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Chromatin Remodeling and Transcriptional Memory: A Dissertation

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CHROMATIN REMODELING AND TRANSCRIPTIONAL MEMORY

A Dissertation Presented

By

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ABSTRACT

Transcriptional regulation of gene expression is critical for all unicellular and multicellular organisms. The ability to selectively induce or repress expression of only a few genes from the entire genome gives cells the ability to respond to changing environmental conditions, grow and proliferate. Multicellular organisms begin life as a single totipotent cell, which undergoes many cell divisions during embryonic and later postnatal development. During this process, the dividing cells of the embryo progressively lose their pluripotency and adopt restricted cell fates. Cell fate restriction leads different cell types to gain unique transcriptional profiles. This transcriptional profile or gene expression pattern not only defines the cell types and restricts the ways in which they can respond to signals, it also has to be faithfully re-established in the progeny of these fate-restricted cells when they divide.

Different mechanisms have evolved in multicellular organisms to propagate transcriptional memory of cell identity. Most of mechanisms involve modifications of chromatin such as epigenetic modification of DNA or alterations of associated histones. In contrast to multicellular organisms which have considerable cellular diversity and a long lifespan for which cell fates and transcriptional memory needs to be maintained, single celled budding yeast, *Sachharomyces cerevisiae* have a life cycle of about 90 minutes in normal nutrient rich conditions. However, even budding yeast have tremendous potential to respond to changing environmental conditions like nutrient availability by inducing expression of various genes. We observed that members of the

GAL gene cluster, which encodes genes induced in response to and for metabolizing the sugar galactose, showed heritable transcriptional memory of previous activation. This dissertation thesis describes the studies I have done for my graduate research to define this phenomenon of transcriptional memory at the yeast *GAL* genes and to determine the mechanism by which it can be formed and inherited.

Chapter I gives an introduction to different mechanisms of establishing transcriptional memory in unicellular and multicellular organisms. Chromatin based mechanisms have been well studied in multicellular organisms but not observed in budding yeast. We compare chromatin based or nuclear inheritance with cytoplasmic inheritance that can be observed in yeast. Chapter II describes work done to define the phenomenon of transcriptional memory at *GAL1* gene. We define this as a faster rate of induction of the *GAL1* gene, compared to a naïve gene, after a brief period of repression. We show that this cellular memory persists through mitosis and can be passed on to the next generation. We also show that chromatin remodeling enzymes appear to be required for rapid reinduction, raising the question if yeast may also possess chromatin associated, nuclear mechanisms for cellular memory. Chapter III describes experiments that show that cellular memory observed at *GAL1* is cytoplasmic in nature and also compares our work with similar examples observed recently by other groups. Finally, Chapter IV offers a perspective of the significance of such cellular memory mechanisms in budding yeast and outlines some potential further experiments to better understand the control of *GAL1* induction kinetics.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BAF	brahma associated factor
bp	base pair
Brg1	brahma related gene 1
Brm	brahma
CAF1	chromatin assembly factor 1
CHD	chromodomain
ChIP	chromatin immunoprecipitation
Chr	chromosome
COOH-terminal	carboxy terminal
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
E(Z)	enhancer of zeste
EED	embryonic ectoderm development
Gal	galactose
Glc	glucose
HAT	histone methyltransferase
HDAC	histone deacetylase
HIRA	histone cell cycle regulation defective homolog A
Hox	homeobox
ICM	inner cell mass
IP	immunoprecipitation
ISWI	imitation SWI
Kb	kilobase
MBD	methyl binding domain
MeCP2	methyl CpG binding protein-2
MLL	mixed lineage leukemia
MNase	micrococcal nuclease
NH_2-terminal	amino terminal
nm	nanometer
NURD	nucleosome remodeling and histone deacetylase
ORF	open reading frame
PAGE	polyacrilamide gel electrophoresis
PcG	polycomb group
PCNA	proliferating cell nuclear antigen
PGC	primordial germ cell
PIC	preinitiation complex
pmol	picomole
PRC	Polycomb repressive complex
PRE	polycomb response element

Raf	raffinose
RNA	ribonucleic acid
RNAP II	RNA Polymerase II
RNase	ribonuclease
RSC	remodels the structure of chromatin
RT-PCR	reverse transcriptase PCR
SAGA	
SANT	Swi3p, Ada2p, N-CoR and $TF_{III}B$
SDS	sodium dodecyl sulphate
SNF	sucrose nonfermenting
SUMO	small ubiquitin related modifier
SWI	mating type SWItching
TBP	TATA binding protein
TRE	trithorax response element
TrxG	trithorax group
UAS	upstream activating sequence
URS	upstream repressive sequence
WT	wild type
YEP	yeast extract peptone media
YEPD	YEP with 2% dextrose

CHAPTER I

INTRODUCTION

ROLE OF CHROMATIN STATES IN TRANSCRIPTIONAL MEMORY

Abstract

Establishment of cellular memory and its faithful propagation is critical for successful development of multicellular organisms. As pluripotent cells differentiate, choices in cell fate are inherited and maintained by their progeny throughout the lifetime of the organism. A major factor in this process is the epigenetic inheritance of specific transcriptional states or transcriptional memory. In this review, we discuss some of these chromatin transitions and mechanisms by which they are inherited by subsequent generations. We also discuss illuminating cases of cellular memory in budding yeast and evaluate whether transcriptional memory in yeast is epigenetically or cytoplasmically inherited.

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Introduction: Transcriptional choice and its inheritance

All organisms regulate their genetic repertoire in response to their environment as well as cell intrinsic cues. Single-celled organisms like yeast can coordinately induce and repress sets of genes as a result of stimuli like nutrient starvation, mating pheromones or DNA damage. In addition to responding to extracellular signals, multicellular organisms can also undergo cell differentiation. Cell differentiation is the culmination of numerous, highly regulated gene expression events that occur during embryonic development and throughout the life of an adult organism, where it controls growth, homeostasis and tissue repair. Some of these gene expression patterns or transcriptional choices become marked by epigenetic alterations of the genome, resulting in a transcriptional memory of gene expression profiles that are inherited by progeny.

Cell fate determination is an integral part of embryonic development in all multicellular organisms. A single-celled zygote undergoes many mitotic divisions till the blastocyst stage, where the inner cell mass (ICM) contains all the totipotent cells that will ultimately give rise to the embryo-proper. As the ICM cells further divide, they reorganize to form the three germ layers – ectoderm, mesoderm and endoderm, which are fated to form distinct tissues and organ systems. During these events, genes that encode pluripotency markers are transcriptionally repressed, and gene products characteristic of particular cell fates begin to be expressed. As these cells further divide and differentiate, such characteristic gene expression states get ‘locked in’ and are

faithfully replicated in all progeny of fate-restricted cells. This transcriptional memory can persist through multiple rounds of cell division, and in many cases throughout the lifetime of the organism even in the absence of the initiation signals. Such cellular memory posits the existence of one or more mechanisms that transmit information of active or silent gene state from mother to daughter cells. Mechanisms for providing cellular memory can be divided into two broad classes: cytoplasmic inheritance and nuclear inheritance (Fig. 1).

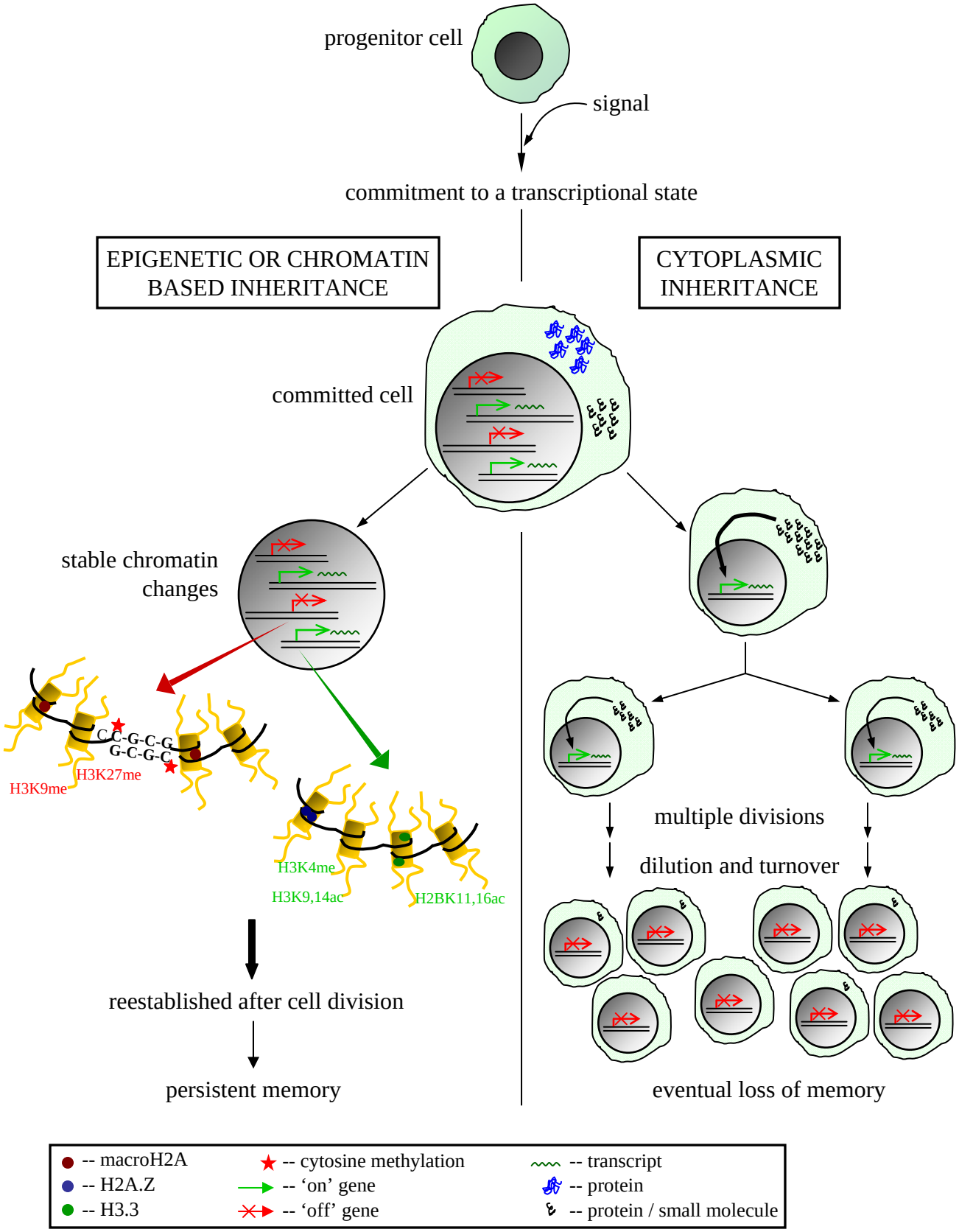
Cytoplasmic inheritance of transcriptional memory involves the presence of a protein or small molecule located in the cytoplasm of the cell that gains memory of a particular transcriptional event. During cell division, such a protein or small molecule signal can be passed on to daughter cells by distribution of the cytoplasm of the mother cell. It can be envisaged that such memory would be relatively short-lived – its duration or persistence is limited by dilution and/or half-life of the cytoplasmically inherited memory factor. In metazoan development, some key contributors to cytoplasmic inheritance via maternal ooplasm include mitochondrial DNA and well as maternally inherited miRNAs. Inheritance of maternal miRNAs is critical for early mouse embryonic development and has been shown to control gene expression profiles during early zygotic divisions (Tang et al. 2007). The cytoplasmic inheritance model will be revisited in a later section of this review.

The nuclear or epigenetic inheritance model of transcriptional memory involves changes in the chromatin state of target genes; changes that can persist through DNA replication and mitosis. These changes can be covalent marks on DNA and/or histones, and therefore would not alter the genomic information of pluripotent cells or their differentiated progeny.

The term "epigenetics" was coined by C.H. Waddington (1957) and 'epigenetic inheritance' classically refers to 'heritable changes in gene expression and phenotypes that does not involve alterations in the DNA sequence'. Over decades, this description has come to encompass many different phenomena such as chromatin modifications and transcriptional control by regulatory RNAs (Holliday 2006; Bird 2007; Goldberg et al. 2007; Ptashne 2007). Currently, there is an active debate on whether 'epigenetic inheritance' should include only phenomena such as DNA cytosine-methylation, where the mechanism of its transmission has been clearly established (Ptashne 2007), or whether it can refer to chromatin-based (such as histone modifications, higher order chromatin folding) or non-chromatin-based (such as RNA, prions) gene regulatory processes as well when the mechanism of inheritance is not yet discovered (Bird 2007; Goldberg et al. 2007; Patel et al. 2009). However, there is growing consensus that changes in chromatin structure that can instruct or perpetuate a pattern of gene expression in the absence of the primary causative signal, can be termed 'epigenetic'.

Where mechanisms for faithful replication of these chromatin marks have evolved, nuclear or epigenetic memory can persist through many generations and indeed throughout life. The substrate for these long-term memory marks is the chromatin and chromatin structure participates directly in transcriptional activation or repression of gene loci. Below we review some chromatin basics, and then we discuss several examples of chromatin based mechanisms for transcriptional memory.

Figure 1. Mechanisms for nuclear and cytoplasmic inheritance of transcriptional memory. A multipotent progenitor cell can respond to a particular signal(s) by altering its transcriptional profile. This step can lead to cell-fate commitment. Memory of adopted cell fate can be transmitted by various epigenetic or other chromatin based mechanism or by cytoplasmic signals. Changes in chromatin state can involve DNA-cytosine methylation, histone modifications and/or histone variants. Cytoplasmic inheritance could involve a signal-induced peptide, RNA or small molecule that maintains target genes in ON or OFF state. Persistence of cellular memory in each case depends on faithful transmission of the 'memory mark' to subsequent generations. See text for details.


progenitor cell
signal
commitment to a transcriptional state
EPIGENETIC OR CHROMATIN BASED INHERITANCE
CYTOPLASMIC INHERITANCE
committed cell
stable chromatin changes
C-C-G-C-G
G-C-G-C
H3K9me
H3K27me
H3K4me
H3K9,14ac
H2BK11,16ac
multiple divisions
dilution and turnover
reestablished after cell division
persistent memory
eventual loss of memory
-- macroH2A
-- H2A.Z
-- H3.3
-- cytosine methylation
-- 'on' gene
-- 'off' gene
-- transcript
-- protein
-- protein / small molecule

Chromatin as a regulator of transcription

The large genome of eukaryotes is packaged into chromatin, a DNA and protein containing complex structure. This structure facilitates compaction of the genome, thereby fitting it into the small volume of a nucleus. Importantly, and in ways still being avidly studied and discovered, it is a critical component of gene regulatory activities. The basic component of chromatin is a nucleosome, generated by wrapping approximately 147bp of DNA ~1.7 times around an octamer of core histone proteins. The canonical histone octamer contains 2 copies each of histones H2A, H2B, H3 and H4. Structurally, these histones consist of a central globular or 'histone-fold' domain and flexible NH_2-terminal and COOH-terminal tail domains. As DNA wraps around the histone octamer, it makes intimate contacts at 14 positions (Luger et al. 1997; Davey et al. 2002; Suto et al. 2003). These tight associations occlude potential transcription factor binding sites and leads to steric hindrance to DNA-binding by transcriptional activators, repressors and the core transcriptional machinery (Hansen and Wolffe 1994). This protection of DNA is also evidenced biochemically by restricted access to DNA cleavage agents like micrococcal nuclease (MNase) and restriction enzymes.

Beyond this primary level of nucleosomal or 'beads-on-a-string' structure are successive higher orders of chromatin folding that ultimately compact DNA into chromatin fibers, with one of the highest degrees of compaction seen in the classical 'metaphase chromosome'. The next clearly distinguishable state of folded chromatin that is more compacted than 'beads-on-a-string' nucleosomes, is the 30nm fiber. It

appears unclear still if the in vitro idealized solenoid or zigzag structure of 30nm fiber is actually found in vivo (Hansen 2002; Horowitz-Scherer and Woodcock 2006). It has been proposed that budding yeast interphase chromatin, which is mostly transcriptionally active is equivalent to a 30 nm fiber (Bystricky et al. 2004). On the other hand, folding of the 30nm fiber upon itself has been proposed to form heterochromatin, with the aid of fiber crosslinking proteins such as linker histones and MeCP2 (Grigoryev et al. 2004; McBryant et al. 2006).

In addition to core histones, metazoans also have a set of unrelated histone called the linker histones (like H1). Mammals appear to have at least eight H1 histone variants five of which are expressed ubiquitously in somatic tissue and some studies indicate that deletion of individual variants can cause distinct phenotypes (Alami et al. 2003; Izzo et al. 2008; Sancho et al. 2008). Like core histones, linker histones are also highly basic in amino acid composition. There is on average 1 linker histone per nucleosome, which protects an additional ~20bp of DNA (Parseghian and Hamkalo 2001; Zlatanova et al. 2008). MNase digestion of H1 containing chromatin generates a ~168bp footprint and this particle has been termed the chromatosome (Simpson 1978). H1 binds to DNA at its entry and exit points in the nucleosome and is important for stabilization of higher order chromatin folding (Hamiche et al. 1996; Zlatanova et al. 2000; Georgel et al. 2003; Woodcock et al. 2006). Like their core histone counterparts, linker histones are also composed of two functional domains – the globular and tail domains. While the globular domain interacts with nucleosomes, the NH_2-terminal and

COOH-terminal tails of linker histone are also sites of post translational modifications, and C-terminal tail is required for stabilizing higher order chromatin folding (Draves et al. 1992; Thomas et al. 1992; Talasz et al. 1996; Ramakrishnan 1997; Vermaak et al. 1998; Dou et al. 1999; Bharath et al. 2002; Garcia et al. 2004; Lu and Hansen 2004; Hale et al. 2006; Wisniewski et al. 2007; Villar-Garea and Imhof 2008). However, whereas removal of H1 has no effect on viability of unicellular organisms like *Tetrahymena*, *Aspergillus nidulans* and *Saccharomyces cerevisiae*, linker histones are essential for cellular differentiation in higher eukaryotes (Shen et al. 1995; Steinbach et al. 1997; Ushinsky et al. 1997; Patterton et al. 1998; Vermaak et al. 1998; Ramon et al. 2000; Ner et al. 2001; Fan et al. 2005). *Xenopus*, mouse, human and others also show cell-type specific variants of histone H1 that control transcription regulation of specific genes and nucleosome spacing (Bouvet et al. 1994; Kandolf 1994; Patterton and Wolffe 1996; Fan et al. 2003; Izzo et al. 2008). There is evidence that H1 subtype switching occurs in *Xenopus* and mammalian embryos during development (Clarke et al. 1998). This suggests a mechanism that may provide memory of cell fate specification during embryonic development.

How can chromatin structure be modified to regulate access to transcriptional regulators and the core transcriptional machinery? Cells employ two general enzymatic strategies that regulate chromatin dynamics. In the first case, ATP-dependent chromatin remodeling enzymes use energy derived from ATP hydrolysis to mobilize nucleosomes, evict some or all of the histones from the nucleosome or to exchange histone variants.

These enzymes all contain ATPases of the Swi2/Snf2 superfamily and are broadly classified into the SWI/SNF-, the ISWI-, the CHD- and the Ino80-subfamilies (Smith and Peterson 2005; Hogan and Varga-Weisz 2007). Some like the SWI/SNF complex are mostly transcriptional regulators; in yeast there are two SWI/SNF family members – RSC and SWI/SNF complexes (Laurent et al. 1992; Peterson and Herskowitz 1992; Kwon et al. 1994; Peterson et al. 1994; Cairns et al. 1996). Members of the SWI/SNF family are primarily involved in transcriptional activation. For instance, the yeast SWI/SNF complex is recruited during activation of many genes during late mitosis as well as some highly inducible metabolic genes like *INO1*, *SUC2*, *GAL1-10*, *PHO5* and *PHO8* (Pollard and Peterson 1997; Krebs et al. 2000; Dhasarathy and Kladde 2005; Adkins and Tyler 2006; Ford et al. 2008). Complex eukaryotes have several different SWI/SNF (also called BAF complexes), with the catalytic subunit being Brg1 or Brm, and a variety of associated subunits (Khavari et al. 1993; Wang et al. 1996; Kadam et al. 2000). Brg1 is essential for mammalian development, as mice harboring a *brg1* deletion show periimplantation lethality (Bultman et al. 2000). Some of the associated subunits are also tissue restricted. For instance BAF60c is restricted to the myocardial lineage, while BAF53b is neuron-specific (Olave et al. 2002; Lickert et al. 2004). Other ATP-dependent remodeling enzymes, like ISWI and Mi-2, usually function in transcriptional repression. Mi-2 complexes, a prominent member of the CHD (chromodomain containing) subfamily play critical roles at different stages of hematopoiesis, and ISWI complexes are critical for normal development and differentiation as evidenced by periimplantation lethality of mice lacking the catalytic

subunit, Snf2h (Stopka and Skoultchi 2003; Williams et al. 2004). The Ino80 subfamily, which includes INO80 and SWR1 complexes are characterized by a split ATPase domain, and the SWR1 complex is required for exchange of the histone variant H2A.Z into nucleosomes.

The second category of chromatin remodeling enzymes is those that mediate posttranslational modifications of histones. Nucleosomal histones can be extensively modified at their N- or C-terminal tail domains, or even at some internal sites. Histone modifications function primarily by influencing the binding of non-histone proteins, like transcription factors and other chromatin remodeling enzymes, to nucleosomes, although some marks (e.g. H4K16ac) directly impact chromatin structure. A wide variety of enzymes have been identified in all organisms that catalyze diverse modifications such as methylation, acetylation, phosphorylation, ubiquitilation and SUMOylation (Khorasanizadeh 2004). Histone modifications are reversible since there are also demethylases and deacetylases dedicated to removal of these groups. Though isolated modifications probably do not significantly affect DNA-histone or nucleosome-transcription factor contacts, they often are found in combinations and act synergistically to recruit or occlude chromatin associated proteins and generate transcriptionally favorable or unfavorable chromatin domains (Ruthenburg et al. 2007). Histone modifying enzymes and ATP-dependent remodelers often act in concert for regulating gene expression.

Finally, the most stable and replicable of chromatin modifications are those on the DNA itself, i.e. cytosine methylation of CpG islands by DNA methyltransferases. Though yeast and *C.elegans* lack DNA methylation, it is critical in other metazoans for development and differentiation (Rountree et al. 2001). In addition there are RNA based chromatin regulatory processes exemplified by X-inactivation, heterochromatin formation and position-effect variegation, which have been discussed in detail elsewhere ((Grewal and Elgin 2007; Riddle and Elgin 2008; White and Allshire 2008) and others).

Strategies to establish transcriptional memory by modifying chromatin state

Establishing cellular memory of a particular transcriptional state is essential in multicellular organisms to get fruitful cell fate specification during development. As fate-restricted cells multiply during organogenesis, various mechanisms have evolved that replicate the transcriptional state of the progenitor cell in its daughters. Therefore genes that confer for example pluripotency or alternate cell fates are stably turned off, while genes characteristic of the chosen cell-fate are maintained in a transcriptionally active or poised state. As a consequence, despite having identical genomes, how cells of different tissues respond to signals that they encounter is determined by their inherited 'lineage identity'. To maintain characteristic identity and function of different tissues during the lifetime of an organism, cells of individual tissues must maintain and propagate their distinct transcriptional profiles or in other words, have transcriptional memory of their gene expression profile. Since chromatin remodeling is often required

to silence or activate genes, transcriptional memory would involve propagation of differential chromatin states in different tissues. Such transcriptional memory can be established and inherited by using various chromatin modifying strategies introduced above. We will discuss some specific enzymatic activities and their roles below.

DNA methylation

DNA methylation, predominantly at symmetrical CpG dinucleotides, is usually associated with gene silencing (Bird and Wolffe 1999; Klose and Bird 2006). Genomic methylation patterns are very stable and heritable in somatic differentiated cells. Once established, they are faithfully replicated at every cell division by the 'methylation maintenance' enzyme DNMT1, which uses hemimethylated DNA substrate to restore symmetrical CpG methylation pattern (Bestor 2000; Pradhan and Esteve 2003). There are two developmental stages – germ cells and preimplantation embryos – where the genomewide methylation pattern is reprogrammed. The fertilized egg undergoes a wave of demethylation during preimplantation development, which erases part of the inherited, parental methylation pattern. After implantation, the embryo then undergoes de novo methylation to establish a new embryonic methylation pattern. DNMT3A/B are the primary de novo DNA methylases that establish new methylation patterns and are expressed in most dividing cell types along with DNMT1 (Li et al. 1992; Okano et al. 1999; Goll and Bestor 2005). Another protein, DNMT3L, is similar to DNMT3A/B in amino acid sequence but lacks enzymatic activity. It is expressed only in germ cells during de novo methylation and is believed to regulate DNMT3A/B (Bourc'his et al.

2001; Suetake et al. 2004). There is also an oocyte-specific form of DNMT1 (DNMT1o) that accumulates to very high levels in the cytoplasm of oocytes and persists in preimplantation embryos. It enters nuclei at the eight-cell stage to maintain imprinted methylation patterns (Howell et al. 2001).

Genomic methylation patterns are largely erased during the proliferation and migration of primordial germ cells (PGCs) and reestablished in sex-specific patterns during gametogenesis (Trasler 2006; Schaefer et al. 2007). These demethylation and de-novo methylation events are critical for generating totipotent cells with broad developmental potential and for establishment of parental-specific methylation marks at imprinted genes (Chaillet et al. 1991; Stoger et al. 1993; Tremblay et al. 1995). During gametogenesis, imprinted genes are epigenetically marked such that they are expressed exclusively from maternal or paternal alleles of the progeny. De novo methylation by DNMT3A and DNMT3B is essential for this pattern of parental imprinting (Okano et al. 1999; Kaneda et al. 2004). Reprogramming is also required for normal development of cloned animals and to generate stem cells as well as for appropriate stem cell differentiation (Reik et al. 2001; Farthing et al. 2008). In mouse PGCs, reprogramming during development coincides with the re-expression of some pluripotency genes, including *Sox2* and *Nanog* (Yamaguchi et al. 2005). However it is not known if demethylation is a requisite for re-expression of pluripotency genes. Overall, DNA methylation has key roles in epigenetic gene regulation and silencing, in particular in

genomic imprinting, X chromosome inactivation, and silencing of retrotransposons (Bird 2002; Li 2002; Reik 2007).

In dividing cells, the maintenance methyltransferase, DNMT1 provides epigenetic memory of transcriptionally silenced loci. This methylation pattern has also been proposed to inhibit transposition as well as recombination and expansion of repetitive elements (Bird 1995; Yoder et al. 1997; Walsh et al. 1998). Maintenance of imprinted silencing by DNMT1 is linked to DNA replication by its association with PCNA and CAF1 (Leonhardt et al. 1992; Chuang et al. 1997; Bestor 2000; Rountree et al. 2000; Sarraf and Stancheva 2004). This association provides the mechanism for propagation of epigenetically silent states but not transcriptionally active states through S-phase to subsequent generations. Experiments suggest that methylation by itself does not prevent transcription (Keshet et al. 1986; Buschhausen et al. 1987; Kass et al. 1997), but instead transcriptional silencing of methylated loci is due to methyl-CpG binding domain proteins (MBDs), that alter chromatin structure. The primary MBDs are MBD1, MBD2, MBD3 and MeCP2 (Hendrich and Bird 1998; Ng et al. 1999; Rountree et al. 2001). The MBD proteins interact with and recruit histone deacetylases (HDACs) such as HDAC1, HDAC2, Mi-2/NURD which deacetylate the associated chromatin to render it transcriptionally incompetent (Nan et al. 1998; Wade et al. 1999; Zhang et al. 1999).

Histone modifications

The NH_2- and COOH-terminal tails of core histones are subject to extensive and dynamic posttranslational covalent modifications. These modifications alter the charge on histone tail residues, for example, lysine methylation or acetylations neutralize its positive charge. This can alter the interaction of modified histones with neighboring nucleosomes or change accessibility of chromatin to transcription factors and other chromatin remodeling enzymes. Prominent among marks associated with the transcriptionally active state are H3K4me, H2BK123Ub and multiple acetylations of H2B-, H3- and H4-lysines (H2BK11,16ac; H3K9,14,18,23,27ac; H4K5,8,12,16ac) (Roth et al. 2001). Some or all of these changes can change chromatin structure leading to a more permissive environment for transcription. Similarly, H3K9me and H3K27me are some of the more common marks associated with repressed loci (Zhang 2003; Martin and Zhang 2007). Different enzymes catalyze these histone modifications and can be conserved across species. Histone modifying enzymes are also important for controlling expression of many developmentally regulated genes. At the same time, histone demethylase and deacetylase enzymes have also been discovered that reverse these marks (Shi et al. 2004; Bradbury et al. 2005; Lee et al. 2005; Shi et al. 2005; Tsukada et al. 2006; Agger et al. 2008). As expected, at most loci in dividing or differentiated cells, certain histone modifications can co-exist or act together to reinforce open or accessible chromatin configuration and vice versa. This observation has led to speculation that particular patterns of histone modifications might predict transcriptional status of genes in different cell lineages (Dover et al. 2002; Ng et al.

2002b; Lewis et al. 2004; Margueron et al. 2005; Nightingale et al. 2006). Interestingly, adult and embryonic stem cells have been shown to possess domains of 'bivalent' chromatin, where positive and negative histone modifications coexist at certain gene loci, which may poise these genes for appropriate regulation when the stem cells differentiate (Bernstein et al. 2006; Attema et al. 2007). Such chromatin domains may be essential for pluripotency and are resolved into active or repressed state during lineage specification.

A key question is the precise mechanism by which histone modifications are transmitted through cell divisions. Unless existing marks are replicated, transcriptional memory cannot be propagated. Many studies have shown that as a replication fork proceeds along the DNA, nucleosomes are disassembled but they are rapidly and efficiently reassembled on the newly replicated strands. As DNA is replicated, old $(H3\text{-}H4)_2$ tetramers are distributed on the daughter strands onto which newly synthesized or old H2A-H2B dimers are added (Jackson 1988; Yamasu and Senshu 1990; Yamasu and Senshu 1993; Henikoff et al. 2004; Groth et al. 2007). Simultaneously, newly synthesized $(H3\text{-}H4)_2$ tetramers are also deposited to fill in the gaps between the old tetramers and subsequently, H2A-H2B dimers are added to these tetramers too. Thus it appears that while hybrid octamers of old tetramers and new dimers can form, $(H3\text{-}H4)_2$ tetramers remain essentially intact through DNA replication and chromatin assembly. Since the redistributed old tetramers contain histone modifications representing active or repressed chromatin, they can potentially recruit enzymatic activities that would

interpret the existing marks and replicate them on the newly formed octamers. Interestingly, there appear to be potential candidates that can 'read' and 'write' H3K4methyl marks on chromatin (Wysocka et al. 2005; Ruthenburg et al. 2007). Another interesting candidate is the bromodomain protein, Brd2, which can bind to H4K14ac and promote transcription. It has been suggested that binding of Brd2 to acetylated chromatin can persist during mitosis, thus providing memory of transcriptional activity across cell divisions (Kanno et al. 2004).

Another intruiging possibility is the semi-conservative model of chromatin assembly (Tagami et al. 2004; Nakatani et al. 2006). According to this model, a tetramer of H3/H4 dimers can be split equally between the two strands of newly replicated DNA and these older H3/H4 dimer can then be used as a template to replicate modifications on the newly assembled nucleosomes. Other alternative models to propagate histone modification patterns have also been proposed and may be evidenced in replication of H3K9me mark by Swi6 (Jackson and Chalkley 1985; Hall et al. 2002).

One of the classic examples of transcriptional memory involves the developmental regulation of expression of several hundred genes in *Drosophila* and other metazoans, including homeotic (*Hox)* genes, by the interplay of two antagonistic sets of gene products that regulate chromatin structure – the Polycomb group (PcG) and the Trithorax group (TrxG). *Hox* genes encode homeodomain transcription factors that

specify cell fates and therefore their transcription must be precisely regulated, since misexpression can lead to severe developmental abnormalities such as formation of appendages at wrong positions. In fact, genes encoding Polycomb proteins were first discovered in *Drosophila* as mutations that led to transformation of body-segmentation patterns along the embryonic anterior-posterior axis, thus transforming the identity of one segment to another.

The initial patterns of homeotic gene transcription are established in response to positional information in the early embryo. During *Drosophila* embryogenesis, the function of PcG and TrxG proteins is maintenance, but not initiation, of homeotic gene expression, even after the regulators that established the precise segmentation patterns have long disappeared. Polycomb and Trithorax proteins repress and activate genes respectively, and together they maintain these gene expression patterns through subsequent cell divisions and thus establish memory of cell fate (Grimaud et al. 2006; Schuettengruber et al. 2007). These complexes bind to and act via Polycomb and Trithorax response elements (PREs/TREs) and control gene expression (Ringrose and Paro 2004; Beisel et al. 2007; Ringrose and Paro 2007). Some PcG and TrxG members possess histone methyltransferase activity. PRC2 complex contains E(Z) (EZH2 in mammals), which is an H3K27 methyltransferase that mediates silencing of *HOX* and other loci. PcG can bind to both H3K9me and H3K27me but have a much stronger preference for the latter. TrxG members TRX (MLL is the mammalian ortholog) and ASH1, in contrast, are H3K4 methyltransferases and are associated with activated gene

expression. TRX protein contains a SET domain, which is very similar to the *S.cerevisiae* Set1p, the first identified H3K4-methyltransferase. Differential H3 lysine methylation status of promoter and coding regions can thus confer a transcriptional ON or OFF status such as at the *Drosophila Ubx* gene (Papp and Muller 2006). In addition to the immediate and local effect, these two complexes have important functions for propagation of the transcriptional state they establish. Once recruited, PcG and TrxG complexes regulate cellular pluripotency and differentiation by maintaining silent or open chromatin states that can be inherited through multiple cell divisions even after decay of the primary silencing or activating signal (Dejardin and Cavalli 2004; Srinivasan et al. 2008). A recent study proposes an elegant mechanism to maintain the PRC2 mediated H3K27me3 mark during DNA replication and thereby propagate transcriptional memory to subsequent cell generations (Hansen et al. 2008). Helin and colleagues show that once EZH2, EED and SUZ12 containing PRC2 complex catalyzes methylation of H3K27 at a particular chromatin locus, the complex itself is recruited to this target locus by binding the H3K27me3 modification. This PRC2 recruitment persists through subsequent rounds of DNA replication and cell division leading to methylation of newly incorporated histones at this locus, thus maintaining the chromatin mark and preserving transcriptional repression in proliferating cells.

Biochemical studies reveal that both the PcG and TrxG complexes are large and have many categories with different functions, all aimed at long term control of chromatin configuration at homeotic and imprinted genes (Shao et al. 1999; Francis

et al. 2004; Terranova et al. 2008). Polycomb group proteins are characterized by two large, multisubunit complexes – PRC1 and PRC2. The PRC2 complex is recruited to PREs where it initiates silencing, whereas the PRC1 complex plays a key role in the maintenance of silencing (Lund and van Lohuizen 2004). PRC2 complex member, E(Z), is the H3K27 methyltransferase. H3K27me is required for targeting to the PREs, while specificity of targeting is modulated by other subunits like EED, Esc and Su(z)12. H3K27me is recognized by the chromodomain of the Polycomb (Pc) subunit of PRC1, a mechanism that is reminiscent of other complexes that use similar mechanisms for targeting to specific gene loci (Levine et al. 2004; Kohler and Villar 2008). PRC2 can also interact with the HDAC, *RPD3*, possibly in a developmentally restricted manner (Tie et al. 2001). Thus one can speculate that local histone deacetylation by RPD3 in the vicinity of PRC2 target sites could reinforce transcriptional repression at these loci. Coupled to this, binding of PRC1 via H3K27me can generate a stably repressive chromatin structure that is refractory to gene expression. In support of this, there is evidence that PRC1 inhibits remodeling by SWI/SNF and transcription by RNA Polymerase II in vitro (King et al. 2002; Otte and Kwaks 2003). Also, PRC1 interacts with TAFs and the transcriptional machinery, suggesting that PRC1 can interact with promoters and providing a direct link to transcriptional control (Breiling et al. 2001; Saurin et al. 2001). Though there are many studies determining the functions and interacting partners of PRC1 and PRC2 complexes, it is still unclear how PRC2 is first recruited to PRE-containing target loci to initiate silencing. However, another group of polycomb proteins like pleiohomeotic (Pho) can bind to certain sequences contained in

PREs with sequence-specificity, and may have a role in PRC2 targeting (Pirrotta et al. 2003; Ringrose et al. 2003; Wang et al. 2004).

Mutations in genes encoding TrxG proteins were isolated as suppressors of polycomb mutants, indicating that TrxG proteins functionally antagonize PcG proteins (Kennison and Tamkun 1988; Daubresse et al. 1999; Levine et al. 2002). One category of TrxG members includes Trx and Ash1, which are SET-domain proteins that catalyze H3K4 methylation (Petruk et al. 2001; Beisel et al. 2002; Byrd and Shearn 2003; Schwartz and Pirrotta 2007). The founding member of the SET-domain proteins is the budding yeast Set1p, which also trimethylates H3K4 for transcriptional activation, showing that these functions have been conserved through evolution. Trx and Ash1 can also be recruited to *Hox* genes but the mechanism for that is still unclear. Ash1 is believed to prevent H3K27 trimethylation by PRC2 at the *Ubx* gene by binding immediately downstream of the promoter, thus keeping the locus transcriptionally active (Papp and Muller 2006). Binding of Trx and Ash1 is also believed to promote transcriptional elongation and this may be stimulated in past by the H3K4me mark. Another group of TrxG members includes subunits of the *Drosophila* SWI/SNF chromatin remodeling complex, like Brahma (BRM), Moira (MOR) and Osa (OSA) (Kennison and Tamkun 1988; Papoulas et al. 1998; Collins et al. 1999; Crosby et al. 1999). Like the SET-domain TrxG members, some of these factors are also conserved across many species, for example, the yeast homolog of BRM is the Swi2/Snf2p ATPase, which forms the catalytic subunit of yeast SWI/SNF. The Kismet (KIS) TrxG

member also encodes an hCHD7 related ATP-dependent remodeling enzyme. Thus TrxG employs both histone methylation and ATP-dependent chromatin remodeling to maintain a heritable transcriptionally ON state. Due to the critical role of PcG and TrxG proteins in maintaining transcriptional memory of developmentally regulates genes, these complexes are extensively studied to determine mechanisms of cell fate choice, stem cell pluripotency as well as cancers.

Histone variants

The canonical histones that make up a core nucleosome particle – H2A, H2B, H3 and H4, are expressed and incorporated into chromatin only during DNA replication. In addition, organisms express variants of canonical histones H2A (H2A.Z, macroH2A, H2A.X and H2ABbd) and H3 (H3.1, H3.2, H3.3, H3.1t and Cenp-A/Cse4) and each of these is thought to have specific properties and function and can establish structurally distinct chromosomal domains in the genome (Wu and Bonner 1981; Malik and Henikoff 2003; Chakravarthy et al. 2004; Tagami et al. 2004; Chakravarthy and Luger 2006; Park and Luger 2008). Unlike their canonical counterparts, nucleosome incorporation of most histone variants is not S-phase restricted but occurs through replication independent mechanisms. Emerging evidence indicates that correct distribution of some histone variants is important for the effective control of gene expression and cell fate decisions. Though the precise mechanisms of targeted deposition are still being worked out, distinct and often dedicated multiprotein

complexes have been discovered that target histone variants for deposition at active genes, centromeres and silent loci. For example, the ATP-dependent chromatin remodeling complex SWR1 is targeted to specific genomic sites like 5′ ends of genes and exchanges canonical H2A-H2B dimers for H2A.Z-H2B at these loci. The histone chaperone, HIRA binds specifically to H3.3–H4 dimers and deposits them on transcriptionally active chromatin (Ray-Gallet et al. 2002; Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004; Tagami et al. 2004).

What are the functions of these histone variants? In yeast, H2A.Z is found within 1-2 nucleosomes that flank all RNA Polymerase II transcribed genes that are both active and inactive. In addition it also prevents ectopic spreading of heterochromatin (Stargell et al. 1993; Meneghini et al. 2003). Thus H2A.Z seems to be important to maintain an open state of chromatin. This premise is supported by the observation that nucleosomes that harbor H2A.Z-H2B dimers are less stable (Abbott et al. 2001; Zhang et al. 2005; Jin and Felsenfeld 2007). H2A.Z also promotes formation of 30nm fibers (Fan et al. 2002). Surprisingly, loss of yeast *HTZ1* has a very mild phenotype in nutrient-rich steady state growth conditions, indicating that transcription of most genes is unperturbed even upon loss of H2A.Z. In contrast, the importance of H2A.Z for regulating chromatin configuration is clearly seen during mammalian embryonic development. Loss of H2A.Z results in preimplantation lethality in mice (Faast et al. 2001; Fan et al. 2002). There is also an active genomewide displacement of H2A.Z from early mouse PGCs that correlates with the timing of genomewide DNA

demethylation, suggesting a role of H2A.Z loss in chromatin decondensation and reprogramming (Hajkova et al. 2008).

A more recently discovered H2A variant, H2A.Bbd (*B*arr *b*ody-*d*eficient) appears to be exclusively associated with transcriptionally active chromatin and was shown to be excluded from inactive X chromosomes in mammalian females (Chadwick and Willard 2001). It is significantly diverged from canonical H2A in amino acid sequence and structure, and its distribution overlaps with regions of histone H4 acetylation, suggesting that this histone variant has evolved to perform a specialized function of stably marking active chromatin, possibly through cell divisions (Bao et al. 2004; Gautier et al. 2004; Gonzalez-Romero et al. 2008). In contrast to the distribution of H2A.Bbd, the H2A variant, macroH2A is enriched on the silenced X chromosome and marks inactive chromatin (Costanzi and Pehrson 1998; Chadwick and Willard 2001). Its enrichment on the inactive X chromosome coincides with *Xist* RNA spreading and therefore with initiation of silencing.

Similar to H2A.Z, H3.3 may also be also involved in maintenance of open, transcriptionally active chromatin. H3.3 differs from canonical H3 at only 4 amino acid positions. However, unlike H3, H3.3 gene is outside the histone gene cluster and synthesized independent of S phase. Thus, unlike the canonical H3, H3.3 deposition is replication-independent and transcription-dependent (Smith 2002; Malik and Henikoff

2003). As a result, H3.3 is also enriched in 'activating' posttranslational modifications such as H3K4me and H3K9,14,18,23ac, whereas canonical H3 preferentially accumulates repressive modifications like H3K9me (McKittrick et al. 2004; Chow et al. 2005; Schwartz and Ahmad 2005; Wirbelauer et al. 2005; Daury et al. 2006).

H3.3 deposition plays critical roles during embryonic development. It replaces canonical H3 during meiotic X chromosome inactivation in mouse germline (van der Heijden et al. 2007). This could provide persistent memory of maternally expressed and paternally imprinted genes at a stage where DNA methylation is reversed. Also in early mouse zygotes H3.3 is incorporated only into paternal chromatin coinciding with its decondensation, soon after gamete fusion (van der Heijden et al. 2005). Consequently, a null mutation of the H3.3 chaperone, HIRA has gastrulation defects in mouse embryos and is early embryonic lethal (Roberts et al. 2002). Also in accordance with the role of H3.3 in decondensation of paternal chromatin, mutations of *Drosophila* HIRA lead to formation of haploid embryos with only maternal chromosomes, which die before hatching (Loppin et al. 2005). An interesting example of the involvement of H3.3 in transcriptional memory comes from studies on *MyoD* gene expression in *Xenopus* embryos (Ng and Gurdon 2008). The authors demonstrate that memory of *MyoD* transcription persists through 24 cell divisions in non-muscle cell lineages of nuclear transplant embryos and this duration coincides with H3.3 occupancy at the MyoD promoter.

It is very tempting to envisage a scenario where histone variants could mark different chromatin domains – euchromatin, facultative heterochromatin and constitutive heterochromatin. As pluripotent cells adopt cell fates during differentiation, inheritance of such chromatin domains via the 'histone variant signature' could faithfully reproduce the transcription profile of a differentiated cell type in its successive progeny. However, some questions still remain. Besides the yet unsolved question of how these histone variants (and their chaperones) are targeted to mark loci, it is also still unclear how information of histone variant occupancy is replicated during S-phase. This knowledge is crucial to understand the mechanism of inheriting memory of transcriptional state. As is the case with inheriting histone modifications, the two popular models of replicating histone variant occupancy are the conservative and semiconservative model of histone deposition during DNA replication (Annunziato 2005; Hake and Allis 2006). Since, extensive labeling and density sedimentation studies do not favor the semiconservative model, another intriguing possibility that could at least be relevant to inheritance of transcriptionally active states and therefore pertinent to H2A.Z and H3.3 is a proposed scenario involving both replication-coupled (RC) and replication-independent (RI) nucleosome assembly (Henikoff et al. 2004). As the replication fork crosses a transcriptionally active locus, there can be RC deposition of canonical H3 containing tetramers. But since both old and new tetramers would be randomly distributed over this locus, the old H3.3 containing tetramers would also be redeposited albeit at lower frequency. However, this lower density of H3.3 containing nucleosomes could still be enough to promote open chromatin and continue

transcription from the locus after replication. Once transcription resumes, there would be RI deposition of H3.3 again at the locus and thus active chromatin would be reconstituted after each round of DNA replication.

RNA-based silencing

Many trans-acting, small non-coding RNAs, including miRNAs and piRNAs that regulate developmental and other gene expression are themselves parentally imprinted (discussed in (Royo and Cavaille 2008)). However, cis-acting non-coding RNAs are implicated in many instances of long term chromatin silencing. The first wave of imprinted X-chromosome inactivation in preimplantation mouse embryos is mediated by the *Xist* RNA. Once transcribed, *Xist* RNA spreads from its origin in *cis* to coat the X-chromosome and recruits other silencing factors (Wutz and Jaenisch 2000; Chaumeil et al. 2006). Not only does *Xist* form a chromosomal memory of X inactivation during differentiation, *Xist* coating recruits further repressive chromatin changes -- histone variant macroH2A, DNA methylation and PcG recruitment to reinforce silencing (Kohlmaier et al. 2004; Masui and Heard 2006). These chromatin changes allow the inactivated X-chromosome to be stably silenced at later stages of development even in the absence of *Xist*.

RNA interference (RNAi) mediated heterochromatin formation has also emerged as a robust means of establishing heritable chromatin states. RNAi regulates heterochromatin formation and spreading at the pericentric *dg* and *dh* repeats in

Schizosaccharomyces pombe (Volpe et al. 2002; Irvine et al. 2006). The process initiates when either of these repeats in transcribed. This is fed into the RNAi pathway, eventually generating siRNAs that get incorporated into the RITS (RNA-induced transcriptional silencing) complex. RITS activity recruits the H3K9 methyltransferase, Clr4 and eventually the heterochromatin protein Swi6 binds to H3K9me to form silenced heterochromatin at centromeres. Inheritence of centromeric silencing through successive generations appears to require a mechanism connecting RNAi and early replication of centromeric repeats (Chen et al. 2008; Kloc et al. 2008).

Cellular memory in budding yeast

Unlike multicellular organisms, unicellular budding yeasts like *Saccharomyces cerevisiae* and *Candida albicans* demonstrate no significant differentiation or functional asymmetry between 'progenitor' mother cells and their progeny. Nevertheless, these cells have complex genetic networks that lend themselves to considerable robustness and sensitivity when a growing population of yeast encounter and respond to environmental changes. Can activation of such transcriptional networks lead to a heritable memory of adaptive response in yeast cells too? There appear to be at least two clear situations where in fact cellular memory is formed. One is the frequency of 'white-opaque' cell type switching in *Candida albicans* (Srikantha et al. 2006; Zordan et al. 2006; Zordan et al. 2007). Another is the rapid reactivation of galactose induced transcription of the *GAL* gene cluster (*GAL1*, *GAL10* and *GAL7*) following a period of

transcriptional repression by glucose (Brickner et al. 2007; Kundu et al. 2007; Zacharioudakis et al. 2007). Interestingly, in both these examples memory of the transcriptional response persists for at least a few generations. What is the mechanism by which transcriptional memory in yeast is inherited by successive generations? Yeast lack DNA methylation and therefore this mode of epigenetic inheritance is absent. For both of the above examples of transcriptional memory, regulators of chromatin structure have been suggested. Studies on white-opaque switching of *C.albicans* indicate that the histone deacetylases *HDAC2* and *RPD3* are involved since deletion of these genes affects switching frequency (Klar et al. 2001; Srikantha et al. 2001). Yet there is still no clear mechanism of how the mark of histone deacetylation is inherited by subsequent generations. For transcriptional memory of *GAL1* induction, chromatin transitions as well as cytoplasmic signaling networks have been proposed and these will be evaluated further.

The yeast *GAL* system and models of transcriptional memory

The *GAL* genes in budding yeast encode enzymes of the Leloir pathway which is activated by galactose and expresses proteins to internalize and metabolize this sugar. *GAL* genes can be broadly separated into two groups – the structural genes (*GAL1*, *GAL5*, *GAL7*, *GAL10*), that encode enzymes to metabolize galactose; and regulatory genes (*GAL2*, *GAL3*, *GAL4*, *GAL80*) that transport galactose and control expression of the structural genes. Expression of Gal1p (galactokinase), Gal7p (galactose-1-phosphate uridyl transferase) and Gal10p (uridine diphosphoglucose epimerase) enzymes is tightly

regulated in the presence of different sugars and they are induced 1000-fold in the presence of galactose (Johnston et al. 1994; Lohr et al. 1995; Bhat and Murthy 2001).

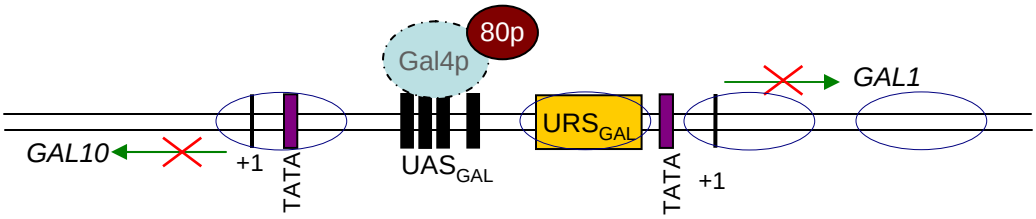
All *GAL* genes are activated by Gal4p, which binds to its target sequences upstream of these genes. *GAL1* and *GAL10* genes are transcribed divergently from their location on chromosome II and share 4 tandem Gal4p activator binding sites (UAS_G) (Fig. 2). When yeast cells are growing in glucose containing media, transcription from *GAL1* and *GAL10* is shut off by 2 mechanisms. Firstly, little Gal4p is produced in glucose and all Gal4p that binds to the UAS_G is inactivated by Gal80p repressor which binds and masks the C-terminal activation domain of Gal4p. Secondly, glucose-responsive repressor proteins Mig1p, Nrg1p and Nrg2p bind to sequences in the URS_G and actively repress transcription (Treitel and Carlson 1995; Wu and Trumbly 1998; Frolova et al. 1999; Zhou and Winston 2001).

When cells grow in 'permissive' carbon sources like raffinose or glycerol, glucose-repression by Mig1p, Nrg1p and Nrg2p is alleviated and only the galactose-specific Gal80p repressor is active at the *GAL* loci. Simultaneously, *GAL4* gene is upregulated and Gal4p activator is synthesized. This transition poises the *GAL1* gene for activation. As a result, when galactose becomes available, induction of *GAL1* transcription occurs much more rapidly and transcripts accumulate within 20 minutes of *GAL1* induction as opposed to 2-3 hours taken for *GAL1* induction, when cells are shifted directly form glucose to galactose.

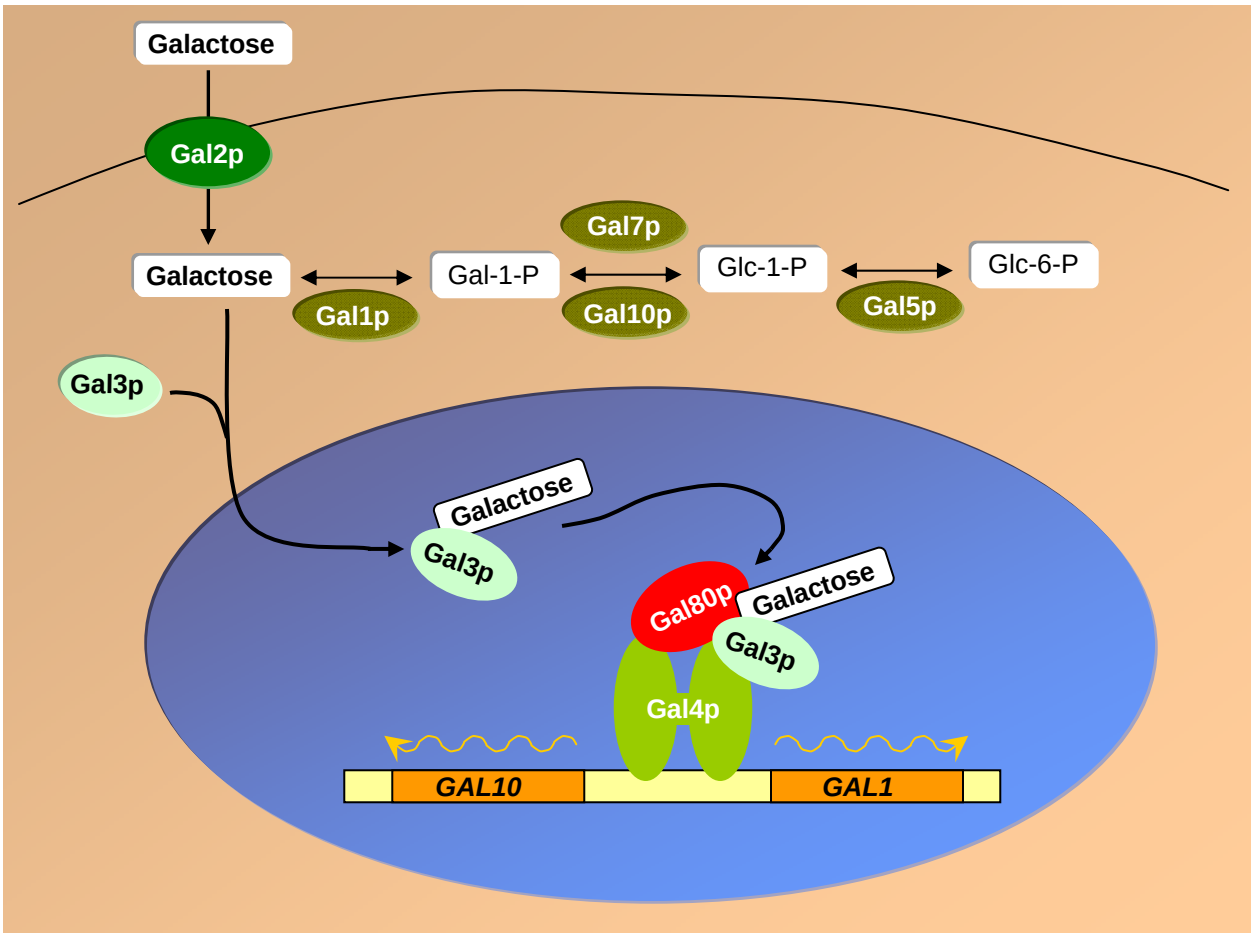
Figure 2. Schematic representation of activation of the *GAL1-10* locus. (Top) *GAL1* and *GAL10* genes are transcribed divergently. They share 4 Gal4p binding sites (black bars). The URS site on *GAL1* promoter is recognized by glucose-repressors like Mig1p. Blue ovals represent nucleosome positions when the genes are repressed in glucose. (Bottom) Transcriptional activation of *GAL* genes requires the presence of galactose sugar. Extracellular galactose is transported to the cytoplasm by Gal2p permease. Intracellular galactose binds Gal3p co-inducer protein and this complex inactivates Gal80p repressor. Inactivation of Gal80p leads to activation of Gal4p activator. Gal4p is a transcription factor that binds to DNA sites upstream of structural *GAL* genes like *GAL1* and *GAL10* and promotes their transcription (wavy arrows). *GAL1* gene product is Gal1p or the galactokinase enzyme that converts galactose to galactose-1-phosphate. Then other enzymes of the *GAL* regulon (Gal7p, Gal10p, Gal5p) further act on this substrate to metabolize galactose for energy production in the cell. See text for details.

Figure 2.

GLUCOSE REPRESSION



ACTIVATION OF GALACTOSE METABOLISM PATHWAY



When galactose is present in the environment, it is transported to the cytoplasm by the Gal2p membrane-bound permease and presence of cytoplasmic galactose is signaled by the co-inducer protein, Gal3p, which binds galactose and translocates to the nucleus (Wightman et al. 2008). The Gal3p-galactose complex can bind and inactivate the Gal80p repressor. Inactivation of Gal80p in turn activates Gal4p, which binds to UAS_G sites upstream of the *GAL* structural genes, recruits the transcriptional machinery and thus turns on transcription (Fig. 2) (Platt and Reece 1998; Peng and Hopper 2002). Besides the core transcriptional machinery, activated Gal4p also recruits the SAGA histone modifying complex and the chromatin remodeling enzyme, SWI/SNF. Neither of these chromatin modifying activities is however required for *GAL1* transcription though Spt20p component of SAGA is essential for *GAL1* activation, suggesting a role of this complex in providing physical contacts between Gal4p and the core transcriptional machinery (Bhaumik and Green 2001; Larschan and Winston 2001; Bhaumik et al. 2004). *GAL1* induction in the presence of galactose leads to translocation of the locus to nuclear pore. Transcription-dependent nuclear pore localization has been observed for many highly induced yeast genes and is believed to aid efficient export of mRNA into the cytoplasm (Casolari et al. 2004; Casolari et al. 2005; Abruzzi et al. 2006; Cabal et al. 2006; Taddei et al. 2006).

When cells are grown in the neutral sugar raffinose and then the naïve *GAL1* gene is induced with galactose, *GAL1* transcripts are detectable by 20 minutes post-

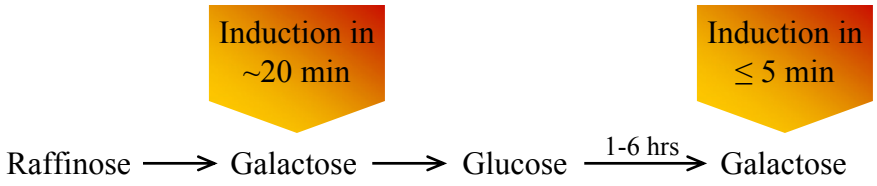
induction. However, if in these cultures, *GAL1* transcription is repressed with glucose till 6 hours and then turned back on, by transferring cells back to galactose containing medium, reinduction occurs with much faster kinetics compared to the initial round of induction. Steady-state level of transcription is observed within 5 minutes of *GAL1* reinduction (Fig. 3, top). This phenomenon of rapid reactivation that follows a period of repression reflects an example of transcriptional memory (Kundu et al. 2007). Such 'short-term' transcriptional memory of *GAL1* activity persists through mitosis, requires chromatin remodeling by SWI/SNF and is ultimately lost by 6 hours of growth in the absence of galactose stimulus, or in other words, persists for approximately 3 cell cycles before being lost.

In another experimental paradigm, a different phenomenon which can be called 'long-term' memory is observed. Here, glucose grown cells are *GAL1*-induced, repressed with glucose and reinduced again by shifting cells back to galactose containing medium (Brickner et al. 2007; Zacharioudakis et al. 2007). In the first round of *GAL1* induction, cells have to overcome not only Gal80p-repression but also glucose-repression by Mig1p, Nrg1p and Nrg2p and have to synthesize Gal4p activator at the same time. Hence induction of the naïve gene requires 3-4 hours. In contrast, *GAL1* reinduction occurs within 2 hours after glucose repression (Fig. 3, bottom). Compared to the 'short-term' memory described above, the transcriptional response during *GAL1* reinduction is slower in this form of memory but persists for 12 hours or longer. Interestingly, different factors have been implicated for 'short-term' and 'long-

term' memory. Studies by Brickner et al. (2007) suggest that formation and inheritance of this 'long-term' memory require the histone variant, H2A.Z and Nup2p mediated nuclear pore association. However, a recent report clearly shows that in budding yeast nuclear pores and proteins or DNA associated with them are retained exclusively within the mother cell while the daughter cell always received new pore complexes (Shcheprova et al. 2008). This argues against the theory of nuclear propagation of memory since any *GAL1* gene that was bound to the pore complexes through extended periods of glucose repression would certainly be segregated asymmetrically to the mother cell and there appears no mechanism to instruct the daughter cell's copy of *GAL1* about its nuclear localization status.

TRANSCRIPTIONAL MEMORY AT YEAST *GAL* GENES

SHORT-TERM MEMORY

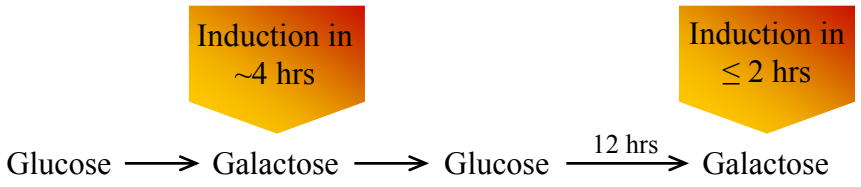


Factors involved :

SWI/SNF

Gal3p/Gal1p?

LONG-TERM MEMORY



Factors involved :

Gal1p

H2A.Z

Figure 3. Schematic to summarize the two forms of transcriptional memory observed at the GAL gene cluster. Two different experimental regimens that lead to memory are shown. 'Short-term' and 'long-term' forms of memory differ in the time for which memory and in factors required to form memory. Short-term memory results in faster reinduction compared to long-term memory, but persists for lesser time. See text for details.

It has also been proposed that H3K4me (catalyzed by Set1p) might provide transcriptional memory of *GAL10* induction since the mark can persist at the locus till 5 hours after glucose repression (Ng et al. 2003). However deletion of Set1p has no effect on 'short-term' transcriptional memory of *GAL* genes (Kundu et al. 2007). On the other hand, our studies have shown that neither histone variants nor nuclear pore localization seem to contribute to short-term *GAL1* memory. Instead, we observed that short-term memory depended on chromatin remodeling by SWI/SNF (Kundu et al. 2007). How could an ATP-dependent chromatin remodeling enzyme promote transcriptional memory? One model proposes that SWI/SNF could be causing short-term *GAL1* memory by generating open chromatin conformation at the promoter, perhaps through alternate nucleosome positioning. However, since memory of *GAL1* transcription is heritable, this alternate chromatin configuration would need to be reestablished after each round of DNA replication even in the absence of SWI/SNF. Interestingly, genetic analysis revealed that deleting either of the two ISWI complexes, ISW1 or ISW2 could rescue short-term transcriptional memory in *swi2Δ* cells. Since ISWI complexes establish repressive chromatin configuration at gene promoters by altering nucleosome positions, it is conceivable that SWI/SNF maintains open chromatin at *GAL1* promoter by preventing ISWI function during short-term glucose repression. Though this scenario can explain the requirement of SWI/SNF for short-term transcriptional memory, the mechanism of its inheritance is still not clear.

Like complex genetic networks of higher eukaryotes, genetic networks of yeast are also controlled at different steps by feedback or feedforward loops. Such loops increase the responsiveness of the network to subtle environmental changes and reduce stochastic noise or cell heterogeneity in gene activity (Ozbudak et al. 2002; Stelling et al. 2004; Kaern et al. 2005). The *GAL* transcriptional network also incorporates signaling loops – positive signaling via Gal3p and negative signaling via Gal80p that can together enhance the robustness of response of the population to galactose (Acar et al. 2005; Ramsey et al. 2006; Ronen and Botstein 2006; Ajo-Franklin et al. 2007). Significantly, long-term memory at *GAL* genes indeed appears to require signaling by Gal1p, high amounts of which can be cytoplasmically distributed to progeny through multiple cell divisions (Zacharioudakis et al. 2007). This is very interesting since Gal1p and Gal3p are very closely related proteins that are believed to have diverged from a common ancestor such as the galactokinase enzyme still seen in *Kluymeromyces lactis*. This means that Gal1p can bind galactose (although with a higher K_m than Gal3p) and therefore when present in the cell in high concentrations, Gal1p functions as a weaker galactose coinducer (Meyer et al. 1991; Platt et al. 2000; Hawkins and Smolke 2006; Hittinger and Carroll 2007).

Two questions arise – firstly, if Gal1p functions as a co-inducer in providing 'long-term' memory to robustly signal the presence of galactose in the environment, then can the actual co-inducer, Gal3p, also not provide memory? Secondly, do Gal1p or Gal3p also contribute to cytoplasmic inheritance of 'short-term' memory?

Zacharioudakis et al. (2007) showed that Gal3p did not contribute to long-term memory at *GAL10* gene. In addition, we observed that Gal1p was not required for short-term memory. Instead, overexpressing Gal3p constitutively increased rate of initial *GAL1* induction almost to the rate of reinduction after short-term repression (our unpublished results). This is significant because the *GAL3* gene is induced 3-5 fold in the presence of galactose. Thus the cellular level of Gal3p co-inducer is responsive to presence of the inducer, galactose and higher cytoplasmic amount of Gal3p can be transmitted to daughter cells during mitosis, even when galactose has been removed from the medium. In the event that galactose again becomes available in the space of a few cell cycles, cells already have higher than basal level of cytoplasmic Gal3p to bind galactose and thus can respond more rapidly by inducing the *GAL* genes than naïve cells. Since Gal3p has a very high affinity for binding galactose, relatively small amounts of the Gal3p-galactose complex can alleviate Gal80p-repression. Together, these results clearly demonstrate that both short-term and long-term memory of *GAL* gene transcription is transmitted to future generations by cytoplasmic distribution of signaling molecules.

Concluding remarks

In all metazoans, and indeed even in yeast, inheritance of transcriptionally active or silenced loci involves chromatin changes. Different mechanisms of inheritance of cellular memory have different half-lives or persistence. At most instances therefore, cells employ more than one strategy cooperatively to regulate both efficiency of cellular memory and also the plasticity of the system. Unlike other organisms, cellular memory

in budding yeast appears so far to be entirely cytoplasmic in nature. Activation of a genetic network builds up a large cellular concentration of signaling proteins, whose half-lives can be longer than the life cycle of yeast. Thus at each round of cell division, cytoplasm of the initial population divides and high levels of signaling proteins (like Gal1p or Gal3p) that were built up are distributed to daughter cells till either the protein is diluted out or is turned over. Since no mechanisms to perpetuate or regenerate these signals are known, cytoplasmically inherited memory is eventually lost and the network is reset.

CHAPTER II

SWI/SNF IS REQUIRED FOR TRANSCRIPTIONAL MEMORY AT THE YEAST *GAL* GENE CLUSTER

Contribution and acknowledgement:

Experiments for data shown in Figures 8a, 8b and 10b were performed by Dr. Peter Horn, in the lab. Other data shown in this chapter are from experiments done by me. I thank Dr. Horn for discussions about many experiments described in this chapter and also for doing initial experiments for this project. I also thank Dr. D. McCollum (UMMS) and Dr. N. Rhind (UMMS) for help with the elutriation studies, Dr. M.R. Green (UMMS) for antibodies, and Dr. F. Winston (Harvard Medical School) for yeast strains.

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Abstract

Posttranslational modification of nucleosomal histones has been suggested to contribute to heritable transcriptional memory. We describe a case of transcriptional memory in yeast where the rate of transcriptional induction of *GAL1* is regulated by the prior expression state. This transcriptional state is inherited by daughter cells, but does not require the histone acetyltransferase, Gcn5p, the histone ubiquitinylating enzyme, Rad6p, or the histone methylases, Dot1p, Set1p, or Set2p. In contrast, we show that the ATP-dependent chromatin remodeling enzyme, SWI/SNF, is essential for transcriptional memory at *GAL1*. Genetic studies indicate that SWI/SNF controls transcriptional memory by antagonizing ISWI-like chromatin remodeling enzymes.

Introduction

Specific patterns of gene expression are established during development, and these gene expression programs can be maintained through many cell divisions. The process of establishing and maintaining a transcriptional state that is heritable to progeny has been termed transcriptional memory. Within eukaryotic cells, chromatin structure plays a key role in establishing and maintaining ON/OFF states of gene expression. In its simplest state, chromatin is composed of long, linear arrays of nucleosomes that contain 147 bp of DNA wrapped about twice around an octamer of the core histones (two each of H3, H4, H2A, and H2B). Within cells, nucleosomal arrays are condensed into higher order structures, and the dynamic folding/unfolding of these structures is associated (and likely causative) with transcriptional activity.

Genetic and biochemical analyses of transcriptional regulatory mechanisms have led to the identification of two classes of highly conserved "chromatin remodeling/modification" enzyme that regulate the dynamic state of chromatin (for reviews see (Becker and Horz 2002; Peterson and Laniel 2004)). One class of chromatin remodeling/modification enzymes catalyzes the covalent attachment or removal of post-translational histone modifications (e.g. lysine acetylation, serine phosphorylation, lysine and arginine methylation, and lysine ubiquitylation). These histone marks can regulate the formation of higher order chromatin structures (e.g. H4-K16Ac; (Shogren-Knaak et al. 2006)), or they can serve as the nucleating event for binding of nonhistone proteins that establish active or inactive chromatin states. For example, methylation of

H3-K9 provides a binding site for the HP1 protein which nucleates formation of repressive, heterochromatic structures (Grewal and Elgin 2002). HP1 can interact with the H3-K9 methyltransferase which has suggested a means for how this chromatin structure can be reestablished following DNA replication (Grewal and Elgin 2002). Likewise, methylation of histone H3 at K4 is associated with transcriptionally active loci in many eukaryotes, and it has been suggested that H3-K4me could provide a memory of previous transcriptional activity (Ng et al. 2003).

In addition to histone modifying enzymes, a distinct class of chromatin remodeling/modification enzyme uses the free energy derived from ATP hydrolysis to enhance the accessibility of nucleosomal DNA or change the histone composition of nucleosomes (Becker and Horz 2002; Smith and Peterson 2005). This family can be subdivided into at least five groups based on their biochemical properties and overall sequence similarity of their ATPase subunits: (1) SWI/SNF, (2) ISWI, (3) Mi-2/CHD, (4) Ino80/Swr1, and (5) Rad54 (Flaus et al. 2006). Whereas many members of the ISWI-like and Mi-2/CHD-like subgroups appear dedicated to transcriptional repression pathways (Kehle et al. 1998; Fazzio et al. 2001; Unhavaithaya et al. 2002), most SWI/SNF-like enzymes play roles in the activation of transcription (Peterson and Workman 2000). Notably, the *Drosophila* SWI/SNF complex harbors the Brm ATPase which is a member of the TrX family of gene products that function as “memory factors” to maintain the transcriptional active state of homeotic genes during embryonic development (Tamkun et al. 1992).

Here we find that transcriptional induction of the yeast *GAL1* gene exhibits "memory" of the preceding transcriptional state. Specifically, the rate of transcriptional induction of a naïve gene is slower than for a *GAL1* gene that was previously transcribed. This ability to re-induce *GAL1* with fast kinetics survives at least one round of DNA replication and mitosis, indicating that this memory phenomenon is inherited by future generations. Previous studies have demonstrated that nucleosomes at the *GAL1* locus are subject to a variety of histone modifications during transcription, but we find that none of these marks are required for memory. In contrast, we find that inactivation of the SWI/SNF remodeling enzyme eliminates transcriptional memory at *GAL1*, such that the rate of transcriptional induction is nearly identical between a naïve gene and a *GAL1* gene that had been previously transcribed. Surprisingly, we find that inactivation of ISWI-based chromatin remodeling enzymes restores transcriptional memory in a *swi/snf* mutant, suggesting that SWI/SNF prevents ISWI-based enzymes from erasing the memory of a previous round of transcription.

Results

Transcriptional memory at GAL1 gene is heritable

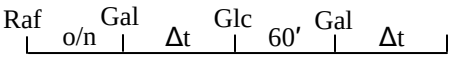
GAL1, which encodes the enzyme galactokinase, can be transcriptionally induced by ~1000-fold when yeast cells are grown in media containing galactose. Addition of glucose leads to rapid and efficient repression of *GAL1* by multiple mechanisms, including a decrease in levels of the Gal4p activator and the galactose permease (Gal2p), and by activating several glucose repressor proteins that act *in cis* at the *GAL1* promoter (Johnston et al. 1994; Carlson 1998). Further, in neutral carbon sources such as raffinose, glycerol, or lactate, *GAL1* is maintained in a poised state due to the masking of the Gal4p activation domain by the Gal80p repressor. We were specifically interested in how the trans-acting glucose repressors function, and therefore, we investigated the re-induction of *GAL1* following a short period of glucose repression (see Fig. 4a). Cells were first grown in raffinose media so that *GAL1* was poised for activation. Upon addition of galactose to the growth medium, *GAL1* transcription commenced and transcripts appeared by 20 minutes post-induction (Fig. 4a, b). However, accumulation of maximum levels of *GAL1* transcripts required > 1 hour of growth in galactose media. Next, *GAL1* expression was repressed by addition of 2% glucose and cells were grown for an additional hour. Surprisingly, when cells were washed into fresh media containing galactose, *GAL1* transcription resumed very rapidly (Fig. 4a, b; Fig. 10). Re-induction of *GAL1* transcription peaked < 10 min after the addition of galactose (Fig. 4a, b). Thus, these results suggest that cells "remember" that *GAL1* was previously transcribed, and consequently they are poised to rapidly re-induce

GAL1 transcription when galactose again becomes the dominant carbon source. Similar results were found for the reinduction of the *GAL7* and *GAL10* genes, indicating that this phenomenon is a general property of the *GAL* gene cluster (data not shown).

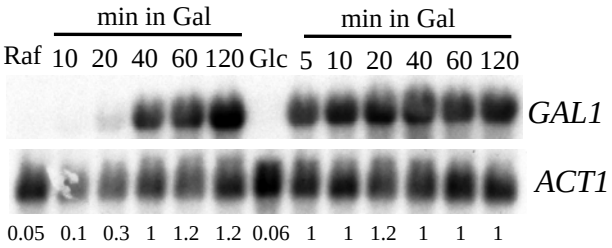
We next tested whether the ability to re-induce *GAL1* with rapid kinetics was a transient state or whether it could survive long term growth in glucose media. Cells were grown overnight in raffinose media and then galactose was added for 60' to induce *GAL1* expression. Glucose was then added to repress *GAL1*, and at varying times after glucose addition, cell aliquots were transferred to galactose media and *GAL1* re-induction kinetics were monitored. Figure 5a illustrates that cells grown for 2 to 4 hours in glucose media retained the ability to rapidly reinduced *GAL1* after subsequent addition of galactose (rapid induction defined as maximal expression at 20' following galactose addition). In contrast, cells grown for 6-8 hours in glucose re-induced *GAL1* with the slower kinetics that mirrored induction kinetics of a naïve gene (compare lanes 10-15 of Fig. 5a with Figure 4a). Since the yeast cell cycle is ~2 hours in glucose media, these results suggest that the ability to rapidly re-induce *GAL1* might survive DNA replication and/or mitosis. To test this idea definitively, we performed an elutriation experiment (Fig. 5b). Cells were grown in galactose media until mid-log phase, and then cells were arrested at the G1/S transition of the cell cycle by treatment with alpha factor. Arrested cells were washed into glucose-containing media and then released from the cell cycle block and allowed to undergo one synchronous cell division in glucose media. Centrifugal elutriation was then performed in glucose media to isolate

small, unbudded daughter cells from this culture. The daughter cell population was then transferred to galactose media and *GAL1* re-induction kinetics were followed. The data shown in Fig. 5b demonstrates that these daughter cells retained the ability to re-induce *GAL1* with rapid kinetics (i.e. peak expression at 20 minutes). These results indicate that memory of *GAL1* gene transcription can be stably inherited.

Figure 4: Transcriptional memory at the *GAL1* gene. (a) Northern analysis of *GAL1* RNA levels. Schematic at top depicts regimen of growth in different carbon sources. Raf, 2% raffinose; Gal, 2% galactose; Glc, 2% glucose. Initial induction of *GAL1* occurs with slower kinetics than when *GAL1* is re-induced following glucose repression. (b) Graph comparing kinetics of *GAL1* induction and re-induction, averaged over three experiments performed as described in panel a. Error bars represent the standard deviation at each point. Slightly different time points were taken in different experiments, so in these cases no error bars are shown. The bottom panel represents an *ACT1* loading control for total RNA levels. The numbers indicate fold induction of *GAL1* transcripts normalized to *ACT1* transcripts, with the maximally induced state set to a value of 1.


Raf
o/n
Gal
Δt
Glc
60′
Gal
Δt

a)


min in Gal
min in Gal
Raf 10 20 40 60 120 Glc 5 10 20 40 60 120
GAL1
ACT1
0.05 0.1 0.3 1 1.2 1.2 0.06 1 1 1.2 1 1 1

b)

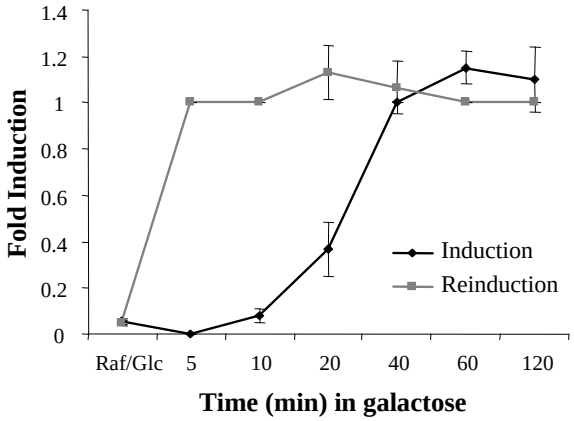
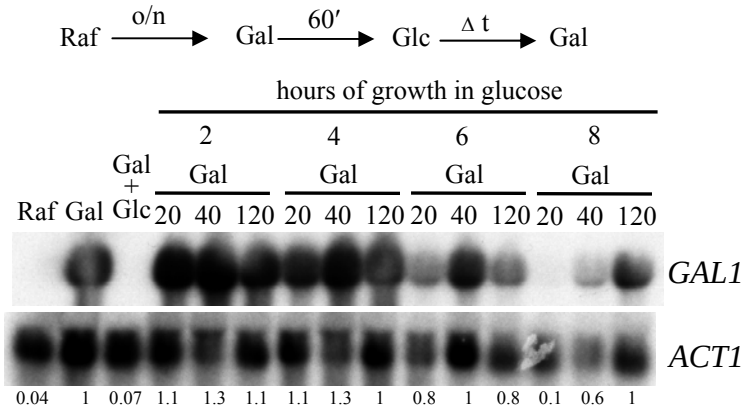
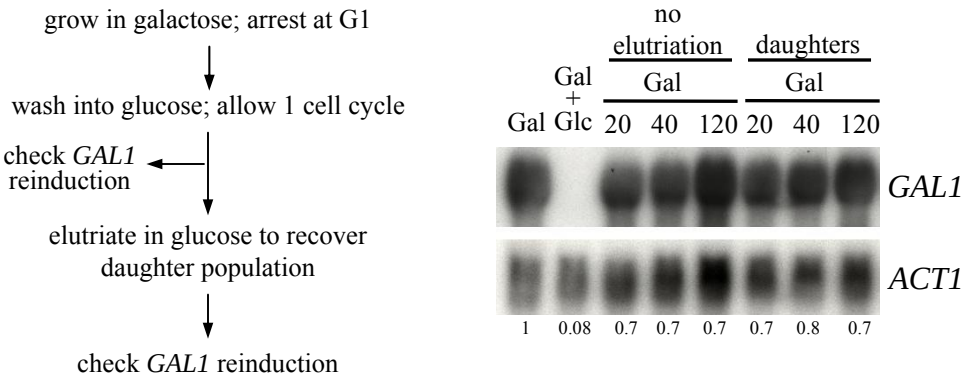

Fold Induction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Induction
Reinduction
Raf/Glc 5 10 20 40 60 120
Time (min) in galactose

Figure 5. Transcriptional memory persists through cell division. (a) Cells were grown overnight in raffinose prior to addition of galactose. After 1 hour, cells were transferred to glucose media. At indicated times, an aliquot of cells was washed into fresh galactose media and *GAL1* reinduction was observed. The "memory" state is maintained through at least 4 hours of repression. (b) Glucose-grown daughter cells retain transcriptional memory. Cells were grown overnight in galactose media and then arrested at the G1/S boundary with alpha factor (lane labeled "Gal"). Arrested cells were then released from alpha factor into glucose medium to repress *GAL1* and simultaneously undergo one synchronous division (lane labeled "Gal+Glc"). An aliquot of these cells were washed into galactose media to monitor re-induction kinetics (lanes labeled "no elutriation"). The remainder of the cells were elutriated to isolate daughter cells that had undergone mitosis in glucose media. Daughter cells were washed into galactose media to follow kinetics of *GAL1* reinduction. The bottom panel represents an *ACT1* loading control for total RNA levels. The numbers indicate fold induction of *GAL1* transcripts normalized to *ACT1* transcripts, with the maximally induced state set to a value of 1.

a)


Raf
o/n
Gal
60′
Glc
Δ t
Gal
hours of growth in glucose
2
4
6
8
Gal
Gal
Gal
Gal
Gal + Glc
Raf Gal
20 40 120 20 40 120 20 40 120 20 40 120
GAL1
ACT1
0.04 1 0.07 1.1 1.3 1.1 1.1 1.3 1 0.8 1 0.8 0.1 0.6 1

b)


grow in galactose; arrest at G1
wash into glucose; allow 1 cell cycle
check GAL1 reinduction
elutriate in glucose to recover daughter population
check GAL1 reinduction
no elutriation
daughters
Gal
Gal
Gal + Gal Glc
20 40 120 20 40 120
GAL1
ACT1
1 0.08 0.7 0.7 0.7 0.7 0.8 0.7

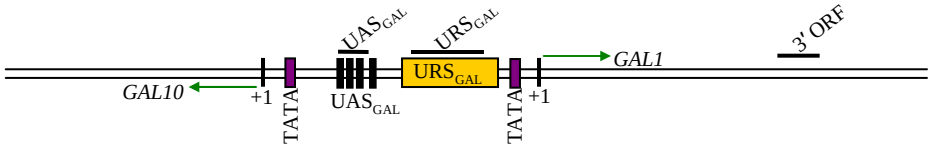
The transcriptional machinery is disassembled during glucose repression

One possibility is that rapid re-induction kinetics involves the persistent association of one or more components of the RNAP II transcription machinery with the *GAL1* promoter during glucose repression. For instance, if TBP remains bound to the promoter during glucose repression, then re-induction might occur with faster kinetics. *GAL1* transcription is activated by Gal4p, which binds to four sites within the UAS_{GAL} between the *GAL1* and *GAL10* genes (Fig 6a). When galactose is added to raffinose-grown cells, Gal80p-dependent inhibition of Gal4p is relieved, and Gal4p recruits a variety of transcription factors, including the SWI/SNF remodeling enzyme, the SAGA HAT complex, and the transcriptional mediator complex (Bhaumik and Green 2001; Larschan and Winston 2001; Lemieux and Gaudreau 2004; Dhasarathy and Kladde 2005). These events are followed by assembly of the pre-initiation complex (PIC) and transcription of *GAL1*. We used chromatin immunoprecipitation to follow the recruitment of these factors to the *GAL1* promoter during growth in galactose and during subsequent glucose repression (Figure 6). As expected, robust levels of Gal4p were detected at UAS_{GAL} when cells were grown in raffinose or galactose media. During glucose repression, Gal4p remained at high levels for the first 1 hour in glucose, and then levels decreased ~2-fold at extended times in glucose media (Fig 6b, top). The lower level of Gal4p was also observed in long-term glucose grown cultures and likely reflects partial occupancy of the multiple Gal4p binding sites (Ren et al. 2000). These ChIP signals are specific to Gal4p since a *gal4Δ* strain showed no enrichment of Gal4p at the UAS_{GAL} (Fig 6b, bottom).

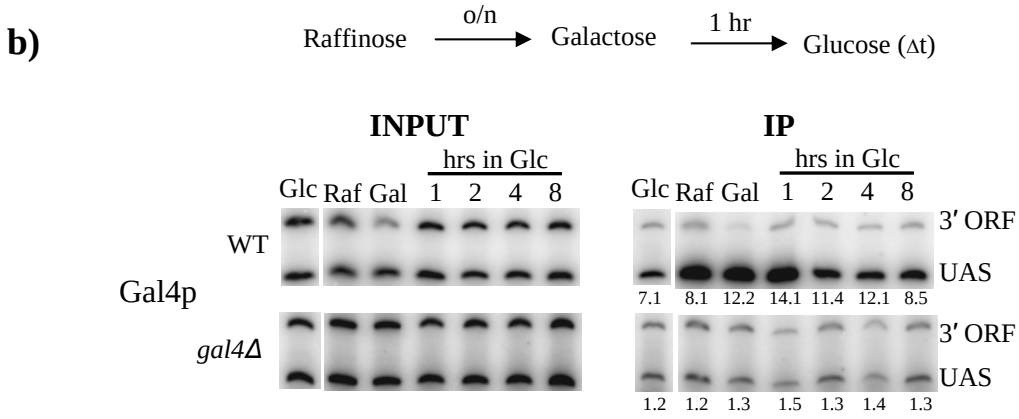
We also found that TBP, Mediator, RNA Polymerase II, SWI/SNF, and SAGA were recruited to the *GAL1* promoter following galactose addition, but unlike Gal4p, none of these factors could be detected at *GAL1* after 1 hour of transferring the cells to glucose-containing medium (Fig. 6c). Thus, components of the transcription machinery rapidly dissociate from the promoter soon after *GAL1* transcription is repressed by glucose addition. Since the ability to rapidly re-induce *GAL1* persists for 2-4 hours in glucose, these results suggest that this phenomenon is not due simply to persistent association of transcription factors with the promoter.

Figure 6. Rapid dissociation of the transcription machinery during glucose repression. (a) Schematic representation of the *GAL1-10* regulatory region. UAS_{GAL} marks the Gal4p binding sites. URS_{GAL} contains the binding sites for the glucose repressor, Mig1p. Regions covered by primers for chromatin immunoprecipitation (ChIP) are shown as horizontal lines. TATA represents the TBP binding sites and +1 represents the transcription start sites. (b) Gal4p ChIP in wild type and *gal4Δ* strains. (c) ChIP for TBP, RNA Polymerase II, Mediator (α-Srb4-13myc), SWI/SNF (α-Snf6) and SAGA (α-Spt3-13myc). For all factors, appropriate strains were grown in raffinose media until mid-log phase, and *GAL1* was induced for 1 hour by adding galactose. Cells were then washed into media with glucose to repress *GAL1* transcription for the indicated times. 3' *GAL1* ORF, telomere (Chr VI-70bp from right end) and *ACT1* PCR primer sets were included as nonspecific controls. Numbers indicate ratio of %IP values to corresponding nonspecific control.

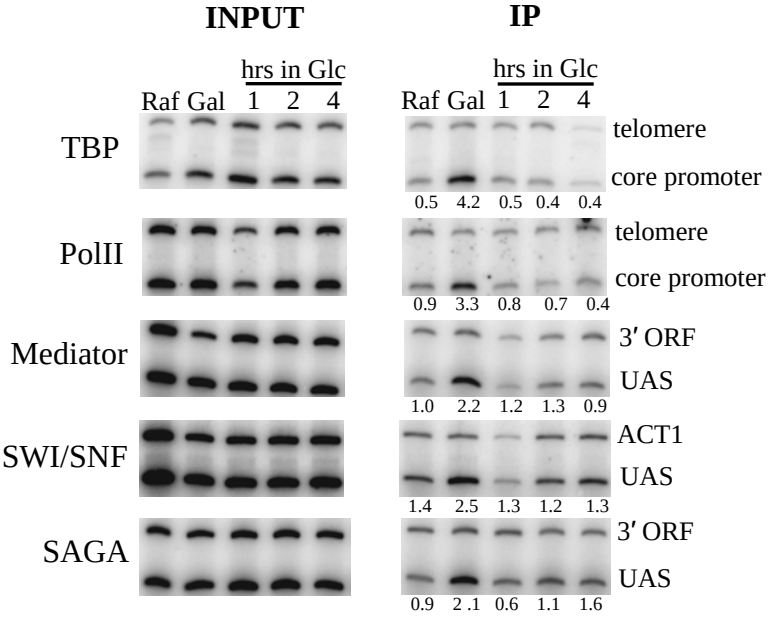
a)


UAS$_{GAL}$
URS$_{GAL}$
GAL1
3′ ORF
GAL10
+1
TATA
UAS$_{GAL}$
URS$_{GAL}$
TATA
+1

b)


Raffinose
o/n
Galactose
1 hr
Glucose (Δt)
INPUT
IP
hrs in Glc
Glc Raf Gal 1 2 4 8
WT
Gal4p
gal4Δ
3′ ORF
UAS
7.1 8.1 12.2 14.1 11.4 12.1 8.5
1.2 1.2 1.3 1.5 1.3 1.4 1.3

c)


INPUT
IP
hrs in Glc
Raf Gal 1 2 4
TBP
PolII
Mediator
SWI/SNF
SAGA
telomere
core promoter
3′ ORF
UAS
ACT1
0.5 4.2 0.5 0.4 0.4
0.9 3.3 0.8 0.7 0.4
1.0 2.2 1.2 1.3 0.9
1.4 2.5 1.3 1.2 1.3
0.9 2 .1 0.6 1.1 1.6

The kinetics of RNA polymerase II recruitment to the *GAL1* promoter was also analyzed throughout an induction/re-induction timecourse (Fig. 2d). In the initial round of *GAL1* induction, high levels of RNAPII was detected at the *GAL1* promoter within 20-40 minutes (Fig. 7), consistent with the appearance of *GAL1* mRNA (Fig. 4a). Strikingly, RNAPII was recruited much faster during re-induction of *GAL1*, with significant amounts of RNAPII detected 5' after galactose addition. Similar results were found for recruitment of TBP (data not shown). Thus, these results indicate that this phenomenon of *GAL1* transcriptional memory occurs at the level of transcription initiation.

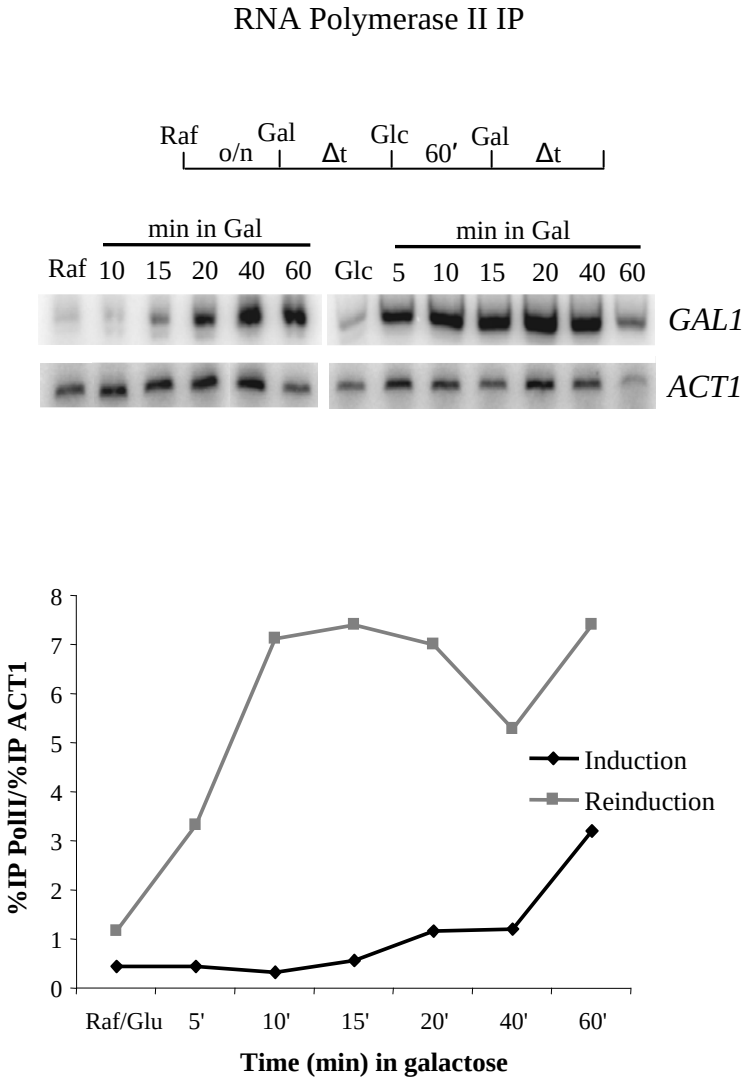
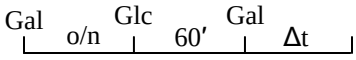


Figure 7. RNA Polymerase is recruited rapidly during *GAL1* reinduction. RNA Polymerase II ChIP in wild type strain comparing induction and re-induction using the regimen shown above panel. Below is shown quantitation of a representative experiment.

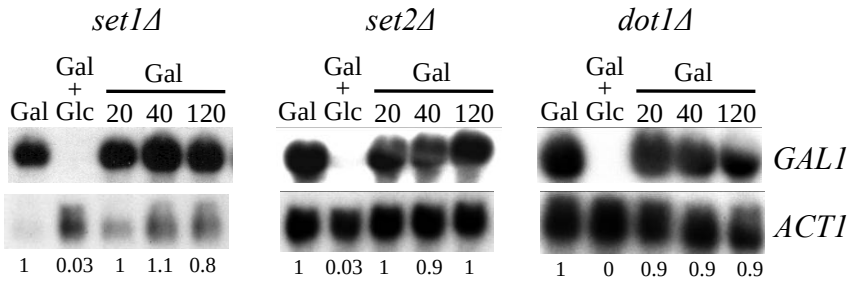
Histone modifications are not required for transcriptional memory

Posttranslational modification of the core histones is an ideal candidate for an chromatin-inheritance mechanism that could contribute to the rapid *GAL1* re-induction kinetics. Histone lysine methylation is particularly attractive as this mark has a much longer half-life as compared to lysine acetylation (Katan-Khaykovich and Struhl 2005). Indeed, the transcription-associated methylation of histone H3-lysine 4 (H3-K4me) has been hypothesized as a possible agent for memory of recent transcriptional activity (Ng et al. 2003). We tested whether Set1p, which is responsible for H3-K4 methylation (Briggs et al. 2001; Roguev et al. 2001), or Set2p and Dot1p which methylate H3-K36 (Strahl et al. 2002) and H3-K79 (Feng et al. 2002; Lacoste et al. 2002; Ng et al. 2002a; van Leeuwen et al. 2002) respectively, are required for transcriptional memory at *GAL1*. Interestingly, none of these methyltransferases are required for the rapid kinetics of *GAL1* re-induction (Fig. 8a). After re-inducing *GAL1* transcription, transcript levels peaked within 20 min in *set1Δ set2Δ* or *dot1Δ*strains (Fig. 8a). We also tested the re-induction kinetics of a *rad6Δ* strain, since Rad6p-dependent ubiquitinylation of histone H2B-K123 is required for H3 methylation (Dover et al. 2002; Ng et al. 2002b; Sun and Allis 2002). In this case as well, *GAL1* re-induction was rapid (Fig. 8b). Finally, we monitored re-induction kinetics in a *gcn5Δ* strain to eliminate SAGA-dependent acetylation of histone H3. In this case as well, no effect was observed on *GAL1* memory (Fig 8c). Thus, posttranscriptional histone modifications do not appear to be responsible for imparting transcriptional memory at the *GAL1* locus.

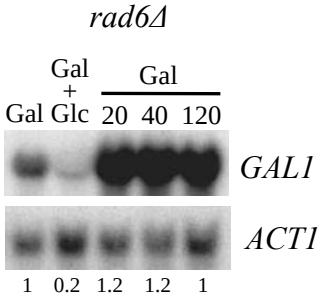
Figure 8. Histone modifying enzymes are not essential for rapid *GAL1* re-induction. Northern blot analyses as in Figure 1. (a) Schematic at top illustrates growth media regimen. RNA was isolated from *set1Δ*, *set2Δ* or *dot1Δ* strains. (b) Identical analysis as in panel a, but with a *rad6Δ* strain. (c) Identical analysis as in panel a, but with a *gcn5Δ* strain. All Northerns were subsequently probed for *ACT1* as a loading control.


Gal
o/n
Glc
60′
Gal
Δt

a)


set1Δ
set2Δ
dot1Δ
Gal + Glc
Gal
20 40 120
GAL1
ACT1
1 0.03 1 1.1 0.8
1 0.03 1 0.9 1
1 0 0.9 0.9 0.9

b)


rad6Δ
Gal + Glc
Gal
20 40 120
GAL1
ACT1
1 0.2 1.2 1.2 1

c)

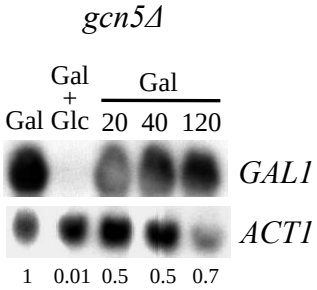

gcn5Δ
Gal + Glc
Gal
20 40 120
GAL1
ACT1
1 0.01 0.5 0.5 0.7

Table 1. List of different histone modifications tested for the requirement in transcriptional memory. *GAL1* induction and reinduction kinetics were compared by Northern Blot in strains listed below. Each of this strain lacks a histone modifying enzyme that is responsible for particular histone modification.

HISTONE MODIFYING ENZYME	FUNCTION	HISTONE MODIFIED	AFFECTS MEMORY?
Rad6	ubiquitylase	**H2B-Lysine 123**	**X**
Set1	methylase	**H3-Lysine 4**	**X**
Set2	methylase	**H3-Lysine 36**	**X**
Dot1	methylase	**H3-Lysine 79**	**X**
Gcn5	acetyltransferase	**H2B-Lysine 11,16 H3-Lysine 9,14,18,23,27**	**X**
Rtt109	acetyltransferase	**H3-Lysine 56**	**X**

Transcriptional memory requires SWI/SNF

After determining that chromatin remodeling via histone modifications might not be required for the faster re-induction kinetics of *GAL1,* we turned to the ATP-dependent chromatin remodeling enzymes. Previous studies have shown that SWI/SNF is recruited by the Gal4p activator to the *GAL1* locus (Lemieux and Gaudreau 2004). Several studies have also shown that inactivation of SWI/SNF does not alter steady state *GAL1* expression (Burns and Peterson 1997; Lemieux and Gaudreau 2004). Likewise, we found that the kinetics of *GAL1* induction in a *swi2Δ*strain are nearly equivalent to those of a wild type strain (Fig. 9a). In contrast, the *swi2Δ* strain was unable to rapidly re-induce *GAL1* following a 1 hour period of glucose repression. Indeed, the kinetics of induction and re-induction were nearly identical (Fig. 9a, b). This defect in re-induction kinetics was observed irrespective of whether raffinose-grown *swi2Δ*cells were switched to galactose for 2 hours (Fig. 9a) or if they were grown overnight in galactose (Fig. 10b). Thus it appears that inactivation of SWI/SNF eliminates the memory of previous *GAL1* activation.

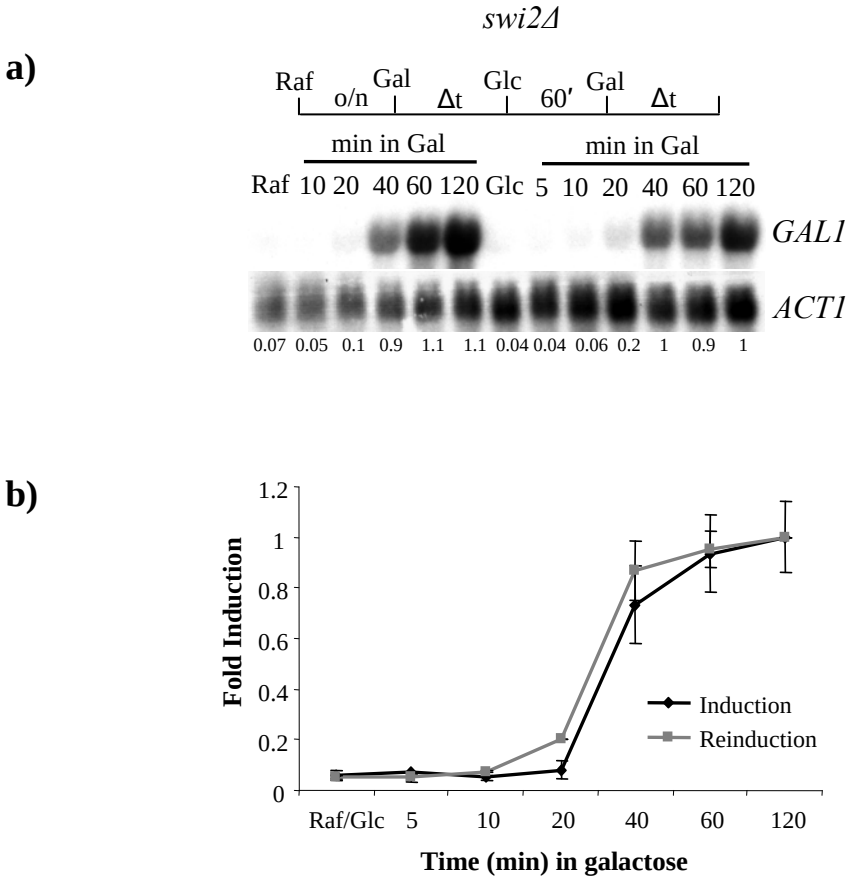


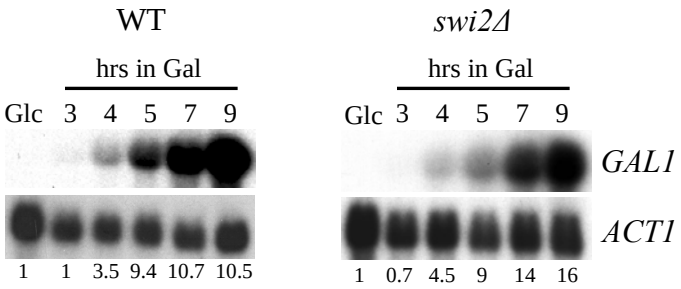
Figure 9. SWI/SNF is essential for *GAL1* memory. (a) Northern analyses. Schematic at top illustrates the growth regimen for *swi2D* cells. (b) Comparison of *GAL1* induction and reinduction kinetics, averaged over three experiments performed as in panel a. Error bars represent the standard deviation at each point. Slightly different time points were taken in different experiments, so in these cases no error bars are shown.

One possibility is that SWI/SNF is not involved in the mechanism of transcriptional memory but that SWI/SNF is generally required to antagonize glucose repression. To test this idea, wild type and *swi2Δ* cells were grown overnight in glucose, and then *GAL1* induction kinetics were monitored after cells were transferred to galactose media. In both the wild type and *swi2Δ*strains, *GAL1* transcripts appeared by 4 hours after galactose addition, demonstrating that SWI/SNF is not required to antagonize glucose repression at *GAL1* during an initial round of transcriptional induction (Fig. 10a).

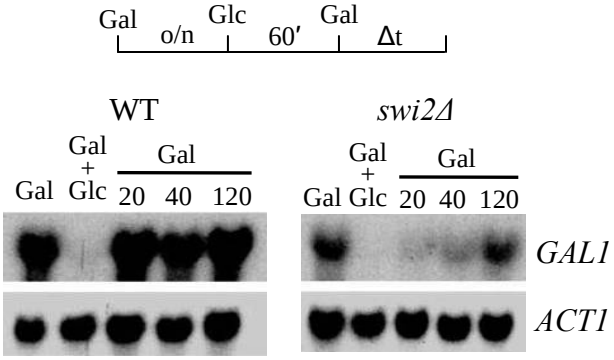
We next tested if the ATPase activity of SWI/SNF was required for transcriptional memory. *GAL1* re-induction kinetics were monitored in a strain harboring the *swi2K798A* allele, which encodes an ATPase-defective version of the Swi2p catalytic subunit. *GAL1* re-induction in the *swi2K798A* strain was much slower than the isogenic wildtype strain (~40' in *swi2K798A* vs ~5' in WT), indicating that the enzymatic activity of SWI/SNF is essential for the ability to rapidly re-induce *GAL1* transcription. We note that re-induction kinetics in the *swi2K798A* strain are reproducibly faster than the isogenic *swi2Δ* strain which may indicate an additional ATP independent role for SWI/SNF in *GAL1* memory (compare Fig. 10c to Fig. 9a).

Figure 10. SWI/SNF has no defect in *GAL1* induction. (a) Isogenic wild type and *swi2Δ* strains were grown overnight in glucose and then cells were transferred to galactose media. Both strains showed similar *GAL1* induction kinetics after overcoming long-term glucose repression. (b) Northern blot showing that *GAL1* transcriptional memory is lost in *swi2Δ* cells even if they are grown in galactose for a long period. Wild type and *swi2Δ* cells were grown overnight in galactose to ensure steady state *GAL1* expression. *GAL1* was repressed by adding 2% glucose and grown for 1 hour. Cells were then washed into galactose media and *GAL1* reinduction was followed. In these experiments, glucose repressed cultures also contained 2% galactose. (c) An intact Swi2p ATPase domain is required for rapid *GAL1* reinduction. Northern analysis of RNA isolated from cells harboring a *swi2K798A* allele which inactivates the ATPase activity of SWI/SNF. Blots were re-probed for *ACT1* as a control.

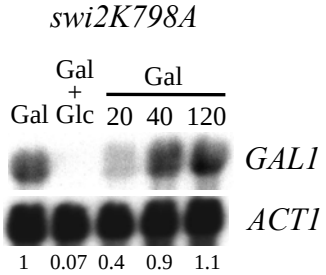
a)


WT
hrs in Gal
Glc 3 4 5 7 9
1 1 3.5 9.4 10.7 10.5
swi2Δ
hrs in Gal
Glc 3 4 5 7 9
1 0.7 4.5 9 14 16
GAL1
ACT1

b)


Gal o/n Glc 60′ Gal Δt
WT
Gal
Gal + Glc
Gal
20 40 120
swi2Δ
Gal
Gal + Glc
Gal
20 40 120
GAL1
ACT1

c)


swi2K798A
Gal
Gal + Glc
Gal
20 40 120
1 0.07 0.4 0.9 1.1
GAL1
ACT1

Loss of ISWI-like enzymes restores memory at GAL1 in the absence of SWI/SNF

Our data suggest that the initial round of *GAL1* transcription establishes a heritable state that is poised for rapid re-induction. Previous studies have identified two changes in *GAL1* chromatin structure that occur during transcriptional induction and that may contribute to this SWI/SNF-dependent memory state: (1) two nucleosomes surrounding the *GAL1* promoter are lost (Lohr and Lopez 1995) and (2) promoter proximal nucleosomes are tri-methylated at H3-K4 by the Set1p methyltransferase (Ng et al. 2003). The methylation of H3-K4 leads to the subsequent recruitment of an Isw1-containing, ATP-dependent chromatin remodeling enzyme (Santos-Rosa et al. 2003). When the transcriptionally active *GAL1* gene is repressed by glucose, the promoter proximal nucleosomes are rapidly re-assembled (Lohr 1984; Lohr and Lopez 1995), and high levels of H3 K4me3 persist (Ng et al. 2003); P.J.H and C.L.P., unpublished results).

One possibility is that SWI/SNF may influence the re-positioning of promoter proximal nucleosomes such that subsequent PIC formation is favored. If this model is correct, then only small changes in nucleosome positioning are required, as Cavalli and Thoma have previously shown that the low resolution view (+/- 50 bp) of *GAL1* promoter nucleosomes is identical when samples are analyzed from cells grown long-term in glucose or from galactose-induced cells that are treated with glucose for less than 1 hour (Cavalli and Thoma 1993). Alternatively, the first round of *GAL1* transcription may lead to a locus with a lower density of nucleosomes, even after

subsequent glucose repression. Indeed previous studies indicate that several hours of glucose repression is required before the entire *GAL1* locus is restored to a regular nucleosomal array (Cavalli and Thoma 1993). SWI/SNF action may favor this chromatin state, whereas ISWI-like enzymes may promote the re-assembly of a more regular nucleosomal array.

Although our data suggest that the *SET1*-dependent recruitment of Isw1p is not essential for *GAL1* re-induction kinetics (Fig. 8a), we tested the possibility that a functional relationship exists between SWI/SNF and ISWI-like remodeling enzymes in transcriptional memory at *GAL1*. Budding yeast contain two distinct ISWI-like enzymes, Isw1 and Isw2 (Vary et al. 2003; Mellor and Morillon 2004), and each functions as the catalytic subunit of distinct multi-subunit remodeling complexes. We created isogenic *isw1Δ*, *isw2Δ*, *isw1Δswi2Δ*, and *isw2Δ swi2Δ* strains and monitored *GAL1* re-induction kinetics. Whereas the *isw1* or *isw2* single mutants had no effect on *GAL1* induction or re-induction kinetics (Fig. 11), we found that deletion of either *ISW1* or *ISW2* restored rapid re-induction kinetics in the *swi2Δ* strain (Fig. 12a, b).

Furthermore, deletion of *ISW2* allowed a *swi2Δ* strain to grow on galactose/antimycin solid media (data not shown). In contrast, inactivation of Set1p did not restore rapid *GAL1* re-induction kinetics in a *swi2* mutant (Fig 12c). Taken together, these results suggest that SWI/SNF controls memory of recent *GAL1* transcription by antagonizing the repressive role of ISWI remodeling complexes. Such functional

antagonism between SWI/SNF and ISWI-like enzymes is not unique to the *GAL1* gene, as deletion of the *ISW2* gene also alleviates the transcriptional requirement for SWI/SNF in the induction of the *INO1* gene when cells are grown in low levels of inositol (Fig. 12d).

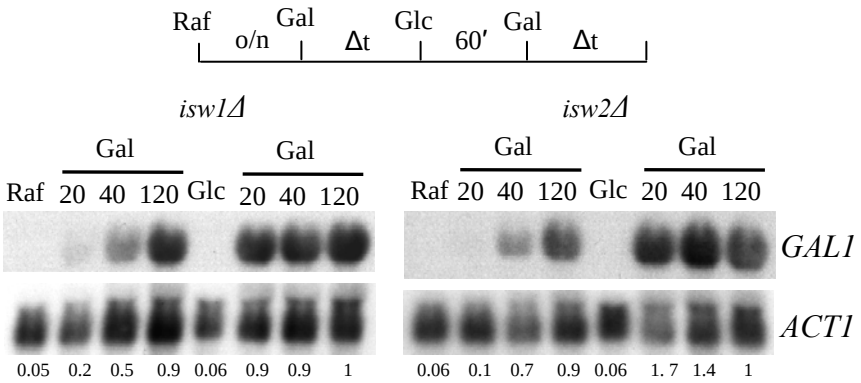
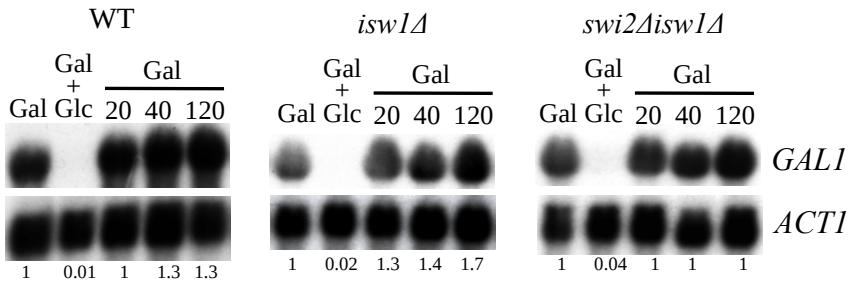


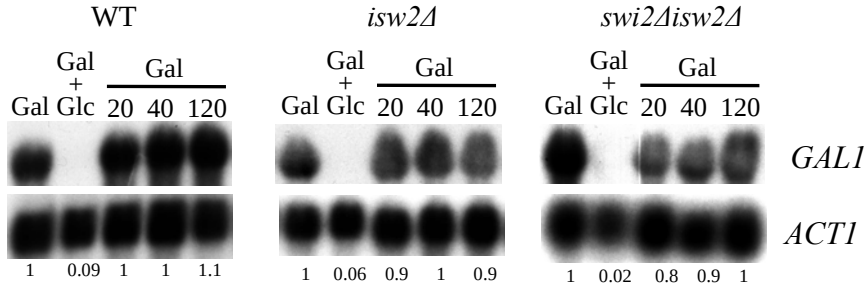
Figure 11. Single mutants of ISWI complexes show transcriptional memory. Induction and re-induction kinetics for *isw1Δ* and *isw2Δ* mutants. The indicated strains were grown as described in the schematic and northern analysis was done with the isolated RNA samples. *ACT1* is the loading control.

Figure 12. SWI/SNF antagonizes ISWI-like remodeling complexes. (a) Northern blot analysis for RNA isolated from WT, *isw1Δ* and *isw1Δ swi2Δ* strains. (b) Northern analysis for RNA isolated from *isw2Δ* and *isw2Δ swi2Δ* strains. (c) Inactivation of Set1p does not restore rapid re-induction kinetics in the absence of SWI/SNF. (d) Analysis of *INO1* expression. The indicated isogenic strains were grown in minimal media containing high (100 μM) or low (10 μM) concentrations of myo-inositol. Blots were re-probed for *ACT1* as a control.

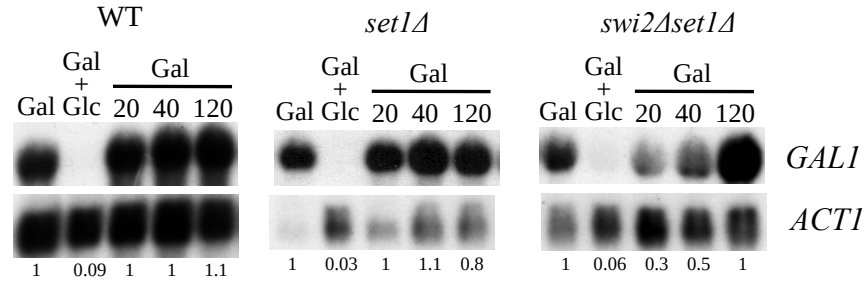
a)


WT
isw1Δ
swi2Δisw1Δ
Gal + Gal Glc
Gal
20 40 120
GAL1
ACT1
1 0.01 1 1.3 1.3
1 0.02 1.3 1.4 1.7
1 0.04 1 1 1

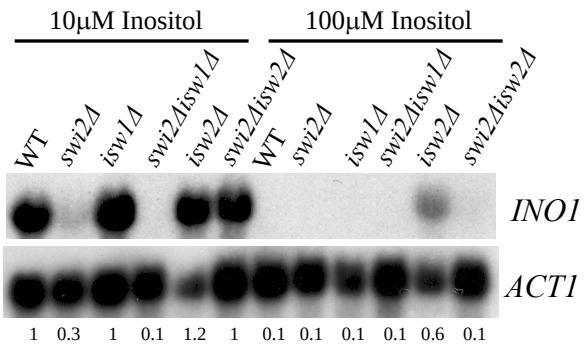
b)


WT
isw2Δ
swi2Δisw2Δ
Gal + Gal Glc
Gal
20 40 120
GAL1
ACT1
1 0.09 1 1 1.1
1 0.06 0.9 1 0.9
1 0.02 0.8 0.9 1

c)


WT
set1Δ
swi2Δset1Δ
Gal + Gal Glc
Gal
20 40 120
GAL1
ACT1
1 0.09 1 1 1.1
1 0.03 1 1.1 0.8
1 0.06 0.3 0.5 1

d)


10μM Inositol
100μM Inositol
WT
swi2Δ
isw1Δ
swi2Δisw1Δ
isw2Δ
swi2Δisw2Δ
INO1
ACT1
1 0.3 1 0.1 1.2 1 0.1 0.1 0.1 0.1 0.6 0.1

Discussion

How do SWI/SNF and ISWI-like complexes function at GAL1?

Our data suggest that the SWI/SNF that is recruited during the first round of *GAL1* transcription potentiates the re-induction of *GAL1* following a period of glucose repression. Furthermore, we found that this requirement for SWI/SNF can be bypassed by removal of the Isw1- or Isw2-based remodeling enzymes. Based on these results, we speculate that these two types of chromatin remodeling enzymes compete with each other to establish a *GAL1* chromatin structure that is permissive for rapid re-induction of *GAL1*. As cells divide, this chromatin state can be inherited initially but is eventually lost. In cells lacking SWI/SNF, the abundant Isw1 and Isw2 enzymes establish an alternative, repressive chromatin state. In this situation, memory of the recent transcriptional activity is erased and slow re-induction kinetics result. Since SWI/SNF and ISWI-like enzymes tend to have opposing effects on the transcription of several yeast genes (including *GAL1* and *INO1*), it is tempting to consider that such direct antagonism may be a more general phenomenon.

Recently van Oudenaarden and colleagues have described a distinct example of *GAL1* transcriptional memory in which cells "remember" whether they were previously exposed to high or low concentrations of galactose (Acar et al. 2005). This particular memory phenomenon requires the *GAL3* and *GAL80* regulatory loops and likely involves the cytoplasmic inheritance of the positive regulator, Gal3p. Cytoplasmic inheritance of the Gal3p that is expressed during the first round of induction may

contribute to the rapid induction kinetics that follows glucose repression. However, SWI/SNF plays a dominant role in transcriptional memory that is independent of Gal3p or the Gal2p permease since SWI/SNF does not affect *GAL3* or *GAL2* expression during the first round of galactose induction (Fig. 13). Thus, transcriptional memory at *GAL1* appears to involve a cytoplasmic mechanism that generally controls *GAL1* expression levels, and a chromatin-based mechanism that specifically regulates the rate of transcriptional reinduction following transient glucose repression.

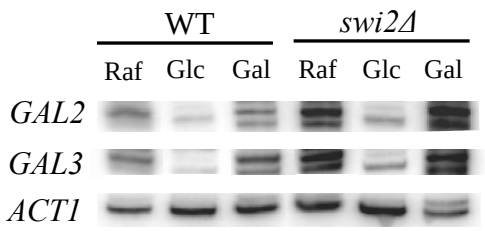


Figure 13: Loss of SWI/SNF does not affect *GAL2* or *GAL3* expression. Reverse transcriptase PCR performed in wild type and *swi2Δ* cells grown overnight in 2% raffinose, 2% glucose, or 2% galactose to detect steady state levels of *GAL2* and *GAL3* transcripts. Galactose expression of *GAL2* and *GAL3* is equal in WT and *swi2Δ* cells. *ACT1* was used as a loading control.

Materials and methods

Yeast strains, media and growth conditions

Strains used in this study are isogenic derivatives of the S288c background. Genotypes are provided in the list of strains used. *S. cerevisiae* liquid cultures were grown at 30°C in YEP (1% yeast extract, 2% bacto-peptone) media supplemented with 2% glucose, 2% galactose or 2% raffinose + 0.2% sucrose depending on whether *GAL1* activation or repression was required. For re-induction studies, aliquots of glucose-grown cultures were centrifuged for 5' at room temperature, cell pellets were washed once with YEP, centrifuged for 5', and pellets resuspended in pre-warmed YEPGal. To activate *INO1* expression cells were grown to mid-log phase in SD medium complete with amino acids. *INO1* expression was repressed by adding 100μM myo-inositol. Deleted strains were made by a PCR-based method using kanamycin resistance cassette (Longtine et al. 1998). Deletions were confirmed by PCR from genomic DNA with primers designed in the ORF of the individual deleted gene.

Centrifugal elutriation

Centrifugal elutriation of wild type cells was performed as described (Johnston and Johnson 1997).

RNA isolation and analysis

Total RNA was isolated from yeast grown to logarithmic phase in appropriate media by hot phenol extraction method. Concentration of RNA was determined by measuring

A_{260} after dissolving in diethyl pyrocarbonate-treated water. 10μg (for *GAL1* expression) or 25μg (for *INO1* expression) of total RNA from each sample was electrophoresed on 1% formaldehyde agarose gels and Northern blotting was done. The housekeeping gene *ACT1* was used as loading control. Radioactively labeled probes for hybridization were generated by PCR amplification of the complete *GAL1*, *INO1* or *ACT1* ORFs from genomic DNA.

Chromatin Immunoprecipitation

Mouse monoclonal antibody to the Gal4-DBD (RK5C1) was obtained from Santa Cruz Biotechnology. Anti-TBP and anti-Snf6 (for SWI/SNF) antibodies were kind gifts from M.R. Green and J. Reese. Mouse monoclonal antibody to RNA Polymerase II (CTD4H8) was obtained from Covance Research Products. SAGA (13-myc tagged Spt3) and Mediator (13-myc tagged Srb4) were immunoprecipitated with mouse monoclonal anti-Myc (9E10) antibody (Santa Cruz Biotechnology). ChIP assays were performed as described (Li et al. 2000). The immunoprecipitated DNA was amplified using quantitative PCR performed with α-32P-dCTP and then electrophoresed on 5% acrylamide gels. Reactions were visualized and quantified by PhosphorImager.

Reverse transcriptase PCR

Wild type and *swi2Δ* cells were grown to mid-log phase in YEP medium with 2% glucose, 2% galactose or 2% raffinose + 0.2% sucrose at 30°C. 10 ml of cells were harvested and total RNA was extracted as described above. First-strand cDNA was

synthesized using 2.5μg RNA, SuperScript II RNase H^- reverse transcriptase (Invitrogen), and 2pmol each of *GAL2*, *GAL3*, or *ACT1* downstream primers, following the manufacturer's instructions. Subsequently, ^{32}P-labeled PCR was performed using 2μl of the first-strand cDNA reaction, and gene-specific primer sets to determine the relative levels of *GAL2*, *GAL3*, and *ACT1* mRNA for each strain. After 14 cycles (for *ACT1*) or 25 cycles (for *GAL2* and *GAL3*) of amplification, PCR products were electrophoresed on 10% acrylamide gels.

CHAPTER III

MECHANISMS GOVERNING SHORT-TERM AND LONG-TERM TRANSCRIPTIONAL MEMORY AT YEAST *GAL* GENES

Contribution and acknowledgement:

All experiments and data described in this chapter were performed by me. I would like to acknowledge Dr. Oliver Rando (UMMS) for providing the *gal1Δ* strain and help with the protocol for nucleosome scanning ChIP. I also thank Dr. Sadanand Vodala (Brandeis University) for discussions regarding nuclear pore localization of *GAL1* and for providing the *nup2Δ* and *sac3Δ* strains.

The data presented in this chapter is being prepared for publication.

Abstract

Several recent studies have shown that yeast *GAL* genes are transcriptionally induced with faster kinetics if the gene had been previously expressed. Depending on the particular experimental regimen, *GAL* gene transcriptional memory can persist for 1-2 cell divisions in the absence of inducer (short-term memory) or for >6 cell divisions (long-term memory). Whereas short-term memory requires the SWI/SNF chromatin remodeling enzyme, long-term memory has been reported to involve numerous factors, including the Htz1 histone variant, components of the nuclear pore, and the product of the *GAL1* gene, the enzyme galactokinase. Here we have evaluated the role of Htz1, SWI/SNF, nuclear pore components, *GAL3*, and *GAL1* in both short-term and long-term memory of *GAL* genes. Our results indicate that transcriptional memory of *GAL* genes is primarily controlled by the cytoplasmic inheritance of the Gal3p and Gal1p signaling factors rather than the propagation of different chromatin states.

Introduction

Adaptability to changing growth conditions is crucial for cell survival. In order to adapt to their microenvironment cells adopt different fates by regulating signaling and transcriptional networks. Many mechanisms are used by cells to form a transcriptional memory of such gene expression changes, some of which are heritable. The ability to pass on this information to their progeny might give the daughter cells an advantage in surviving in altered environmental conditions. Transcriptional memory is often associated with changes in the chromatin of these cells and two commonly observed heritable changes involve DNA methylation and histone modification patterns at gene loci. Chromatin structure is a key component is determining the ON/OFF state of genes and has been widely implicated in metazoans as well as microbes to regulate gene expression.

Microorganisms have in their repertoire, a multitude of genes and metabolic pathways that gives them versatility in responding to nutrient stress. Microorganisms, including yeast, can turn on or off different genetic pathways that allow them to best utilize available nutrients. Such pathways consist of chromatin regulating and transcription factors as well as signaling proteins that can communicate with the environment to sense carbon sources, phosphate concentrations etc. and also internal levels of these nutrients. Often times, feedback regulation mechanisms are built into these pathways which promote systems properties like homeostasis, increased sensitivity and faster kinetics of gene activation to reach steady state expression levels.

The *GAL* gene cluster of budding yeast has been extensively studied in these respects. *GAL* genes can be broadly separated into two groups – the structural genes (*GAL1*, *GAL5*, *GAL7*, *GAL10*), that encode enzymes to metabolize galactose; and regulatory genes (*GAL2*, *GAL3*, *GAL4*, *GAL80*) that transport galactose and control expression of the structural genes. Expression of Gal1p (galactokinase), Gal7p (galactose-1-phosphate uridyl transferase) and Gal10p (uridine diphosphoglucose epimerase) enzymes is tightly regulated in the presence of different sugars and they are induced 1000-fold in the presence of galactose (Johnston et al. 1994; Lohr et al. 1995; Bhat and Murthy 2001). While the Gal4p activator and the Gal80p repressor respond specifically to presence of galactose sugar, other repressor proteins like Mig1p, Nrg1p and Nrg2p are involved in broader glucose-sensitive repression of *GAL* and other carbon-utilizing genes (Treitel and Carlson 1995; Treitel et al. 1998; Wu and Trumbly 1998; Frolova et al. 1999; Zhou and Winston 2001). The membrane-bound galactose permease Gal2p and co-inducer Gal3p regulate galactose entry and gene activation respectively. Thus Gal3p is a key signaling protein participating in feedback loops and homeostasis of the system. A very interesting property of *GAL* genes like *GAL1* galactokinase, that has become evident over the past year, is transcriptional memory of previous activation. This memory is heritable and lasts for few generations before it is lost. Studies from our and other groups have implicated factors involved in chromatin regulation, nuclear localization of *GAL1* as well as cytoplasmic pools of signaling proteins as to participate in transcriptional memory at *GAL1*, which results in faster reinduction of the gene following a brief period of glucose repression (Brickner et al.

2007; Kundu et al. 2007; Zacharioudakis et al. 2007). Interestingly, using different experimental regimens, these studies have found differences in duration of persistence of this memory.

When cells are grown in the neutral sugar raffinose and then the naïve *GAL1* gene is induced with galactose, *GAL1* transcripts are detectable by 20 minutes post-induction. However, if in these cultures, *GAL1* transcription is repressed with glucose till 6 hours and then turned back on, by transferring cells back to galactose containing medium, reinduction occurs with much faster kinetics compared to the initial round of induction. Steady-state level of transcription is observed within 5 minutes of *GAL1* reinduction. This phenomenon of rapid reactivation that follows a period of repression reflects an example of transcriptional memory (Kundu et al. 2007). Such 'short-term' transcriptional memory of *GAL1* activity persists through mitosis, requires chromatin remodeling by SWI/SNF and is ultimately lost by 6 hours of growth in the absence of galactose stimulus, or in other words, persists for approximately 3 cell cycles before being lost.

In another experimental paradigm, a different phenomenon which can be called 'long-term' memory is observed. Here, glucose grown cells are *GAL1*-induced, repressed with glucose and reinduced again by shifting cells back to galactose containing medium (Brickner et al. 2007; Zacharioudakis et al. 2007). In the first round of *GAL1* induction, cells have to overcome not only Gal80p-repression but also

glucose-repression by Mig1p, Nrg1p and Nrg2p and have to synthesize Gal4p activator at the same time. Hence induction of the naïve gene requires 3-4 hours. In contrast, *GAL1* reinduction occurs within 2 hours after glucose repression. Compared to the 'short-term' memory described above, the transcriptional response during *GAL1* reinduction is slower in this form of memory but persists for 12 hours or longer. Thus there is a distinct possibility that different molecular mechanisms are at work to provide heritable, cellular memory of recent *GAL1* transcription.

Here we re-evaluate the role of chromatin remodeling factors and cytoplasmic signaling molecules in both 'short-term' and 'long-term' transcriptional memory of *GAL* genes. We find that the cytoplasmic inheritance of the Gal1p and Gal3p signaling molecules are the primary determinant of transcriptional memory. The histone variant HTZ1 and the SWI/SNF chromatin remodeling enzyme are required for the rapid transcriptional response of the reinduced state, but this appears to be due to a change in the rate-limiting steps for *GAL1* induction.

Results

Long-term memory at GAL1 is independent of SWI/SNF or components of the nuclear pore but requires Gal1p

Transcriptional memory at *GAL1* has been defined as the ability to reinduce *GAL1* transcription with much faster kinetics compared to initial induction. We and others (Brickner et al. 2007; Kundu et al. 2007; Zacharioudakis et al. 2007) have shown that memory persists through several cell divisions during glucose repression. We previously reported that the capacity for rapid *GAL1* reactivation, where steady state levels of transcript could be detected within 5 minutes of reactivation, persisted only for 6 hours of growth in glucose. However, Brickner and colleagues (Brickner et al. 2007) reported a 'long-term' memory phenomenon, where the capacity for rapid reactivation persisted longer than 12 hours. In this set of experiments, glucose grown cultures were shifted to galactose, whereas in our case raffinose grown cultures were shifted to galactose to measure induction kinetics. Given the discrepancy in duration of memory between the two reports, we first tested if our strains (S288c background) could recapitulate the 'long-term' memory. *GAL1* transcription was induced following overnight glucose growth (Glc→Gal) in both wild type and *swi2Δ* strains to measure rates of initial induction. These cultures were then glucose repressed for 12 hours and *GAL1* and *GAL10* reinduction rate was measured again (Glc→Gal→Glc→Gal). Wild type cells showed long-term memory for *GAL10* (Fig. 1b) and *GAL1* transcription (data not shown). Surprisingly, *swi2Δ* also showed long-term memory at both *GAL1* and *GAL10* genes similar to wild type (Fig. 14a).

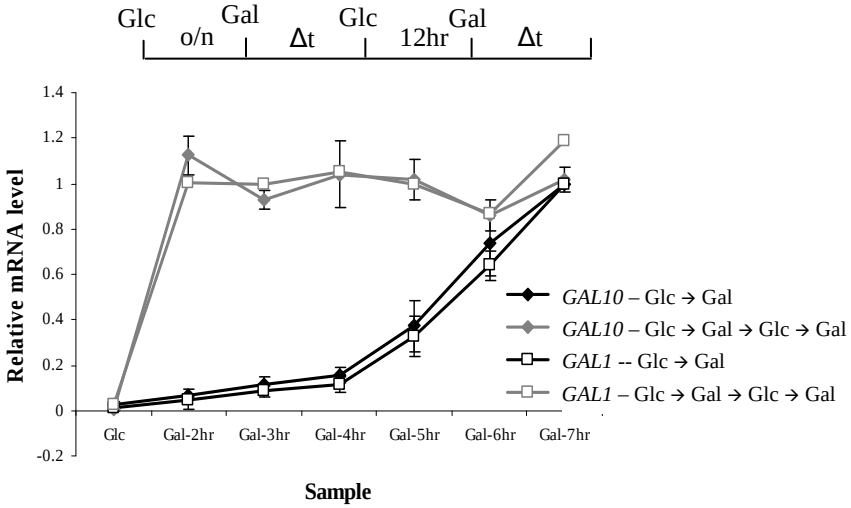
In another report (Zacharioudakis et al. 2007), Tzamarias and colleagues showed that the *GAL1* gene product, galactokinase enzyme, is essential for long term memory. It is possible that high levels of cytoplasmic Gal1p allow it to function as a weak counterpart of Gal3p co-inducer and thus provide long-term cytoplasmically heritable memory of previous *GAL1* induction. We tested their model in our strains and found that indeed *gal1Δ* lost long-term transcription memory. We monitored induction (Glc→Gal) as well as reinduction (Glc→Gal→Glc→Gal) in wild type and *gal1Δ* strains and observed that *gal1Δ* cells showed very similar rates of *GAL10* induction and reinduction following 12 hours of glucose repression suggesting that *GAL1* contributed to memory of its own transcription (Fig. 14b).

The *GAL1* locus has been shown to migrate to the nuclear periphery when it is actively transcribed and associates with the nuclear pore complex (Casolari et al. 2004; Casolari et al. 2005; Abruzzi et al. 2006; Cabal et al. 2006; Taddei et al. 2006). Also Brickner and colleagues (Brickner et al. 2007) reported that a component of the nuclear pore complex, Nup2p was required for prolonged retention of *GAL1* gene to the nuclear periphery and suggested that this could lead to long-term memory of *GAL1* transcription. We tested if Nup2p is required for long-term memory of *GAL* genes by comparing the rates of initial induction (Glc→Gal) and reinduction (Glc→Gal→Glc→Gal) of GAL10 following 12hrs of glucose repression in a *nup2Δ* strain (Fig. 14c). From our experiments, we observed that *nup2Δ* had no defect in transcriptional memory.

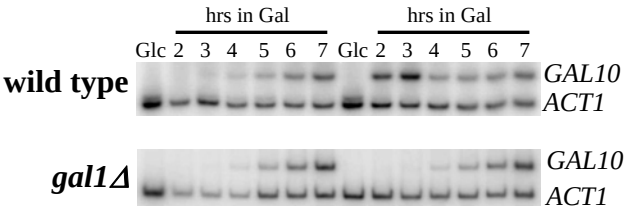
Figure 14. Long-term memory at *GAL1* requires its gene product but not SWI/SNF or nuclear pore localization. Schematic at top depicts regimen of growth in different carbon sources. Gal, 2% galactose; Glc, 2% glucose. (a) RT-PCR of *GAL1* and *GAL10* mRNA levels from a *swi2Δ* mutant strain. *GAL1* and *GAL10* reinduction is fast compared to initial induction following long term glucose repression (12hr). SWI/SNF is not required for long-term memory. (b) RT-PCR analysis of *GAL10* mRNA levels following initial induction and reinduction after long-term glucose-repression (12hr). Wild type culture showed a memory of previous *GAL10* induction, which was lost in *gal1Δ* strain. Top panel shows a representative experiment, bottom panel is an average of three experiments. (c) RT-PCR analysis of *GAL10* induction and reinduction kinetics in *nup2Δ* following long-term glucose-repression (12hr). Long-term memory does not require Nup2p. Data for above panels is averaged over three independent experiments and represented as relative fold increase over *ACT1* mRNA control normalized to a maximum value of 1. Error bars represent the standard deviation at each point.

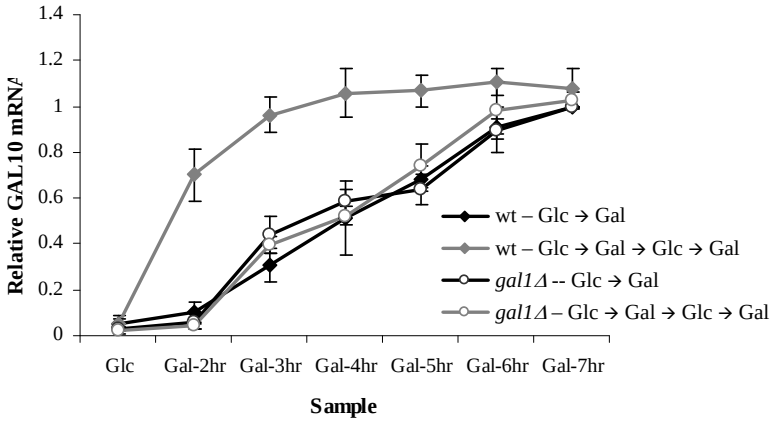
a)

swi2Δ



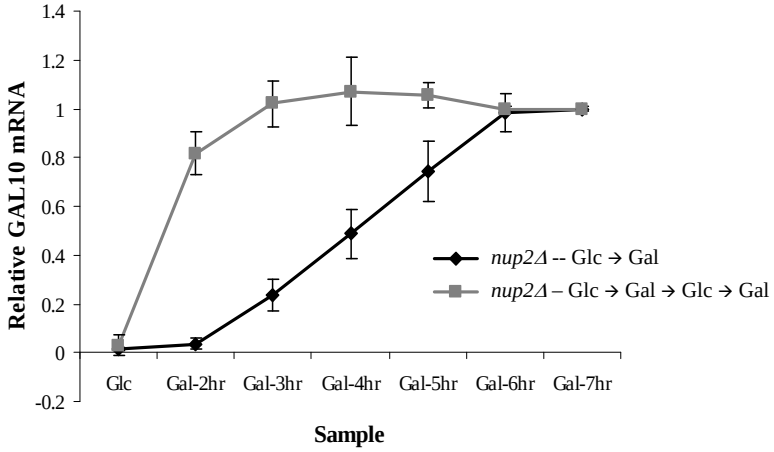
b)





c)

nup2Δ



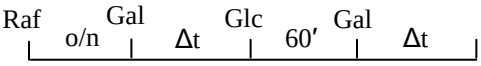
Gal1p and H2A.Z contribute to long-term memory but not to short-term memory

Since we found that SWI/SNF is required for short-term memory but dispensable for long-term memory, we wanted to determine if other factors reported to be required for long-term memory also played a role in short-term memory which was observed in the following experimental conditions – Raf→Gal→Glc→Gal, with 1 hr of glucose repression. Brickner et al. (2007) reported that long-term memory of *GAL1* transcription required the histone variant H2A.Z. After confirming these results in our strain background, we asked if H2A.Z was also required for short-term memory. Interestingly, we found that *htz1Δ* strains could rapidly reinduce *GAL1* transcription suggesting that H2A.Z was not required for short-term memory (Fig. 15a).

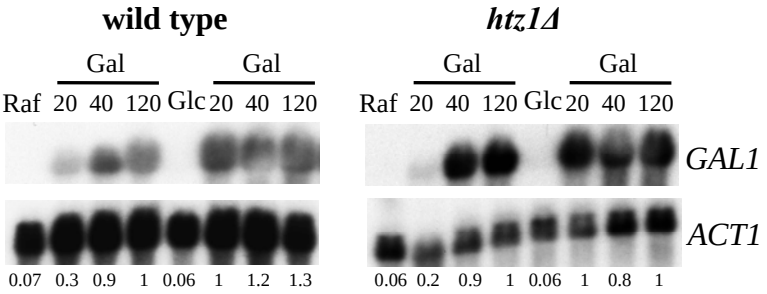
Since Brickner et al. also reported that long-term memory of transcription required Nup2p, we sought to determine the role of nuclear pore association for long-term and short-term memory. We already observed that long-term memory was intact in *nup2Δ* (Fig. 14c). Next we used two deletion strains, *nup2Δ* and *sac3Δ*, to test if nuclear pore localization was required for short-term memory. While Nup2p is a component of the nuclear pore complex, Sac3p is a nuclear pore associated protein that is involved in mRNA export from the nucleus. It has been reported (Cabal et al. 2006; Kohler et al. 2008) that localization of the active *GAL1* gene to the nuclear periphery is compromised in both these strains. Significantly, we observed no defect in either *nup2Δ* or *sac3Δ* strains for rapid *GAL1* reactivation or short-term memory (Fig. 15b).

Next, we tested if Gal1p was also required for short-term memory. As expected, wild type cells showed robust short-term memory at *GAL10* (Fig. 15c). However when we tested the *gal1Δ* strain, we observed this strain rapidly reactivated *GAL10* transcription following 1hr of glucose repression. Thus we concluded that a high cytoplasmic level of Gal1p is not essential for transcriptional memory of *GAL10* expression after a brief period of repression. These results revealed that the factors required for short-term and long-term memory are distinct.

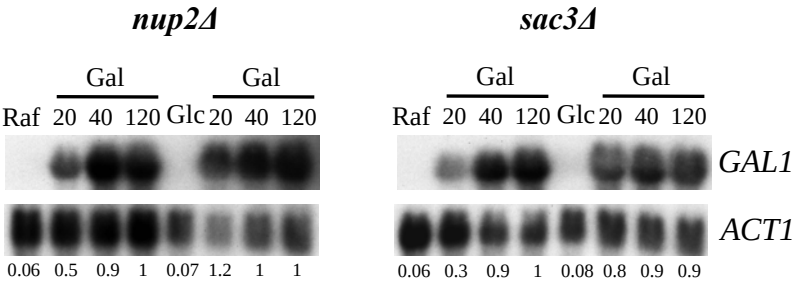
Figure 15. Short-term memory does not require H2A.Z, nuclear pore localization or Gal1p. Schematic at top depicts regimen of growth in different carbon sources. Raf, 2% raffinose; Gal, 2% galactose; Glc, 2% glucose. (a) Northern analysis of wild type (left) and *htz1Δ* (right) showing rapid *GAL1* reinduction following short term glucose repression (1hr). (b) Short-term *GAL1* memory also does not require localization of the *GAL1* gene to nuclear pores. Nuclear pore-localization of actively transcribed *GAL1* locus is defective in *nup2Δ* (left) and *sac3Δ* (right) mutants. (c) Northern analysis of *GAL10* RNA levels in wild type (left) and *gal1Δ* (right) strains during initial induction and reinduction, following short-term glucose repression (1hr). *gal1Δ* strain also can rapidly reinduce *GAL10* transcription and is thus not required for short-term memory. Top panel shows a representative experiment, bottom panel is an average of three experiments. Error bars represent standard deviation. In (a), (b) and (c), the bottom panel represents an *ACT1* loading control for total RNA levels. The numbers indicate fold induction of *GAL1* or *GAL10* transcripts normalized over *ACT1* transcripts with the maximally induced state set to a value of 1 and are averaged over three experiments.


Raf
o/n
Gal
Δt
Glc
60′
Gal
Δt

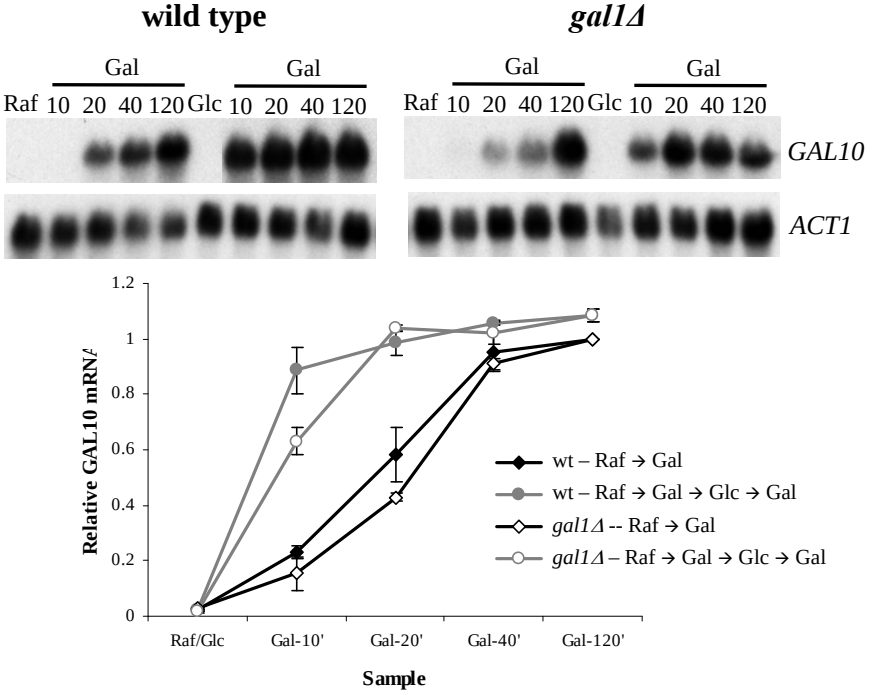
a)


wild type
htz1Δ
Gal
Gal
Raf 20 40 120 Glc 20 40 120
GAL1
ACT1
0.07 0.3 0.9 1 0.06 1 1.2 1.3
0.06 0.2 0.9 1 0.06 1 0.8 1

b)


nup2Δ
sac3Δ
Gal
Gal
Raf 20 40 120 Glc 20 40 120
GAL1
ACT1
0.06 0.5 0.9 1 0.07 1.2 1 1
0.06 0.3 0.9 1 0.08 0.8 0.9 0.9

c)


wild type
gal1Δ
Gal
Gal
Raf 10 20 40 120 Glc 10 20 40 120
GAL10
ACT1
Relative GAL10 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
Raf/Glc
Gal-10'
Gal-20'
Gal-40'
Gal-120'
Sample
wt – Raf → Gal
wt – Raf → Gal → Glc → Gal
gal1Δ -- Raf → Gal
gal1Δ – Raf → Gal → Glc → Gal

SWI/SNF promotes rapid PIC loading but does not generate alternate nucleosome positions

We had reported (Kundu et al. 2007) that chromatin remodeling by SWI/SNF is required for short-term transcriptional memory at *GAL1*. We proposed a model in which SWI/SNF could be establishing alternate nucleosome positions at *GAL1* promoter during glucose repression. If so, then this would facilitate faster PIC assembly on the promoter during reinduction and thus faster appearance of transcripts. To test if chromatin remodeling by SWI/SNF could facilitate faster PIC assembly on the *GAL1* promoter at the time of reinduction, we monitored loss of RNA Polymerase II during glucose repression and reloading (during *GAL1* reinduction). We observed that wild type cells were able to recruit RNA Polymerase II much faster to the promoter than *swi2Δ* (Fig. 16a, left). While we saw greater than 10-fold enrichment for RNA Polymerase II within 10 minutes of *GAL1* reinduction in wild type, this was severely compromised in *swi2Δ*. We also observed that both TBP and SWI/SNF were recruited faster to the *GAL1* locus during reinduction (data not shown). From these results it can be concluded that the transcription machinery was loaded faster during reinduction in wild type cells but not in *swi2Δ*. This was not due to a defect in reloading of nucleosomes during glucose repression in *swi2Δ* strain. A ChIP for histone H3 showed that promoter nucleosomes were regained with equal efficiency in both wild type and *swi2Δ* (Fig. 16a, right).

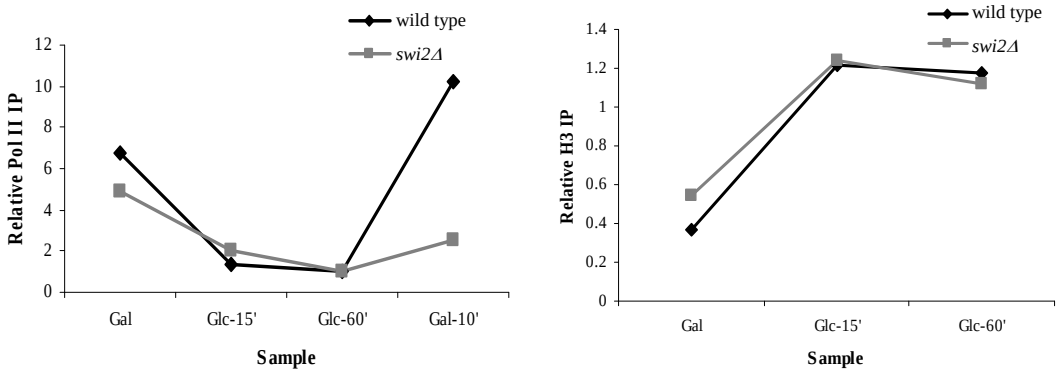
Since SWI/SNF indeed seemed to aid PIC re-loading, we decided to test our model that SWI/SNF dependent chromatin remodeling was generating alternate nucleosome positions at the *GAL1* promoter at the time of short-term glucose repression. We mapped the positions of the two promoter proximal nucleosomes at *GAL1* locus (see Fig. 16b for schematic representation) by nucleosome scanning ChIP. Wild type cells were grown overnight in glucose media (long-term repressed) and collected after crosslinking. Alternatively, raffinose grown cells were shifted to 2% galactose for 2hrs to fully induce *GAL1*. They were then shifted to 2% glucose for 1hr to repress *GAL1* gene (short-term repressed) and then collected after crosslinking. Mononucleosomal chromatin was prepared from both samples and used for ChIP with α-histone H3 antibody. Quantitative PCR was done with primer pairs scanning approximately 300bp around the predicted dyads of nucleosome B (Fig. 16c, left) and nucleosome C (Fig. 16c, right).

In long-term repressed cells, nucleosomes B and C were found to be positioned around their predicted dyads protecting ~160bp of DNA. When mononucleosomal chromatin was prepared from cells growing in galactose, these nucleosomes could not be seen. However, we observed that both nucleosomes returned to their original positions when the gene was briefly repressed. This was shown by the near overlap in the profiles of both nucleosomes B and C when compared between long-term repressed and short-term repressed cultures. Thus no alternate nucleosome positions could be detected (Fig. 16c). We repeated the same set of experiments with a *swi2Δ* strain and

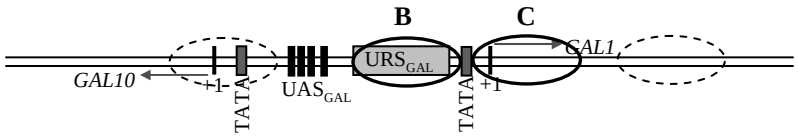
obtained similar results confirming that SWI/SNF was not involved in nucleosome positioning at the *GAL1* promoter during glucose repression (Fig. 16d). Thus we concluded that the role of SWI/SNF in promoting rapid *GAL1* reinduction was not by generating a novel pattern of nucleosome positions at the *GAL1* promoter.

Figure 16. SWI/SNF promotes nucleosome loss and RNA Polymerase II loading at *GAL1* promoter. (a) Left, RNA Polymerase II ChIP in wild type and *swi2Δ* strains showing that faster recruitment during *GAL1* reinduction is dependent on SWI/SNF. Right, histone H3 ChIP in wild type and *swi2Δ* strains to measure nucleosome occupancy at *GAL1* promoter. Loss of SWI/SNF does not inhibit kinetics of promoter nucleosome regaining during glucose repression. However SWI/SNF promotes nucleosome loss and RNA Polymerase II loading during reinduction. RNA Polymerase II and H3 levels were tested at the *GAL1* promoter and normalized to a telomere sequence (Chr VI-70bp from right end). (b) Schematic representation of the *GAL1-10* regulatory region. UAS_{GAL} marks the Gal4p binding sites. URS_{GAL} is the binding site for glucose dependent repressor, Mig1p. Ovals represent previously mapped nucleosome positions. Ovals shown in solid lines represent *GAL1* promoter nucleosomes that are mapped in (c) and (d). TATA represents the TBP binding sites and +1 represents the transcription start sites. (c) Nucleosome scanning ChIP with histone H3 antibody in wild type strain for promoter nucleosomes **B** (left) and **C** (right). Black lines represent overnight glucose grown cultures and grey lines represent short-term (1hr) glucose repressed cultures following brief *GAL1* induction. On X axes of graphs, B1-B10 represent 10 primer pairs spanning positions (-302) to (+3) from translation start site. C1-C10 represent 9 primer pairs spanning positions (-148) to (+160) from translation start site. On the Y axis is plotted the relative H3 %IP normalized to a maximum value of 1. (d) Same as in (c) but with *swi2Δ* strain.

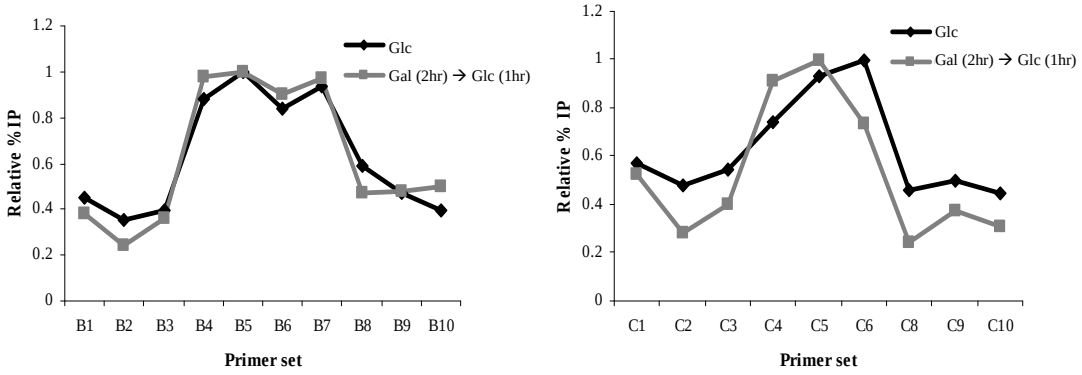
a)



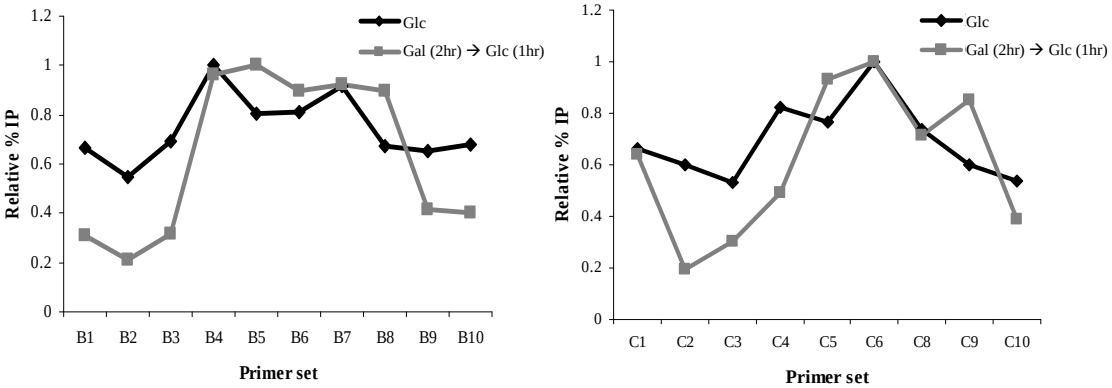
b)



c) wild type



d) *swi2Δ*



Gal3p co-inducer overexpression leads to rapid GAL1 induction in wild type

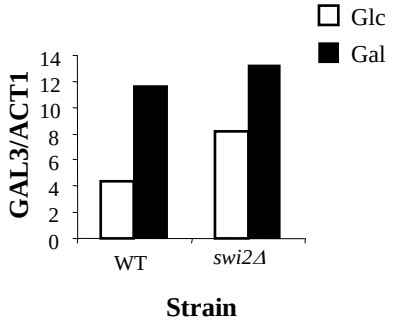
We considered an alternate hypothesis in which the different rates of *GAL1* induction are controlled by the rates of signaling molecule accumulation (k_1) and chromatin remodeling (k_2) (see model in Fig. 20). In this model, transcriptional memory is due to the cytoplasmic inheritance of the Gal3p or Gal1p signaling molecules which are induced to high levels in the first round of expression. In the initial round, the signaling step is slow compared to remodeling (i.e. $k_2 > k_1$). But in the second round, signaling is very rapid due to already high levels of Gal3p or Gal1p which had built up in the cytoplasm during initial induction. Hence the signal amplification step becomes fast and results in rapid PIC assembly and gene activation (i.e. $k_2 < k_1$).

To rule out the simple explanation that induction of Gal3p expression in the first round was defective in *swi2Δ* strains, we did RT-PCR to measure induction of *GAL3* (Fig. 17a) gene expression in wild type and *swi2Δ* and saw that expression of signaling proteins was not compromised in *swi2Δ*. One prediction of this model is that increasing levels of signaling molecules prior to the first round of induction should uncover a kinetic role for SWI/SNF. To test this idea, both wild type and *swi2Δ* strains were transformed with a high copy number plasmid driving Gal3p expression from a constitutive *ADH1* promoter or with the appropriate vector control and Gal3p expression levels from these transformants were monitored by RT-PCR (Fig. 17b). Northern analyses were performed to compare *GAL1* induction and reinduction kinetics. The induction and reinduction kinetics of wild type and *swi2Δ* strains transformed by

vector plasmid controls were similar to the untransformed strains (Fig. 18a). As predicted by our model, constitutive expression of Gal3p in wild type greatly increased the rate of initial induction such that accumulation of *GAL1* transcripts was nearly indistinguishable form rate of reinduction (Fig. 18b, left). Strikingly, rapid *GAL1* reinduction was not rescued in *swi2Δ* strain even by constitutive expression of Gal3p (Fig. 18b, right) clearly suggesting the requirement of a SWI/SNF dependent chromatin remodeling step for efficient *GAL1* activation (k_2 in Fig. 20). We did observe, as expected, that the general *GAL1* activation rate in both rounds was equally improved in Gal3p overexpressing *swi2Δ* strain as compared to the one containing the vector control.

Figure 17. RT-PCR analyses for *GAL3* mRNA levels from strains in different growth media. (a) Both wild type and *swi2Δ* strains express basal levels of *GAL3* mRNA in glucose. In galactose, expression is induced to similar levels. (b) *GAL3* mRNA levels were measured in strains indicated on the X axis of the graph. 2% raffinose grown and 2% galactose grown cultures were compared. '+ Gal3' indicates strain transformed with P_{ADH1}-*GAL3* plasmid. '+ empty' indicates strain transformed with appropriate vector control. *GAL3* mRNA levels were normalized to *ACT1* mRNA levels. No. of PCR cycles for *GAL3* mRNA – 25; no. of cycles for *ACT1* mRNA – 12.

a)


Glc
Gal
GAL3/ACT1
14
12
10
8
6
4
2
0
WT
swi2Δ
Strain

b)

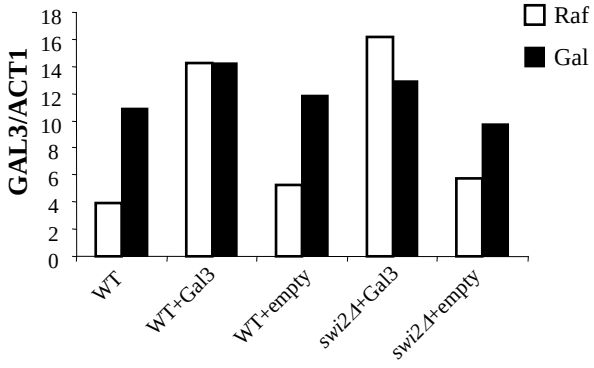
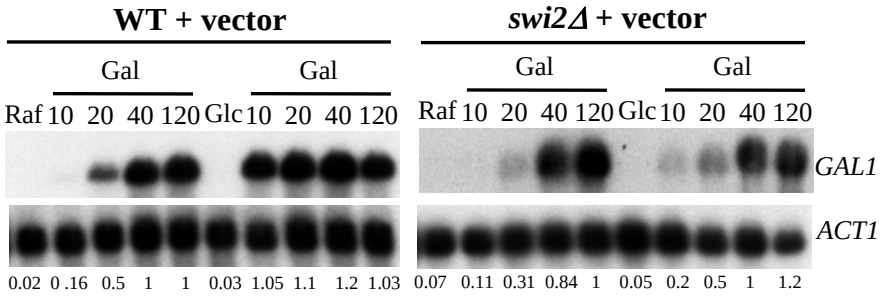
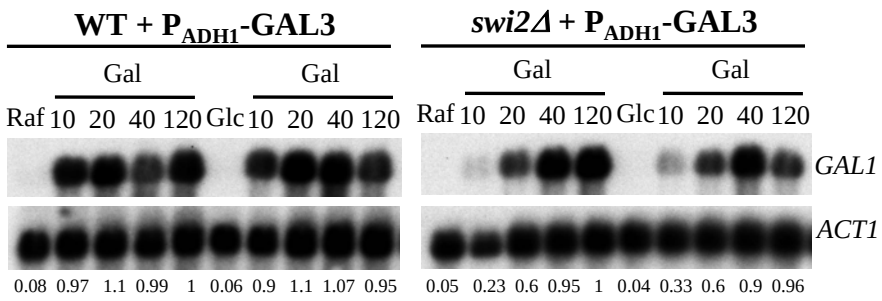

Raf
Gal
GAL3/ACT1
18
16
14
12
10
8
6
4
2
0
WT
WT+Gal3
WT+empty
swi2Δ+Gal3
swi2Δ+empty

Figure 18: Gal3p co-inducer overexpression leads to rapid *GAL1* induction in wild type. (a) Northern analysis in wild type (left) and *swi2Δ* strain (right) transformed with empty plasmid vector control. (b) Northern analysis in wild type (left) and *swi2Δ* strain (right) constitutively overexpressing Gal3p from a plasmid. Constitutive expression of Gal3p rapidly turns on *GAL1* transcription even upon initial induction in wild type. Memory is not rescued in *swi2Δ* though general rates of induction and reinduction are elevated. All Northerns were subsequently probed for *ACT1* as a loading control. The numbers indicate fold induction of *GAL1* transcripts over *ACT1* and averaged over three experiments.

a)


WT + vector
swi2Δ + vector
Gal
Gal
Raf 10 20 40 120 Glc 10 20 40 120
GAL1
ACT1
0.02 0 .16 0.5 1 1 0.03 1.05 1.1 1.2 1.03
0.07 0.11 0.31 0.84 1 0.05 0.2 0.5 1 1.2

b)

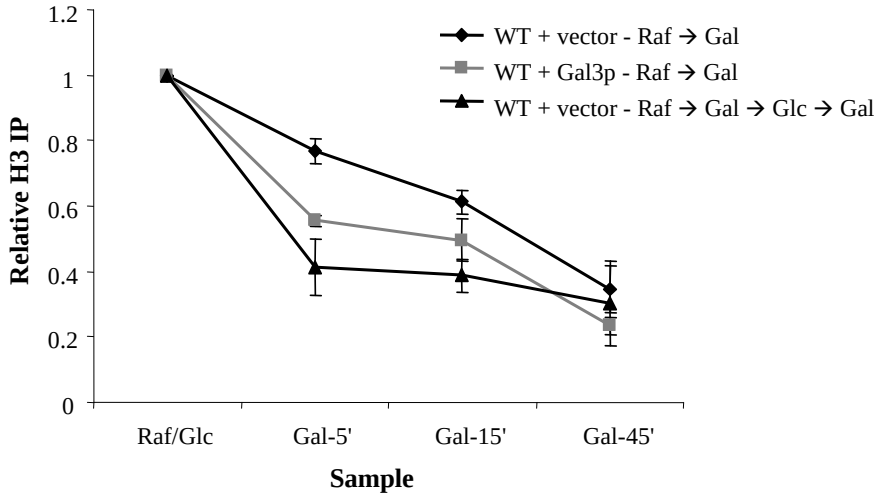

WT + P ADH1 -GAL3
swi2Δ + P ADH1 -GAL3
Gal
Gal
Raf 10 20 40 120 Glc 10 20 40 120
GAL1
ACT1
0.08 0.97 1.1 0.99 1 0.06 0.9 1.1 1.07 0.95
0.05 0.23 0.6 0.95 1 0.04 0.33 0.6 0.9 0.96

Rate of nucleosome loss reflects faster GAL1 induction rate with constitutive Gal3p

To measure the rate of activation dependent nucleosome loss from *GAL1* promoter, histone H3 ChIPs were performed using different strains. As expected, nucleosome loss was much rapid during *GAL1* reinduction compared to initial induction in wild type (Fig. 19a). Whereas complete nucleosome loss after initial induction required upto 45 min (Raf→Gal), this was achieved within 5 min during reinduction (Raf→Gal→Glc→Gal). Constitutive expression of Gal3p greatly enhanced nucleosome loss even during initial induction (Raf→Gal) suggesting that robust Gal3p signaling promoted rapid recruitment of chromatin remodelers like SWI/SNF and the PIC to the promoter. In contrast, loss of promoter nucleosomes was slower in *swi2Δ* compared to wild type and enhanced only slightly by constitutive Gal3p expression (Fig. 19b). Taken together, these results clearly indicated that a high residual level of Gal3p alone from previous activation events was not sufficient to promote rapid reactivation of *GAL1* transcription. Instead, rapid reactivation following short-term repression of *GAL1* showed a clear dependence on chromatin remodeling by SWI/SNF in addition to optimum cytoplasmic signaling to achieve efficient rates of transcription.

Figure 19: Rate of nucleosome loss reflects enhanced *GAL1* induction kinetics with constitutive expression of Gal3p. (a) H3 ChIP in wild type with and without constitutive expression of Gal3p. Promoter nucleosomes are lost more rapidly during GAL1 reinduction. Constitutive Gal3p enhances rate of nucleosome loss during initial induction nearly to rate of nucleosome loss seen during reinduction in wild type (compare 'wild type+Gal3p' with 'wild type+vector – reinduction'). (b) H3 ChIP in *swi2Δ* with and without constitutive expression of Gal3p. Rate of nucleosome loss is increased only slightly compared to appropriate strains in wild type background during initial induction. In all ChIPs, H3 %IP was normalized to a telomere sequence and then normalized to a maximum value of 1.

a)


1.2
1
0.8
0.6
0.4
0.2
0
Relative H3 IP
WT + vector - Raf → Gal
WT + Gal3p - Raf → Gal
WT + vector - Raf → Gal → Glc → Gal
Raf/Glc
Gal-5'
Gal-15'
Gal-45'
Sample

b)

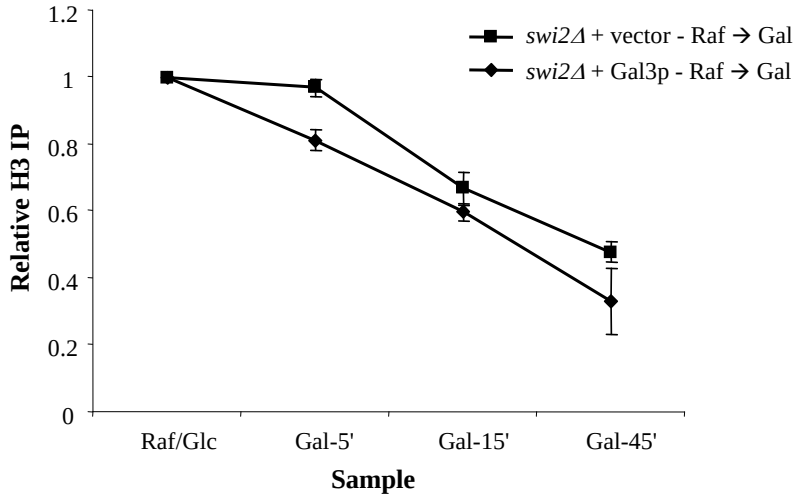
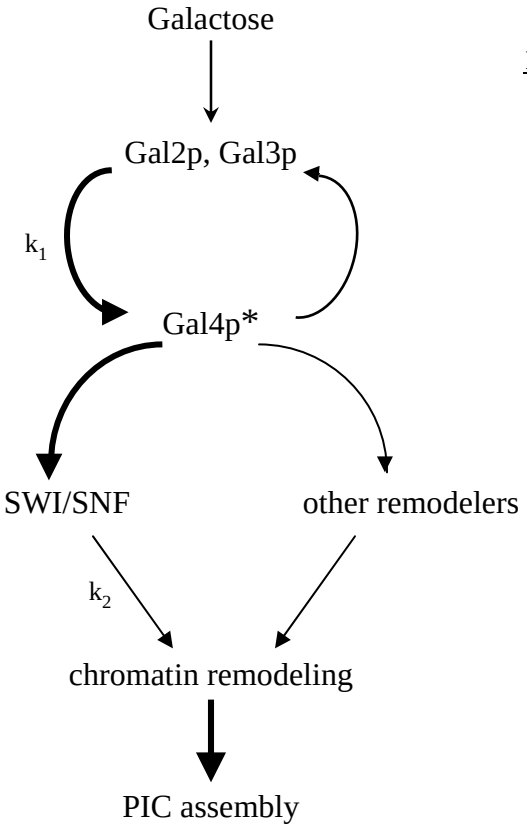

1.2
1
0.8
0.6
0.4
0.2
0
Relative H3 IP
swi2Δ + vector - Raf → Gal
swi2Δ + Gal3p - Raf → Gal
Raf/Glc
Gal-5'
Gal-15'
Gal-45'
Sample

Figure 20: Two-step model for optimum induction kinetics of *GAL1* transcription. Extracellular galactose in transported into the cell nucleus via signaling proteins Gal2p and Gal3p. This activates Gal4p activator allowing it to recruit SWI/SNF. Chromatin remodeling by SWI/SNF leads to rapid loss of promoter nucleosomes and PIC assembly, resulting in *GAL1* induction. Gal4p activation (shown as Gal4p*) leads to increased production of Gal2p and Gal3p, thereby amplifying the signal. k_1 and k_2 represent the rate determining steps of this pathway. In the absence of SWI/SNF, other non-specific chromatin remodelers may cause promoter nucleosome loss. This eventually opens up the chromatin and allows slower PIC assembly and transcription initiation. In the initial round of induction, Gal2p and Gal3p levels need to be built up through expression of those genes, making k_1 the rate determining step. After a brief period of glucose repression, when *GAL1* is reinduced, cytoplasmic levels of signaling proteins is already high. Hence k_1 becomes fast making k_2 (chromatin remodeling) the slowest and thus rate limiting step.



1st Round Induction

$k_2 > k_1$

Re-induction

$k_1 > k_2$

Discussion

Heritable memory of transcriptional choice and cell fate is a hallmark of differentiated tissue and cell types in metazoans. Various mechanisms, primarily DNA methylation and chromatin modifications have been implicated for such memory. While the unicellular budding yeast does not have the potential to undergo differentiation into multiple cell types, it does have tremendous capacity to adapt to changes in its immediate environment by changing its transcriptional profile. Many groups have studied the dynamic regulation of metabolic genes of *Saccharomyces cerevisiae* in conditions of nutrient stress (Ramsey et al. 2006; Ronen and Botstein 2006; Ajo-Franklin et al. 2007; Acar et al. 2008). The galactose utilization (*GAL*) genes have been particularly well studied (Lohr and Lopez 1995; Bhat and Murthy 2001). More recently, we and others have shown that in addition to the intricate positive and negative control of the *GAL* gene cluster, these genes exhibit a unique property which is cellular memory of previous gene activation (Acar et al. 2005; Brickner et al. 2007; Kundu et al. 2007; Zacharioudakis et al. 2007).

For our studies of *GAL1* induction, we have induced cells which had been growing in log phase in a neutral sugar, raffinose. The advantage of this approach is that growth in raffinose relieves *GAL1* of the additional layer of active glucose repression. Thus, when we measured *GAL1* induction, we were able to interrogate the kinetics of the galactose specific steps exclusively. Since non-specific glucose repression is already relieved in raffinose-grown cells, *GAL* genes can respond more rapidly to addition of

galactose in the medium. This approach also enabled us to detect the very rapid reactivation of *GAL1* (within 5 minutes) after brief repression, which we called transcriptional memory. We also demonstrated that this phenomenon was stable through mitosis, lasted for about 6 hours and required chromatin remodeling by SWI/SNF, suggesting that regulating chromatin organization was at least in part responsible for memory. However, the groups of Brickner and Tzamarias measured *GAL1* induction kinetics starting with a glucose-repressed system. Thus in their case, activation required not only *GAL* system-specific steps, but also (i) alleviation of glucose repression by Mig1p, Nrg1p, Nrg2p; and (ii) synthesis of Gal4p activator. By this approach, while both *GAL1* induction and reinduction occurred in the time scale of hours instead of minutes, transcriptional memory persisted longer – at least 12hrs. In this study we sought to distinguish between this 'long-term' memory and the 'short-term' and SWI/SNF dependent memory that we observed.

First we asked if SWI/SNF, we had previously shown to be required for short-term memory (Kundu et al. 2007), also played a role in long-term memory and found to our surprise that in fact long-term memory was independent of SWI/SNF dependent chromatin remodeling (Fig. 14a). Recently, Tzamarias and colleagues showed through elegant experiments that Gal1p provided 'long-term' memory of its own recent transcriptional activity. *S.cerevisiae* Gal1p (galactokinase) and Gal3p (galactose-binding co-inducer) are believed to have diverged from a common ancestral protein that is still present in *Kluveromyces lactis* (Meyer et al. 1991; Platt et al. 2000; Hawkins and

Smolke 2006; Hittinger and Carroll 2007). As a result, while *S.cerevisiae* Gal3p has lost galactokinase activity due to a point mutation in its catalytic domain, Gal1p has a much lower affinity for binding galactose compared to Gal3p. Thus Gal1p can act as a weak co-inducer protein when present in relatively large amounts in the cell. Considering that *GAL1* is induced ~1000-fold by growth in galactose (Johnston et al. 1994), high Gal1p concentrations likely can autoregulate gene expression. As reported (Zacharioudakis et al. 2007) we were able to see loss of long-term memory from *gal1Δ* cells (Fig. 14b). However to our surprise, *gal1Δ* had no defect in short-term memory (Fig. 15c).

We then tested if we could also detect H2A.Z dependence of 'long-term' memory in our strains, to rule out the simple possibility of background specific peculiarities. As reported by Brickner and colleagues, we did observe failure of rapid *GAL1* reinduction in *htz1Δ* cells. Nevertheless, we determined if *htz1Δ* also lost short-term memory and saw that these strains were as competent as wild type cells in our experimental regimen (Fig. 15a). We also ruled out the need for nuclear pore localization of actively transcribed *GAL1* gene as a requirement for short-term memory since mutants previously reported to have defects in pore localization of *GAL1* had no effect on memory (Fig. 15b).

We proceeded to elucidate the mechanism for short-term memory at *GAL* genes. We tested two models for this purpose. Our first model was based on the requirement of SWI/SNF for rapid reactivation. We hypothesized that the function of SWI/SNF was to

establish alternate positions for *GAL1* promoter nucleosomes that were reloaded during glucose repression. To test this model, we compared promoter nucleosome positions between short-term (1hr) repressed and glucose-grown cells. Nucleosome positions at *GAL1-10* locus have been extensively mapped (Lohr 1984; Lohr and Lopez 1995). We used this information for doing higher resolution nucleosome scanning ChIPs but surprisingly found that promoter nucleosomes returned to their original (as mapped in glucose-grown cells) positions within 1hr of glucose repression in both wild type and *swi2Δ* cells (Fig. 16b, c). Thus contrary to our hypothesis, SWI/SNF was not involved in maintaining alternate nucleosome positions at the *GAL1* locus. Instead, presence of SWI/SNF seemed to aid faster RNA Polymerase II loading (Fig. 16a) and therefore faster PIC assembly.

These results led us to our second model that *GAL1* activation was a process whose rate was determined by two slow steps, with the rate of the slowest step determining the kinetics of the final output, i.e. appearance of *GAL1* transcripts (Fig. 20). During initial induction, signal transduction (via Gal2p and Gal3p signaling proteins) was the slowest and rate determining step. On the other hand at reinduction, sufficient cytoplasmic levels of signaling proteins made this step a rather fast one. We hypothesized that the new rate determining step during *GAL1* reinduction was chromatin remodeling by SWI/SNF (possibly to evict promoter nucleosomes). The simplest test for this hypothesis was to constitutively express the Gal3p co-inducer and compare *GAL1* induction and reinduction rates. As we expected, wild type cells

overexpressing Gal3p could rapidly lose promoter nucleosomes and turn on *GAL1* transcription within minutes of adding galactose to the growth medium, thus clearly suggesting that building up the galactose signal was indeed the rate limiting step for initial *GAL1* induction (Figs. 18 and 19). Strikingly, *swi2Δ* cells overexpressing Gal3p continued to have slow *GAL1* induction as well as reinduction. This result signified that SWI/SNF dependent chromatin remodeling was indeed the second rate determining step in this two-step process.

What then is 'memory' of previous activation? Based on our and others' findings we believe that while chromatin remodeling enhances efficiency of gene activation, the heritable memory of this event is cytoplasmic in nature, i.e. cellular levels of signaling proteins like Gal3p. In this context, it is intriguing to note that the half-life of Gal3p is approximately 4-5 hours, which corresponds very well with the duration for which we observe 'transcriptional memory'. From a more general perspective we speculate that such systems evolved to give yeast cells better adaptability and growth advantage in an unpredictable and rapidly changing natural environment.

Materials and methods

Yeast strains, media and culture conditions

Strains used in this study are isogenic derivatives of the S288c background. Genotypes are provided in the list of strains used. *S. cerevisiae* liquid cultures were grown at 30°C in YEP (1% yeast extract, 2% bacto-peptone) media supplemented with 2% glucose, 2% galactose or 2% raffinose + 0.2% sucrose depending on whether *GAL1* activation or repression was required. For Gal3p overexpressing strains, wild type and *swi2Δ* strains were transformed with 2-micron plasmids expressing full length Gal3p from a constitutive *ADH1* promoter or the relevant vector control. Transformants were selected and grown on SD plates under URA selection.

RNA isolation and analysis

Total RNA was isolated from yeast grown to logarithmic phase in appropriate media by hot phenol extraction method. Concentration of RNA was estimated by measuring A_{260} after dissolving it in diethyl pyrocarbonate-treated water. 10µg of total RNA from each sample was electrophoresed on 1% formaldehyde agarose gels and Northern blotting was done. The housekeeping gene *ACT1* was used as loading control. Radioactively labeled probes for hybridization were generated by PCR amplifying the complete *GAL1*, *GAL10* or *ACT1* ORF from genomic DNA.

Reverse transcriptase PCR

Cells were grown to mid-log phase in YEP medium with 2% glucose, 2% galactose or 2% raffinose + 0.2% sucrose at 30°C. 10 ml of cells were harvested and total RNA was extracted as described above. First-strand cDNA was synthesized using 2.5µg RNA, SuperScript II RNase H^- reverse transcriptase (Invitrogen), and 2pmol each of downstream primers designed for genes of interest, following the manufacturer's instructions. Subsequently, ^{32}P-labeled PCR was performed using 2µl of the first-strand cDNA reaction, and gene-specific primer sets to determine the relative levels of *GAL1*, *GAL3*, *GAL10* and *ACT1* mRNA for each strain. After 12 cycles (for *GAL1*, *GAL10* and *ACT1*) or 25 cycles (for *GAL3*) of amplification, PCR products were electrophoresed on 10% acrylamide gels. Reactions were visualized by PhosphorImager.

Chromatin Immunoprecipitation

Rabbit polyclonal antibody to C-terminus of Histone H3 (ab1791) was obtained from Abcam Inc. Mouse monoclonal antibody to RNA Polymerase II (CTD4H8) was obtained from Covance Research Products. ChIP assays were performed as described by (Li et al. 2000). The immunoprecipitated DNA was amplified using quantitative PCR performed with α-^{32}P-dCTP and then electrophoresed on 5% acrylamide gels. Reactions were visualized and quantified by PhosphorImager.

Mononucleosome preparation

Samples (from 100 ml of culture at A_{600} ~ 0.7) were crosslinked for 30 min with 37% formaldehyde (final concentration of 2%) at 30°C. Reactions were quenched by adding 2.5M Glycine to a final concentration of 125mM. Cell pellets were collected and washed with water to remove residual media. Mononucleosomes were prepared as described by (Dion et al. 2007). An aliquot of this sample was de-proteinized and crosslinks were reversed. Phenol-chloroform extraction was done and samples were ethanol precipitated. The resulting pellet was resuspended and treated with RNaseA (1μg for 1hr at 37°C) to remove all RNA. Samples were then electrophoresed on 1.5% agarose gels to determine the best titration that yielded mononucleosomal DNA.

Nucleosome scanning analysis

This method was adapted from Sekinger et al., (Sekinger et al. 2005), with modifications. Briefly, mononucleosomal chromatin was prepared as described above. This material was used for IP with Histone H3 antibody as previously described. The immunoprecipitated DNA was amplified using quantitative PCR and a set of overlapping primer pairs that were staggered 20bp relative to each other and covering an approximately 300bp region of DNA. The products of all primer pairs were approximately 100bp long. Efficiency of each primer pair was assayed by performing quantitative PCR with genomic DNA.

CHAPTER IV

CONCLUDING REMARKS

Cellular memory of transcriptional choice

There is an emerging concept of cellular memory in budding yeast, in the light of current studies on genes of the *GAL* regulon in *S.cerevisiae* (and also studies on other systems such as white-opaque colony color switching in *C.albicans*). One can imagine that cellular memory of gene expression patterns in response to a change in environmental conditions can be very useful information for survival of a yeast population. A mechanism to propagate such transcriptional memory to daughter cells through mitosis could give a significant growth advantage to this population over another 'naïve population' facing similar environmental changes for the first time. Typical environmental changes could be nutrient stress eg. availability of a new carbon source; or others like heat shock and cell damaging agents.

What could be the mechanism for inheritance of transcriptional memory in yeast? There are two possibilities – first, cytoplasmic inheritance, where a small molecule or protein or RNA expressed in response to the environmental cue has a half-life longer than the yeast life cycle and thus can be transmitted to daughter cells as the cytoplasm of the mother cells divides during mitosis. The second possibility is inheritance of a chromatin state where chromatin remodeling enzymes, histone modifications and/or histone variants can be used to stably mark transcriptionally active or silenced loci. Metazoans have evolved a wide variety of mechanisms such as DNA-cytosine methylation, incorporation of histone modifications and histone variants, to alter chromatin configuration in order to stably mark the transcriptional status of loci or

in other words, to maintain transcriptional memory. Some of these mechanisms allow chromatin modifications to be replicated on newly synthesized DNA at the time of DNA replication, leading to inheritance of chromatin states as a mechanism of transmitting transcriptional memory.

As described in Chapter I (Introduction), studies from our group and others on the *GAL1*, *GAL7* and *GAL10* genes of *S.cerevisiae* have led to discovery of transcriptional memory at these genes, which is defined as rapid reinduction of transcription from these genes compared to the initial round of induction, following a period of transcriptional repression (Brickner et al. 2007; Kundu et al. 2007; Zacharioudakis et al. 2007). Depending on the experimental conditions, two forms of transcriptional memory can be observed that I have distinguished as 'short-term' (lasting 4-6 hours) and 'long-term' (lasting 12 hours) memory. They can be separated by the factors required for forming memory at each case. The aim of this work has been to characterize this phenomenon and describe its mechanism.

What does transcriptional memory at *GAL1* mean?

One of the first questions that intrigued me was whether transcriptional memory at *GAL1* was just a property of cells that were growing in galactose or could this memory be passed on to future generations even in the absence of the memory-inducing signal? Two lines of evidence suggested that memory was heritable. Firstly, cultures growing asynchronously in glucose, where *GAL1* was induced briefly and then

repressed, retained the ability for rapid *GAL1* reactivation till ~6 hours. Secondly, when synchronized cells were briefly galactose-induced and then repressed, they could grow and divide after glucose repression to produce daughter cells that inherited memory of their mothers' galactose exposure. We explored potential mechanisms that could lead to rapid reactivation and also lead to its persistence through S-phase. There is a lot of exciting evidence regarding the contribution of chromatin state, especially histone modifications and histone variants, to stable transcriptional memory during development in higher eukaryotes (Henikoff et al. 2004; Grimaud et al. 2006; Hake and Allis 2006; Nightingale et al. 2006; Schuettengruber et al. 2007). Since the yeast *GAL1* locus also recruits chromatin remodeling enzymes and exhibits transcription associated chromatin modifications, we explored the possibility that one or more of these chromatin modifications during the initial round of gene activation could potentiate subsequent reinduction and memory. We tested different transcription associated histone modifications but found that none could cause transcriptional memory at *GAL1*, including Set1p mediated histone H3K4-me mark, which is known to mark transcriptionally active loci in metazoans (Roth et al. 2001; Boa et al. 2003). The histone H2A variant H2A.Z, which occupies *GAL1* promoter proximal nucleosomes, also did not appear to play a role in short-term memory. However, interestingly, H2A.Z appears to be required for long-term memory though the mechanism for this is not clear (Brickner et al. 2007); and our unpublished results in Chapter III).

Surprisingly, the ATP-dependent remodeling enzyme SWI/SNF seemed to be required for short-term *GAL1* memory. This was interesting because it had previously been shown that though SWI/SNF is recruited to the *GAL1* locus during transcriptional activation, it is dispensable (Krebs et al. 2000; Lemieux and Gaudreau 2004). We proposed that SWI/SNF may play a role not during the initial round of induction but probably at very early stages of glucose repression to establish alternate nucleosome positions for the returning promoter nucleosomes. Support for this hypothesis came from our genetic analysis of transcriptional memory in *isw*$^{-}$ mutants (Chapter II). ISWI complexes are believed to aid establishment of repressive nucleosome positions over promoters (Mellor and Morillon 2004). We found that both *isw1Δ* and *isw2Δ* mutants could rescue transcriptional memory in a *swi2Δ* background strain suggesting that these complexes could be competing to establish alternate nucleosome positions at the *GAL1* locus. However, mapping of promoter nucleosome positions indicated that no alternate nucleosome positions were established during glucose repression (Chapter III). Curiously, though SWI/SNF is required for short-term memory, it is not required for long-term memory.

An alternate hypothesis was that transcriptional memory was caused by cytoplasmic inheritance of a galactose-induced signaling molecule. Presence of galactose could induce high cytoplasmic levels of this molecule, which can be passed on through some rounds of cell division even in the absence of galactose. The duration of memory would then be determined by turn-over and dilution of this factor. Two

candidates for cytoplasmic memory factors are the co-inducer protein Gal3p and the product of *GAL1* gene itself – Gal1p or galactokinase enzyme. Gal3p is a protein that binds to galactose when the sugar enters a cell's cytoplasm. This complex then translocates to the nucleus where it binds and inactivates Gal80p repressor protein. Inactivation of Gal80p in the presence of galactose in turn activates Gal4p activator protein, which is a sequence specific transcription factor that binds to recognition sites upstream of structural genes of the *GAL* regulon. *GAL1*, which has 4 upstream binding sites for Gal4p can be induced ~1000 fold in the presence of galactose. The *GAL3* gene, on the other hand, has only 1 Gal4p binding site and is modestly induced by about 3-5 fold (Johnston et al. 1994; Lohr et al. 1995). Thus activation of Gal4p (and hence *GAL* gene transcription) by Gal3p-galactose complex forms a feedforward loop. Interestingly, the Gal1p enzyme, which phosphorylates galactose (galactokinase), can also bind galactose act as a Gal3p-like co-inducer (Meyer et al. 1991; Platt et al. 2000; Hawkins and Smolke 2006; Hittinger and Carroll 2007). But since the K_m of Gal1p for galactose is much higher than Gal3p, Gal1p must be present in much higher concentration in the cytoplasm to function as a co-inducer protein. Yet, the large induction of *GAL1* expression suggests that there could indeed be very high concentration of Gal1p in a cell's cytoplasm even after glucose repression of *GAL1* expression.

If galactose-induced cells are subsequently shifted to glucose media, transcription of *GAL1* would stop and transcription of *GAL3* would go back to basal

levels. But one can imagine that if galactose becomes available in the environment after a brief period, there would be significant cytoplasmic levels of Gal3p and Gal1p to rapidly bind galactose and reinduce *GAL* gene expression. This second round of induction would then be much faster than the initial round since the slow step of synthesizing co-inducer protein to have optimal galactose signaling is now eliminated. This kind of cytoplasmic memory would persist as long as daughter cells receive Gal3p and/or Gal1p from the mother cell's cytoplasm at a level above the threshold required for rapid reinduction. Indeed Gal1p is required for long-term memory though not for short-term memory (Zacharioudakis et al. 2007); and our unpublished results in Chapter III).

What steps distinguish the kinetics of *GAL1* induction vs. reinduction?

Based on our experiments, we propose that induction of *GAL1* transcription is governed primarily by two rate-determining steps – accumulation of signaling proteins to activate Gal4p and chromatin remodeling at *GAL1* promoter by SWI/SNF. During initial induction of the naïve gene when cells are shifted from raffinose to galactose, accumulating sufficient amount of signaling proteins to have robust induction becomes the rate limiting step and masks the other slow step of chromatin remodeling. Thus the slow activation kinetics (~20 minutes to detect transcripts) possibly reflects the slow accumulation of optimal levels of Gal3p signaling protein. During reinduction, cytoplasmic Gal3p levels are already high. Therefore signaling is rapid and *GAL1* transcription can reach steady state levels within 5 minutes of reinduction. SWI/SNF

can remodel chromatin at the *GAL1* promoter during both rounds of induction in wild type cells, possibly promoting rapid loss of promoter nucleosomes. However, in the absence of SWI/SNF (*swi2Δ* strain), remodeling is slow and thus during reinduction, even though Gal3p signaling can rapidly activate Gal4p, opening up of chromatin at the promoter can be slow, thereby slowing PIC assembly and reinduction kinetics (~20 minutes to detect transcripts). Thus loss of SWI/SNF exposes the requirement of chromatin remodeling for optimal kinetics of *GAL1* induction. It is notable that long-term memory has no SWI/SNF dependence. This can be explained by the fact that rapid *GAL1* reinduction in this situation requires 2 hours (opposed to 3-4 hours for initial induction), which is longer than the time taken to remodel promoter chromatin by alternate means in the absence of SWI/SNF (possibly about 20 minutes, as evidenced by our experiments for short-term memory).

How can we test if the cytoplasmic level of signaling proteins governs *GAL1* induction kinetics? This can be done by artificially altering the expression of *GAL3* gene and make it unresponsive to upregulation by galactose. In experiments described in Chapter III, I transformed wild type and corresponding *swi2Δ* strains with a high copy number plasmid that expressed *GAL3* under the control of a constitutive *ADH1* promoter. Thus cells are constantly producing elevated amounts of Gal3p. Comparing *GAL1* induction and reinduction kinetics in these strains led to two important conclusions. Firstly, overexpressing Gal3p in wild type strain increased the rate of *GAL1* induction, upon shifting from raffinose to galactose, to the rate of reinduction.

GAL1 transcription reached steady-state levels within 10 minutes of both induction and reinduction, clearly indicating that galactose signaling was indeed the rate limiting step during initial induction. The second significant clue came from the Gal3p overexpressing *swi2Δ* strain. Even though induction and reinduction rates were slightly higher in this strain compared to the empty vector control, this strain was still clearly slow in both inducing and reinducing *GAL1* transcription compared to wild type. This result demonstrated that chromatin remodeling by SWI/SNF is the second rate-determining step in the series of events that lead to *GAL1* transcription. In the absence of SWI/SNF, transcription eventually occurs and reached optimum levels, but initiation is delayed possibly because eviction of promoter nucleosomes becomes inefficient.

It would be interesting to test the scenario where chromatin remodeling is intact but enhancement of Gal3p/Gal1p signaling is compromised. One would imagine that in such a strain memory, or rapid reinduction would be lost. Zacharioudakis et al. (2007) tested this possibility for long-term memory and found that loss of Gal1p but not Gal3p led to loss of memory. However, when we tested the requirement of Gal1p for short-term memory, we found that it was dispensable. This was perhaps because both Gal3p and Gal1p levels are sufficiently high in the duration of short-term memory and therefore they were redundant. However, careful observation suggests that *gal1Δ* strain has a mild defect in the rate of *GAL10* reinduction (Chapter III, Figure 15c). One cannot do similar experiments with *gal3Δ* strains because they have a severe defect in *GAL1* induction (Bhat and Venkatesh 2005). However, we plan to circumvent this problem by

using a P_{tet}-*GAL3* strain, where Gal3p expression is under control of a *Tet-ON* system and non-responsive to galactose since its sole Gal4p binding site has been deleted (Acar et al. 2005). We plan to grow this strain in conditions where the cellular Gal3p levels are always maintained at basal levels found in a wild type strain growing in glucose. Under these conditions we plan to test the kinetics of *GAL1* induction and reinduction. Preliminary experiments comparing *GAL1* induction and reinduction rates suggest that there is a slight defect in *GAL1* reinduction compared to wild type, even though re-induction is clearly faster than the initial induction (Fig.21). It appears that this defect could be stronger than the reinduction defect seen in the *gal1Δ* strain. However, faster rate of reinduction still suggests that Gal1p and Gal3p are redundant in their function of rapid signaling. Hence the next step will be to delete the *GAL1* gene in this strain and test whether short-term memory can be completely lost in the absence of enhanced signaling from Gal3p and Gal1p.

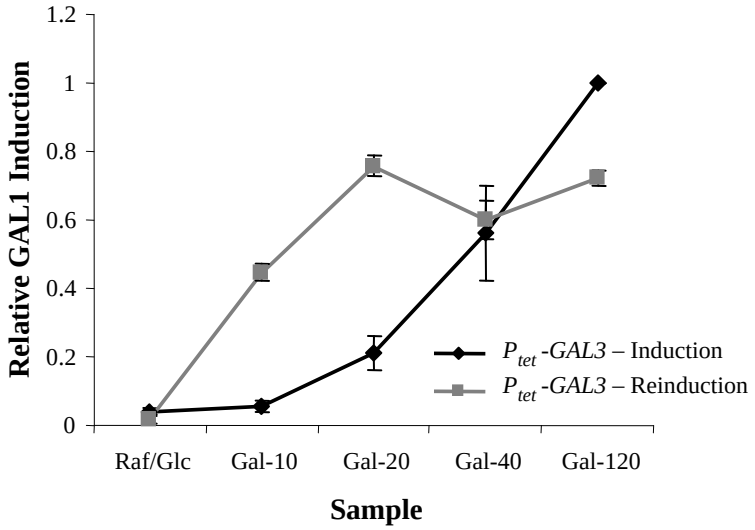


Figure 21. Inability to induce Gal3p expression causes slight defect in *GAL1* reinduction. P_{tet}-*GAL3* construct containing strain was grown overnight in media containing 2% raffinose and then shifted to 2% galactose. Samples were taken till 2 hrs after induction to monitor *GAL1* induction. Cells were then washed into media containing 2% glucose to repress *GAL1*, for 1 hour and then shifted back to 2% galactose-containing media to monitor *GAL1* reinduction. RNA was prepared from all samples and RT-PCR was performed to measure *GAL1* mRNA levels. Values were normalized to *ACT1* mRNA and expressed as a fraction of maximal induction value (set to 1).

In summary, the yeast *GAL1* gene has provided an excellent system to dissect molecular mechanisms of transcriptional memory. Yeast have the ability to adapt to an alternate carbon source like galactose by generating a transcriptional response. We saw that they also form a memory of this transcriptional response that can be passed on to a few generations. Though this mechanism does not involve any chromatin changes, it is propagated cytoplasmically by a signaling factor that gets progressively diluted in subsequent generations. It should be noted that though more stable, chromatin based memory mechanisms have not yet been discovered in budding yeast, such cytoplasmic memory appears to last a few generations and therefore can be sufficient given the budding yeast's short lifespan. It might be interesting if such mechanisms have also evolved in transiently dividing or amplifying populations of cells in higher organisms, possibly as a mechanism of providing a short-term memory of a transient transcriptional state.

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LIST OF STRAINS

STRAIN	GENOTYPE
CY1032	BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 rad6Δ::KanMX
CY1069	FY32 S288c MATΔ snf2Δ1::HIS3 ura3-52 his3Δ200
CY1072	FY1856 MATα his3Δ200 leu2Δ0 lys2-128Δ ura3Δ0
CY1102	BY4741 MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 set2Δ::KanMX
CY1192	FY631 Mat**a** his4 917-δ leu2Δ1 lys2-173R3 trp1D63 ura3-52 SGY1 Spt3-13myc
CY1193	FY631 Mat**a** his4 917-δ leu2Δ1 lys2-173R3 trp1D63 ura3-52 SGY212 Srb4-13myc
CY1267	BY4741 MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dot1Δ::KanMX4
CY1268	MATα ura3-52 leu2-3,112 trp1 his3 set1Δ::KanMX4 Δ[HHT1-HHF1] Δ[HHT2-HHF2] / pRS314-copyII (TRP1, HHT2-HHF2)
CY1270	FY1370 α gcn5Δ::HIS3 his3Δ200 leu2Δ1 ura3-52 (in FY1369)
CY1272	CY1069 with CP350 (swi2K798A in YIP5) integrated URA+
CY1273	CY1069 with isw1Δ::KanMX6
CY1287	CY1269 with isw1Δ::KanMX6
CY1288	CY1072 with isw2Δ::KanMX6
CY1289	CY1069 with isw2Δ::KanMX6
CY1290	CY1069 with set1Δ::KanMX6
CY1291	BY4741 MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gal4Δ::kanMX4

LIST OF STRAINS

STRAIN	GENOTYPE
CY1406	MAT**a** W303 a leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100 bar1-1 rtt109Δ::kanMX
CY1198	MAT**a** W303 a leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100 bar1-1 asf1Δ::kanMX
CY1422	BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gal1Δ::kanMX4
CY1087	BY4741 MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 htz1Δ::kanMX4
CY1421	MATα ade2::ADE2P_{GAL1}-YFP ura3::URA3P_{tet}-GAL3 gal3Δ::KanMX
CY1377	BY4741 MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 nup2Δ::kanMX4
CY1491	BY4741 MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sac3Δ::kanMX4