). Finally, while inactivation of a conditional allele of Nup2 using the Anchor Away technique [179] led to rapid loss of peripheral localization under both activating and memory conditions, inactivation of Nup100 disrupted peripheral localization only during memory (Figure 2.4B & C). 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 12h of repression (Figure 2.3 B & C). This suggests that targeting to the nuclear periphery is a product of *GAL* memory, but the interaction with the NPC is not essential to promote faster *GAL* gene reactivation.

2.D. 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 2.3A). 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 2.3A). This suggested that MRS$_{GAL1}$-mediated peripheral localization during *GAL* transcriptional memory either required growth in glucose or is inhibited in galactose.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 non-repressing and non-activating condition. Whereas induced *GAL1* in cells grown in galactose (G) localized at the nuclear periphery, uninduced *GAL1* in cells grown in raffinose (R) localized to the nucleoplasm (Figure 2.3D). This result conflicts with previous work showing that *GAL1* localizes at the nuclear periphery in cells growing in raffinose [178]. However, we find that expression of the ER/nuclear envelope marker used in that study (RFP-HDEL) is responsible for the discrepancy (not shown).

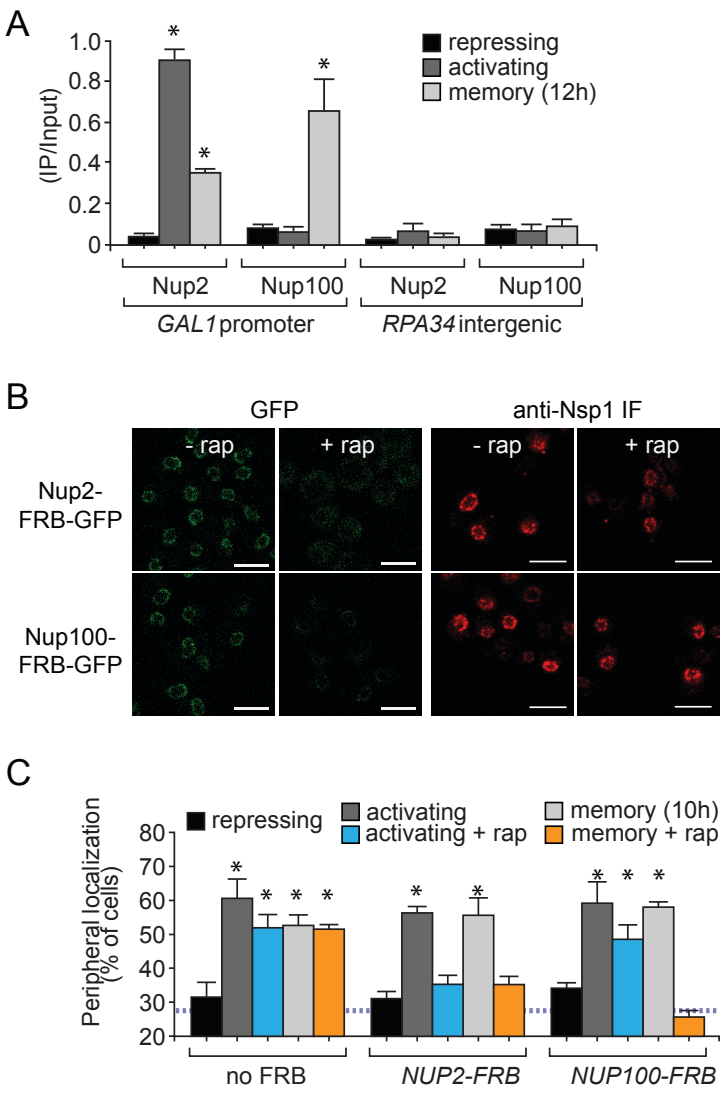

Figure 2.4. Nup100-dependent *GAL1* peripheral localization during transcriptional memory. The experiments were done under repressing (glucose), activating (galactose) and memory (galactose → glucose, 12h) conditions. A. ChIP of TAP-tagged Nup2 and Nup100. The enrichment for *GAL1* promoter and *RPA34* (negative control) in the IP was quantified relative to the input fraction by qPCR. B. Confocal images of cells with Nup2-FRB-GFP or Nup100-FRB-GFP before and after 1 h rapamycin treatment. Left: GFP fluorescence in live cells, imaged with identical settings. Right: immunofluorescence against Nsp1 shows that NPC number or structural integrity is not altered by anchor away of Nup2 or Nup100. Scale bar = 5 µm. C. Peripheral localization of *GAL1* in live cells depleted of Nup2 and Nup100 by Anchor Away [244]. The hatched line represents the level of co-localization with the nuclear envelope predicted by chance. Error bars represent SEM from at least 3 independent replicates of 30-50 cells. * $p \leq 0.05$ (Student's t-test) relative to the repressing condition.

Unlike *GAL1* in cells shifted from galactose to glucose, which remained at the periphery (D 14h, Figure 2.3D), *GAL1* in cells shifted from galactose to raffinose for either 4h or 14h

localized in the nucleoplasm (R, Figure 2.3D). This was not due to lower Gal1 protein levels in cells shifted to raffinose; 4h after shifting from galactose to raffinose, Gal1-mCherry levels were slightly higher than in cells shifted from galactose to glucose for 4h (not shown). 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 4h and then shifted to glucose for 10h, *GAL1* relocalized to nuclear periphery (R 4h → D 10h; Figure 2.3D). Therefore, Gal1 and glucose together promote targeting of *GAL* genes to the nuclear periphery during memory.

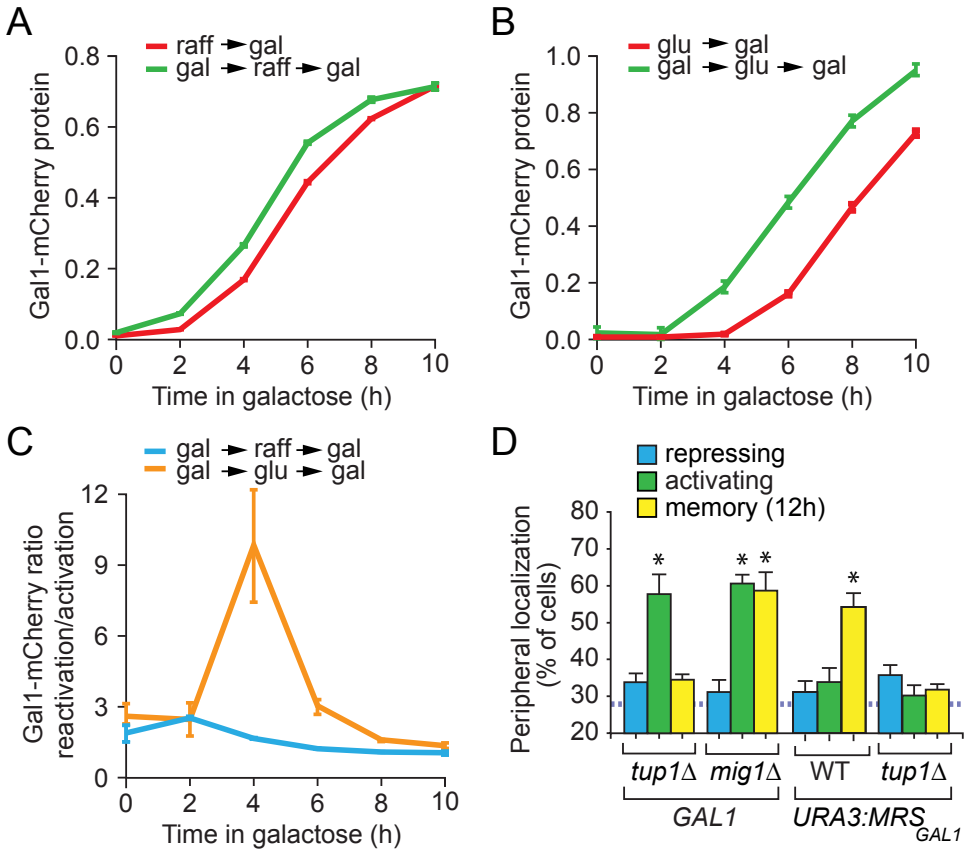

Figure 2.5. The adaptive value of memory in cells grown in non-repressing and repressing carbon sources. A and B. Gal1-mCherry expression, normalized to the constitutively expressed CFP (P_{TDH}-*CFP*), during activation and reactivation, measured by flow cytometry. Activation: cells were shifted to galactose from either a non-repressing carbon source, raffinose (A), or a repressing carbon source, glucose (B). Reactivation: cells were shifted from galactose to either raffinose (A) or glucose (B) for ~7 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 *tup1Δ* and *mig1Δ* mutant strains. The hatched line represents the level of co-localization with the nuclear envelope predicted by chance. * $p \leq 0.05$ (Student's t-test) relative to the repressing condition. Error bars represent SEM for ≥ 3 biological replicates.

The rate of activation of *GAL* genes is much slower in cells shifted from glucose than in cells shifted from a non-repressing carbon source like raffinose [144, 147]. 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 2.5). In cells shifted from raffinose to galactose, the rates of activation (raff → gal) and reactivation (gal → raff, 7 divisions → gal) were similar (Figure 2.5A). 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, 7 divisions → gal; Figure 2.5B). The difference between these two repressive sugars was also evident from the reactivation:activation ratio of Gal1-mCherry during induction (Figure 2.5C). This ratio was maximal (~11) in cells shifted from glucose back to galactose for 4h, illustrating the much greater impact of memory in cells grown in glucose.

In glucose, the Mig1 repressor and the co-repressors Tup1 and Cyc8 bind to the *GAL* gene promoters to repress transcription [180, 181]. Therefore, we asked if these factors played a role in *GAL1* localization during transcriptional memory by scoring *GAL1* localization in *mig1Δ*

and *tup1Δ* cells. The *cyc8Δ* mutant showed 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 2.5D). Thus, Tup1 is required for MRS$_{GAL1}$-mediated peripheral localization of *GAL1* during memory.

2.E. 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 pre-initiation RNAPII to the promoter [22, 23, 30]. To test if *GAL1* transcriptional memory involves a similar mechanism, we used ChIP to monitor binding of RNAPII at *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* coding sequence was quantified by real-time quantitative PCR (Figure 2.6A). RNAPII occupancy was low over both the *GAL1* promoter and coding sequence under repressing conditions and was high over both under activating conditions (Figure 2.6A). 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 coding sequence (Figure 2.6A). However, between 2 and 4 hours of repression, RNAPII association with the promoter increased (Figure 2.6A). Binding of RNAPII during memory was unaffected by loss of Nup100 or mutations in the MRS$_{GAL1}$ (Figure 2.6D). However, loss of Tup1 specifically blocked RNAPII binding to the *GAL1* promoter during memory (Figure 2.6A). 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 reverse transcriptase quantitative PCR to measure mRNA levels (Figure 2.6B). In the wild-type strain, the rate of reactivation of *GAL1* was much faster than the rate of initial activation (Figure 2.6B, green vs red). Consistent with a role in glucose repression, the rate of *GAL1* activation was slightly faster in absence of Tup1 (Figure 2.6B, cyan). However, following 12h of repression, the rate *GAL1* reactivation was significantly slower in the *tup1Δ* strain (Figure 2.6B, orange) and the rates of *GAL1* activation and reactivation were quite similar. This was not true under conditions of short-term *GAL1* memory; after 1h of repression in glucose, *tup1Δ* cells showed very rapid reactivation that was faster than the wild type cells (Figure 2.7). During osmotic stress, the Hog1 kinase converts the Tup1-Cyc8-Sko1 repressor complex into an activator [182, 183]. However, loss of Sko1 had no effect on *GAL* memory (not shown). Thus, Tup1 plays a role in both glucose repression and in long-term *GAL* gene memory.

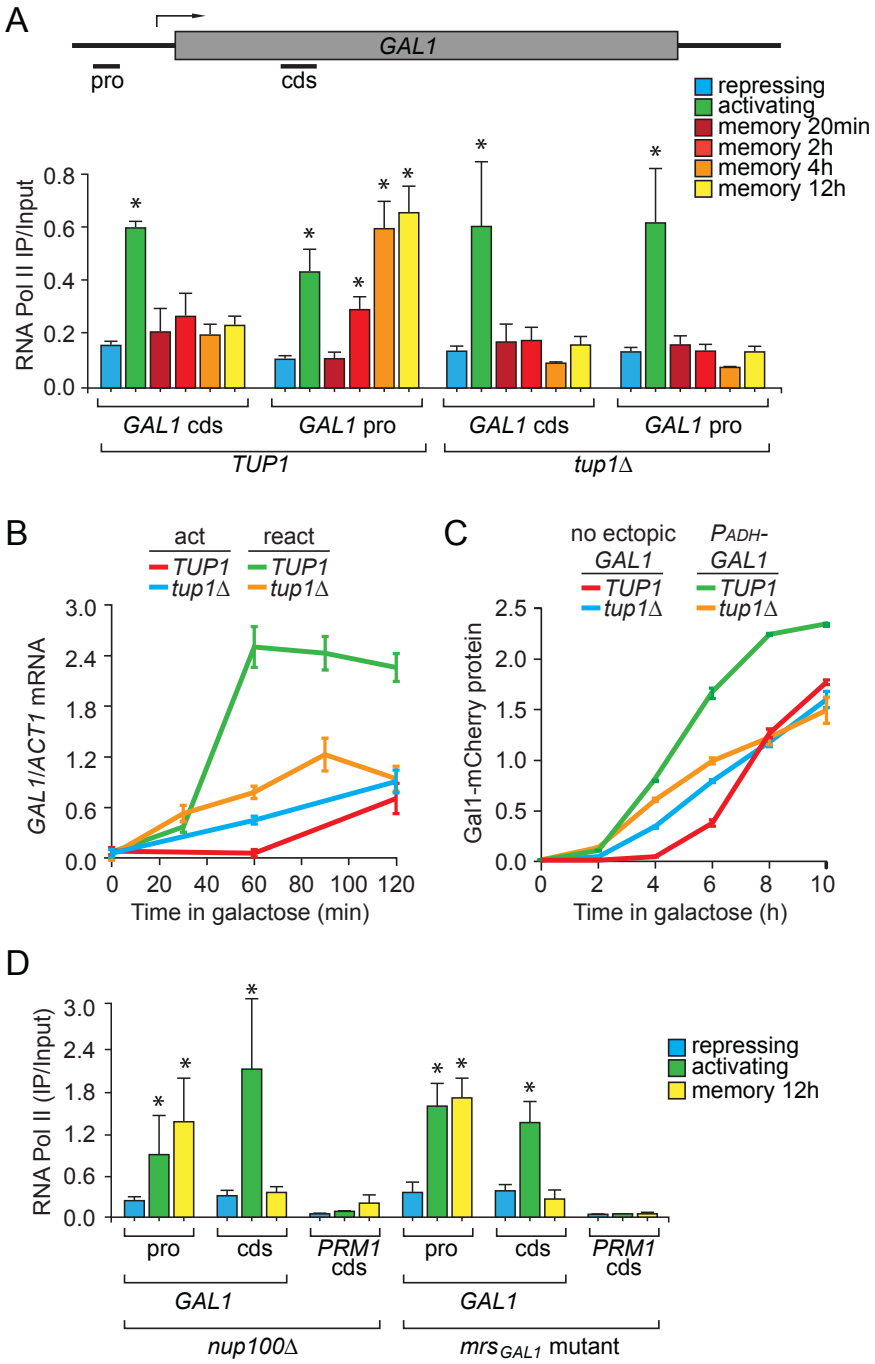

Figure 2.6. Tup1 functions downstream of Gal1 to promote binding of RNAPII to the promoter and faster reactivation of *GAL1* during memory. A. RNAPII ChIP from wild-type and *tup1Δ* cells under repressing (glucose), activating (galactose) and at different times during memory (galactose → glucose, 20min-12h) conditions. Recovery of the *GAL1* promoter (pro) and coding sequence (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 12hour → galactose) in wild-type and *tup1Δ* cells. C. Gal1-mCherry expression, normalized to the constitutively expressed CFP (P_{TDH}-*CFP*) internal

reference, measured by flow cytometry during activation in wild-type and *tup1Δ* cells with or without P_{ADH}-*GAL1* integrated at the *TRP1* locus. D. RNAPII ChIP under repressing (glucose), activating (galactose) and memory (galactose → glucose, 12h) conditions for mrs_{GAL1} and *nup100Δ* mutant. Error bars represent SEM for ≥ 3 biological replicates. * $p \leq 0.05$ (Student's t-test) relative to the repressing condition.

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 *tup1Δ* cells in the presence and absence of P_{ADH}-*GAL1* (Figure 2.6C). In wild-type cells, P_{ADH}-*GAL1* led to a dramatic increase in the rate of activation of *GAL1-mCherry* (Figure 2.6C, green vs red). As observed with mRNA quantification, activation of Gal1-mCherry was slightly faster in the *tup1Δ* strain (Figure 2.6C, cyan vs red). However, loss of Tup1 blocked the effect of ectopic expression of Gal1 (Figure 2.6C, orange vs cyan). This suggests that Tup1 functions downstream of Gal1 to promote faster *GAL* gene reactivation.

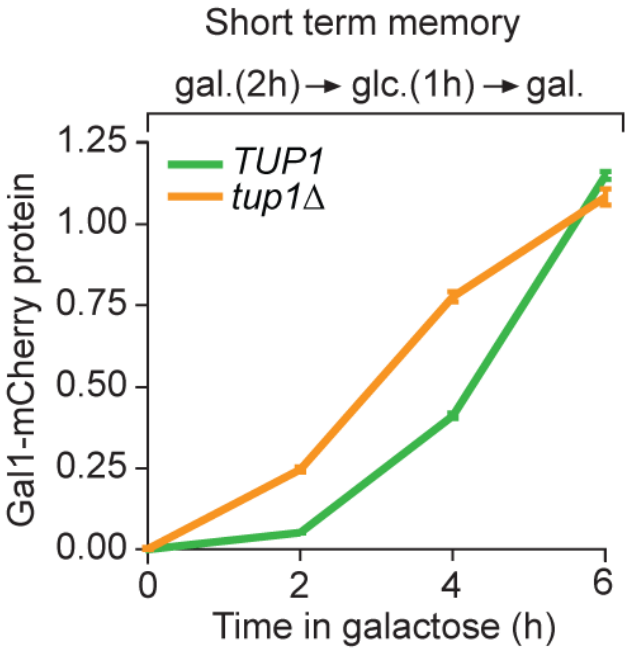

Figure 2.7. Tup1 is not required for short term *GAL1* memory. Gal1-mCherry levels, normalized to the constitutively expressed CFP (P_{TDH}-*CFP*) upon reactivation during short-term memory in wild-type and *tup1Δ* cells, measured using flow cytometry. To induce short-term *GAL* memory, cells were shifted from glucose to galactose for 2h, back to glucose for 1h and

then to galactose for reactivation. Error bars represent SEM from at least 3 independent replicates.

2.F. 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 repression [184]. 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 [22, 24]. 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 2.8A and B; [185]). To test if H2A.Z plays a specific role 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 2.8C) and binding of poised RNAPII to the promoter during memory (Figure 2.8D), 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 2.8E). 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.

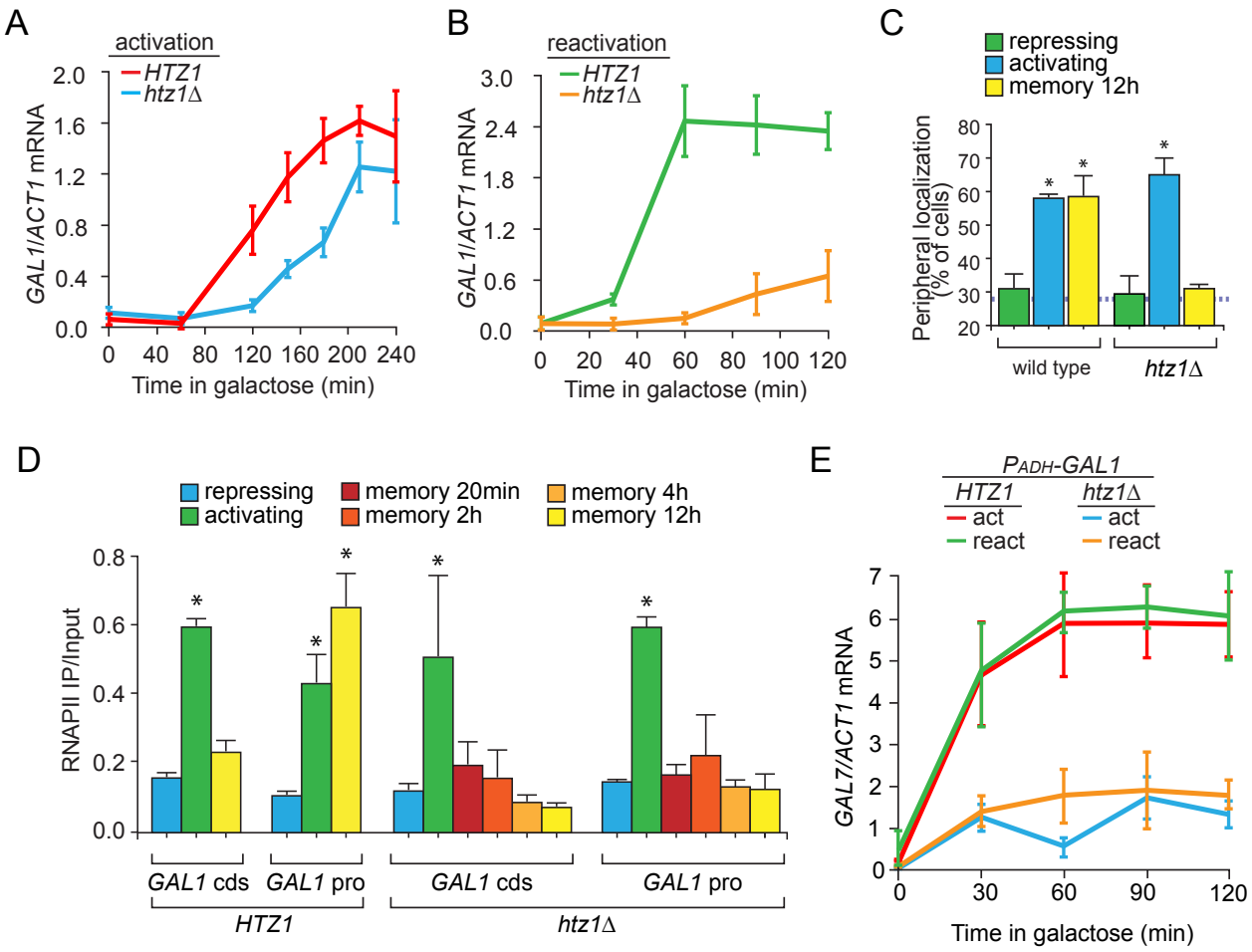

Figure 2.8. H2A.Z functions downstream of Gal1 to promote *GAL* transcriptional memory. A and B. *GAL1* expression, relative to *ACT1*, measured by RT-qPCR over time in wild-type and *htz1Δ* cells during activation (A) and reactivation after 12h of repression (B). C. Peripheral localization of *GAL1* under repressing (glucose), activating (galactose) and memory (galactose → glucose, 12h) conditions in wild-type and *htz1Δ* cells. The hatched line represents the level of co-localization with the nuclear envelope predicted by chance. D. RNAPII ChIP from wild-type and *htz1Δ* cells under repressing, activating and at different times during memory (galactose → glucose, 20min-12h) conditions. E. *GAL7* expression, relative to *ACT1*, measured by RT-qPCR during activation or reactivation in wild-type and *htz1Δ* cells transformed with P_{ADH}-*GAL1*. Error bars represent SEM from at least 3 independent replicates. * $p \leq 0.05$ (Student's t-test) relative to the repressing condition.

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

The *INO1* memory requires both persistent H2A.Z incorporation and H3K4me2 chromatin modification at the promoter [22, 23, 30]. Therefore, we tested if *GAL* gene transcriptional memory is associated with these chromatin alterations. The recovery of the coding sequence 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 [22, 30].

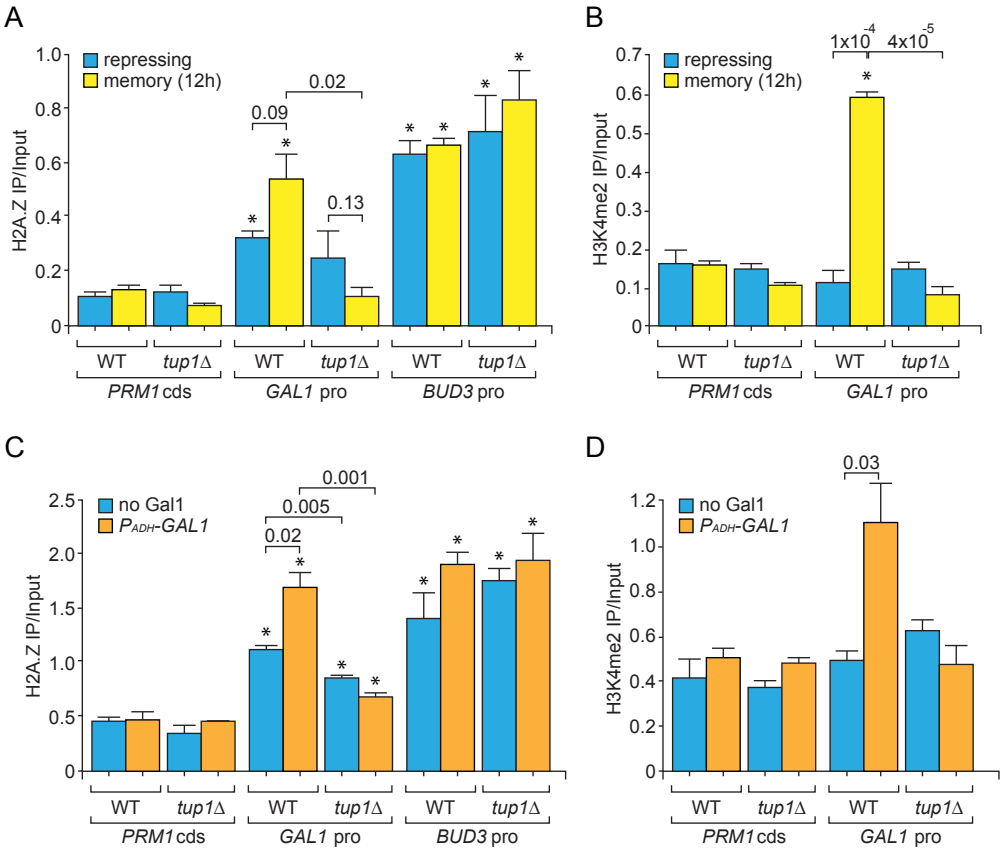

Figure 2.9. Tup1 promotes H2A.Z incorporation and H3K4me2 modification during *GAL* memory. A and C. H2A.Z ChIP in wild-type and *tup1Δ* cells under repressing (glucose), and memory (galactose → glucose, 12 h) conditions (A) or under repressing conditions with P_{ADH}-*GAL1* (C). The recovered DNA fragments 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 wild-type and *tup1Δ* cells, performed as described in A and C. Error bars represent SEM from at least 3 independent replicates. * $p \leq 0.05$ (Student's t-test) relative to the repressing condition.

During memory, both H2A.Z occupancy and dimethylation of H3K4 increased significantly at the *GAL1* promoter, relative to the repressed condition (Figure 2.9A & B). Likewise, expression of P_{ADH}-*GAL1* under repressing conditions also led to an increase in both H2A.Z occupancy and H3K4me2 (Figure 2.9C & 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 2.9). 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.

2.H. Discussion

The yeast *GAL* genes localize to the nuclear periphery and physically interact with the NPC during both activation and memory [24]. During activation, peripheral localization of *GAL1* requires the GRS4 and GRS5 DNA zip codes and is necessary for full expression [51]. 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.

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 *GAL* genes expression via the Mig1-Tup1-Cyc8 repressor complex [186]. Although Mig1 recruits the Tup1-Cyc8 co-repressor to the *GAL1* promoter in glucose [187], Tup1 is also recruited to the active *GAL1* promoter in a Mig1-independent manner [142]. 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 2.10), loss of Tup1 specifically disrupted *GAL1* peripheral localization during memory, 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 co-repressor [188] that masks activation domains [123], binds hypoacetylated histones [189], recruits histone deacetylases [190], interacts with mediator subunits [142, 191] and repositions nucleosomes [192]. However, Tup1 can also function as a co-activator, facilitating recruitment of SAGA or SWI/SNF to promote transcription [142, 166, 193-195]. 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.

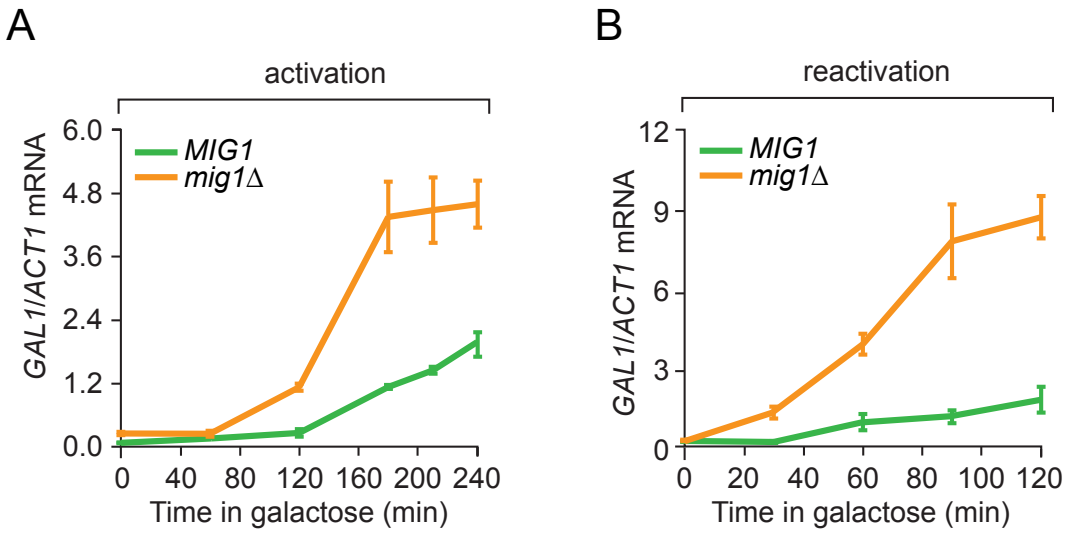

Figure 2.10. Loss of Mig1 promotes faster/stronger expression of *GAL1* under all conditions. A & B. Time course of RT-qPCR for *GAL1* expression relative to *ACT1* during activation (glucose → galactose, A) and reactivation (galactose → glucose, 12 h → galactose, B) in wild-type and *mig1Δ* cells. Error bars represent SEM from at least 3 independent replicates.

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 [142, 184]. Loss of H2A.Z leads to a defect in both the rate of activation and reactivation of *GAL1,* but leads to specific defects in both RNAPII binding at *GAL1* promoter and *GAL1* peripheral localization during memory [24, 185]. 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 ≥ 7 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.

Note:

This chapter was adapted from "Sood. *et al.* Epigenetic Transcriptional Memory of GAL Genes Depends on Growth in Glucose and the Tup1 Transcription Factor in Saccharomyces cerevisiae. *GENETICS* (2017)". The due permission was taked from the hournal *GENETICS*. The experiments in this seaction were done my be and Dr. Cajigas.

Chapter 3. Genetic and epigenetic strategies potentiate Gal4 activation to enhance fitness in recently diverged yeast species

3.A. Introduction

Transcriptional adaptation to fluctuations in nutrient availability contributes to fitness [196-199] and transcriptional memory leads to heritable increase in the rate of transcriptional induction of certain genes [14, 20, 22-24, 26, 27, 29, 30, 200, 201]. While transcriptional memory is observed from yeast to humans, however, it is unknown how this phenomenon evolved or its effects on fitness. Also, while some aspects of transcriptional memory are deeply conserved, gene-specific features also occur [23, 25, 27, 202] suggesting that gene-specific regulatory systems can be regulated by transcriptional memory. In *S. cerevisiae, GAL* genes exhibit transcriptional memory. When cells are shifted from glucose to galactose, the initial rate of induction of *GAL* genes is very slow; for example, the Gal1 protein reaches steady state levels after ~10h in galactose (Figure 3.1B). Induction is slow in part because it is initially heterogeneous within the population, with some cells responding and others not [147]. However, in cells that have previously grown in galactose, the population induces *GAL* genes rapidly and uniformly, resulting in faster average expression [24-26, 144]. Like other genes that show memory, *GAL* transcriptional memory is associated with changes in chromatin structure, leading to a poised state [25, 29, 30]. Thus, *GAL* transcriptional memory increases the uniformity of the behavior of the population and the rate of induction. Here, we explored the adaptive value, evolutionary history and molecular mechanism of *GAL* gene transcriptional memory.

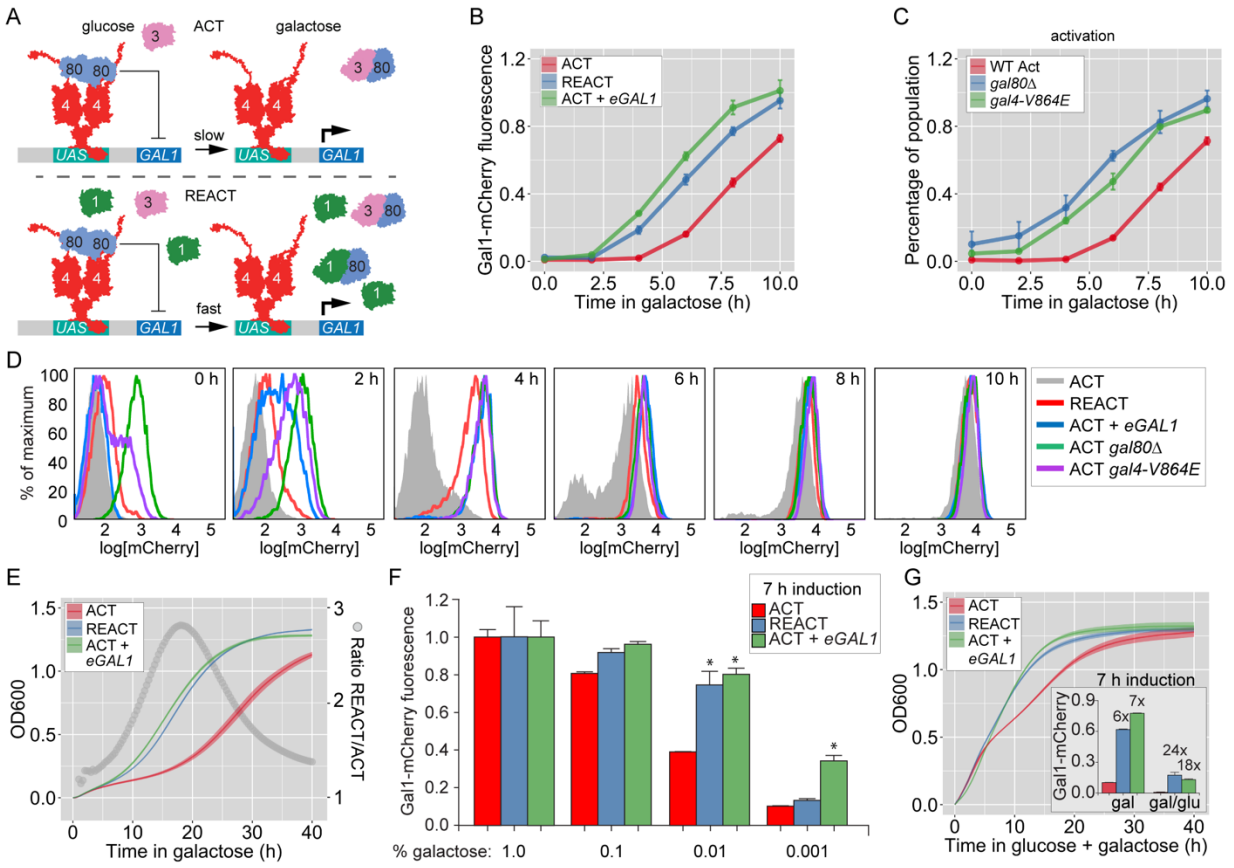

Figure 3.1. Fitness benefit of *GAL* memory. A. Model for *GAL1* regulation and memory. Upper panel: during activation, Gal3 sequesters the Gal80 repressor from the Gal4 activator, leading to *GAL* gene expression. Lower panel: during reactivation, residual Gal1 augments Gal3 co-activation, leading to faster expression kinetics. **B - G.** Naïve cells (ACT), naïve cells expressing ectopic Gal1 (ACT + e*GAL1*), or cells that were grown in galactose overnight and shifted to glucose for 12 hours (REACT) were shifted to galactose (**B-F**) to measure either GAL1-mCherry fluorescence (**B-D**) or follow growth kinetics by plotting OD_{600} (**E** and **G**). GAL1-mCherry fluorescence, relative to constitutively expressed *CFP*, at the indicated times using flow cytometry for wild-type cells (**B**) and mutant cells (**C**). **D**. Concatenated histograms for Gal1-mCherry from time points in **B** and **C**. **E.** At time = 0, all cultures were diluted to an OD_{600} of 0.1 in galactose and relative increase plotted every 20 minutes using 96-well plate reader. Open circle represents the ratio of OD_{600} between REACT and ACT. **F.** Gal1-mCherry levels relative to CFP control at 7 hours in different concentration of galactose, plotted as fraction of expression in 1% galactose. **G.** Growth kinetics and Gal1-mCherry expression (Inset) similar to **E** and **F**, respectively, but in 0.2% glucose + 1.8% galactose. Error bar represents SEM from ≥ 3 biological replicates for **B**, **C**, **F** and **G inset**. The line and the surrounding envelope is the mean and SEM from ≥ 6 biological replicates for **E** and **G**.

3.B. Transcriptional memory enhances fitness by promoting uniform, rapid activation of *GAL* genes.

Because Gal1 is both necessary and sufficient to promote faster induction of *GAL* genes during memory, Gal1 likely interacts with Gal80 to allow rapid de-repression of *GAL* genes (Figure 3.1A). The relative rates of *GAL1* transcription can be compared by measuring Gal1-mCherry fluorescence expressed using flow-cytometry [25]. In cells that hadn't been previously exposed to galactose (*i.e.* naïve cells), Gal1-mCherry was undetectable for the first 4h after shifting from glucose to galactose (Figure 3.1B & 3.1D; ACT). Between 4h and 8h after switching cells to galactose, expression of Gal1-mCherry was apparent in a subset of cells in the population (*i.e.* bimodal expression; Figure 3.1B & D, ACT). After 10h in galactose, the entire population expressed Gal1-mCherry (Figure 3.1D; ACT). In contrast, in cells that were previously grown in galactose and then repressed for 12 hours (~7-8 cell divisions; *i.e.* memory), the entire population responded rapidly and Gal1-mCherry fluorescence was measurable within 4h after shifting back to galactose (unimodal expression; Figure 3.1B & D, REACT). Likewise, ectopic expression of *GAL1* promoted both rapid and unimodal accumulation of Gal1-mCherry (Figure 3.1B and D; ACT + *eGAL1*; refs 16,18,26,35). Furthermore, consistent with the model in Figure 3.1A, either loss of Gal80 or a point mutation in Gal4 (V864E) that disrupts the interaction with Gal80 [203] also resulted in rapid, unimodal expression of Gal1-mCherry (Figure 3.1C and D). Thus, memory leads to faster and more uniform *GAL1* transcriptional activation, likely by promoting rapid and uniform relief of Gal80 repression.

To quantify the adaptive effect of faster reactivation of *GAL* genes during memory, we followed the growth kinetics upon shifting cells from glucose to galactose (Figure 3.1E). Naïve

cells exhibited a long growth lag before entering exponential phase (Figure 3.1E; ACT). In contrast, during memory or in cells ectopically expressing Gal1, adaptation was much faster (Figure 3.1E; REACT, ACT + e*GAL1*). Although the growth rates were ultimately similar once cells reached exponential phase, memory confers a large fitness benefit by decreasing the growth lag after shifting cells from glucose to galactose (Figure 3.1E, grey circles).

Rapid *GAL* gene activation in fungal species is also associated with increased responsiveness to low concentrations of galactose [204-206]. During memory or in cells expressing ectopic Gal1, Gal1-mCherry was expressed at higher levels in media with low concentrations of galactose (Figure 3.1F). Because yeast cells are likely exposed to mixtures of sugars in nature, we asked if this higher sensitivity for galactose also impacts the expression of Gal1-mCherry in the presence of glucose. *S. cerevisiae* normally does not induce *GAL* genes in the presence of low levels of glucose (0.2% glucose and 1.8% galactose; Figure 3.1G, inset). However, memory or ectopic Gal1 promoted stronger Gal1-mCherry expression in the presence of glucose (Figure 3.1G; inset). Gal1-mCherry expression correlated with a fitness benefit in 0.2% glucose + 1.8% galactose medium. In this medium, once glucose is exhausted after ~6h of growth, naïve cells exhibited a significant lag during which they adapted to galactose (Figure 3.1G; [205, 207]). However, during memory or in the presence of ectopic Gal1, this lag was absent and cells adapted immediately to galactose (Figure 3.1G). Thus, transcriptional memory provides a strong potential adaptive advantage in both galactose and glucose-galactose mixtures.

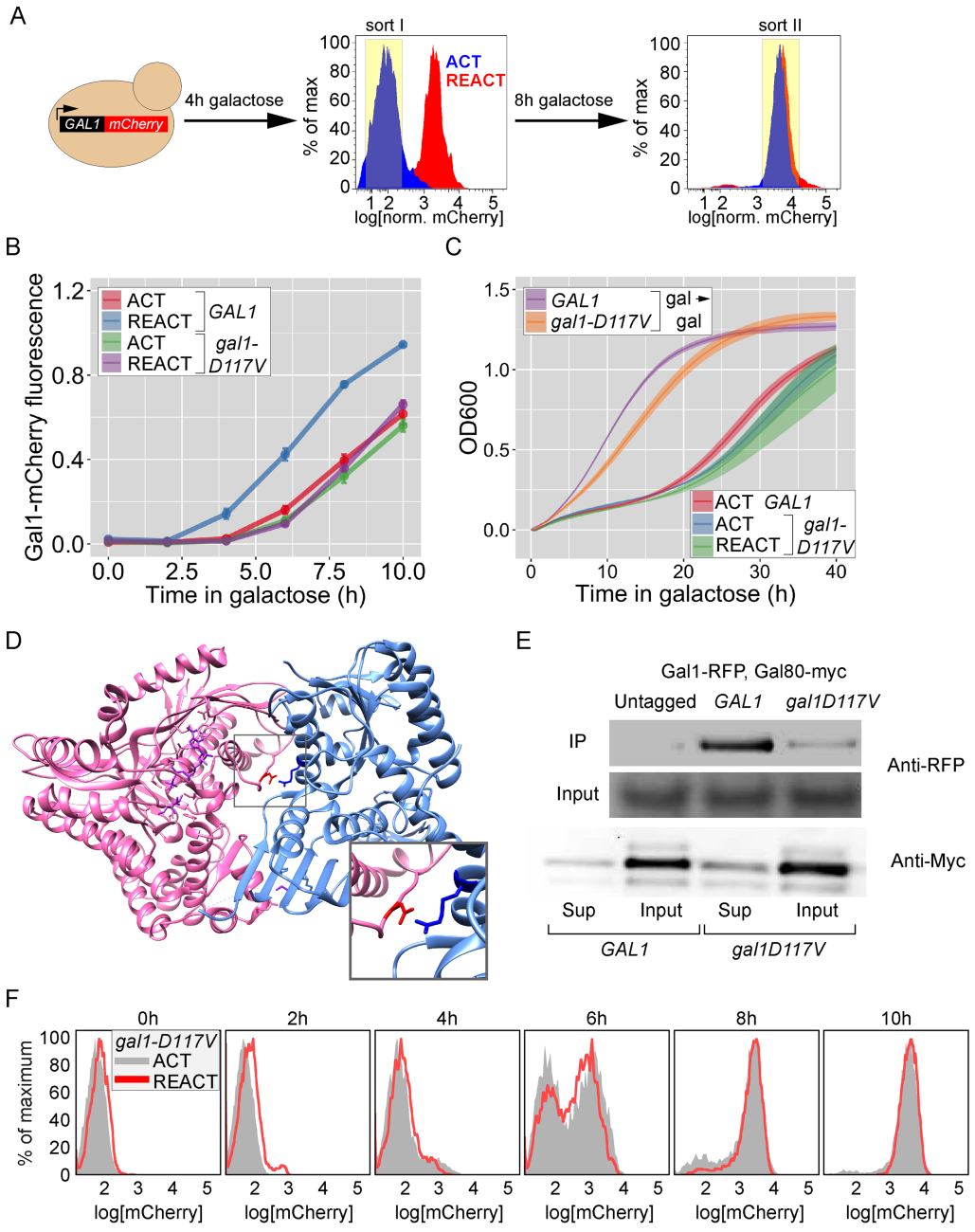

Figure 3.2. Genetic screen for mutants defective for *GAL* memory identifies *gal1-D117V*. A. Schematic of the 2-step FACS based screen (see Methods for details). **B.** Gal1-mCherry intensity relative to CFP internal control in wild-type and *gal1-D117V* mutant, measured by flow-cytometry. Cells were shifted from glucose to galactose for activation (ACT) or grown in galactose overnight, shifted to glucose for 12 hours and then shifted to galactose for reactivation (REACT). Error bar represents SEM from ≥ 3 biological replicates. **C.** Growth curve of wild-type and *gal1-D117V* mutant cells assayed by plotting OD_{600} every 20 minutes during

exponential growth in galactose (gal → gal), during activation (ACT) or reactivation (REACT) after 12hours of repression. The line represents the mean and the envelope represent the SEM from ≥ 4 biological replicates. **D.** Co-crystal structure between Gal3 (pink) and Gal80 (blue), highlighting the salt bridge between the Gal3-Asp111 and Gal80-Arg367 (inset). **E.** Lysates from strains expressing Gal80-13xmyc and Gal1-mCherry were subjected to co-immunoprecipitation using anti-myc antibody. The immunoprecipitated fractions (IP; top), input (middle), supernatant after immunodepletion (bottom) were resolved by SDS PAGE and immunoblotted against either mCherry (top two panels) or the myc epitope tag (bottom panels). **F.** Overlay of concatenated histograms for ACT and REACT of *gal1D117V*.

3.C. Gal1-D117V disrupts the interaction with Gal80, specifically blocking *GAL* transcriptional memory.

To explore the molecular basis of faster reactivation of *GAL* genes during memory, we performed a genetic screen based on fluorescence activated cell sorting (FACS). After 4 hours in galactose, strong expression of Gal1-mCherry occurs during reactivation but not during activation (Figure 3.2A). We exploited this difference to screen for *GAL* memory mutants; UV-mutagenized cells that failed to express Gal1-mCherry after 4h of reactivation were collected (Figure 3.2A; sort I), followed by a second sort at 12h for cells that expressed Gal1-mCherry (Figure 3.2A; sort II). This second sort removed Gal^- mutants or those that had lost Gal1-mCherry expression. The recovered cells were colony-purified and screened by flow cytometry to identify those that specifically lost rapid *GAL1* reactivation during memory.

Based on the model in Figure 3.1A, we expected to identify alleles of Gal1 that specifically blocked memory. Indeed, the screen produced an allele of *GAL1* (*D117V*) that specifically reduced the rate of Gal1-mCherry reactivation during memory (Figure 3.2B). Reconstruction of the *gal1-D117V* mutation into the *GAL1* locus recapitulated this phenotype (not shown), confirming that this mutation is causative. As expected, *gal1-D117V* cells also lost the apparent adaptive fitness associated with memory; the growth of *gal1D117V* during

reactivation closely resembled the growth of naïve wild-type cells during activation (Figure 3.2C). This mutation had no effect on Gal1-mCherry stability (Figure 3.3B) or the rate of activation (Figure 3.3B) and only slightly affected the rate of exponential growth in galactose (Figure 3.2C). Finally, ectopic expression of Gal1 was epistatic to *gal1-D117V* for both faster Gal1-mCherry expression (Figure 3.3C) and growth rate (Figure 3.3D). Thus, Asp117 in Gal1 plays a critical and specific role in promoting epigenetic transcriptional memory.

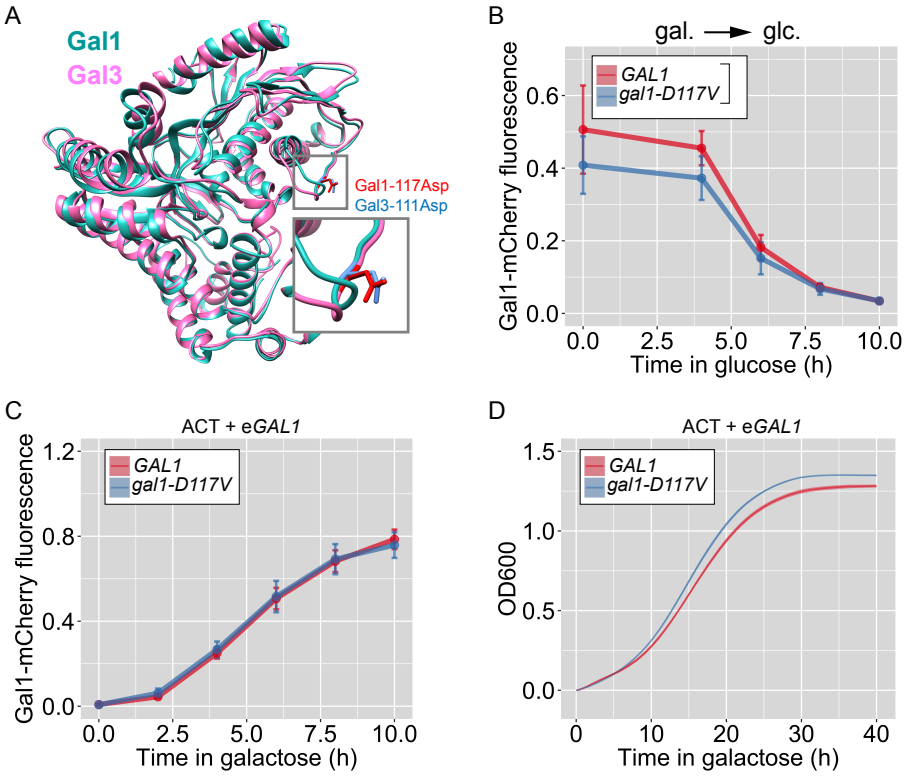

Figure 3.3. Characterization of the *gal1-D117V* mutant. A. Structural superposition of the Gal1 (blue) and Gal3 (pink) crystal structures. Inset: Gal1-117Asp and the structurally equivalent Gal3-111Asp. **B.** Wild-type and *gal1-D117V* strains were shifted from galactose to glucose and Gal1-mCherry fluorescence, normalized to CFP, was measured over time. **C** and **D**. Expression of Gal1-mCherry relative to CFP (**C**) and OD_{600} (**D**) over time upon shift from glucose to galactose in wild-type and *gal1-D117V* mutants containing ectopically expressed *GAL1* (*eGAL1*). Error bars represent the SEM from ≥ 3 biological replicates, represented as bars (**B** and **C**) or envelope (**D**).

The structures of Gal1, Gal3 and Gal3-Gal80 are known [148, 149]. Gal1 and Gal3 show 74% sequence identity and were structurally superimposable with a root mean square deviation of ~ 1.1 Angstroms (Figure 3.3A; [148, 149]). Aspartate 117 maps to the predicted interaction surface between Gal1 and Gal80. In the Gal3-Gal80 structure, Gal3-Asp111 is at the structurally equivalent position to Gal1-Asp117 and forms an ionic bond with the Gal80-Arg367 (Figure 3.2D and 3.3A; [149]). To test if disrupting this salt bridge reduces the affinity between Gal1 and Gal80, we performed co-immunoprecipitation of wild-type and D117V Gal1-mCherry with Gal80-myc. Although these proteins were expressed at similar levels, immunoprecipitation of Gal80 recovered only ~20% of Gal1-D117V compared with wild-type Gal1 (Figure 3.2E). This reduced affinity for Gal80 lead to slow, bimodal expression of Gal1-mCherry during both activation and reactivation (Figure 3.2F). Further, a complementary mutant in Gal80 (R367L) predicted to disrupt the salt bridge between Gal80 and both Gal3 and Gal1 led to a Gal^- phenotype (not shown). Thus, interaction between Gal1 and Gal80 plays a critical role in *GAL* gene transcriptional memory and the *gal1-D117V* mutation specifically disrupts memory without affecting other functions of Gal1.

3.D. Constitutive *GAL* gene poising in *S. uvarum* is due to higher basal expression of Gal1.

S. uvarum diverged from *S. cerevisiae* ~20 million years ago and has evolved a distinct strategy for adapting to growth in galactose (Figure 3.5A; [205, 208-210]). We asked if this species benefits from previous growth in galactose. Although the rate of Gal1-mCherry reactivation during memory was slightly faster than the rate of activation in *S. uvarum* (Figure 3.4A & 3.5B), this difference was much smaller than that observed in *S. cerevisiae* (Figure 3.4B). Moreover, in *S. uvarum*, previous growth in galactose did not lead to a fitness benefit during

memory (Figure 3.4C). Activation of Gal1-mCherry and adaptation to galactose in *S. uvarum* was constitutively fast (Figure 3.4A & C). This suggests that in *S. uvarum*, the rate of *GAL* gene induction is sufficient to provide maximal fitness benefit and that increasing this rate provides no additional effect.

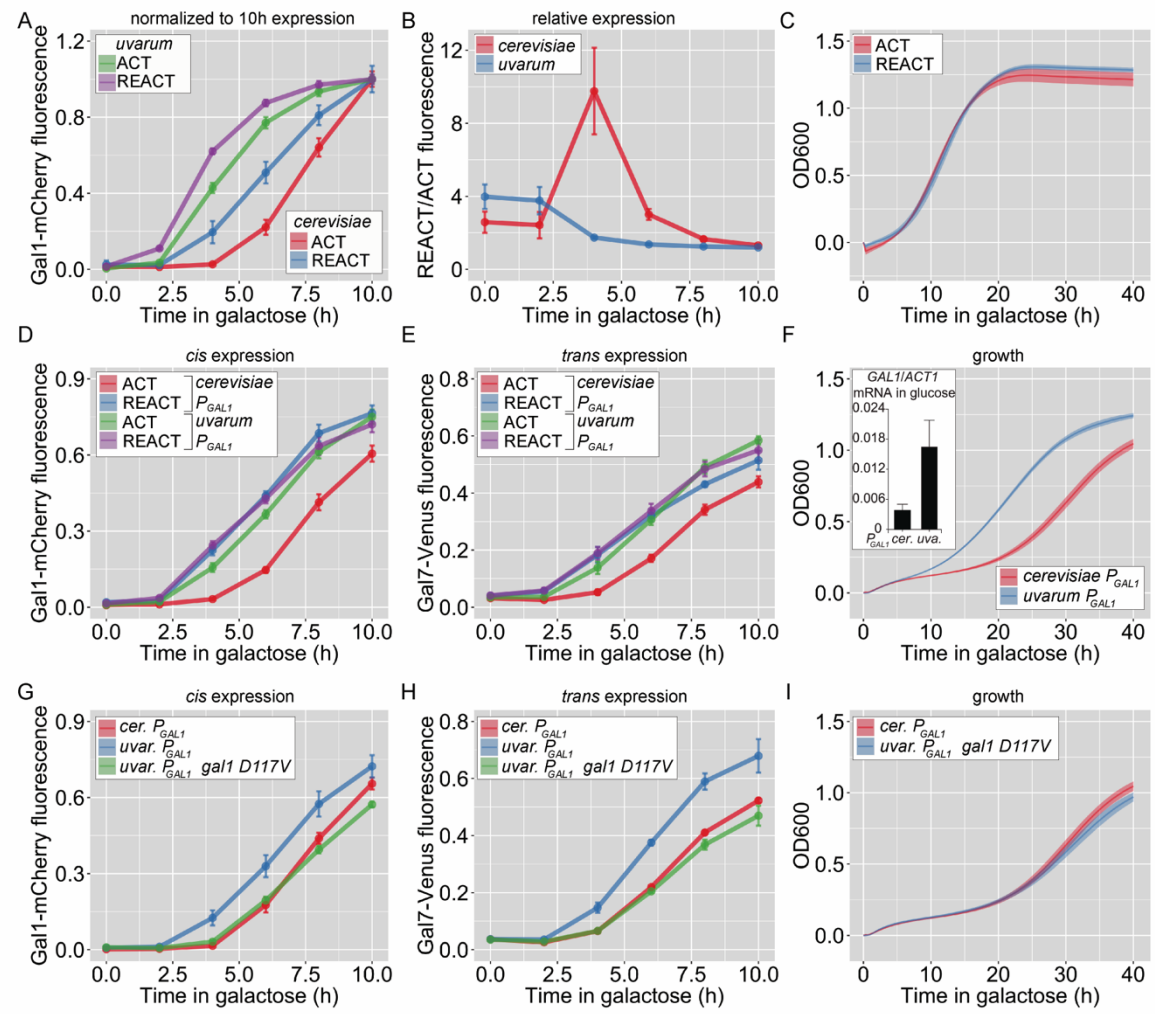

Figure 3.4. Recently diverged *Saccharomyces* species utilize genetic and epigenetic switches to adapt to growth in galactose. **A-I.** Cells were shifted from glucose to galactose for activation (ACT) or grown in galactose overnight, repressed for 12h (*S. cerevisiae*) or 18h (*S. uvarum*) in glucose and then shifted to galactose for reactivation (REACT). **A.** Gal1-mCherry fluorescence during activation and reactivation in *S. cerevisiae* and *S. uvarum*, normalized to expression at 10 h. **B.** Ratio of reactivation to activation from the time course in **B.** **C.** OD_{600} of *S. uvarum* during activation and reactivation. **D-F.** The *GAL1* promoter from *S. uvarum* was introduced in place of the endogenous *GAL1* promoter in *S. cerevisiae*. Gal1-mCherry (**D**) and

Gal7-Venus (**E**) fluorescence relative to CFP and OD_{600} (**F**) was measured during activation (ACT) and reactivation (REACT). Inset: Basal *GAL1* mRNA, relative to *ACT1*, transcribed from the P_{GAL1} from *S. cerevisiae* and *S. uvarum* in glucose media. **G-I.** The *gal1-D117V* mutation was introduced downstream of the *GAL1* promoter from *S. uvarum* in place of the endogenous *GAL1* gene in *S. cerevisiae*. Gal1-mCherry (**G**) and Gal7-Venus (**H**) fluorescence relative to CFP and OD_{600} (**I**) was measured during activation (ACT) and reactivation (REACT). Error bars represent SEM from ≥ 3 biological replicates for expression and ≥ 4 biological replicates for growth.

Several differences between *S. uvarum* and *S. cerevisiae* might explain the difference in their response to previous growth in galactose; *S. uvarum* has higher basal *GAL* gene expression, except *GAL80*, which shows lower expression [205, 208]. Thus, differences in *cis*-acting elements, *trans*-acting factors or both could lead to constitutive *GAL* gene poising. To investigate these possibilities, we substituted the *GAL1* promoter (P_{GAL1}) in *S. cerevisiae* with P_{GAL1} from *S. uvarum*. In this strain, induction of Gal1-mCherry during both activation and reactivation was as fast as reactivation in wild-type cells (Figure 3.4D). Thus, P_{GAL1} from *S. uvarum* is sufficient to induce constitutive *GAL1* poising in *S. cerevisiae* without any other *uvarum* factors.

The effects of P_{GAL1} from *S. uvarum* are consistent with this promoter being more easily induced. Hybrid *cerevisiae-uvarum* promoters suggest that this effect is largely explained by differences in the UAS_{GAL} elements and *GAL1*-proximal sequences (Figure 3.5C-E; [211]). However, because epigenetic *GAL* gene transcriptional memory in *S. cerevisiae* requires only a few hundred molecules of Gal1 per cell [25], very low basal expression of Gal1 might produce memory-like effects. To distinguish between these possibilities, we asked if introducing P_{GAL1} from *S. uvarum* into *S. cerevisiae* also promoted faster activation of other *GAL* genes in *trans.* In cells bearing the *S. uvarum* P_{GAL1}, the rate of Gal7-Venus activation and reactivation was as

fast as that observed during reactivation in wild-type cells (Figure 3.4E). Likewise, *S. uvarum* P_{GAL1} promoted faster adaptation to galactose (Figure 3.4F). Thus, the *S. uvarum* P_{GAL1} is sufficient to induce constitutive *GAL* gene poising and faster adaptation to galactose, likely through basal Gal1 production.

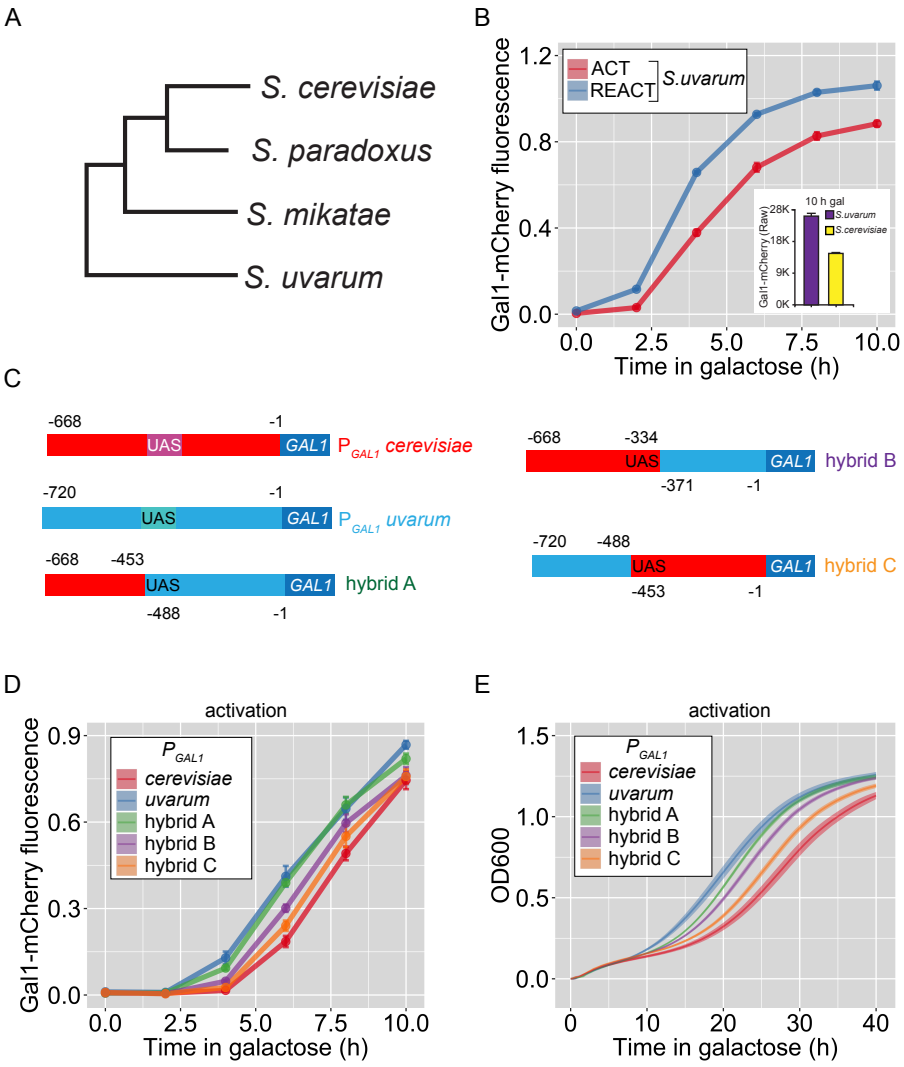

Figure 3.5. Kinetics of *GAL1* expression in *S. uvarum*. A. Dendrogram depicting the evolutionary relationship between different species in the *Saccharomyces* genus [44]. **B.** *S. uvarum* cells harboring *GAL1-mCherry* and P_{TDH1}*-VENUS* were either shifted from glucose to galactose (ACT) or grown overnight in galactose, shifted to glucose for 18 hours and then shifted to galactose (REACT). Gal1-mCherry fluorescence was measured relative to constitutively expressed Venus by flow cytometry. Inset: the raw Gal1-mCherry levels between *S. cerevisiae* and *S. uvarum* after 10 hours in galactose. **C.** Schematic of hybrid promoters studied inserted in *S. cerevisiae*, shown in **D** and **E**. The UAS_{GAL4} in P_{GAL1} for both *S.cerevisiae*

and *S. uvarum* is shown for reference. Gal1-mCherry fluorescence relative to CFP (**D**) and OD_{600} (**E**) were measured in the indicated strains after shifting cells from glucose to galactose. Error bars represent SEM from ≥ 3 biological replicates, depicted as bars (**B** and **D**) or envelope (**E**).

Unfortunately, we were unable to measure basal Gal1 protein in these cells using either flow cytometry or immunoblot. However, if low-level expression of Gal1 from the *S. uvarum* promoter were responsible for faster *GAL* gene expression, then disrupting the interaction between Gal1 and Gal80 would block this effect. Indeed, introduction of the *gal1-D117A* mutation into the *S. cerevisae* strain harboring the *S. uvarum* P_{GAL1} blocked the *cis* and *trans* effects of this promoter on expression (Figure 3.4G & H) and the growth (Figure 3.4I). Thus, constitutive poising of *GAL* genes in *S. uvarum* is due to genetically encoded basal expression of Gal1 that impinges upon the same molecular mechanism employed during epigenetic transcriptional memory in *S. cerevisiae*.

3.E. Fitness costs of constitutive *GAL1* expression.

If faster *GAL* genes expression promotes adaptation to galactose, why is it restricted to reactivation during memory in *S. cerevisiae*? Basal Gal1 expression is detrimental for growth in glucose-galactose mixtures because the galactose-1-phosphate generated by the galactokinase activity of Gal1 inhibits phosphoglucomutase and slows glycolysis [205, 212]. Consistent with this model, both *S. uvarum* and *S. cerevisiae* expressing ectopic *GAL1* showed a measurable growth disadvantage upon shifting to a 1:1 glucose – galactose mixture (1% each sugar; Figure 3.6A & B). Thus, basal *GAL1* expression is a double-edged sword; it promotes growth upon shift from glucose to galactose but leads to small but significant defect in glucose-galactose mixtures.

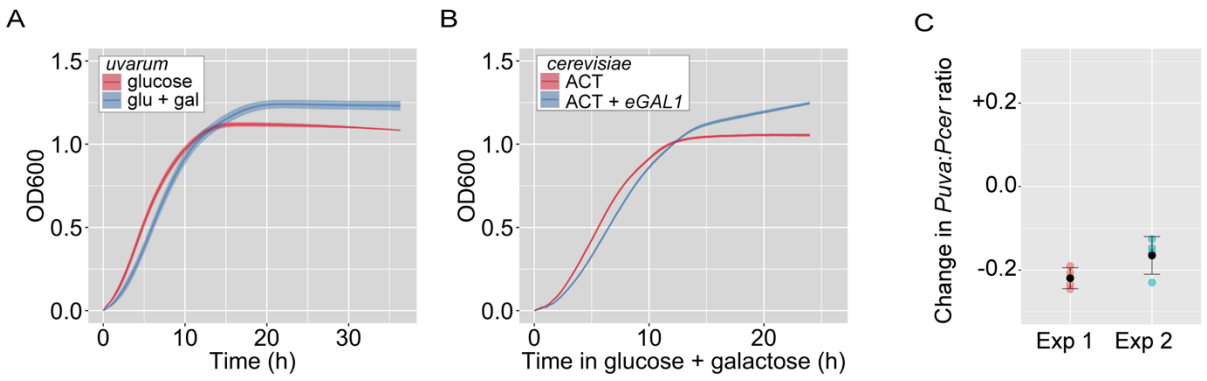

Figure 3.6. Basal *GAL1* expression leads to growth defects in mixed sugars. OD_{600} was measured every 20 minutes. **A** and **B.** Wild-type *S. uvarum* cells (**A**) or *S. cerevisiae* cells with and without ectopic *GAL1* (**B**) were shifted from glucose to either a mixture of 1% glucose + 1% galactose or to glucose. Growth curves represent the average and the envelopes represent the SEM from ≥ 4 biological replicates. **C.** Competitive growth assay between *S. cerevisiae* cells containing native P_{GAL1}*cerevisiae* and P_{GAL1}*uvarum* in 1% glucose + 1% galactose. Venus fluorophore was constitutively expressed in either P_{GAL1}*cerevisiae* cells (Exp1) or P_{GAL1}*uvarum* cells (Exp2). Four independent biological replicates were performed for each experiment. Cells were mixed 2:1 *cerevisiae*:*uvarum* promoters for experiment 1 and 1:1 *uvarum*:*cerevisiae* promoters for experiment 2. The fraction of cells expressing Venus were measured over time by flow cytometry. Plotted is the change in the ratio P_{GAL1}*uvarum* to P_{GAL1}*cerevisiae*, normalized to the initial ratio, after 36 h growth. The Malthusian selection coefficient for the strain having the *uvarum* P_{GAL1} was -0.005 ± 0.0007 in experiment 1 and -0.007 ± 0.0006 in experiment 2.

3.F. The Gal4 central domain promotes stronger transcription during *GAL* memory

In addition to the *gal1-D117V* mutant, which showed specific loss of memory without strong effects on activation, the flow cytometry screen also identified a mutation in Gal4 (*L282P*) that both blocked memory and led to defective activation of Gal1-mCherry (Figure 3.8A). This mutation likely destabilizes the Gal4 protein, leading to lower protein levels (Figure 3.8A, inset). However, this mutation was interesting because the *gal4-L282P* mutant was also unaffected by ectopic expression of Gal1 (Figure 3.8A), confirming that the loss of memory in *gal4-L282P* cells was not an indirect effect of lower levels of Gal1 during reactivation. Thus, although Gal4-L282P shows a defect in activation, it blocked memory downstream of Gal1.

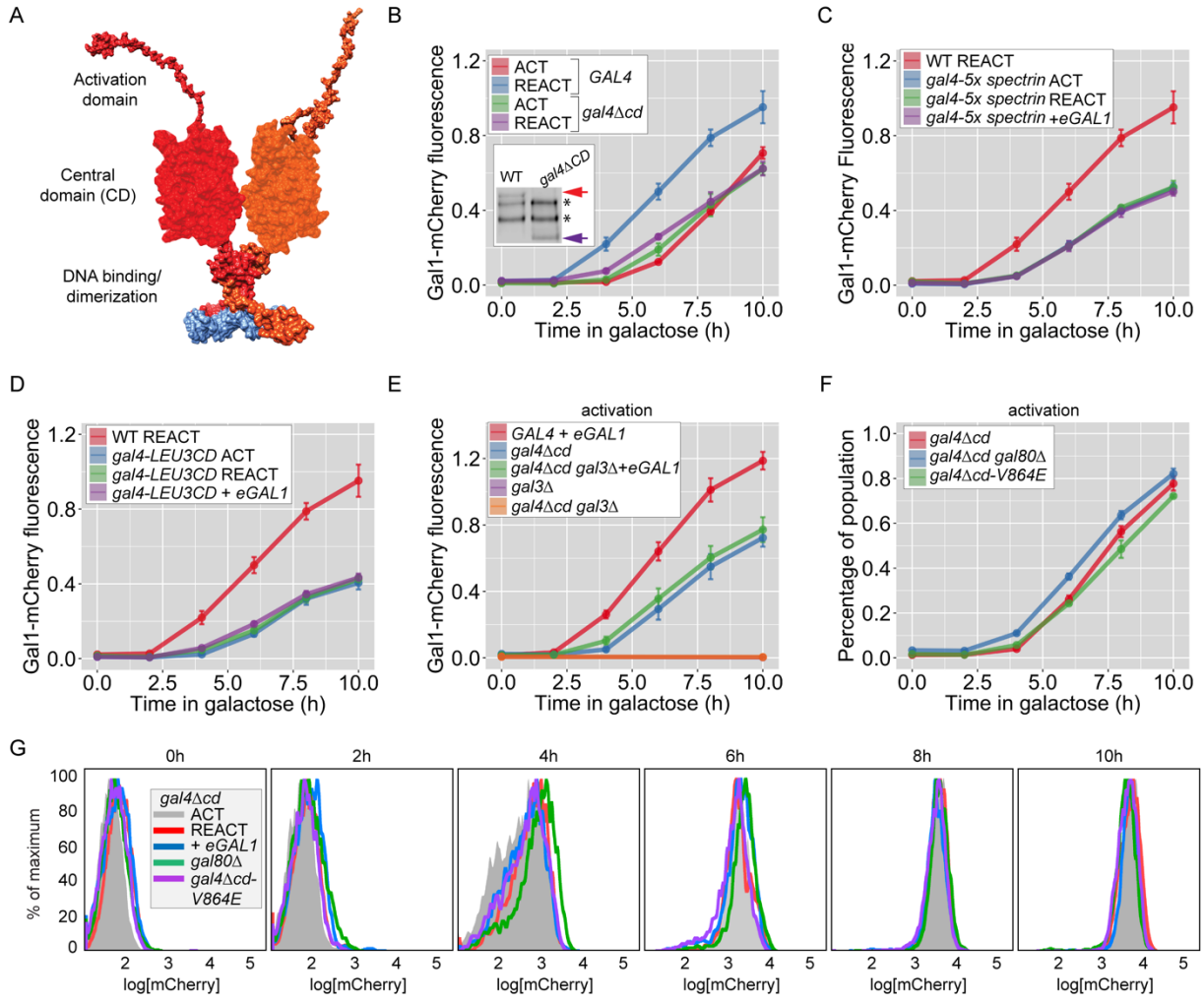

Figure 3.7. The Gal4 central domain is required for *GAL* memory. **A.** Schematic of the putative domain organization with a large central domain of Gal4 (based on a structural prediction), between the N-terminal DNA binding domain and an unstructured C-terminal activation domain. **B-F.** Naïve cells (ACT), naïve cells expressing ectopic *GAL1* (ACT+e*GAL1*), or cells that were grown in galactose overnight and shifted to glucose for 12 hours, were shifted to galactose (REACT) to assay the Gal1-mCherry fluorescence relative to constitutively expressed CFP. **B.** Wild type and *gal4Δcd* mutant. Inset: immunoblot of Gal4-myc immunoprecipitated from Wild type and *gal4Δcd* mutant cells; arrows: Gal4, asterisks:non-specific band. **C.** and **D.** Central domain of Gal4 was replaced with 5-tandem repeats of Spectrin domain (**C**) or central domain from Leu3 (**D**). **E.** Wild-type, *gal3Δ*, *gal4Δcd* and *gal4Δcd gal3Δ* strains with or without e*GAL1*. Only the 0h and 10h time points are plotted for *gal3Δ* and *gal4Δcd gal3Δ* mutants. **E.** *gal4Δcd* strains with and without *gal80Δ* and *gal4V864E* mutation. **G.** Overlay of concatenated histograms of biological replicates for data in **B** and **F**. Error bars represent SEM from ≥ 3 biological replicates.

The *gal4-L282P* mutation lies within the central domain of Gal4 (CD; Figure 3.7A). In other members of the zinc binuclear cluster transcription factor family, the central domain has been proposed to have a regulatory function [164, 165, 167-170]. However, the role of CD is unclear; deletion of this domain produces a largely functional Gal4 activator but certain point mutations in this domain disrupt Gal4 function [151, 155, 157, 158, 213, 214].

To explore the role of the Gal4 central domain, we tested how deletion of this domain affected memory and the response to Gal1. Unlike Gal4-L282P, Gal4Δcd protein levels were similar to full length Gal4 protein levels (Figure 3.7B; Inset) and the rate of Gal1-mCherry activation was similar in *gal4Δcd* and wild-type cells (Figure 3.7B; [151, 155, 213]). However, cells lacking the central domain showed no memory (Figure 3.7B) and were unaffected by ectopic expression of either Gal1 (Figure 3.8B). Thus, *gal4Δcd* mutant blocked memory downstream of Gal1 and independent of the small difference in the steady state Gal1-mCherry expression, relative to wild-type (Figure 3.8D).

Given the weak sequence conservation of the central domain, we asked if CD promotes memory by acting as a spacer to increase the access of the activation domain to co-activators. The CD was replaced with either domains 12-16 of human β-spectrin, which functions as an inert spacer of similar size to the CD [215, 216] or the central domain from Leu3, a related transcription factor [165, 167, 217-219]. Although these hybrid proteins supported Gal1-mCherry expression, they blocked memory and were unresponsive to Gal1 (Figures 3.7C and D). Thus, the Gal4 central domain has a sequence-specific function in potentiating expression and neither a simple spacer, nor a generic, swappable domain. Because Gal3 has a higher affinity for Gal80 than Gal1 [220], loss of memory could result if Gal4Δcd is de-repressed normally by

Gal3, but is unresponsive to Gal1 (Figure 3.1A). To test this hypothesis, we asked if Gal1 could replace Gal3 to promote activation of Gal1-mCherry. In cells lacking Gal3, Gal1-mCherry is not expressed (Figure 3.7E; *gal3Δ* and *gal4Δcd gal3Δ*). However, ectopic expression of Gal1 complemented this defect in *gal4Δcd* cells, allowing Gal1-mCherry expression (Figure 3.7E), but at levels observed during initial activation. This argues that Gal4Δcd responds to both Gal1 and Gal3, but is limited in its activity, leading to slower/lower expression of Gal1-mCherry.

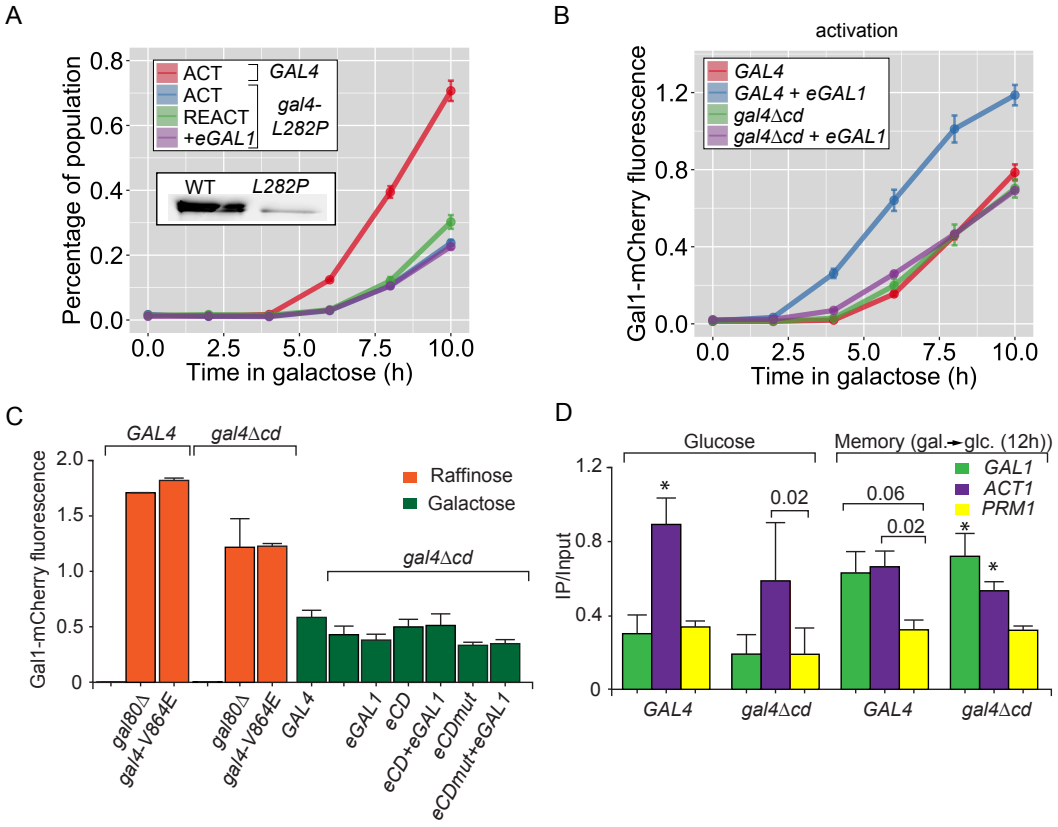

Figure 3.8. Gal4 central domain regulates potentiation downstream of *GAL1*. A-D. Naïve cells (ACT), naïve cells with ectopically expressed *GAL1* (*eGAL1*), or cells that were grown in galactose overnight and shifted to glucose for 12 hours (REACT), were shifted to galactose and Gal1-mCherry fluorescence was measured relative to CFP control. **A.** The *gal4-L282P* mutant strain; Inset: immunoblot for immunoprecipitated Gal4 and gal4-L282P mutant. **B**. Wild-type and *gal4Δcd* mutant with and without ectopic *GAL1*. **C**. Steady state Gal1-mCherry levels relative to CFP in strains derived from *GAL4* cells and *gal4ΔCD* mutant cells, grown in raffinose and galactose. **D.** ChIP against H3K4me2 in wild-type and *gal4Δcd* mutants under long-term repressed (glucose) and memory (gal. → glc., 12h) conditions. Recovery of the *GAL1* promoter, positive control locus (*ACT1*) and negative control locus (*PRM1*), were quantified relative to

input by real time quantitative PCR. * $p \leq 0.05$ (Student's t-test) relative to the ChIP enrichment of *PRM1*.

3.G. The Gal4 central domain is a target of Gal80 repression

Loss of the Gal4 central domain also altered Gal80 repression. During both activation and reactivation, *gal4Δcd* cells showed *unimodal* Gal1-mCherry expression (Figures 3.7G & 3.9). Hence, loss of the central domain had two effects: it both reduced the strength of Gal1-mCherry expression (as measured by average expression in the population; Figure 3.7B) and led to a more uniform responsiveness of the population (Figures 3.7G & 3.9). Because either loss of Gal80 or transcriptional memory also leads to unimodal activation (Figure 3.1D), this implied that the central domain is required for proper Gal80 repression. If so, then loss of Gal80 should not further increase the rate of activation. Indeed, neither loss of Gal80 nor disruption of the Gal4-Gal80 interaction (*gal4-V864E*) increased the rate of activation in the *gal4Δcd* cells (Figure 3.7F & G). Thus, the Gal4 central domain is required for both proper Gal80 repression and maximal expression during memory.

If loss of the Gal4 central domain completely blocked Gal80 repression, this should lead to expression of Gal1-mCherry in raffinose medium in cells lacking the central domain, in which Gal80 is the sole regulator of *GAL* gene expression. However, in raffinose medium, while either loss of Gal80 or loss of the interaction between Gal4 and Gal80 (*gal4-V864E*; [203]] led to de-repression of Gal1-mCherry, deletion of the central domain alone did not (Figure 3.8C). Thus, loss of the Gal4 central domain increases the rate of Gal80 de-repression.

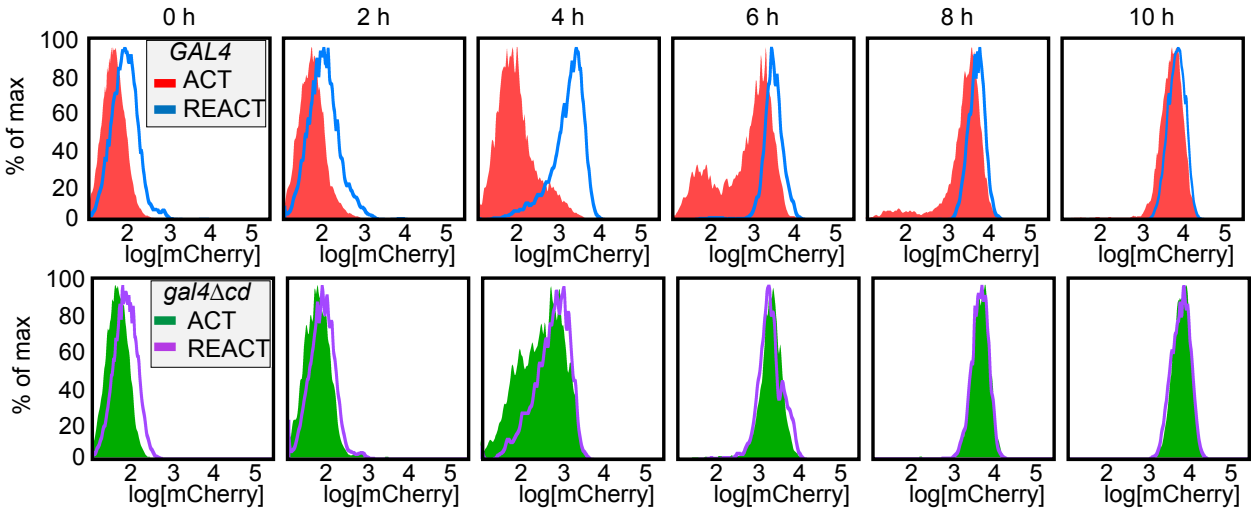

Figure 3.9. Transcriptional activation in *gal4Δcd* is more uniform but lacks potentiation. Naïve cells (ACT) and cells that were grown in galactose overnight and shifted to glucose for 12 hours (REACT), were shifted to galactose. Overlap of concatenated histograms of Gal1-mCherry between ACT and REACT, for wild-type cells (**A**) or *gal4Δcd* mutant (**B**).

3.H. An inter-domain interaction potentiates Gal4 activation

In the other members of the Gal4 transcription factor family, the central domain directly interacts with the activation domain to allosterically regulate activation [164, 169, 170, 219]. To test if the central domain (CD) interacts with the rest of Gal4, we asked if this domain could potentiate Gal4Δcd activation in *trans* (Figure 3.10B; schematic). Ectopically expressed CD localized in the nucleus (Figure 3.10A), independent of Gal4 (Figure 3.11A). Ectopic CD increased the rate of Gal1-mCherry activation in *gal4Δcd* strains (Figure 3.10C and D). However, this effect required either expression of ectopic Gal1 (Figure 3.10C) or loss of Gal80 (Figure 3.10D). Under these conditions, ectopic CD was recruited to the *GAL1* promoter (Figure 3.10E). This suggests that CD physically interacts with Gal4Δcd to potentiate activation and this interaction is regulated by Gal1-Gal80.

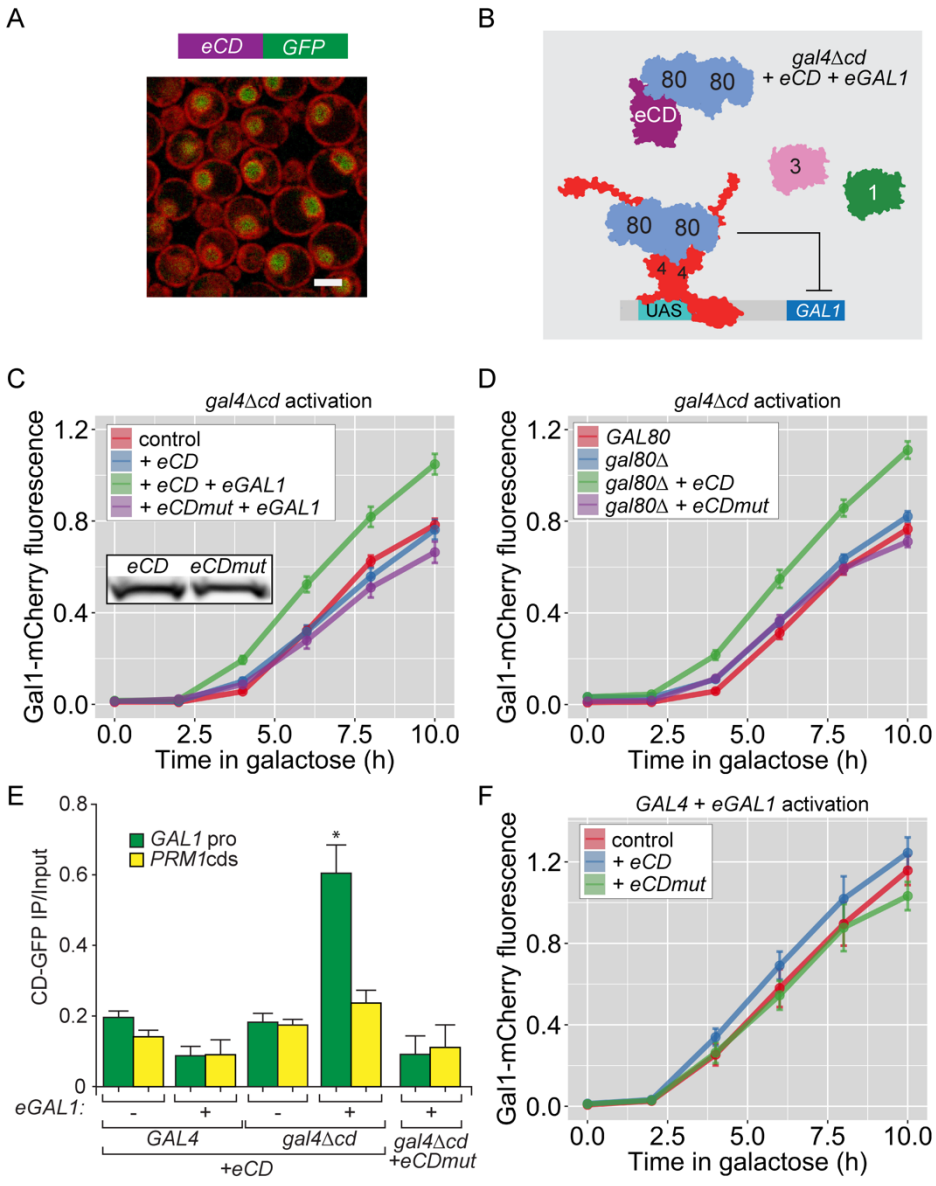

Figure 3.10. The Gal4 central domain is required for *GAL* memory. **A.** Schematic of the putative domain organization with a large central domain of Gal4 (based on a structural prediction), between the N-terminal DNA binding domain and unstructured C-terminal activation domain. **B-F.** Naïve cells (ACT), naïve cells expressing ectopic *GAL1* (ACT+e*GAL1*), or cells that were grown in galactose overnight and shifted to glucose for 12 hours, were shifted to galactose (REACT) to assay the Gal1-mCherry fluorescence relative to constitutively expressed CFP. **B.** Wild-type and *gal4Δcd* mutant. Inset: immunoblot of Gal4-myc immunoprecipitated from wild-type and *gal4Δcd* mutant cells; arrows: Gal4, * = non-specific bands. **C.** and **D.** Central domain of Gal4 was replaced with either 5-tandem repeats of β-spectrin domain (**C**) or the central domain from Leu3 (**D**). **E.** Wild-type, *gal3Δ*, *gal4Δcd* and *gal4Δcd gal3Δ* strains with or without e*GAL1*. Only the 0h and 10h time points are plotted for *gal3Δ* and *gal4Δcd gal3Δ* mutants. **F.** *gal4Δcd* strains with and without *gal80Δ* and *gal4V864E* mutation. **G.** Overlay of

histograms of biological replicates from the indicated strains and time points in **B** and **F**. Error bars represent SEM from ≥ 3 biological replicates.

This effect was highly specific; in the absence of ectopic Gal1, ectopic CD neither upregulated Gal1-mCherry expression nor bound to the *GAL1* promoter (Figure 3.10C & E, 3.11B & C). Furthermore, ectopic CD did not potentiate activation from full-length Gal4 (Figure 3.10F, 3.11C) and ectopic *L282P* mutant CD (CDmut) had no effect (Figure 3.11C and D), despite similar expression levels as CD (Figure 3.10C, Inset). Thus, the mutation of L282P in the CD blocks either the inter-domain interaction or its effects on Gal4 function.

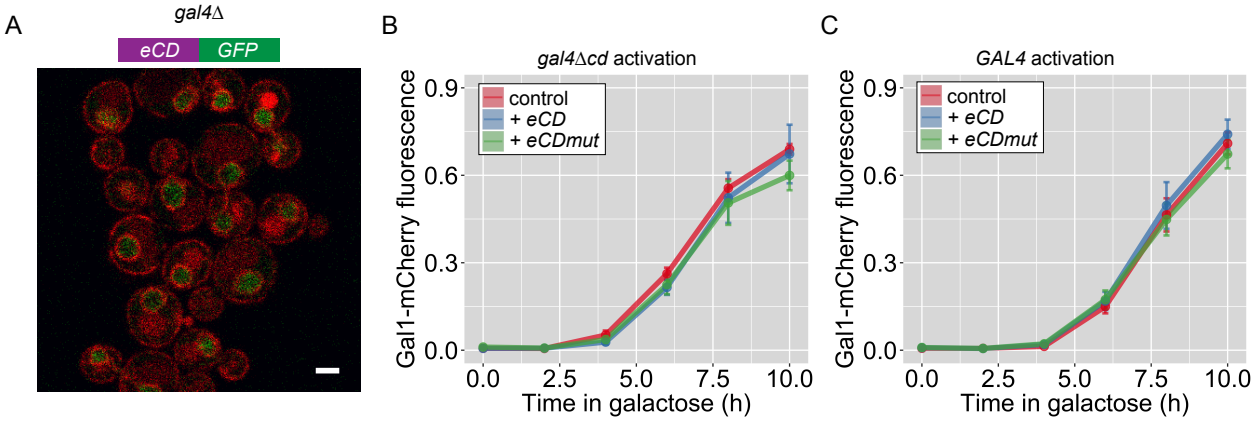

Figure 3.11. Localization and function of Gal4 central domain. A. Confocal micrograph showing localization of GFP fused central domain of Gal4 in *gal4Δ* cells expressing ER/nuclear envelope-targeted RFP. Gal1-mCherry fluorescence relative to CFP control in wild-type cells (**B**) and *gal4Δcd* mutant (**C**), expressing ectopic eCD or mutant eCDmut (L282P).

3.I. Discussion

This study provides important new insights into both the molecular mechanism of epigenetic *GAL* gene transcriptional memory in *S. cerevisiae* and an illustration of the evolutionary logic whereby the same molecules can produce either conditional, epigenetic poising or constitutive, genetic poising. Our current model for both is shown in Figure 3.12. Gal80 physically interacts with both the activation and central domains of Gal4 [140, 159-162]. Although it is not yet clear if the Gal80 that binds to the Gal4 activation domain is the same molecule as the Gal80 that interacts with the central domain, our results suggest that both interactions are required for proper repression. Early during activation, Gal3 interacts with Gal80, permitting Gal4-mediated transcriptional activation in a subset of the cells in the population (Figure 3.12A). In these cells, the central domain potentiates activation, leading to high-level expression. During memory, or in *S. uvarum*, the population shows uniform, rapid transition to high-level expression of *GAL* genes (Figure 3.12B). However, in cells lacking the Gal4 central domain, the population responds uniformly, but the level of expression is low. These cells lack memory both because they are less well repressed by Gal80 (and therefore do not benefit from previous expression of Gal1) and because they are unable to achieve full activation.

Slight differences in the degree of repression of *GAL1* lead to two different strategies that favor growth under different conditions. Low-level basal *GAL1* expression in *S. uvarum* leads to rapid adaptation to galactose but also encumbers a fitness cost in glucose-galactose mixtures. On the other hand, tight *GAL1* repression restricts fitness in galactose, but leads to optimal utilization of glucose in the presence of other sugars. Glucose is the most efficiently

utilized sugar through glycolysis and *S. cerevisiae* has a clear preference for it; expression of several genes is optimized for growth in glucose over other carbon sources [221, 222]. Epigenetic memory in *S. cerevisiae* allows cells to benefit from previous growth in galactose without compromising the preference for glucose. In other words, memory provides a mechanism for reclaiming a fitness benefit from repeated exposures to galactose while maintaining a growth advantage in glucose over longer time scales.

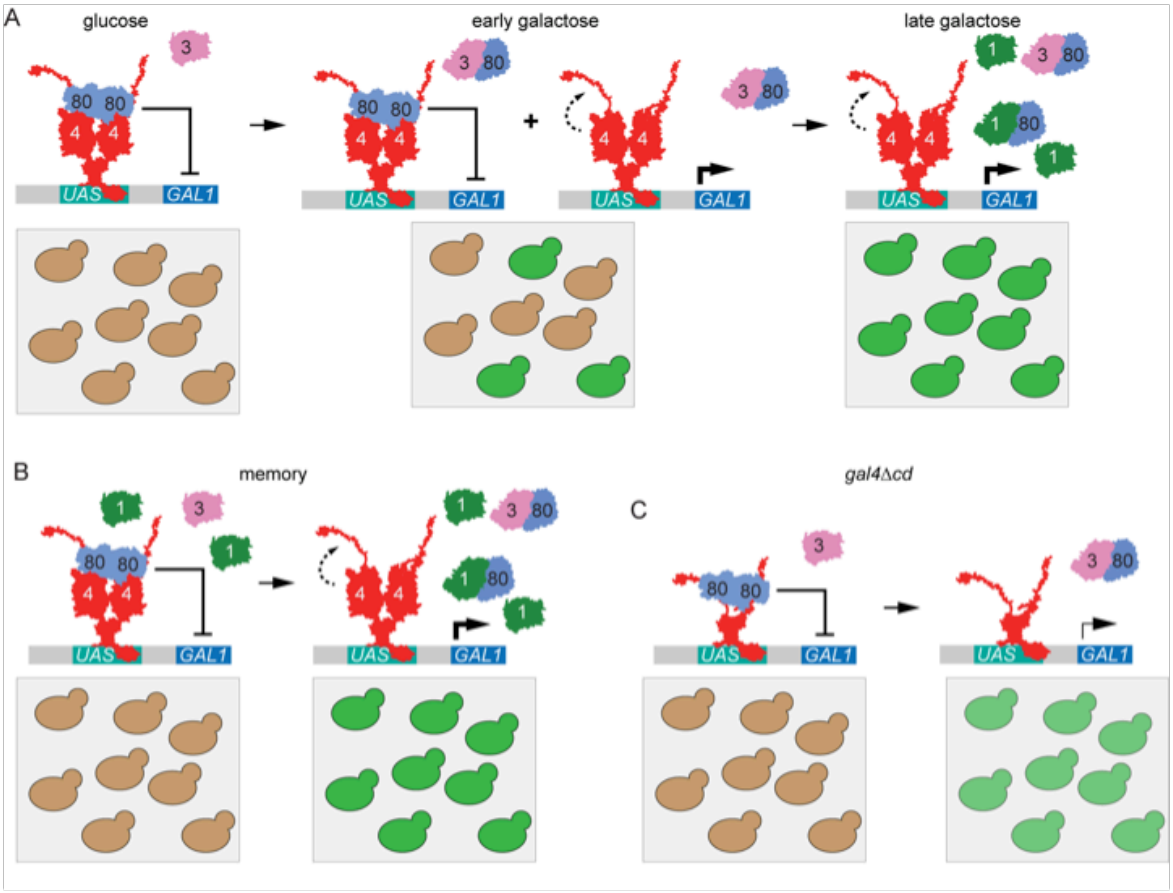

Figure 3.12. Model for epigenetic potentiation of Gal4 activation through inter-domain potentiation. A. In wild-type cells during early activation, Gal80 repression is relieved in subset of population, leading to lower-level expression. Inter-domain interaction between central domain and activation domain potentiates higher activation levels in cells relieved of Gal80. **B.** During memory (or in the presence of basal Gal1 expression), Gal80 repression is relieved early in whole population leading to uni-modal and potentiated *GAL* gene expression. **C.** *gal4Δcd*

cells show uniform but unpotentiated activation that does not achieve high-level expression unless the CD is provided in *trans* either along with eGal1 or in the absence of Gal80.

A whole-genome duplication during *Saccharomyces* evolution has led to specialization of function between duplicated paralogs Gal1 and Gal3 [211, 223-226]. Different species have followed different evolutionary paths toward subfunctionalization of these proteins. Species such as *K. lactis* and *C. albicans* that diverged from *Saccharomyces* before the whole genome duplication also exhibit constitutive poising of *GAL* genes [132, 204, 225]. This is because these species lack Gal3, they express basal levels of Gal1 to permit expression of the *GAL* genes, suggesting that basal *GAL1* expression is the ancestral regulatory scheme that has been maintained in *S. uvarum* [204, 211, 226-228]. Replacing P_{GAL1} in *S. cerevisiae* with the P_{GAL1} from the more closely related *Saccharomyces* species *S. mikatae* and *S. paradoxus* did not lead to constitutive poising (Figure 3.13A & B). This suggests that basal *GAL1* expression due to promoter differences persisted in *S. uvarum*, but was lost in *S. cerevisiae*, *S. paradoxus* and *S. mikitae*. Tighter *GAL1* repression has been accompanied by evolution of *GAL3* as a specialized co-activator: constitutively expressed Gal3 from *S. cerevisiae* has lost galactokinase activity and has 10-fold higher affinity for Gal80 repressor that Gal1 [172, 205, 208, 211, 220]. Thus, *GAL* transcriptional memory in *S. cerevisiae* is a product of the parallel evolution of tighter *GAL1* repression and specialization of the *GAL3* paralog as co-activator.

Using a FACS-based genetic screen, we identified two mutations that provide important insight into the molecular mechanism of *GAL* transcriptional memory. The *gal1-D117V* mutation maintains galactokinase function but reduces affinity for Gal80 and specifically

disrupts memory. This mutation has a modest defect in growth in galactose, likely due to a defect in the ability to co-activate Gal4. Furthermore, *gal1-D117V* blocked *GAL* gene poising caused by the *S. uvarum* P_{GAL1}, confirming that these effects are mediated by low level expression of Gal1.

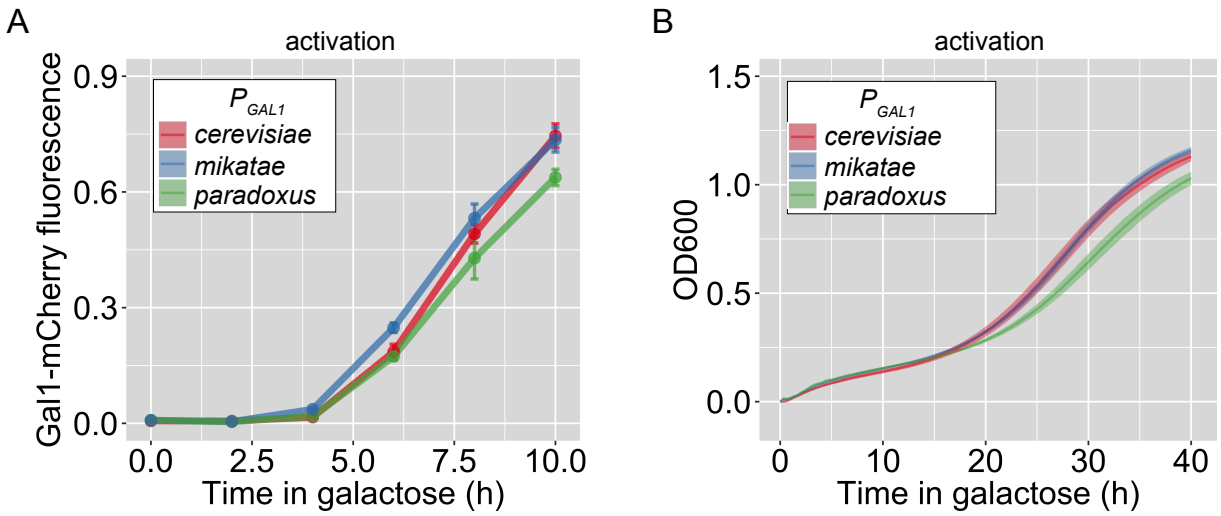

Figure 3.13. Testing basal expression activity of P_{GAL1} from other *Saccharomyces* species. The *GAL1* promoters from *S. mikatae* and *S. paradoxus* were introduced into *S. cerevisiae* in place of the endogenous *GAL1* promoter and mCherry fluorescence (**A**) and OD_{600} (**B**) were measured after shifting cells from glucose to galactose.

The screen also identified *gal4-L282P*, a mutation in the central domain of Gal4 that blocks the ability of Gal4 to respond to Gal1. Deletion of the central domain also disrupted memory, without strongly altering Gal4 protein levels or the rate of activation. The central domain has two functions: it promotes tighter Gal80 repression and it promotes stronger Gal4 activity (Figure 3.12C). Disrupting these functions resulted in a qualitative change in the *GAL1* transcriptional output, leading to a more uniform population of cells that transitioned to a weaker level of expression (Figure 3.12C). In other words, unimodal induction is necessary, but not sufficient, for the rapid expression observed during memory. Because Gal80 interacts with

both the central domain and the activation domain [140, 159-162], we propose that the central domain either enhances Gal80 recruitment to Gal4 or inhibits dissociation of Gal80 from Gal4. If so, then Gal80 would likely also regulate the potentiation of Gal4 activation by the central domain. We envision two mechanisms by which such potentiation might occur. First, the central domain might physically interact with the Gal4 activation domain, allosterically altering its ability to promote transcription. Second, because transcriptional memory also leads to changes in the chromatin structure of the *GAL1* promoter [25], central domain might recruit co-factors that mediated these changes, stimulating stronger transcriptional output. The Gal4 central domain plays a critical role in *GAL* gene transcriptional memory.

GAL transcriptional memory is a manifestation of ongoing resolution of expression levels of the partially redundant paralogs, Gal1 and Gal3. Among different *Saccharomyces* species, the degree of repression of *GAL1* in glucose dictates whether faster adaptation to galactose is regulated through either a constitutive, genetic mechanism or a conditional, epigenetic mechanism. Although leaky *GAL1* expression is advantageous in galactose, it compromises fitness in mixtures of sugars. *S. cerevisiae* has traded faster kinetics of *GAL* gene activation for optimal growth in glucose-galactose mixtures. During memory, cells both switch from a heterogeneous to uniform population and employ an inter-domain potentiation of Gal4 activation to more rapidly adapt to a challenge that they have experienced recently (Figure 3.12B).

Note: This chapter was adapted with my permission from my manuscript, which is under review “Sood and Brickner”. Genetic and epigenetic strategies potentiate Gal4 activation to enhance fitness in recently diverged yeast species”.

Chapter 4. Summary and future directions

4.A. Summary

Transcriptional memory promotes adaptation by accelerating the transcriptional response to the changing environment. Studies prior to this thesis have shown that several aspects of the mechanism for transcriptional memory are conserved [22, 30]. However, gene-specific variations in the mechanism also exist [25, 224]. The ongoing question at the start of this work was what are the relative contributions of the conserved *vs* gene-specific mechanisms for faster reactivation. The widespread occurrence of transcriptional memory and the conservation of its mechanisms suggest an evolutionary pressure for retaining this epigenetic phenomenon. However, how this phenomenon evolved and whether it confers any fitness benefits were not known. Work in this thesis addressed these questions using the *GAL* gene transcriptional memory as model.

GAL genes exhibit the conserved molecular features of transcriptional memory: interaction with Nup100 at the nuclear periphery, incorporation of H2A.Z, H3K4me2 modification, and binding of poised RNAPII at the promoter. However, peripheral localization and interaction with Nup100 is dispensable for faster reactivation. Furthermore, loss of faster reactivation is not associated with a loss of H3K4me2 modification (Figure 3.7 and 3.8). Thus, conserved mechanisms for transcriptional memory seem dispensable for *GAL* transcriptional memory, which rather requires three sequential steps. First, a uniform expression of *GAL* genes promoted by the cytoplasmically inherited Gal1 protein. Second, an allosteric upregulation of transcriptional activation by the central domain of Gal4. Third, switching of Tup1 from a repressor to a co-activator. This study also addressed the adaptive role and evolution of

memory. *GAL* transcriptional memory confers a huge growth advantage upon shift to galactose, relative to naïve cells. Comparison of this advantage across related *Saccharomyces* species revealed how *GAL* transcriptional memory has evolved. Gal1 needed for memory is produced at high basal level in a related species, *S. uvarum*. As a result, *S. uvarum* shows constitutively fast *GAL* gene induction and shorter growth lag upon shift to galactose. In *S. cerevisiae*, *GAL1* is rather tightly repressed. The higher levels of Gal1 are restricted to the transcriptional memory phase in *S. cerevisiae*. This thesis has thus revealed that *GAL*-gene-specific determinants play a dominant role in transcriptional memory. Further, *GAL* memory is a recently evolved phenomenon that resulted from a tighter repression of *GAL1*. Finally, memory has a huge adaptive value. These results open up lots of questions about both the general and *GAL*-gene-specific mechanisms of transcription memory.

4.B. Peripheral localization and *GAL* transcriptional memory

Peripheral localization and association with NPC is essential for transcriptional memory, except in the case of *GAL* genes [22, 25, 27, 30]. It would be worthwhile to explore the evolution of peripheral localization of *GAL* genes and its effect on transcriptional memory in other *Saccharomyces* species. It is possible that peripheral localization and interaction with Nup100 affects expression in other species and it is rather a vestige in *S. cerevisiae*. If this were true, the hybrid diploids between such a species and *S. cerevisiae* would provide a good system to dissect how Nup100 promotes *GAL* memory. However, given that both a zip code (MRS_{GAL1}) and Nup100 specifically regulate peripheral localization, two potential roles are still conceivable. First, peripheral localization could play a role under certain condition, which we

have not explored e.g. in glucose-galactose mixtures. *GAL* genes are expressed in glucose-galactose mixtures only during *GAL* memory (Figure 3.1G). Furthermore, glucose is essential for peripheral localization during *GAL* memory (Figure 2.1D). Thus, peripheral localization could play a role in *GAL* gene expression in glucose-galactose mixture. This hypothesis can be tested using growth or expression assay in mrs_{GAL1} or *nup100Δ* mutant cells. Second, peripheral localization could act in a redundant pathway to promote *GAL* memory. This possibility can be explored through a genetic screen for loss of *GAL* memory in *nup100Δ* cells. Presence of mutants that are rescued by adding back *NUP100* would support a redundant role for peripheral localization.

4.C. Uniform, potentiated expression during *GAL* memory

Intuitively, switch from a bi-modal to a uni-modal expression would be expected to increase the average rate of expression. However, Chapter 3 highlighted that Gal1 mediated uni-modal *GAL* gene expression is necessary but not sufficient for faster reactivation. It also requires potentiation of transcription activation levels by the Gal4 central domain. Thus, *gal4Δcd* unraveled new model of kinetic regulation. This raises the obvious question, is transcriptional memory of other genes associated with a more uniform expression or an increase in the levels of transcription within each cell? This can be addressed by flow-cytometric analysis of expression during activation and transcriptional memory of these genes. This approach would also reveal what aspects of kinetic upregulation are controlled by the conserved mechanisms of transcriptional memory.

4.D. Allosteric potentiation by Gal4 central domain

The central domains in transcription factors related to Gal4, like Leu3, regulate the switch between active and inactive transcription factor [164, 169, 170, 219]. These transcription factors activate constitutively in the absence of their native central domain. The Gal4 central domain, however, is unique. It regulates the switch between slow and fast kinetics of transcriptional activation. This raises the obvious question, what is its mechanism and whether a similar mechanism operates in other systems. It is clear that CD_{GAL4} does not act through chromatin (Figure 3.8). However, unlike central domains of other transcription factors, ectopic CD_{GAL4} can accelerate kinetics in *trans*. Thus, a series of ectopic hybrids between CD_{GAL4} and CD_{Leu3} could delineate the subdomain for this allosteric upregulation. This subdomain could then be tested for general function through fusion with other transcription factors. In parallel, this approach could also identify subdomain that regulates Leu3 switch to an active form. Given how little is known about what regulates activation output from transcription factors, ectopic CD_{GAL4} provides a good system to define such factors.

4.E. Transcriptional repressors hysteresis during transcriptional memory

The faster reactivation during transcriptional memory can also be conceived has a hysteresis of repression function. Hysteresis is used to describe the phenomenon in which the physical property lags behind changes in the effect causing it. In case of transcriptional repressors, it would imply a delay in gaining complete repressor potential i.e. the same repressor has a weaker repressor potential during transcriptional memory relative to activation. During *GAL* memory, neutralization of Gal80 repressor by Gal1 decreases its repressor potential. But, what

if the Gal80 that was removed away from Gal4 does not immediately regain its Gal4 binding and repression potential, upon shift to glucose media. During this period Gal80 would have weaker repression i.e. hysteresis. However, the interaction with Gal1 makes it harder to assay this effect. Mig1 repressor, on the other hand, shows such hysteresis. Two hours of galactose induction primes *GAL* genes for faster reactivation (Figure 4.1) [56, 57, 144]. This phenomenon does not involve Gal1 [144]. I found that it is not the galactose treatment, but the absence of glucose that actually causes this priming (Figure 4.1). Mig1 is deactivated through phosphorylation in the absence of glucose [143]. Thus, it is likely that the faster reactivation results from a delay in dephosphorylation that gives rise to a window of weaker Mig1 repression. It would be worth testing if other Mig1 regulated genes show this effect and whether other transcriptional repressors show similar hysteresis. A comparative study of factors that show *vs* don't show hysteresis would be useful to uncover the determinants of this short-term memory.

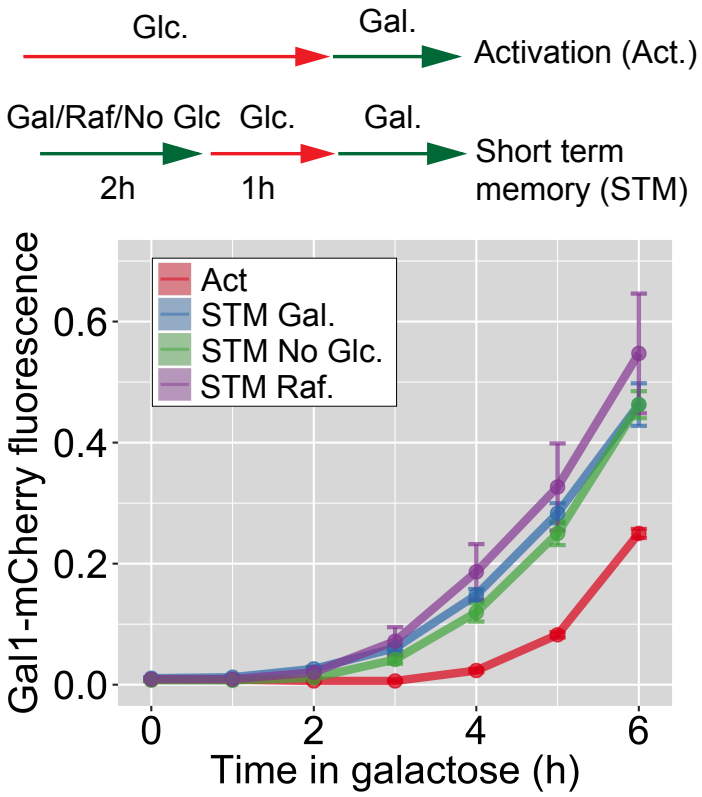

Figure 4.1. Short-term *GAL* memory requires growth in absence of glucose. Top panel: Schematic of media shifts during activation and short-term memory. Bottom panel: For activation, cells were shifted from glucose to galactose. For short-term memory, cells were grown in the indicated media for 2 hours, followed by growth in glucose media for 1 hour and then shifted to galactose media. The relative Gal1-mCherry intensity was plotted over time. Error bar represents SEM from ≥ 3 biological replicates.

Chapter 5. Materials and Methods

5.A. Reagents

All chemicals unless noted otherwise were from Sigma Aldrich (St. Louis, MO). Yeast media components were from Sunrise Science Products (San Diego, CA). Restriction enzymes were from New England Biolabs (Ipswich, 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), rabbit anti-H2A.Z (4626) and rabbit anti-H3K4me2 (32356) were from AbCam. Rapamycin was from Millipore.

5.B. Plasmids, yeast strains, and molecular biology

Plasmids pAFS144 [229], p6LacO128-GAL1, p6LacO128-GAL1-10prom have been described previously [24, 38, 51]. 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 [38]. pRS304-ADH1-GAL1 was created by ligating P_{ADH1}-*GAL1,* excised from pGREG700, into *Sac*I and *Kpn*I digested pRS304 [230]. pGREG700 in turn was generated from pGREG600 [231] by swapping *GAL1* promoter with *ADH1* promoter using the *Sac*I and *Spe*I sites. Promoter fragments and MRS variants were integrated at *URA3:p6LacO128* using the pZIPKan plasmid [175] or by cloning in p6LacO128 [22, 37]. The plasmids were linearized by digestion and integrated at the desired locus.

S. cerevisiae cells containing Nup2–TAP, Nup100-TAP and Gal1-GFP [146, 176] were from S288c background, all strains were constructed from CRY1 or CRY2 [232], derived from the W303 background (*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*). *S. uvarum* strains were generated from JRY8153 strain from the Hittinger lab Cells were grown in Synthetic Dextrose Complete (SDC), Synthetic Galactose Complete (SGC) or Synthetic Raffinose Complete (SRC) at 30°C [233] for localization, qRT-PCR and ChIP experiments. For flow cytometry of the Gal1-mCherry, cells were grown in either Yeast Peptone Dextrose (YPD) or Yeast Peptone Galactose (YPG).

A PCR-based system was used for deletion [234] 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 were transformed with either pAFS144 [229] or pRS305-GFP-LacI for GFP-LacI expression, pRS304-Sec63myc for immuno-labeling the nuclear envelope and derivatives of p6LacO128 plasmid to tag the locus of interest [24]. For live cell localization assays, the 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 [179]. Cells were treated with 1µg/ul rapamycin for depletion of FRB tagged proteins for 1 hour before imaging.

5.C. Chromatin Localization Assay

Chromatin localization experiments using immunofluorescence with fixed cells [174] and with live cells [175] 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 top or bottom of the nucleus. Error bars represent the standard error of the mean for three biological replicates of 30–50 cells each.

5.D. Chromatin Immunoprecipitation

Cells were fixed in 1% formaldehyde for 15 minutes at room temperature, 150mM Glycine was added to quench the formaldehyde reaction and ChIP was performed as described previously [22, 37, 38, 175]. For Nup2 and Nup100 ChIP, cells were fixed at room temperature for 1 hour. 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 q-PCR as described previously [38] using primers listed in Table II. Error bars represent the SEM from three biological replicates.

5.E. 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 and diluted to OD_{600} ~0.01 in SDC and grown for 12h. 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 [24]. *GAL1*, *GAL2* and *GAL7* mRNA levels were quantified relative to *ACT1* levels using the *GAL1* CDS, *GAL2* CDS and *GAL7* CDS primers, respectively. For experiments using the *gal1Δ* strain, cells were grown in SRC, shifted to SGC for 4 hours and then shifted to SDC for 12 hours. Error bars represent the SEM of three biological replicates.

5.F. Flow cytometry

Cells with *GAL1-mCherry* were induced in YPG and maintained at $OD_{600} \leq 0.3$ throughout the induction. 1 ml of culture was harvested at different times of induction and the cells were frozen in 10% glycerol and stored at -80˚C. For flow cytometry, cells were thawed on ice and analyzed a BD LSRII flow-cytometer. mCherry and CFP were excited with 561nm and 405nm lasers, respectively. For detecting mCherry emission a 600nm long pass dichroic mirror and 610/20nm band pass filter set was used, while for CFP emission 505nm 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.

5.G. Genetic Screen

Exponentially growing wild-type cells in SGC were mutagenized by exposure to 254nm ultra violet (UV) light using a hand-held lamp (UVGA-25, UVP Inc). 10ml of cells at OD_{600nm} = 0.1 in a 10 cm petri plate were exposed to UV for 60 seconds from 15 cm in a closed container, which killed 30% of the cells [235, 236]. The mutagenized cells were transferred to YPD for 12 hours before reactivation in YPG, 4h. Fluorescence activated cell sorting for non-fluorescent cells was done using the BD FACSAria SORP 5 at the Northwestern Flow Cytometry Core Facility. Approximately two million cells were harvested in YPG. Cells were harvested and resuspended in fresh YPG for additional 8 hours and then subjected to a second sort. Cells collected from the second sort were plated for single colonies on galactose plates. *GAL1-mCherry* activation and reactivation kinetics was individually assayed for each colony. Complementation with wild-type *GAL* genes was used for mapping mutations that lead to specific reactivation defects followed by subsequent Sanger sequencing of the mutant loci to identify the mutation.

5.H. Growth Assay

Exponentially growing cells were diluted to an OD_{600} = 0.1, washed with media containing no sugar and then resuspended in the appropriate media in a 96 well plate. Growth was monitored by measuring OD_{600} every 20 minutes for 40h using a 96-well plate reader (BioTek Synergy™), normalized to media without cells. The cell density at t = 0 was subtracted from all measurements. Multiple biological replicates were done for each condition on different days.

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Appendix

Yeast strains used in the study

Strain Name	Genotype
ICY165	*MATalpha ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 htz1Δ::HIS5 URA3:ADH1pro-GAL1*
yHMK 65	MATa ho D::NatMX
VSY164	*MAT alpha ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:HIS3 gal4-L282P URA3:pADH1pro-GAL1*
VSY163	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX PGAL1HybridC GAL1-mCHERRY:KanMX6*
VSY162	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX PGAL1HybridB GAL1-mCHERRY:KanMX6*
VSY161	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX PGAL1HybridA GAL1-mCHERRY:KanMX6*
VSY160	*MAT alpha ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX gal1D117V-mCHERRY:HIS3 URA3:pADH1pro-GAL1*
VSY159	MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD
VSY158	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX PGAL1uvarum gal1D117V-mCHERRY:KanMX6 GAL7-VENUS:URA3*
VSY157	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX PGAL1uvarum GAL1-mCHERRY:KanMX6*
VSY156	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX PGAL1uvarum GAL1-mCHERRY:KanMX6 GAL7-VENUS:URA3*
VSY155	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6 GAL7-VENUS:URA3*
VSY142	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX pGAL1paradoxus GAL1-mCHERRY:KanMX6*
VSY141	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX pGAL1mikatae GAL1-mCHERRY:KanMX6*
VSY139	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD-CDLEU3 URA3:pADH1pro-GAL1*
VSY138	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD-5XSPECTRIN URA3:pADH1pro-GAL1*
VSY137	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD-CDLEU3*

VSY136	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD-5XSPECTRIN*
VSY135	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD mig1Δ::HIS3*
VSY134	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 mig1Δ::HIS3*
VSY133	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD gal80Δ::HIS3*
VSY132	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal80Δ::HIS3*
VSY131	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD gal80Δ::HIS3 URA3:pADHpro-$CD_{GAL4L282P}$*
VSY130	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD gal80Δ::HIS3 URA3:pADHpro-CD_{GAL4}*
VSY129	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD TRP1:pADH1pro-GAL1 URA3:pADHpro-CD_{GAL4}-GFP*
VSY128	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD URA3:pADHpro-CD_{GAL4}-GFP*
VSY127	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 URA3:pADHpro-CD_{GAL4}-GFP*
VSY126	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD TRP1:pADH1pro-GAL1 URA3:pADHpro-$CD_{GAL4L282P}$*
VSY125	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD TRP1:pADH1pro-GAL1 URA3:pADHpro-CD_{GAL4}*
VSY124	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 TRP1:pADH1pro-GAL1 URA3:pADHpro-$CD_{GAL4L282P}$*
VSY123	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 TRP1:pADH1pro-GAL1 URA3:pADHpro-CD_{GAL4}*
VSY122	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD URA3:pADHpro-$CD_{GAL4L282P}$*
VSY121	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD URA3:pADHpro-CD_{GAL4}*
VSY120	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 URA3:pADHpro-$CD_{GAL4L282P}$*

VSY119	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 URA3:pADHpro-CD$_{GAL4}$*
VSY118	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 TRP1:dsRed-HDEL URA3:pADHpro-CDgal4-GFP gal4Δ::HIS3*
VSY117	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 TRP1:dsRed-HDEL URA3:pADHpro-CDGAL4-GFP*
VSY115	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD gal3Δ::HIS3*
VSY114	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD gal3Δ::HIS3 URA3:pADH1pro-GAL1*
VSY113	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6 URA3:pADH1pro-GAL3*
VSY112	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD URA3:pADH1pro-GAL3*
VSY111	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6 gal4ΔCD TRP1:pADH1pro-GAL1*
VSY110	*MAT alpha ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX gal1D117V-mCHERRY:HIS3*
VSY110	*MAT alpha ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:HIS3 gal4-L282P*
VSY109	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6* GAL80-Myc:Trp1
VSY108	*MAT alpha ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX gal1D117V-mCHERRY:HIS3* GAL80-Myc:Trp1
VSY106	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL1-GFP:KanMx*
VSY103	*MATa ade2-1 can1-100, his3-11,15 HO::TDH1prm-CFP-NatMX GAL1-mCHERRY:KanMX6 tup1Δ::HIS6 URA3:ADH1prom-GAL1*
VSY102	*MATa ade2-1 can1-100, his3-11,15 HO::TDH1prm-CFP-NatMX GAL1-mCHERRY:KanMX6 tup1Δ::HIS5*
VSY100	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Pho88-mCherry:HIS5 LEU2:LacI-GFP GAL1:URA3p6LacO128*
VSY099	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Pho88-mCherry:HIS5 LEU2:LacI-GFP URA3:p6LacO128Ab2.2 TRP1:ADHprom-GAL1*
VSY098	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Pho88-mCherry:HIS5 LEU2:LacI-GFP URA3:p6LacO128Ab2.2 tup1Δ::KanMX*
VSY097	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Pho88-mCherry:HIS5 LEU2:LacI-GFP URA3:p6LacO128Ab2.2 nup100Δ::KanMX*
VSY096	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Pho88-mCherry:HIS5 LEU2:LacI-GFP URA3:p6LacO128Ab2.2*

VSY095	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 GAL1:URA3p6LacO128 SEC63-13myc:KanMX HIS3:LacI-GFP TRP1:dsRed-HDEL*
VSY094	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 GAL1:URA3p6LacO128 SEC63-13myc:KanMX HIS3:LacI-GFP TRP1:Heh2-L-mCHERRY*
VSY092	*MATalpha tor1-1 fpr1::NAT RPL13A-2×FKBP12::TRP1 LEU2:LacI-GFP Pho88-mCherry:HIS5 GAL1:URA3p6LacO128 NUP100-FRB:KanMX6*
VSY091	*MATalpha tor1-1 fpr1::NAT RPL13A-2×FKBP12::TRP1 LEU2:LacI-GFP Pho88-mCherry:HIS5 GAL1:URA3p6LacO128 NUP2-FRB:KanMX6*
VSY090	*MATalpha tor1-1 fpr1::NAT RPL13A-2×FKBP12::TRP1 LEU2:LacI-GFP Pho88-mCherry:HIS5 GAL1:URA3p6LacO128*
VSY089	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Pho88-mCherry:HIS5 LEU2:LacI-GFP GAL1:URA3p6LacO128 nup100Δ::KanMX6*
VSY088	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6 pGAL1::URA3-SUP4-o*
VSY069	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Pho88-mCherry:HIS5 LEU2:LacI-GFP GAL1:URA3p6LacO128*
VSY060	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 TRP1:pADH1pro-GAL1*
VSY057	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 URA3:ADH1pro-GAL1*
VSY057	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-TDH-CFP-NatMX GAL1-mCHERRY:KanMX6 URA3:pADH1pro-GAL1*
VSY048	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 LEU2:LacI-GFP mrsGAL1:URA3p6LacO128 TRP1:Sec63-13XMyc SEC63-13XMyc:KanMX6*
VSY047	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 LEU2:LacI-GFP GAL1:URA3p6LacO128 TRP1:Sec63-13XMyc SEC63-13XMyc:KanMX6*
VSY043	*MATa ade2-1 can1-100, his3-11,15 ura3-1 TRP1:Sec63-13XMyc LEU2:LacI-GFP URA3:p6LacO128AmpΔ::mrsGAL1prom-KanMX6*
VSY042	*MATa ade2-1 can1-100, his3-11,15 ura3-1 TRP1:Sec63-13XMyc LEU2:LacI-GFP URA3:p6LacO128AmpΔ::GAL1prom-KanMX6*
VSY040	*MAT a ade2-1 can1-100, his3-11,15 ura3-1 TRP1:Sec63-13XMyc LEU2:LacI-GFP URA3:p6LacO128AmpΔ::mrs-KanMX6*
VSY039	*MAT a ade2-1 can1-100, his3-11,15 ura3-1 TRP1:Sec63-13XMyc LEU2:LacI-GFP URA3:p6LacO128AmpΔ::mrs4-KanMX6*
VSY039	*MAT a ade2-1 can1-100, his3-11,15 ura3-1 mrsGAL1*
VSY038	*MAT a ade2-1 can1-100, his3-11,15 ura3-1 TRP1:Sec63-13XMyc LEU2:LacI-GFP URA3:p6LacO128AmpΔ::mrs3-KanMX6*
VSY037	*MAT a ade2-1 can1-100, his3-11,15 ura3-1 TRP1:Sec63-13XMyc LEU2:LacI-GFP URA3:p6LacO128AmpΔ::mrs2-KanMX6*

VSY036	*MAT a ade2-1 can1-100, his3-11,15 ura3-1 TRP1:Sec63-13XMyc LEU2:LacI-GFP URA3:p6LacO128AmpΔ::mrs1-KanMX6*
VSY034	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6*
VSY034	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 HO::P-$_{TDH}$-CFP-NatMX GAL1-mCHERRY:KanMX6*
VSY003	*MAT a ade2-1 can1-100, his3-11,15 ura3-1 nup100Δ:KanMX6*
Nup2-TAP	*MATa his3Δ1 leu2Δ0 met15Δ1 ura3Δ0 Nup12-TAP::His5+*
Nup100-TAP	*MATa his3Δ1 leu2Δ0 met15Δ1 ura3Δ0 Nup100-TAP::His5+*
KVY001	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 SEC63-13myc:Kan^r HIS3:LacI-GFP URA3:GAL1prom-p6LacO128*
JRY8153	*MATa hoΔ::NatMX his3-11 lys2 trp1-1 ura3-1*
JRY8153	*MATa hoΔ::NatMX his3-11 lys2 trp1-1 ura3-1 GAL1-mCHERRY:KanMX6 URA3:pTDH-VENUS*
ICY63	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 tup1Δ::His5*
ICY39	*MATalpha ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 htz1Δ::HIS5*
ICY29	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 tup1::His5*
ICY195	*MATa ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128AmpΔ::Ab2.2-KanMX6*
ICY194	*MAT A ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128AmpΔ::Ab2.1-KanMX6*
ICY193	*MAT A ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128 SUP4-o*
ICY192	*MAT A ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128AmpΔ::GALproUAS1,2,4mut-KanMX6*
ICY191	*MAT A ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128AmpΔ::GALproAa2-KanMX6*
ICY190	*MAT A ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128AmpΔ::GALproAa1-KanMX6*
ICY189	*MAT A ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128AmpΔ::GALproAa-KanMX6*
ICY188	*MAT A ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128AmpΔ::GALproAb-KanMX6*
ICY187	*MAT A ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128AmpΔ::GALproIB-KanMX6*
ICY186	*MAT A ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128AmpΔ::GALproIA-KanMX6*
ICY185	*MATa ade2-1 can1-100, his3-11,15 ura3-1 Sec63-myc::TRP1 LEU2:LacI-GFP URA3:p6LacO128-KanMX6*

ICY176	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1Δ::His5+ LEU2:LacI-GFP GAL1:URA3p6LacO128*
ICY167	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 LEU2:LacI-GFP GAL2:URA3p6LacO128 TRP1:ADH1pro-GAL1*
ICY150	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 His5::gal1Δ LEU2:LacI-GFP GAL2:URA3p6LacO128*
ICY083	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 LEU2:LacI-GFP GAL1:URA3p6LacO128 TRP1:ADH1pro-GAL1*
ICY075	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 LEU2:LacI-GFP GAL2:URA3p6LacO128*
DBY051	*MATalpha ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 htz1Δ::HIS5 SEC63-13myc::KANMX HIS3:LacI-GFP GAL1:URA3p6LacO128*
DBY032	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 SEC63-13myc:Kan^r HIS3:LacI-GFP GAL1:URA3p6LacO128*
CRY2	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
CRY1	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
CEY346	*MATalpha tor1-1 fpr1::NAT RPL13A-2×FKBP12::TRP1 NUP2-FRB-GFP:HIS5*
AFY28	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1,HIS3: LacIGFP SEC63-13myc:TRP1 nup100Δ::KANMX GAL1:URA3p6LacO128*
ADY046	*MATalpha tor1-1 fpr1::NAT RPL13A-2×FKBP12::TRP1 NUP100-FRB-GFP:HIS5*