The Dynamic Interactions of the α2 Repressor Engender Robust Repression of Developmentally Regulated Genes while Priming them for Rapid Activation

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Abstract

In budding yeast, mating-type identity is determined genetically by the *MAT* locus, which encodes a set of transcriptional regulators that direct the cell to execute a specific genetic program. Even though mating type is genetically encoded, yeast cells are able to switch their phenotype by replacing the allele present at *MAT* with an allele encoding a different set of regulators. After a switch, the regulators that were expressed in the previous type must be inactivated for the cell to properly establish its new phenotype. To accomplish this, each of the mating-type regulators is constitutively targeted for rapid degradation in the proteasome. However, for the α2 repressor, rapid destruction would seem to be incompatible with its function. α2 represses a-cell specific genes (asg) by recruiting the Tup1/Ssn6 co-repressor complex, and in spite of its instability, repression engendered by α2 is both stable and robust. I have investigated the functionally engaged pool of α2 and found that interaction with Ssn6 altered the dynamics of α2-target gene interactions, allowing it to remain bound to its targets while the unbound pool was degraded. This effect depended on Isw2, suggesting that a repressive chromatin structure may shield the DNA-bound pool from destruction, and allow the formation of a stable co-repressor complex. In spite of its apparent stabilization, I found that once α2 dissociated from its targets, a-specific gene expression was activated extremely rapidly. Activation kinetics could not be explained by the pre-loading of RNA polymerase II or TBP. Unexpectedly, I found that each of the asg were acetylated on lysine 9 of histone H3 while they were in the repressed state. Both histone acetylation

and asg activation kinetics were dependent on Gcn5, suggesting that the acetylation of repressed asg primes them for rapid activation. Furthermore, both the acetylation of repressed asg, and the binding of Gcn5 to asg, depended on the Tup1/Ssn6 complex, suggesting that the co-repressor complex directs the priming of asg. Similar results were observed for the rapidly induced *GRE2* gene. I conclude that co-repressor directed acetylation of histones might be a commonly used mechanism to enable the rapid activation of repressed genes.

Prologue

Master regulators and phenotypic switching

Multicellular organisms are typically composed of countless different cell types, each with a particular set of characteristics that allow it to accomplish specialized tasks. In order for these diverse cell types to arise from a single cell, there must be mechanisms that allow cells to switch their phenotype from one type to another during development. For example, self-renewing hematopoietic stem cells are able to change their phenotypes and become a number of different, terminally differentiated immune cells (reviewed in Welner et al., 2008). Immune cell precursors are stimulated to change their phenotype by factors that activate signal transduction pathways, which ultimately change the activity of several master transcription factors, resulting in large-scale changes in gene expression (reviewed in Marshall et al., 2000). The process of phenotypic switching is a fundamental aspect of the biology of multicellular organisms, but the mechanisms that underlie the ability of cells to switch phenotypes remain relatively poorly understood.

Even though it is a single-celled organism, the yeast *Saccharomyces cerevisiae* provides an excellent model system for the study of phenotypic switching. Yeast are able to differentiate into a number of phenotypic states, and the factors that influence switching, as well as the transcriptional regulators that determine the gene expression profile of each type, have been well characterized for some time (reviewed in Herskowitz, 1985). Also, a more recent genomic study has allowed the complete gene expression profiles of each type to be

determined (Galgoczy et al., 2004). Yeast therefore provide an exceptionally well characterized system for the study of the phenotypic switching process.

While the characteristics and gene expression profiles of each of the yeast cell types have been examined thoroughly, the processes that take place as a cell transitions from one type to the next have only begun to be investigated. In this thesis, I have investigated the role of α2, a master transcriptional repressor that helps to establish one of the cell type-specific genetic programs (Johnson and Herskowitz, 1985; Keleher et al., 1988; Keleher et al., 1989; Strathern et al., 1981). α2 functions by recruiting the Tup1/Ssn6 co-repressor complex to a specific set of genes (Keleher et al., 1992; Komachi et al., 1994; Smith et al., 1995). The mechanism of transcriptional repression by the Tup1/Ssn6 complex has been studied extensively (for reviews see Malave and Dent, 2006; Smith and Johnson, 2000b), which makes the repression of cell type-specific genes an ideal subject for the study of the switching of gene expression programs. I have found that even though α2 engenders robust repression of a set of cell type-specific genes, its dynamic interactions with its target genes, as well as with the Tup1/Ssn6 complex, also help the cell to change its gene expression profile by priming cell type-regulated genes for rapid activation during cell-type switching.

Chapter 1

Introduction to *Saccharomyces cerevisiae* mating-type biology and the biochemistry and function of the α2 repressor protein

Part 1: Yeast Mating Biology

The utility of *Saccharomyces cerevisiae* as a model system for the study of phenotypic switching and cellular differentiation arises from its unique life cycle and mating biology. Through their normal life cycle, yeast cells display differentiation into multiple cell types with specific functions, the ability to switch between different cell types, and multiple levels of transcriptional regulation (for reviews see Herskowitz, 1985; Nasmyth and Shore, 1987). In this section, I will discuss much of what is known about yeast mating biology, and the molecular mechanisms that enable phenotypic switching. Taken together, these processes provide an excellent example of how a cell may differentiate into a specialized cell type, and how it may switch between cell types through the action of a small set of master regulators.

Cell-type determination in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, commonly known as baker's or budding yeast, can exist as three distinct cell types. Two of the cell types are haploid and are referred to as a and α. These cell types display specific mating behaviors: each is competent to specifically mate with the cell of the other type to form the third cell type, which is a diploid. Diploid cells also display a specific phenotype in that they are unable to mate with cells of any type, but can undergo meiosis to generate two a and two α haploid cells. The determining factor that commits a haploid cell to a particular fate is the allele present at a single genetic locus

called *MAT*, which encodes a set of master regulators that directs the cell to express one genetic program or the other (figure 1.1) (Galgoczy et al., 2004; Herskowitz, 1985; Nasmyth and Shore, 1987). The expression program in a cells is the simplest of the cell types and is the default state. The *MAT*a allele encodes 2 transcripts: *MAT*a*1* and *MAT*a*2* (or simply a1 and a2) (Astell et al., 1981). However, genes expressed only in the a cells (a-specific genes or asg) are expressed without any direct influence of the *MAT*a allele due to the presence Mcm1, a transcriptional activator common to all cell types (Bender and Sprague, 1987; Keleher et al., 1989; Passmore et al., 1989). The genetic program in α cells is somewhat more complex. The *MAT*α allele contains two genes termed *MAT*α*1* and *MAT*α*2* (or α*1* and α*2*) (Strathern et al., 1981). The α1 protein activates transcription of genes that are specific to the α cell type by interacting with Mcm1 (Bender and Sprague, 1987; Passmore et al., 1989; Sprague et al., 1983; Strathern et al., 1981; Tan et al., 1988). The α2 protein also interacts with Mcm1, but it completes the α cell genetic program by repressing the a-specific genes (Johnson and Herskowitz, 1985; Keleher et al., 1988; Keleher et al., 1989; Strathern et al., 1981). In diploid cells, which contain both *MAT*a and *MAT*α alleles, the a1 protein interacts with α2 and alters its DNA-binding specificity such that it binds to and represses a set of haploid-specific genes as well as α1, thereby establishing the diploid cell type genetic program (Goutte and Johnson, 1988; Siliciano and Tatchell, 1986).

Mating type interconversion/switching

The key property that makes yeast a useful model system for the study of phenotypic switching is that even though the mating type of a particular cell is determined genetically, the phenotype of a particular haploid cell is not necessarily static. In some strains, cells are able to switch their mating type from α to a, or from a to α, in a very characteristic pattern (figure 1.2) (Strathern and Herskowitz, 1979). Any cell that has already gone through a cell cycle (referred to as a "mother" cell) is able to change the genetic information at the *MAT* locus and give rise to two cells of the opposite mating type. Switching occurs at high frequency in mother cells, with approximately 70-80% changing their phenotype. However, buds or "daughter" cells are unable to switch until they have gone through an additional cell division (thereby becoming mother cells). This pattern of switching leads to a distinct mitotic lineage that will produce cells of both mating types. Interestingly, this lineage will include one line of cells that has never undergone a switch and has retained the mating type of the original cell.

The ability of yeast to change their mating type is made possible by the fact that copies of both the *MAT*α and *MAT*a alleles are stored at sites on the left and right arms of chromosome 3, respectively (Harashima et al., 1974). These loci, termed *HML* and *HMR*, contain all of the coding sequences of the mating type regulators, but are not transcriptionally active (Klar et al., 1981; Nasmyth and Tatchell, 1980). The structures of *HML* and *HMR* (figure 1.3) are complex, each containing several sequences of homology to the *MAT* locus as well as sequences that are unique to each allele (Nasmyth and Tatchell, 1980; Tatchell et al., 1981). All three loci have been divided into 4 distinct regions denoted as

W, X, Y, and Z. The W region is present at both *HML* and *MAT*. It is immediately followed by the X region, which contains much of the coding sequence for α2, but lacks a small portion of the 5' sequences (which are contained in the Y_α region). The X region is also present at *HMR*. The adjacent Y region contains the sequences unique to each allele, with α sequences typically stored at *HML*, and a sequences typically stored at *HMR*. The final, Z, region is common to all three loci, with the exception of a small sequence that is absent from *HMR*, known as the Z_2 region. The Z region contains the 3' end of the *α1* coding sequence. The allele-specific information in the Y segment is arranged similarly for both alleles. A small intergenic region acts as the promoter for both transcripts, which are transcribed divergently with *α2* and *a2* transcribed to the left and *α1* and *a1* transcribed to the right (Klar et al., 1981; Siliciano and Tatchell, 1984). Interestingly, the *a2* gene, which has no apparent function, is actually the same sequence as *α2* save for a relatively small 5' deletion (Tatchell et al., 1981). The structure of these loci is instrumental in the actual mating-type switching process. A switching event begins when the product of the *HO* gene makes a double-stranded cut at the junction between the Y and Z regions at *MAT* (Kostriken et al., 1983; Strathern et al., 1982). The cut is then healed by homologous recombination between the X region and the Z region of *MAT*, and the corresponding regions in either *HML* or *HMR*. The result is the replacement of the Y region of *MAT* with the Y region from the *HML/R* loci carrying the opposite mating-type information with no change made to the donor loci (Klar et al., 1982).

Regulation of switching events

The ability of cells to switch is dependent on the *HO* gene product- if switching strains lose a functional copy of this gene they cease to switch (Hicks and Herskowitz, 1976). Expression of the *HO* gene is tightly regulated: it is only expressed in mother cells during late G_1, and both *HO* mRNA and *HO* activity disappear before entry into mitosis (Nasmyth, 1983). Because the expression of *HO* is a key, highly regulated step in the mating-type switching process, the study of *HO* serves as a model for both regulation of gene expression and for phenotypic switching. *HO* expression has been found to be dependent on the products of the *SWI1* (*swi*tch) through *SWI10* genes, each of which plays a distinct role in *HO* activation (Breeden and Nasmyth, 1987; Stern et al., 1984). Many of these genes are thought to encode general transcriptional activators, as they are necessary for the expression of multiple genes (Peterson and Herskowitz, 1992; Peterson et al., 1991). The most important factor in determining the cell-cycle dependence of *HO* expression is the product of the *SWI5* gene (Nasmyth et al., 1987a; Nasmyth et al., 1987b). The Swi5 protein is a zinc-finger DNA-binding protein that binds to two specific UAS regions in the *HO* promoter (Nagai et al., 1988; Stillman et al., 1988; Tebb et al., 1993). *SWI5* is only transcribed in the S, G_2, and M phases of the cell cycle, after switching events have already occurred. Thus, the Swi5 protein that activates *HO* was synthesized in the previous cell cycle. Swi5 is prevented from entering the nucleus by phosphorylation of its nuclear localization signal by Cdc28 (Moll et al.,

1991). It is able to enter the nucleus in late anaphase when Cdc28 activity declines and it is dephosphorylated by Cdc14 (Visintin et al., 1998). When it enters the nucleus, Swi5 associates with the *HO* promoter and sets in motion an ordered recruitment of several activators, including the other Swi proteins, which will initiate *HO* transcription later in G_1 (Cosma et al., 1999). Association of Swi5 with *HO* is transient, lasting only about 5 minutes due to its rapid degradation after entering the nucleus (Tebb et al., 1993). Presumably, the degradation of Swi5 prevents the ectopic activation of *HO* transcription in S, G_2, or M phase.

While the regulation of Swi5 expression, localization, and degradation explains the cell-cycle dependency of *HO* expression, it cannot be responsible for the mother-daughter regulation since it is present in both cells. The daughter specific repression of *HO* expression is controlled by Ash1, a zinc finger DNA binding protein that can bind to the UAS of *HO* (Bobola et al., 1996; Cosma et al., 1999; Sil and Herskowitz, 1996). The association of Ash1 with the *HO* promoter inhibits the ability of Swi5 to recruit the other Swi proteins resulting in a failure of *HO* activation (Cosma et al., 1999). Ash1 protein accumulates primarily in the daughter cell, with only a small amount being expressed in the mother cell (Bobola et al., 1996). The accumulation of Ash1 in the daughter is accomplished by the localization of *ASH1* mRNA to the distal tip of the budding daughter cell following anaphase (Takizawa et al., 1997). Localization depends on an additional set of 5 factors known as the She proteins (Jansen et al., 1996). She2 binds to a localization signals in the 3'UTR of Ash1 mRNA causing them to form a large RNA-protein particle (Takizawa et al., 1997). It then binds the adaptor

protein She3, which attaches it to She1, a myosin motor protein that then moves it into the daughter cell (Beach and Bloom, 2001; Bohl et al., 2000; Jansen et al., 1996; Takizawa et al., 1997). The She4 protein associates with She1 and is necessary for its proper function (Toi et al., 2003; Wesche et al., 2003). The final component is She5, which is thought to be involved in anchoring *ASH1* mRNA to the distal tip of the bud, thereby preventing it from diffusing back into the mother cell (Beach et al., 1999).

Donor preference in switching

In order for mating-type switching to occur at high frequency, there must be a mechanism that controls which silent loci will be used as the donor to repair the double stranded break at *MAT*. If the donor were simply chosen randomly, 50% of switching events would replace the allele at *MAT* with the same allele. Quite surprisingly, the choice of the donor does not depend on the sequences present at *HML* or *HMR*. If *HML* sequence is replaced by *HMR* sequence at the same locus, a *MAT*<u>a</u> cell will still use the sequence at the *HML* locus as the donor (Wu et al., 1996). The same is true if *HMR* sequences are replaced with *HML* sequences in an α cell, or if only the Y region sequences are transposed (Klar et al., 1982). The positions of the mating type cassettes seem to be more important. If the *HML* cassette is deleted and an *HM* cassette is inserted at a new locus within about 40 kb of the end of the left arm of chromosome III, the donor preference of an <u>a</u> cell is maintained, but if it is moved outside of this region, preference is lost (Wu and Haber, 1995). Position is also important for

donor selection in α cells, but the dependence on position is distinct. α cells will recombine efficiently if the donor cassette is placed nearly anywhere on chromosome III as long as it is to the right of *MAT,* while moving it to the left side of *MAT* will stop recombination altogether (Wu et al., 1996). α and a cells also show unique behaviors when the preferred donor loci is removed. If *HML* is not present, a cells will recombine with *HMR* in its place, whereas if *HMR* is absent, α cells cannot recombine with *HML* (Wu et al., 1996).

The dependence on position of the *HM* cassette appears to be largely due to a 700 bp segment of chromosome III that has been termed the recombination enhancer (RE) (Wu and Haber, 1996). The RE is located approximately 17 kb to the right of *HML,* and moving an *HM* cassette progressively further away from it results in a progressive loss in donor preference in a cells. Deletion of the RE results in a cell donor preference switching to *HMR*. The RE contains two binding sites, termed *DPS1* and *DPS2*, for the mating-type regulator α2 and its cofactor Mcm1 (Szeto et al., 1997; Wu and Haber, 1996). These loci are genuine α2 operators, as they are able to confer mating-type dependent transcription on reporter genes (Szeto et al., 1997). In a cells, *DPS1* and *DPS2* produce transcripts that themselves have no apparent function. However, transcription of this region is critical for the proper donor preference in a cells. If expression is lost by deletion of the loci, deletion of certain transcription factors, or by expression of α2 in a cells, donor preference is lost. Conversely, if *DPS1* and *DPS2* are expressed in α cells, donor preference is reversed (Coic et al.,

2006b; Szeto and Broach, 1997; Szeto et al., 1997; Wu et al., 1998; Wu and Haber, 1995, 1996; Wu et al., 1996).

The exact mechanism whereby transcription of in the RE leads to the preference for *HML* recombination in switching remains unclear. Since transcription of the RE creates an open chromatin structure around the RE, it has been proposed that the function is to allow the binding of the recombination machinery or other factors that enhance recombination (Ercan and Simpson, 2004; Weiss and Simpson, 1997). This hypothesis is supported by the fact that Fkh1 transcription factor is able to bind the RE when it is transcribed, and that it is necessary for donor preference, even though it does not contribute to RE transcription (Ercan et al., 2005). However, this hypothesis is complicated by the fact that the disruption of chromatin remains local to the RE and does not include *HML* or the rest of the left arm of chromosome III (Ercan and Simpson, 2004). An interesting possibility is that transcription from *DPS1* and *DPS2* allows the recruitment of the DNA damage repair enzyme yKu80. yKu80 is able to bind to both the double strand break made by the *HO* gene product and to the RE (Frank-Vaillant and Marcand, 2002; Ruan et al., 2005). Its association with the RE is dependent on the presence of Mcm1, suggesting a dependence on transcriptional state, and it is necessary for proper donor preference (Ruan et al., 2005). Interestingly, when the *HO* gene is induced, successive chromatin immunoprecipitations can be used to observe yKu80 move across the left arm of chromosome III until it associates with *HML*. The interpretation of this result has been that that the RE provides an entry point for HO-cut *MAT*, and that it then

scans the arm of chromosome III until it finds a homologous sequence (Ruan et al., 2005).

An alternate hypothesis to explain the function of the RE is that it removes a physical barrier to recombination such as tethering of the left arm of chromosome III to a particular location that makes it less accessible to *MAT* (Bressan et al., 2004; Coic et al., 2006a). This hypothesis is based on the observation that fluorescently-labeled *HML* displays much more mobility in a cells than it does in α cells (Bressan et al., 2004). Mobility in a cells is lost upon deletion of the RE, suggesting that its function might be in overcoming the restrictions in the movement of the chromosome arm. Evidence for this model is provided by the fact that if *MAT*a is moved to the left arm of chromosome III and the RE is deleted, it is able to use *HML* as a donor, suggesting that having the two regions be in close proximity allows them to interact (Coic et al., 2006a).

Silencing of the donor loci

In order for a proper mating type to be established, the alleles present at *HML* and *HMR* must be kept silent, so that the regulators encoded therein do not interfere with the establishment of the proper genetic program. Because of this, understanding the mechanisms that engender silencing is an important aspect of the study of yeast mating-type biology. At both *HML* and *HMR*, sequences flanking the loci direct the silencing of the mating-type information (Abraham et al., 1984; Feldman et al., 1984). The sequences at each loci are termed the E (essential) silencer, which is required for silencing, and the I (important) silencer,

which is necessary for maximal silencing but, has a milder phenotype when removed. The ability of these sequences to silence expression is not specifically limited to the mating-type regulator genes; non-mating-type genes cloned into the silenced area are not transcribed (Brand et al., 1985). Also, the E silencer can be moved 2.6 kb away from *HMR* without loss of silencing (Brand et al., 1985). Each of the E silencers contains ARS sequences that are necessary for repressive function (Abraham et al., 1984; Brand et al., 1987; Feldman et al., 1984) and which are bound by three proteins, Rap1, ABF1, and ORC1, all of which are necessary for silencing (Bell and Stillman, 1992; Brand et al., 1987; Loo et al., 1995; McNally and Rine, 1991; Shore and Nasmyth, 1987).

The actual silencing function depends on the four Sir proteins (*s*ilent *i*nformation *r*egulator) (Rine and Herskowitz, 1987). ORC1 interacts directly with Sir1 and recruits it to the silencer (Triolo and Sternglanz, 1996), and Sir1 in combination with Rap1 recruits Sir3 and Sir4 (Moretti et al., 1994). Once this structure is nucleated, Sir3 and Sir4 spread out from the ARS and cover the adjacent areas of the chromosome and bind to the tails of histones H3 and H4 (Hecht et al., 1995; Rusche et al., 2002). The spreading of Sir3 and Sir4 require the recruitment of Sir2, a nicotinamide adenine dinucleotide-dependent (NAD) histone deacetylase (HDAC) that removes the acetylation marks from lysine 9 and lysine 14 of histone H3, and from lysine 16 of histone H4 (Hoppe et al., 2002; Imai et al., 2000; Rusche et al., 2002). The deacetylation of histone tails is thought to assist in the spreading of the Sir proteins by enhancing the ability of histone H4 to bind to Sir3 (Carmen et al., 2002). Once the spreading is

complete, the result is the formation of heterochromatin that prevents the binding of RNA polymerase II (Pol II) and the general transcription factors TFIIB and TFIIE (Chen and Widom, 2005).

The spreading of heterochromatin presents a problem to the cell. If it is not checked, it could extend into nearby genes and inactivate them. This is prevented by the presence of barrier elements that block the extension of heterochromatin (Donze et al., 1999). Like the silencers themselves, barrier elements are not specific to one particular location. Moving a barrier element closer to the nucleation site of the silenced chromatin will prevent the spread past that point (Donze et al., 1999). The composition of barriers is not necessarily uniform. Promoters of specific RNA polymerase III-transcribed genes, specific Pol II-transcribed genes, and the *Drosophila gypsy* element have all been demonstrated to function as barriers in yeast (Donze and Kamakaka, 2001). Rather than particular sequences, barrier elements appear to depend more on modifications to chromatin, as chromatin remodeling enzymes such as RSC, and histone acetyltransferase enzymes such as SAGA, among other factors, appear to be necessary for barrier element function (Jambunathan et al., 2005; Oki and Kamakaka, 2005).

An additional striking feature of silencing of the mating type loci is the heritability of their expression state. In early experiments, it was observed that in strains carrying mutant Sir1, individual cells could have either silent or active *HML* and *HMR* loci, and their progeny would maintain their expression state through several generations (Pillus and Rine, 1989). While the mechanism

allowing inheritance is still unclear, the apparent heritability of silencing is likely a result of its tight cell-cycle dependent regulation. Early evidence that the establishment of silencing was linked to the cell cycle came from the simple observation that once silencing was disrupted, it could not be re-established until the cell passed through at least one S phase (Miller and Nasmyth, 1984). More recently, it has been observed that if silencing is disrupted by loss of Sir3 activity, and then Sir3 activity is restored, an individual cell may re-establish repression after passing through a single cell cycle, or it may require passage through several cell cycles (Katan-Khaykovich and Struhl, 2005; Osborne et al., 2009). Surprisingly, the requirement of passage through S-phase is not dependent on DNA replication, as genes carried on non-replicating segments of DNA still have this requirement (Kirchmaier and Rine, 2001; Li et al., 2001). In subsequent molecular analysis, it was discovered that following disruption of Sir protein complexes with a temperature-sensitive Sir3 allele, Sir2, Sir3, and Sir4 were able to robustly rebind to the *HMR* E silencer and to spread from it by the G_2/M boundary, but rebound only weakly during S phase (Lau et al., 2002). However, rebinding was not sufficient to restore silencing until passage through the following S phase and arrival at telophase. Unexpectedly, re-establishment of silencing was dependent upon the loss of cohesion between sister chromatids. The dependence on the loss of cohesion, combined with previous observations that the cohesin proteins bind to the barrier elements (Donze et al., 1999; Laloraya et al., 2000), suggested the possibility that cohesins bind to the *HM* loci and inhibit silencing. This hypothesis was supported by the observation that

depleting cohesin proteins lead to early establishment of silencing (Lau et al., 2002). Subsequent work strengthened the connection between cohesin proteins and silent chromatin when it was observed that the localization of cohesin proteins was dependent on the presence of Sir proteins (Chang et al., 2005). A possible mechanism whereby the binding of cohesin could prevent silencing is through the inhibition of the HDAC of Sir2. It has been observed that in G_1-arrested cells, Sir2 is able to bind to the *HMR* E silencer and is competent for spreading, but it was unable to deacetylate the histones within the *HMR* locus (Kirchmaier and Rine, 2006), a critical component of silencing.

An alternate explanation for the cell cycle dependence of silencing was recently proposed when the cell-cycle regulated Mcm2 through Mcm7 replicative helicase complex (function reviewed in Forsburg, 2004) was found to interact with Sir2 via an associated factor Mcm10. Ablation of these interactions led to a defect in maintaining repression of *HM* loci (Liachko and Tye, 2005, 2009). Mutations were found in Mcm10 that could specifically derepress the silent loci without affecting the ability of the Sir proteins to bind to the silencers or to spread. Additionally, interactions between Mcm10 and Sir2 were not dependent on DNA, and point mutants that affected Mcm10-Sir2 interactions did not affect replication (Liachko and Tye, 2009). The authors proposed that Mcm proteins act on Sir2, perhaps by recruiting a modifying factor, resulting in the activation of silencing.

Part 2: Biochemical Properties of α2

In the work I have presented here, I have focused on the role of the α2 regulator in the process of mating-type switching. The experiments I have completed imply that the biochemical properties of α2, and the interactions between α2 and several cofactors, are important in enabling mating-type switching. In this section, I will discuss the characterization of the biochemical properties of α2 that appear to be relevant to the process of switching.

Degradation of α2

Even though there are several mechanisms to ensure that mating-type switching is executed faithfully, the ability of haploid cells to switch their mating type presents an interesting problem. After a genetic switching event, the master regulators that it encoded would still be present and would prevent the cell from executing its new genetic program, thereby preventing switching on a phenotypic level. This issue is resolved by the fact that all of the mating-type regulators have extremely short half-lives, and disappear from cells within a few minutes of a genetic switch (Chen et al., 1993a; Hochstrasser and Varshavsky, 1990; Johnson, 1997; Nixon and Laney, unpublished observations). The importance of destroying the regulators in this way is highlighted by the fact that if the α2 regulator is made to persist following an α-to-a genetic switch, cells fail to complete the phenotypic switch and become sterile (Laney and Hochstrasser,

2003). Destruction of each of the factors is mediated by the ubiquitin-proteasome system. In this system, specific proteins are marked for degradation in the proteasome, a large multi-subunit protease, through the covalent attachment of chains of a small protein called ubiquitin (Ub). Marking proteins for destruction is carried out by a three-step system (reviewed in Hershko and Ciechanover, 1998). First, an ubiquitin-activating enzyme (also known as E1) binds to a molecule of ubiquitin and charges it for use. A class of enzymes known as ubiquitin-conjugating enzymes (E2) then transfers activated ubiquitin molecules to target proteins by covalently attaching it to a lysine residue in the target protein. Ubiquitin-protein ligases (E3) provide the specificity for the system by binding to degradation signals in the sequences of proteins known as degrons, and directing them to the E2.

Of the mating type regulators, the degradation of α2 has been by far the best studied. Early pulse-chase analysis revealed α2 to be remarkably unstable, with a half-life of only about 4 to 5 minutes (Hochstrasser and Varshavsky, 1990). Through use of a fusion construct, it was also observed that α2 could confer instability on the normally stable β-galactosidase (β-gal) protein. Furthermore, by fusing various α2 deletion proteins to β-gal, it was discovered that α2 contained at least two degrons, one within its first 67 amino acids that has been named *Deg1*, and a second one located in the final 75 amino acids. Further mapping identified residues 53-67 as being particularly important for the function of *Deg1*. An additional study also examined the effects of point mutants within *Deg1*, and found that several substitutions within amino acids 14-32 were particularly

important for maintaining turnover of α2 and other *Deg1* containing substrates (Johnson et al., 1998). Interestingly, many of these point mutations were in the hydrophobic face of a predicted α helix, raising the possibility that recognition of an amphipathic helix may be important in identifying α2 as an ubiquitin substrate. Using epitope-tagged and mutant versions of ubiquitin, it was demonstrated that α2 turnover depended on functional ubiquitin, that α2 could be modified by the addition of Ub chains, and Ub could be conjugated to at least two different sites simultaneously (Hochstrasser et al., 1991). Further studies demonstrated that the proteasome was also necessary for rapid α2 degradation (Richter-Ruoff et al., 1994).

Pathways targeting α2 for degradation have also been well characterized. Examination of α2 degradation kinetics in strains deleted for ubiquitin-conjugating enzymes (Ubc proteins) revealed that α2 turnover was moderately (3-fold or less) impaired in *ubc4, ubc5, ubc6,* or *ubc7* strains (Chen et al., 1993a). Because the effect of single mutations on α2 stability was fairly modest, the authors of this study then examined combinations of the *ubc* deletions to determine if they regulated α2 turnover via two or more separate pathways. They observed that these four mutants could be broken down into two epistatic groups- *UBC4* and *UBC5* formed one group while *UBC6* and *UBC7* formed the other. Combing an *ubc4* mutation with an *ubc7* mutation led to a much greater increase in α2 half life than any of the single mutants (10 to 15-fold), suggesting that Ubc4/5 and Ubc6/7 target α2 for degradation through separate pathways. They also found that a *Deg1*-β-gal fusion protein was strongly stabilized in an *ubc7* strain, while it

was not affected by *ubc4* or *ubc5* mutations. Thus, it appears that the Ubc6/7 pathway targets α2 for degradation by recognizing the *Deg1* signal. In a subsequent genetic screen for ubiquitin-protein ligases that target *Deg1* containing proteins for degradation, it was found that mutation of the gene coding for the E3 Doa10 caused a similar impairment on turnover of a *Deg1* containing substrate as a *ubc7* mutation (Swanson et al., 2001). Furthermore, *ubc6 doa10* double mutants did not show any further decrease in degradation kinetics, confirming that they function in the same pathway. However, *ubc4 doa10* double mutants showed severe inhibition of degradation, suggesting that they work in separate pathways. The E3 enzyme that works via the Ubc4/5 pathway has recently been identified as a complex of the Slx5 and Slx8 proteins (Yie and Hochstrasser, personal communication).

An interesting property of α2 degradation kinetics is that they can be greatly altered without disruption of the Ub-targeting system. For example, the kinetics of α2 turnover are quite different in diploid cells than in haploids (Johnson et al., 1998). In pulse-chase experiments in diploids, α2 was degraded with bi-phasic kinetics. In the first 10-15 min of the chase α2 was degraded rapidly, but approximately one quarter to one third of the protein remained present after one hour. Kinetics were highly responsive to levels of a1 protein, suggesting that dimerization of α2 with a1 might affect turnover. Notably, a1 binds to α2 within the same putative amphipathic α helix that appears to be necessary for turnover (Ho et al., 1994). Indeed, the stabilization of α2 was dependent on its ability to interact with a1, suggesting that a1/α2 interaction

masks α2 from recognition by the ubiquitin machinery (Johnson et al., 1998). In another example of this phenomenon, Tup1, which binds directly to *Deg1* (Komachi et al., 1994), can cause the stabilization of *Deg1* containing substrates, including α2 (Laney et al., 2006). Over-expression of Tup1 has been observed to greatly increase the half-lives of *Deg1*-β-gal or *Deg1*-Ura3 fusion proteins. Stabilization was relieved by point mutations that prevent interaction of Tup1 with *Deg1*, confirming that the change in degradation kinetics was not a secondary effect of Tup1 over-expression. Similarly, over-expression of Tup1 and its co-factor Ssn6 caused significant stabilization of α2, and this effect was also relieved by point mutations that disrupt α2-Tup1 or α2-Ssn6 binding.

The ability of α2 turnover rate to change when it is in different complexes raises the possibility that the functionally engaged fraction of α2 might be degraded differently than the fraction that remains unbound to target genes. The previous observation that Tup1/Ssn6 binding can mask degradation signals suggest this might be the case, since functionally engaged α2 interacts with Tup1/Ssn6. Using chromatin immunoprecipitation assays, we have observed that following the silencing of α2 synthesis, dissociation of α2 is measurably slower than the overall rate of its destruction, suggesting the presence of a DNA-specific turnover pathway (Wilcox and Laney, submitted, this study figure 2.3A). Removal of α2 from its target genes was found to be dependent on the known E2 and E3 enzymes that target if for ubiquitination, but quite surprisingly, removal of α2 was dependent on the ubiquitin-selective Cdc48 chaperone (Wilcox and Laney, submitted). Therefore, independent of its involvement in α2 degradation,

the ubiquitination machinery also plays an important role in α2-target gene dynamics.

α2 structure and effects of protein-protein interactions on DNA binding

The ability of α2 to bind to and repress its targets is highly influenced by both its globular structure and several protein-protein interactions that it makes with its cofactors (summarized in figure 1.4). In early biochemical characterizations, it was observed that the protein contained two protease-resistant domains spanning amino acids 1-102 and 132-210 (Sauer et al., 1988). Using SDS-PAGE in both reducing and non-reducing conditions, as well as sedimentation analysis and gel filtration, it was found that α2 forms homodimers in solution by creating disulfide bonds between cysteines in the N-terminal fragment. The formation of dimers was determined to be important for protein-DNA interaction. Using DNase I protection experiments, the authors observed that α2 dimers bound the *STE6* operator with a K_d of 3 nM, approximately 20 times lower than monomers. Interestingly, they also observed that in DMS and hydroxyl-radical protection experiments, α2 binding protected two segments on the periphery of the operator. Each segment was protected symmetrically, with one G being protected from DMS modification and several surrounding bases being protected from hydroxyl-radical attack. The protected segments were termed α2 half-sites to distinguish them from the center of the operator, which was left exposed. The pattern of protection could be reproduced using only the C-terminal domain of α2. They concluded from this data that α2 binds to its

operator as a dimer, but leaves the center of the operator open for interaction with other proteins. Additionally, the unbound region in the middle of the operator could be deleted, thus altering the space between the protected sites, without affecting the binding affinity. It was suggested that the ability to alter spacing without affecting affinity indicated that α2 was flexible enough to adopt slightly different geometries. Finally, they concluded that α2 binds to the operator through its C-terminal domain, which was expected since sequences in the C-terminal domain are homologous to homeodomain proteins (Laughon and Scott, 1984; Shepherd et al., 1984). Crystallographic study of a C-terminal fragment of α2 bound to its operator confirmed much of this biochemical data (Wolberger et al., 1991). Most notably, α2 does form a homeodomain and it makes direct contacts with many of the same DNA bases that were protected in DMS or hydroxyl-radical experiments. Additionally, base-specific contacts are made in the two nucleotides flanking the DMS-protected G, identifying the TGT motif as a particularly important feature of the α2 operator. A NMR solution structure also confirmed the secondary structures in α2, including the homeodomain (Phillips et al., 1991).

Further biochemical studies revealed the importance of an additional protein in the interaction between α2 and the *STE6* operator. These studies were based on the observation that Mcm1 (also known as general regulator of mating type or GRM) could bind to and protect the center region of the α2 operator from DNase I digestion (Keleher et al., 1988). Using gel-shift and DNase I protection assays, they observed that Mcm1 and α2 can co-occupy the

same operator, and that Mcm1 binding did not hinder the binding of α2. In fact, in a key result of this study and in a follow-up study, they observed that Mcm1 and α2 bind to the operator cooperatively, with the presence of Mcm1 increasing the affinity of α2 for the *STE6* operator by between 50 and 500 times (Keleher et al., 1988; Keleher et al., 1989). It is important to note that several groups have attempted to measure the affinity of α2/Mcm1 for its target sites, but a wide range of K_d values have been reported ranging from 640 pM to 50 nM (Mead et al., 1996; Smith and Johnson, 1992; Vershon and Johnson, 1993; Zhong et al., 1999; Zhong and Vershon, 1997). Additional characterization of the Mcm1-α2 cooperativity interaction revealed that this could be achieved with only a small fragment of Mcm1 containing amino acids 18-96 (Mead et al., 1996). The critical region for this interaction in α2 was contained within amino acids 110-128. This segment, when attached to another homeodomain protein, was sufficient to confer cooperative binding with Mcm1. This segment of α2 lies within the unstructured linker region between the N- and C-terminal domains, highlighting the importance of the flexible linker region in α2-Mcm1 and α2-DNA interactions. Further analysis revealed that a short hydrophobic patch consisting of the sequence LVFNVVT was located just outside of the α2 homeodomain region and was particularly important for α2-Mcm1 binding. Mutations in this patch led to loss of Mcm1 binding, but did not affect α2-DNA binding in the absence of Mcm1, confirming that the loss of Mcm1 binding was not a consequence of altering α2-DNA interactions. This result was further refined by co-IP of α2 and Mcm1 in the absence of DNA, confirming that the disruption in α2-Mcm1 binding was direct.

The interaction between α2 and Mcm1 is thought not to merely increase the affinity of α2 for its binding sites, but also to ensure that α2 binds only to the proper operators and in the correct orientation. In the absence of Mcm1, neither small changes in the distance between α2 half-sites, nor inversion of the half-sites, change the affinity of α2 for its target, and even a 100 bp insert between the operators did not ablate binding (Smith and Johnson, 1992). However, small changes in the spacing between half-sites did severely affect the ability of α2 and Mcm1 to bind to the operator cooperatively. Similarly, inverting the direction of one half-site also disrupted cooperativity. These results were interpreted to mean that a function of Mcm1 is to ensure that α2 only binds strongly to proper operators and not to random sequences that may resemble one. They confirmed the physiological relevance of this model by determining that α2 could repress a reporter gene by 100-fold, but if the spacing or orientation of the half-sites in the operator were improper, repression was only 2-fold. In a similar set of experiments, the ability of α2 to bind to randomly selected DNA sequences in the presence or absence of Mcm1 was measured, and it was found that Mcm1 caused the sequence requirements for α2 binding to became more stringent. Therefore, in addition to selecting operators with proper spacing and orientation, Mcm1 also helps to ensure that α2 binds to proper sequences (Zhong and Vershon, 1997).

The DNA-binding properties of α2 can also be greatly altered through interactions with the mating-type regulator a1. Using electrophoretic mobility shift assays (EMSA) and 2-dimensional electrophoresis, it was observed that a

complex consisting of one molecule of α2 and one molecule of a1 could bind to the α1 promoter (Dranginis, 1990). Mapping the promoter by the methylation-interference binding assay revealed a pattern of contacts that was distinct from the pattern that had been observed previously for α2-Mcm1 binding reporter Sauer et al. (1988). Further analysis confirmed that a1/α2 could bind to the genetically identified haploid specific gene (hsg) operator, which is distinct from the α2 operator (Miller et al., 1985). Using purified α2 and a1 protein in the EMSA assay, it was determined that the two proteins bind cooperatively to the hsg operator with an overall K_d of 10^{-16} M^2 (Goutte and Johnson, 1993). Also, by using a UV crosslinking assay, it was observed that both a1 and α2 bind directly to DNA.

The domain of α2 responsible for interacting with a1 was first determined using limited proteolysis (Mak and Johnson, 1993). When the C-terminal domain of α2 was exposed to V8 protease, a 22 amino acid peptide was removed from the C-terminus. An α2 construct missing only the final 22 amino acids was able to repress expression of asg fully, however it was not able to repress α1 expression, suggesting that the interaction with a1 had been lost. This was supported by the fact that in an EMSA, α2 carrying this C-terminal deletion required 100-fold more a1 to bind to an hsg operator than did wild-type α2. The difference in binding was largely due to an approximately 30-fold increase in the dissociation rate of a1/mutant α2 complex from an hsg operator. This effect was not likely to be a result of the mutation affecting α2-DNA binding directly because in DNase protection assays mutant α2 showed similar protection to wild-type α2.

In a crystal structure of a1-α2 bound to an b, the C-terminal 16 amino acids, which were disordered in the α2 crystal structure, formed a small helix that interacted directly with the a1 homeodomain, possibly enabling it to make DNA contacts (Li et al., 1995).

Further investigation of a1-α2 was undertaken based on a putative coiled-coil interaction that was inferred from the sequences of the two proteins (Ho et al., 1994). α2 contains two predicted amphipathic helices that were potentially important in the formation of a protein-protein interaction with a hydrophobic helix in a1. Deletion of either of the two regions, from amino acid 22-43 or 67-95 resulted in significant loss of interaction with a1 as judged by loss of repression of a reporter construct. Similarly, multiple point mutations introduced into these sequences also caused derepression. Importantly, the point mutations that caused the most derepression of the reporter construct fell on the predicted protein-protein interaction face of the helices. This was confirmed in the a1/α2/DNA crystal structure, which demonstrated that the mutations fell on the face of a helix that made hydrophobic interactions with a helix from a1 (Li et al., 1995).

α2-Tup1/Ssn6 interactions and the formation of the co-repressor complex

In addition to its DNA-binding ability, protein-protein interactions between α2 and the co-repressors Tup1 and Ssn6 (also known as Cyc8) are critical for its repression activity. Mutants of both Tup1 and Ssn6 have been associated with a loss of repression of asg and sterility, among other phenotypes (Lemontt et al.,

1980; Schultz and Carlson, 1987). Co-immunoprecipitation studies revealed that Tup1 and Ssn6 make physical interactions, which combined with their similar mutant phenotypes, suggested that they function as a complex (Williams and Trumbly, 1990). Additional studies found that α2/Mcm1- and a1/α2-regulated reporter constructs required Ssn6 to bring about repression, even though α2 can bind to its operator in an *ssn6Δ* strain (Keleher et al., 1992). Tethering of Ssn6 to a reporter construct by using LexA fusions revealed that it had repressive activity, even though no α2 operator was present. This activity was dependent on Tup1 however, providing further evidence that the two proteins function as a complex. These results led to the hypothesis that Tup1/Ssn6 form a general co-repressor complex that is directed to function at specific genes via recruitment by a sequence-specific DNA-binding protein.

Further evidence to support the model that α2 brings about repression of asg by recruiting the general repression complex came from the isolation of a set of α2 point mutations that were defective for repression, but were not defective for DNA-binding (Komachi et al., 1994). Specifically, four point mutations, I4T, L9F, L10S, and G71K, all caused a 50% or greater loss in repression of β-Gal expressed from the promoter of the asg *MFA2*. Each of the mutations could be at least partially suppressed by over-expression of Tup1, suggesting a defect in α2-Tup1 interactions. The physical interaction between α2 and Tup1 was confirmed when α2 was retained in a column containing a Tup1-GST fusion. Retention was lost or decreased in each of the α2 point mutants, as well as in α2 deleted for its first 10 amino acids. Binding required the C-terminal domain of

Tup1 as well. The Tup1 C-terminus contains seven repeats of a ~40 amino acid motif rich in Trp-Asp repeats (WD motif) (Williams and Trumbly, 1990). Analysis of several mutants revealed that a single WD motif was sufficient to allow binding to α2, but removal of all of them abrogated binding (Komachi et al., 1994). Computer modeling of the 3-dimentional structure of the Tup1 C-terminal domain, and later a crystal structure of the WD repeat domain, indicated that the seven WD motifs would fold into a seven-bladed propeller structure that might function as a protein-protein interaction domain (Komachi and Johnson, 1997; Sprague et al., 2000). This was supported by mutagenesis studies that revealed that point mutations that impaired Tup1-α2 interactions mapped to the face of the propeller structure (Komachi and Johnson, 1997).

Similar analysis has delineated the sequences that allow the interaction between α2 and Ssn6. The N-terminus of Ssn6 contains ten repeats of the tetratricopeptide (TPR) motif. These repeats are required for repressive activity and have been proposed to facilitate protein-protein interactions (Schultz et al., 1990; Sikorski et al., 1990). Columns containing GST-Ssn6 retain α2, confirming that the two proteins interact physically (Smith et al., 1995). Similarly to the WD repeats of Tup1, individual TPR motifs can bind α2, although multiple repeats make stronger interactions. By digesting α2 into its two domains and passing them through the GST-Ssn6 column, the C-terminal domain was found to be necessary for binding, and further digestion revealed that the interaction specifically required the α2 homeodomain. The finding that Ssn6 binds to α2 within its homeodomain raised the possibility of a link between α2-DNA binding

and α2-Ssn6 binding. However, analysis of the R173A point mutant in α2, which decreased α2-Ssn6 binding significantly, revealed that it did not affect α2-DNA binding, or the cooperativity of α2 and Mcm1 or of α2 and a1, suggesting that α2-DNA binding and α2-Ssn6 binding are independent processes (Smith and Johnson, 2000a).

The domains in Tup1/Ssn6 that are necessary for them to associate with each other have also been examined. Using the yeast 2-hybrid assay, only the first 72 amino acids of Tup1 were found to be necessary for physical interaction with Ssn6 (Tzamarias and Struhl, 1994). Furthermore, tethering Tup1 to a reporter construct through a LexA fusion revealed that Tup1 has multiple separate repression domains. The N-terminal 200 amino acids were sufficient to mediate complete repression of the reporter construct. Shortening this fragment to the N-terminal 142 or 127 amino acids still showed repression, although it became progressively less efficient. Fragments with only the N-terminal 91 amino acids or less did not repress. Removal of the first 148 amino acids while leaving the rest of the C-terminal regions intact showed only a modest decrease in repression. Removal of the first 288 or 316 amino acids did not completely prevent repression, but removal of the N-terminal 389 amino acids lost all repressive activity. Together, these results demonstrate that there is a repression domain in the first 200 amino acids of Tup1 and a second between amino acids 288 and 316. Interestingly, full-length Tup1 was able to repress in strains with *ssn6Δ* mutations, suggesting that Tup1 is more important in repression than Ssn6, which requires Tup1. Similar analysis has mapped the

functional domains in Ssn6 (Tzamarias and Struhl, 1995). Only the first three TPR motifs (amino acids 1-175) were required for interaction with Tup1. The effects of systematic deletion of each of the TPR motifs on different Tup1/Ssn6 repressed genes found that some targets were derepressed by particular deletions, while others were not, suggesting that Ssn6 makes a number of different interactions at different promoters. In addition to mapping the Tup1/Ssn6 interaction, the stoichiometry of their binding in solution has also been investigated by sedimentation analysis and determined to be four molecules of Tup1 for every one molecule of Ssn6 (Varanasi et al., 1996). Similar observations were made using sedimentation equilibrium analytical ultracentrifugation on a 91 amino acid N-terminal fragment of Tup1, which was found to associate into tetramers (Jabet et al., 2000). Circular dichroism experiments also indicated that this fragment forms a helical structure, and low-resolution crystallography indicated that it would form a coiled-coil domain likely to mediate tetramerization.

Part 3: Mechanisms of Tup1/Ssn6 Repression

α2 must recruit Tup1 and Ssn6 to asg in order to effect repression (Keleher et al., 1992; Komachi et al., 1994; Smith et al., 1995). Therefore, Tup1 and Ssn6 also play key roles in the establishment of the α mating type, and they are also likely to have roles in mating-type switching. The Tup1/Ssn6 complex represses transcription through several proposed mechanisms (reviewed in Malave and Dent, 2006; Smith and Johnson, 2000b), and repression must be relieved following a mating-type switch. Because Tup1/Ssn6 has a central role in the establishment of asg repression, understanding the mechanisms that it uses to repress asg is important for understanding the changes that must take place during a mating-type switch. In this section I will discuss what is known about how Tup1/Ssn6-mediated repression of asg is accomplished, as well as how repression may be relieved.

Repression of asg by α2/Tup1/Ssn6-chromatin interactions

The exact mechanism that Tup1/Ssn6 uses to repress asg has been thoroughly investigated, leading to a wide array of interactions and processes being proposed to explain its activity, many of which are still under investigation. Perhaps the best-characterized mechanism that has been put forth is the organization of nucleosomes into a repressive chromatin structure. Early experiments using an α2 operator inserted into a high-copy plasmid and micrococcal nuclease footprinting of the downstream DNA demonstrated that in α

cells nucleosomes would arrange in a stable and orderly formation, while in a cells they remained disordered (Roth et al., 1990). Higher resolution mapping revealed that a nucleosome would bind 13 bp from the end of the α2 binding site in α cells but not in a cells, again suggesting that α2 plays a role in positioning nucleosomes (Shimizu et al., 1991). Similar experiments on genomic DNA showed that the nucleosomes arranged in an array beginning near the α2 binding site in *STE6* and *BAR1*, and would cover much of the promoter region and extend into the coding regions of the genes. Further studies using DNase I footprinting reported similar ordering of nucleosomes across the *STE2* promoter and coding region in α cells but not in a cells (Ganter et al., 1993). Additionally, the positioning of the nucleosomes in α cells covered the *STE2* TATA box, suggesting that one mechanism for asg repression could be to make the TATA box inaccessible to TATA-binding protein. Footprinting analysis done in cells carrying deletion mutations at *TUP1* and *SSN6* revealed that nucleosome positioning had been largely lost, demonstrating that Tup1/Ssn6 carries out the positioning activity. Also, chromatin immunoprecipitation (ChIP) experiments have shown that TBP is unable to bind to Tup1/Ssn6-repressed promoters, while deletion of *TUP1* allows it to bind (Kuras and Struhl, 1999).

Even though these footprinting analyses clearly demonstrated that α2/Tup1/Ssn6 co-repressor binding to the α2 operator resulted in ordering of nucleosomes, they did not demonstrate how the repressor effected this change. Additional footprint analysis in strains carrying N-terminal mutations in histone H4 revealed that nucleosome positioning had been disrupted (Roth et al., 1992).

This was true using both short deletions from amino acid 4 through 19, or point mutations at amino acid 16, 17 or 18. This raised the possibility that the co-repressor complex might order nucleosomes by interacting directly with the amino terminal region of histone H4. Consistent with this, Tup1 was found to be able to bind to both histone H3 and H4 (Edmondson et al., 1996). Additionally, the residues in Tup1 that are necessary for binding mapped to the pre-determined Tup1 repression domain (Tzamarias and Struhl, 1994). Importantly, Tup1-histone binding was highly dependent on the acetylation state of the histone tails. Tup1 preferentially bound to H3 and H4 that was either non-acetylated or acetylated once, but bound very poorly to histones acetylated multiple times (Edmondson et al., 1996).

The observation that Tup1 associates with histone tails and that binding is dependent on acetylation state led to the hypothesis that nucleosome phasing, and therefore asg repression, is carried out by direct interactions between Tup1/Ssn6 and histone tails. Much additional evidence indicates that this is the case. ChIP experiments have revealed that insertion of the α2 operator into a high-copy plasmid results in a loss of acetylation of histone H3 and H4 in α cells relative to a cells, and that acetylation levels in α cell increase considerably in *tup1*Δ cells (Bone and Roth, 2001). Similar results were observed at the genomic *STE6* locus (Davie et al., 2002). Additionally, mutations in histone tails, or increasing histone tail acetylation by deletion of HDACs greatly diminished Tup1-asg binding *in vivo*, and also abolished asg repression (Davie et al., 2002; Watson et al., 2000). Interestingly, Tup1 and Ssn6 have also been demonstrated

to physically interact with multiple HDACs (Davie et al., 2003; Wu et al., 2001). Taken together, these results raise an interesting possible model about Tup1/Ssn6-mediated repression: it seems possible that after being recruited to an asg promoter, the co-repressor complex might then recruit HDACs, which modify histones and allow Tup1/Ssn6 to bind stably and to repress asg expression by altering nucleosome positioning.

While the association of Tup1/Ssn6-directed histone deacetylation and nucleosome positioning has been well reported, the details about the roles of specific HDACs and the mechanisms of nucleosome phasing have remained fairly controversial. For example, Tup1 has been found to interact physically with the HDAC Hda1, and deletion of *HDA1* leads to increased acetylation of at least two different asg, but it appears to be dispensable for asg repression (Green and Johnson, 2004; Wu et al., 2001). Also, the nature of the Tup1/Ssn6 interaction that is necessary for nucleosome positioning is unclear. It has been reported that once Tup1 is recruited to the *STE6* promoter, it spreads into the coding region of the gene, with 2 molecules of Tup1 binding to each histone, and then abruptly stops at the end of the coding region (Ducker and Simpson, 2000). This result implies a mechanism for the ability of Tup1 to organize chromatin that is analogous to the action of the Sir proteins at the silent loci. The abrupt stop in spreading even suggests the possibility of a barrier element at the end of the gene. However, another study reported that the spreading of Tup1 into the *STE6* coding region proceeds from the α2 operator, but rapidly declines and is undetectable more than 750 bp past the translation initiation codon (Davie et al.,

2002), suggesting that Tup1 exerts its effects in the promoter or on the first few nucleosomes of the gene. A third group reported that Tup1 binds only to the α2 operator and does not spread, which is completely inconsistent with a Sir-like repression model (Wu et al., 2001). An additional hypothesis posits that Tup1/Ssn6 might not actually position nucleosomes at all, but rather prevents disorganization of chromatin by Mcm1 (Gavin et al., 2000). This hypothesis is a result of the observation that in a cells that carry mutations in the Mcm1 binding site in the *STE6* α2 operator, a nucleosome is positioned directly adjacent to the α2 operator, just as it is in wt α cells. From this data, it appears that α cell-like positioning of nucleosomes is the default state of asg chromatin, and Mcm1 disrupts positioning when it activates transcription. It follows then, that the function of Tup1 is to prevent activation by Mcm1, and the ordering of nucleosomes is in fact an indirect effect.

More recently another mechanism for Tup1/Ssn6 positioning of nucleosomes has been implicated. A complex composed of Isw2 and Itc1, in addition to other factors, is required for the organization of chromatin into a repressive structure at certain genes in yeast (Gelbart et al., 2001; Goldmark et al., 2000; McConnell et al., 2004; Morohashi et al., 2006; Zhang and Reese, 2004b). α cells mutated for *ITC1* display a number of phenotypes associated with derepression of asg. Reverse transcriptase PCR analysis shows that at least three of the seven asg, *BAR1*, *ASG7*, and *STE2* are partially derepressed in an *itc1* strain (Ruiz et al., 2003). Genomic footprinting of the *BAR1* gene revealed that in an *isw2Δ* strain chromatin organization resembled that of an a

cell (Morohashi et al., 2006). Interestingly, in spite of loss of chromatin organization, *BAR1* showed only a very minor amount of derepression, being expressed at about 2% of the levels in a cells. This is consistent with Tup1/Ssn6 having other repressive functions in addition to chromatin remodeling. In addition to the aforementioned genetic results, physical evidence of Isw2-Itc1 acting at asg has been observed. Using a catalytically-inactive mutant of Isw2 that associates stably with target genes, significant enrichment of Isw2 has been found at the *STE6* α2 operator in α cells relative to a cells (Gelbart et al., 2005).

Repression of asg by α2/Tup1/Ssn6-RNA polymerase II interactions

In addition to the histone-dependent repression activity described above, the α2/Tup1/Ssn6 co-repressor complex also has chromatin-independent repression activities. In *in vitro* experiments conducted by adding bacterially-produced α2 to preparations made either from whole-cell extracts or preparations containing a protein complex made by co-purifying Tup1/Ssn6-interacting proteins, α2 was able to moderately repress transcription of reporter constructs (Herschbach et al., 1994; Redd et al., 1997). Since these preparations did not contain histones bound to the reporters, the authors believed that Tup1/Ssn6 was able to repress transcription by directly interacting with the general transcriptional machinery. In subsequent genetic experiments, asg repression was found to be partially lost in strains carrying mutations in the mediator complex, a multi-subunit complex that assists in activation (reviewed in Kornberg, 2005), or in subunits of RNA polymerase II holoenzyme (Chen et al., 1993b;

Gromoller and Lehming, 2000; Jiang and Stillman, 1992; Wahi and Johnson, 1995). The best characterized of these components with respect to repression is Srb7, a subunit of the Pol II holoenzyme (Hengartner et al., 1995). In a split-ubiquitin screen for Pol II components that interact with Tup1, only Srb7 was isolated (Gromoller and Lehming, 2000). Using the same assay to map the amino acid sequences in Tup1 that can bind to Srb7, it was shown that binding could be mediated through the constructs containing amino acids 111-221 and 222-331. These segments overlap with the previously studied Tup1 repression domains between amino acids 1-200 and 288-316 (discussed above). Deletion of the first 7 amino acids of Srb7 prevented interaction with Tup1 and led to mild derepression of *MFA1*, and severe derepression of *STE2*. The loss of repression at both asg confirmed that Tup1 can mediate repression by contacting Pol II. This result is also of particular interest because it appears to function differently at two asg. Furthermore, this study provided a likely mechanism whereby Tup1 could repress transcription. They found that Srb7 also interacted with Med6, a mediator complex component that communicates interactions between transcriptional activators and mediator to the Pol II (Han et al., 1999), and that this interaction required the first 7 amino acids of Srb7. Again using the split ubiquitin assay, they demonstrated that over-expression of Tup1 prevented interaction between Srb7 and Med6 (Gromoller and Lehming, 2000). Based on this observation they proposed that Tup1 and Med6 compete for binding to Srb7, and when Tup1 is bound, Med6 cannot bind and stimulate transcription through Srb7.

Global regulation of gene expression by the Tup1/Ssn6 complex

In addition to asg, the Tup1/Ssn6 co-repressor complex regulates a large fraction of the genome. Recent genomic analysis indicated that over 300 genes are derepressed in *tup1Δ* strains (Green and Johnson, 2004). The mechanisms employed by Tup1/Ssn6 to repress several sets of genes other than asg have been examined extensively by a number of groups. Comparisons between the regulation of such genes can be a useful tool in better understanding how asg are repressed. However, much care should be taken in drawing conclusions from these studies, because there is an ample body of evidence showing that mechanisms employed by Tup1/Ssn6 can differ significantly from one class of genes to the next. For example, serial deletion of several of the TPR domains in Ssn6 showed different patterns of derepression of each of the four different promoter types tested (Tzamarias and Struhl, 1995). In another striking example, a reporter construct containing the a1/α2 operator did not show ordering of nucleosomes in the repressed state (Huang et al., 1997). Genomic analysis of Tup1-based repression has confirmed that this is a wide spread phenomena. Deletion of *TUP1* has been observed to derepress 334 genes, while only 96 of these same genes are derepressed in cells deleted for the Tup1-associated HDAC *HDA1* (Green and Johnson, 2004). Similarly, only 54 Tup1-repressed genes were derepressed in cells carrying a mutation in the Pol II subunit Srb10. Additionally, genomic analysis of derepression of four different Tup1 point mutations revealed that each mutant derepressed a distinct, only

partially overlapping set of genes (Green and Johnson, 2005). With these limitations in mind, comparisons between asg repression and repression of other Tup1/Ssn6-regulated gene sets will be considered below.

Repression of glucose-regulated genes by Mig1/Tup1/Ssn6

One of the best-studied classes of genes that are regulated by Tup1/Ssn6 is the glucose-repressed genes. The representative gene *SUC2* contains sites in its promoter for the sequence-specific binding protein Mig1 (Nehlin et al., 1991; Nehlin and Ronne, 1990). *GAL1* also contains Mig1 binding sites, and is a well-characterized target for Mig1-dependent repression, but results from *GAL1* are harder to interpret because it is also regulated by glucose and galactose through a complicated system involving additional activators and repressors (reviewed in Johnston, 1987). Mig1 is generally thought to be the functionally analogous factor to α2 at glucose-regulated genes because LexA fusions can confer glucose-dependent and Tup1/Ssn6-dependent repression on reporter constructs. There are however, key differences between its behavior and that of α2. Most notably, *mig1* mutations only result in partial loss of glucose repression, while *ssn6* mutations result in complete loss, and there are therefore likely to be additional factors that recruit Tup1/Ssn6 to these promoters (Vallier and Carlson, 1994). One possibility for such a factor is Mot3. Mot3 has been observed to enhance Tup1/Ssn6 interactions at *ANB1,* and deletion of *MOT3* causes partial derepression of *SUC2* (Kastaniotis et al., 2000). However, the role of Mot3 at *SUC2* has not been characterized further. Another key difference between α2

and Mig1 behavior is that Mig1 is not regulated by degradation, but instead when glucose is absent Mig1 function is regulated by phosphorylation by the Snf1 kinase (Treitel et al., 1998). It was originally thought that Mig1 phosphorylation caused derepression of its target genes by signaling it to be rapidly shuttled out of the nucleus, thereby preventing it from binding its operator (De Vit et al., 1997). This also seemed supported by the observation that mutants that could not be phosphorylated were constitutive repressors (Treitel et al., 1998). Quite unexpectedly, ChIP experiments have revealed that Mig1 remains bound to its targets under derepressing conditions (Papamichos-Chronakis et al., 2004). Furthermore, Tup1 and Ssn6 remain bound to glucose-repressible genes under derepressing conditions as well, but do not require Mig1 to remain bound, suggesting the existence of an as-yet undiscovered DNA binding protein or histone interactions (Papamichos-Chronakis et al., 2004; Papamichos-Chronakis et al., 2002). However, using co-immunoprecipitation experiments in repressing or derepressing conditions, it has been observed that phosphorylated Mig1 loses its ability to make physical interactions with Ssn6 (Papamichos-Chronakis et al., 2004). The authors of this work hypothesized the loss of interaction between Mig1 and Ssn6 might cause a conformational change in the Tup1/Ssn6 complex that inactivates its repressive function. From this work it is quite clear that Mig1 and Tup1/Ssn6 behave quite differently at glucose-repressed genes than α2 and Tup1/Ssn6 are thought to at asg.

In contrast to the interactions between Mig1 and Tup1/Ssn6, the mechanism of repression of glucose genes seems to be more similar to that of

asg. Like at asg, repressed glucose-regulated genes have positioned nucleosomes over their promoters, and this positioning is lost under inducing conditions or in *mig1* or *ssn6* mutants (Matallana et al., 1992: Perez-Ortin et al., 1987). Isw2 has been found to bind to the promoter of *SUC2*, suggesting that it may be involved in ordering nucleosomes (Gelbart et al., 2005). Also, repressed genes displayed a Tup1-dependent loss of acetylation in the repressed state (Bone and Roth, 2001). Deletion of multiple redundant HDACs lead to hyperacetylation of histones in the promoter of *SUC2*, much as it did at *MFA2* (Watson et al., 2000). HDAC deletions also resulted in a nearly complete loss of repression. This is in contrast to *MFA2*, which only showed mild derepression. This difference in dependence of HDAC may be a result of different interactions formed between Mig1 and Ssn6, since different mutations in the TPR region of Ssn6 affect repression of these genes differently than asg (Tzamarias and Struhl, 1995). Deletion of HDACs also have similar effects on *ENA1*, another glucose-regulated gene. Deletion of *HDA1* and *RPD3* both cause the hyperacetylation of specific histone residues, as well as derepression (Wu et al., 2001). Interestingly though, epistasis experiments show that Tup1 only mediates deacetylation by Hda1. *ENA1* also exhibits Tup1- and Isw2-dependent organization of chromatin into a repressive structure (Zhang and Reese, 2004b). It is important to note that this characterization of *ENA1* might not be representative of glucose-repressed genes as a whole. *ENA1* may be thought of as a special case among glucose-repressed genes because it is also regulated by the salt-stress pathway through the action of another DNA-binding protein, Sko1 (Proft and Serrano, 1999).

Like at asg, Tup1/Ssn6 has been observed to interact with several mediator and Pol II subunits to inhibit transcription of glucose-repressible genes (Chen et al., 1993b; Kuchin et al., 1995; Lee et al., 2000). Most notably, blocking interactions with Srb7 leads to significant, but not complete, derepression of both *GAL1* and *SUC2*, much as it does for asg (Gromoller and Lehming, 2000). At least one set of interactions between Tup1/Ssn6 has been observed to affect repression of *SUC2* that has not yet been seen at asg. Yeast 2-hybrid and GST-fusion experiments have demonstrated that the repression domain of Tup1 and the TPR repeats of Ssn6 can interact physically with the mediator subunit Hrs1 (Papamichos-Chronakis et al., 2000). In a reporter construct containing a *SUC2* promoter fragment including the Mig1 binding site, Tup1/Ssn6 repression could be mostly overcome by over-expression of Hrs1, suggesting that the co-repressor complex may repress *SUC2* by inhibiting the mediator complex. Additionally, cells over-expressing Hrs1 showed the clumpy growth and slow growth phenotypes that are typical of cells deleted for *TUP1* or *SSN6*, suggesting that interaction with Hrs1 might be a widely used mechanism in repression of Tup1/Ssn6-regulated genes.

Repression by the high osmolarity-glycerol (HOG) pathway

Many genes activated in response to salt-stress are repressed by Tup1/Ssn6 under non-stress conditions (Marquez et al., 1998). In most cases, Tup1/Ssn6 repression depends on the DNA-binding protein Sko1 (Garcia-Gimeno and Struhl, 2000; Proft et al., 2001; Proft and Serrano, 1999; Rep et al.,

2001). Like Mig1 and α2, Sko1-binding sites are located in the promoters of many of the genes it regulates (Proft and Serrano, 1999) and Sko1 is able to physically associate with the repression domain of Tup1 (Pascual-Ahuir et al., 2001). The repression activity of Sko1 is regulated by phosphorylation in much the same way that Mig1 is. When cells are exposed to salt stress, SkoI-based repression is inactivated by phosphorylation by the MAP kinase Hog1, and similar to Mig1, mutants that cannot be phosphorylated are constitutive repressors (Proft et al., 2001). Phosphorylation causes Sko1 to be rapidly shuttled out of the nucleus and to lose its ability to bind its target sites *in vitro* (Pascual-Ahuir et al., 2001). In spite of loss of its binding activity *in vitro*, ChIP assays reveal that Sko1 remains bound to promoters following a switch to derepressing conditions, as do Tup1 and Ssn6 (Proft and Struhl, 2002). Also, using GST fusion proteins, it was demonstrated that phosphorylated Sko1 has a much lower binding affinity for the Tup1/Ssn6 complex (Proft et al., 2001).

The common pattern of regulation between Tup1/Ssn6 at glucose-repressed and salt stress-regulated genes suggests that Tup1/Ssn6 works quite similarly at these promoters and also has implications for the regulation of Tup1/Ssn6 at asg. In the cases of both Mig1 and Sko1, a phosphorylation event causes interactions between the DNA-binding protein and the Tup1/Ssn6 complex to be diminished, leading to loss of repression. Importantly, repression is lost in spite of the persistence of the co-repressor complex, which is consistent with the hypothesis that loss of interaction with a DNA-binding protein causes a physical change in the complex that changes its function. While α2 is not known

to be phosphorylated, its removal from asg promoters by Cdc48 likely accomplishes this same task. Also, both Mig1 and Sko1 are rapidly removed from the nucleus under inducing conditions. While a function for this has not yet been demonstrated, one possible explanation is that physical removal of the DNA-binding protein could prevent rebinding to promoters and the re-establishment of repression. In the case of α2, this is accomplished by its rapid degradation rather than its localization.

The specific mechanisms of repression of Sko1 have not been investigated as extensively as repression of Mig1- or α2-regulated genes have. As mentioned above, *ENA1* has been observed to be regulated by HDACs, and displays Tup1- and Isw2- dependent phasing of nucleosomes (Wu et al., 2001; Zhang and Reese, 2004b). However, *ENA1* is regulated by both Mig1 and Sko1, indicating that its regulation might be unique. Genome wide analysis has identified some Sko1-regulated genes as bound by Isw2, but others were found not to be (Gelbart et al., 2005). There are therefore likely multiple different mechanisms that regulate Sko1-repressed genes.

Repression of hypoxia-inducible genes

Many genes that are only expressed when the cell is challenged by hypoxic conditions are actually repressed in oxygen-replete conditions by Rox1, a DNA-binding repressor that functions through Tup1/Ssn6 (Balasubramanian et al., 1993; Lowry and Zitomer, 1984; Tzamarias and Struhl, 1995; Zhang et al., 1991). Control of hypoxic genes is exerted through a pathway quite different

than those discussed for asg, glucose-, or salt-regulated genes. In the presence of oxygen, Rox1 expression is driven by Hap1 (Keng, 1992). Strikingly, using a reporter construct containing the *ROX1* promoter, it was found that Rox1 represses its own transcription in the presence of oxygen (Deckert et al., 1995). This is thought to create a feedback loop whereby Rox1 prevents is own levels from accumulating beyond a level where becomes toxic, since it was observed that over-expression of Rox1 inhibits cell growth. When oxygen is depleted from the media, Hap1 becomes a repressor and levels of Rox1 decline, allowing induction of the hypoxia genes. This regulating mechanism is quite different from that effected by Mig1 or Sko1, which are quickly inactivated by phosphorylation. Similar to α2, Rox1 is degraded upon a switch to hypoxic conditions, but its decay is slow, taking greater than one hour before it is cleared completely (Mennella et al., 2003).

Rox1-dependent regulation has been best studied at the *ANB1* gene, and again Rox1 interactions with Tup1/Ssn6 are quite different than for the previously discussed regulators. *ANB1* contains two pairs of Rox1-binding sites termed OpA and OpB (Deckert et al., 1998; Lowry et al., 1990). Maximal repression requires both binding sites to be present, and single base mutations that lower Rox1 binding affinity or incremental deletion of the Rox1 binding sites lead to increasing derepression (Deckert et al., 1998). Gel shift experiments have revealed that Rox1 binding to these sites is not cooperative. Furthermore, OpA contributes significantly more to repression than OpB (Lowry et al., 1990). This inequity in repression activity has been shown to be a result of the binding of an

additional factor, Mot3, to the OpA sequence (Kastaniotis et al., 2000). Reporter assays revealed that OpA functions seven times more effectively in wild-type strains than it does in *mot3Δ* strains. Rox1 and Mot3 each contribute to the recruitment of Tup1/Ssn6 to *ANB1*. ChIP assays have demonstrated that both are required to recruit Ssn6 completely, and deletion of both results in a mildly more severe defect than deletion of either one alone (Mennella et al., 2003). Tup1 recruitment is completely dependent on Rox1, but deletion of *MOT3* does cause a moderate defect in Tup1 recruitment. Interestingly, Ssn6 must be present for Tup1 to bind, suggesting that Rox1/Mot3 may recruit Tup1 indirectly. Unlike Mig1 and Sko1, upon induction of hypoxic genes, Rox1 and Mot3 dissociate from *ANB1*, although dissociation is slow and it takes several hours for the promoters to be cleared of these factors. Comparison of the rate of degradation of Rox1, as measured by immunoblotting, to the rate of dissociation from its promoters, as measured by ChIP, suggests that its degradation rate is rate-limiting for Rox1 dissociation from *ANB1*. In spite of the loss of Rox1/Mot3, Tup1 remains associated with *ANB1* for at least four hours after gene induction.

Analysis of the Tup1/Ssn6-dependent repression mechanisms employed at *ANB1* also reveals unique properties. Results from *ANB1*-driven reporter constructs show that mutations in *SRB7* and *SRB10*, two subunits of the Pol II holoenzyme that have been implicated in asg and glucose repression, have only miniscule effects on repression, if at all (Kastaniotis et al., 2000; Mennella et al., 2003). Micrococcal nuclease assays have been performed at *ANB1* and revealed that ordered nucleosomes are present, and that a nucleosome does

reside over the TATA box (Kastaniotis et al., 2000). The ordering is disrupted in *mot3Δ, rox1Δ,* and *tup1Δ* strains. Quite surprisingly however, mutations in the tails of histones H3 and H4 that led to disorganization of chromatin showed virtually no derepression of *ANB1* reporters (Deckert et al., 1998; Kastaniotis et al., 2000; Mennella et al., 2003). Simultaneous deletion of *SRB10* and *HDA1* do cause a roughly 50% loss of repression, suggesting that these mechanisms may be involved, but that they are functionally redundant rather than additive (Zhang and Reese, 2004a). Additionally, ChIP experiments demonstrated that in strains with mutant histone tails, TBP is able to bind *ANB1* under inducing conditions, but remains unable to bind under repressing conditions, leading to the conclusion that there is an as-yet undefined Tup1/Ssn6-dependent mechanism contributing to exclusion of TBP that does not involve positioning nucleosomes (Mennella et al., 2003).

Repression of DNA damage-induced genes

Genetic selections for genes that regulated the expression of factors that are induced in response to DNA damage identified Crt1, Tup1, and Ssn6 as the primary negative regulators (Zhou and Elledge, 1992). Loss of function of any of these three proteins leads to constitutive expression of the genes encoding ribonucleotide reductase (Rnr). Crt1 binds to sequences in the promoters of its target genes, and in a reporter construct that carries its binding site, it was found to confer Tup1/Ssn6 dependent repression in the absence of a DNA-damaging agent (Huang et al., 1998). Like Rox1, Crt1 can bind and repress its own

promoter, although repression in this case is only 2-fold. GST-fusion experiments have shown that that Crt1 physically associates with both Tup1 and Ssn6, and it appears to bind them in the same 4:1 Tup1:Ssn6 molar ratio that has been observed previously (Varanasi et al., 1996). Crt1 is unique among the aforementioned repressors however, as a C-terminal fragment of the protein has been shown to display Tup1/Ssn6-independent repression activity as well (Zhang and Reese, 2005). When cells are exposed to a DNA-damaging agent, Crt1 is phosphorylated by the DNA-damage response proteins, and its ability to bind to its targets is greatly reduced, leading to the release of its target genes from repression (Huang et al., 1998). The regulation of Crt1 therefore resembles that of Mig1 and Sko1. However, the behavior of the co-repressor complex during induction of the *RNR* genes is somewhat different than at glucose-regulated or salt stress-regulated genes. Crt1 dissociates completely from promoters within 2 hours of exposure to a DNA-damaging agent (Huang et al., 1998; Li and Reese, 2001). Similarly, exposure to DNA-damaging agents causes the release of Tup1/Ssn6 from the *RNR3* promoter (Zhang and Reese, 2004b, 2005). This is distinct from the behavior of glucose-, salt-, or oxygen-regulated genes where Tup1/Ssn6 remains bound under activation conditions.

The Tup1/Ssn6-dependent repression mechanisms have been extensively studied at the DNA-damage inducible genes and have led to interesting observations about the nature of Tup1/Ssn6-based repression. Tup1 binding to the *RNR* genes is highly dependent on the tails of histone H3 and H4 being intact, and its binding leads to loss of acetylation (Davie et al., 2002).

Repression of reporter constructs is dependent on Rpd3, Hos1, and Hos2 HDAC activity, but appears to be independent of Hda1 (Zhang and Reese, 2005). Micrococcal nuclease and DNase I mapping have revealed that under repressing conditions, the chromatin at *RNR3* is arranged in an orderly array, and positioning is lost in *tup1Δ*, *ssn6Δ*, or *crt1Δ* strains (Li and Reese, 2001). As seen at other promoter types, the nucleosomes cover the TATA box at *RNR3*. Nucleosome positioning was also found to be dependent on Isw2 in addition to Tup1, as has been discussed previously for other promoter types (Zhang and Reese, 2004b). Surprisingly, deletion of *ISW2* was not observed to cause significant derepression. ChIP assays revealed that even in an *isw2Δ* strain with disordered chromatin, TBP and Pol II were unable to bind the *RNR3* promoter. This suggests that a similar chromatin-independent mechanism for exclusion of TBP may be at work at DNA-damage regulated genes as at oxygen-regulated genes. The continued repression of *RNR3* in *isw2Δ* strains led to further striking observations about their Tup1/Ssn6-mediated repression. Many reported Tup1/Ssn6-dependent activities can be lost without significant derepression of *RNR3*: single *isw2Δ*, *rpd3Δ*, *hda1Δ*, *med3Δ*, and *srb10Δ* mutations show very little, if, any derepression of physiological targets (Zhang and Reese, 2004a). However, several combinations of these mutations do cause significant derepression of *RNR3*, as well as a second Crt1 target *HUG1*. It therefore appears that several independent mechanisms may repress DNA damage-inducible genes, but that any one or two of them are sufficient for near-complete repression.

Activation by Tup1/Ssn6

While the role of Tup1/Ssn6 as a repressor has been well defined, evidence has now indicated that they may also act as a co-activator complex that reverses the effects of its own repression activities. The first indication that Tup1/Ssn6 could act as an activator came when it was observed that tethering Ssn6 to a reporter plasmid with a LexA fusion led to seven-fold activation in cells deleted for *TUP1* or for Tup1-interacting components of the mediator complex (Conlan et al., 1999). It was also observed that Ssn6 could interact with Rtg3, a transcriptional activator that regulates expression of *CIT2* (Jia et al., 1997). They then observed that *CIT2* could not be activated in cells carrying dysfunctional Ssn6, leading to the conclusion that Ssn6 acts as an activator at the *CIT2* promoter (Jia et al., 1997).

Other examples of Tup1/Ssn6 acting as an activator have also been documented. It has been observed that at multiple Sko1-regulated promoters, Tup1 remains bound to target genes and is necessary for maximal recruitment of the SAGA and Swi/Snf complexes (Proft and Struhl, 2002). In this case, the switch from being a repressor to an activator is dependent on the phosphorylation of Sko1 by the Hog1 kinase. It is likely that the loss of direct interaction between Sko1 and Tup1 that results from phosphorylation (Proft et al., 2001) changes the activity of Tup1 and allows it to interact with a new set of proteins. The authors of this work conclude that because the activities of SAGA and Swi/Snf are the opposite of those normally carried out by Tup1, by becoming

an activator Tup1 serves to undo its own actions (Proft and Struhl, 2002). They support this with the observation that preventing repression by mutating *TUP1* relieves the need for SAGA or Swi/Snf activity during activation.

The activation of glucose-regulated genes has also been observed to require Tup1. Activation of glucose-regulated genes was found to require the Tup1/Ssn6-interacting protein Cti6 (Papamichos-Chronakis et al., 2002). Also, like at the Sko1-regulated genes, ChIP experiments revealed that Tup1/Ssn6 remains bound to glucose-regulated genes under inducing as well as repressing conditions, and that Cti6 binding to glucose-regulated genes depended on physical interactions with Ssn6. Cti6 was found to be necessary for recruitment of SAGA and TBP to a glucose-regulated gene, and this depended on the presence of Ssn6. Therefore, Tup1/Ssn6 assisted in the activation of a glucose-regulated gene by recruiting Cti6. As was the case for Sko1, phosphorylation of Mig1 upon induction causes the physical interaction between Mig1 and Tup1/Ssn6 to be lost, consistent with the hypothesis that the change in the contacts between the sequence-specific DNA-binding protein and Tup1/Ss6 (Papamichos-Chronakis et al., 2004) signals the change from a repressor to an activator. Similar to Sko1-regulated genes, the need for the recruitment of SAGA was relieved in strains carrying *tup1* or *ssn6* mutations, suggesting that Tup1/Ssn6 is involved in relieving it own activity.

The role of α2/Tup1/Ssn6 in the repression and activation of asg

In this thesis, I have further investigated both the mechanisms of repression utilized by α2/Tup1/Ssn6 at asg, and the process of activation following withdrawal of α2. I will present evidence that the repression of different asg is not controlled by a common set of mechanisms, but that different pathways are used for different asg. Notably, α2 appears to have Tup1/Ssn6 independent repression activity at some asg, which may be the result of sterically hindering the binding of the Ste12 activator. Using time-resolved ChIP and reverse transcriptase assays, I have observed that dynamic interactions take place at asg promoters during induction, and found that they promote the activation of asg with surprising speed. I will also present evidence that the kinetics of asg induction are due in part to the Tup1/Ssn6 complex acting as an activator as well as a repressor. Finally, I will demonstrate that Tup1/Ssn6 promote the extreme kinetics of asg induction by acting partly as an activator even while still engendering robust asg repression by priming asg for activation by directing the partial acetylation of histone H3 in the promoters of repressed asg.

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Figure Legends

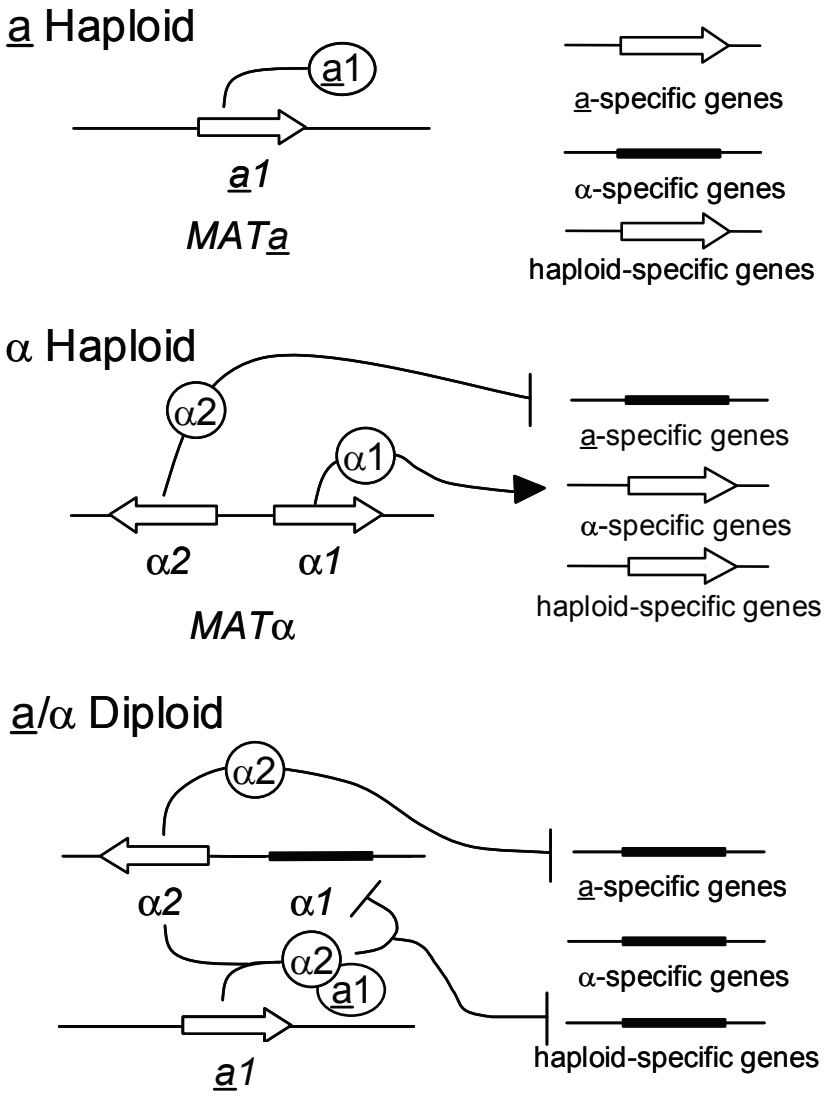
Figure 1.1 | Schematic of the mating-type regulatory circuit. Mating-type regulators are expressed from *MAT*, depicted on the left. The genetic program executed in each cell type is depicted on the right. Open arrows denote actively transcribed genes, while closed boxes denote inactive genes. The a2 gene does not produce a protein and has been omitted.

Figure 1.2 | Mother-daughter asymmetry during switching. The first cell represents a spore that has not yet completed a cell cycle. For all subsequent divisions, the mother cell is positioned to the left and the daughter cell is positioned to the right.

Figure 1.3 | The arrangement of the mating-type loci on chromosome III. The elements present at each of the loci are shown. Regions of homology are indicated by common color. Horizontal bars and arrows indicate the position of the expressed and repressed genes. The HO cut site is indicated by an arrow.

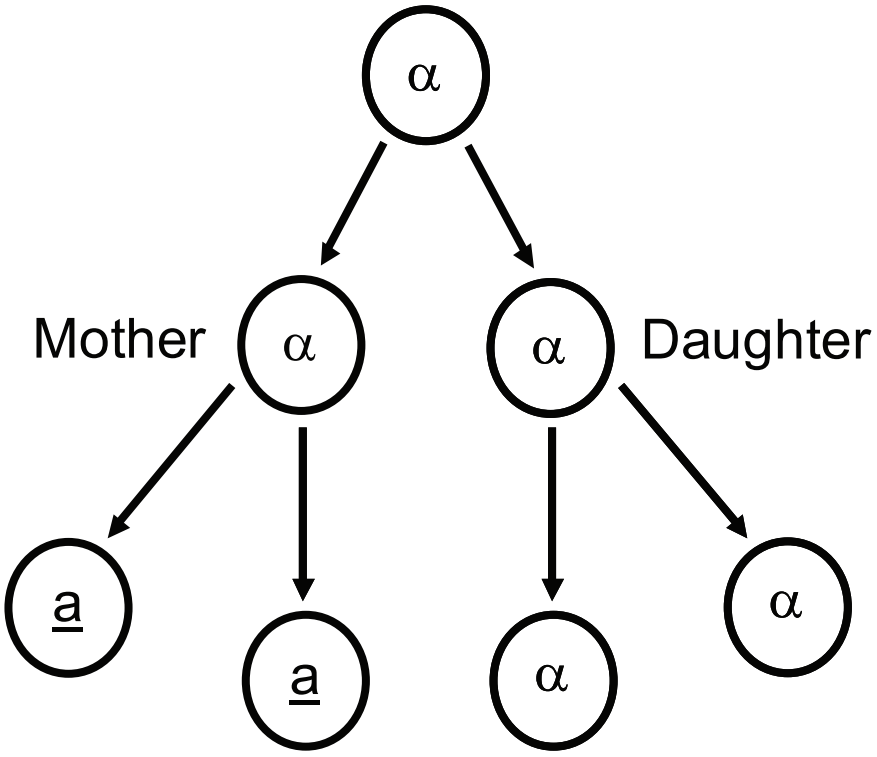
Figure 1.4 | Interactions of α2. **A**, Diagram showing the domains in α2 and the regions necessary for interaction with its cofactors. **B**, Model of the α2/Mcm1/Tup1/Ssn6 co-repressor complex based on the known interactions and proposed stoichiometry. Note that Ssn6 interacts with the homeodomain of α2, but not with DNA, and Tup1 interacts with the N-terminus of α2.

Figure 1.1



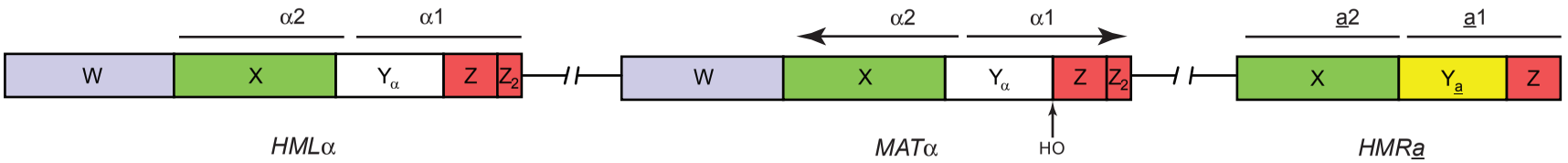
Adapted from Herskowitz, 1985

Figure 1.2



Adapted from Herskowitz, 1985

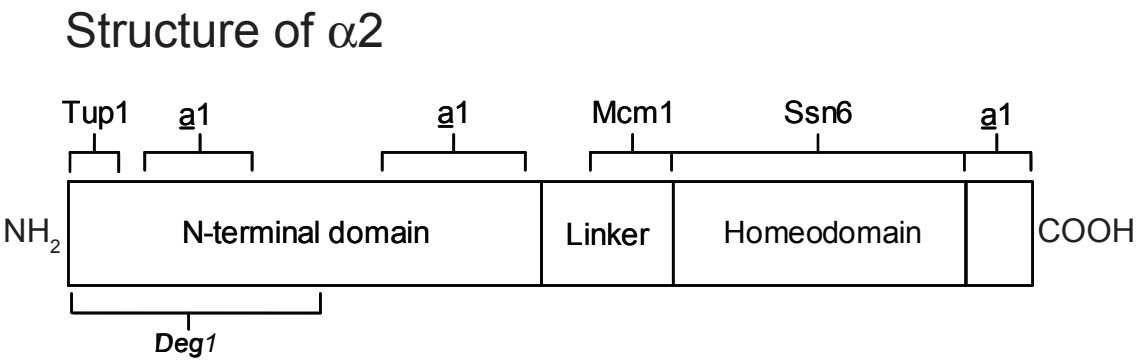
Figure 1.3



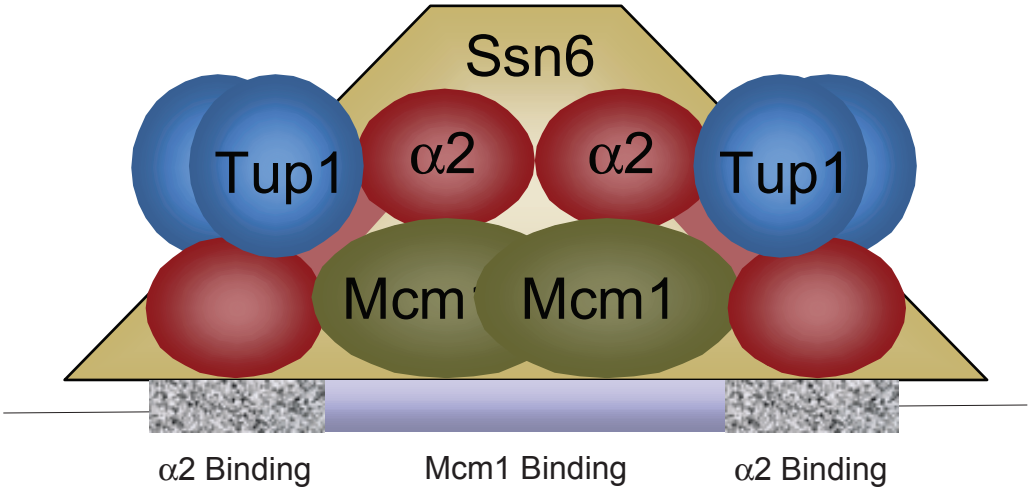
Adapted from Abraham et al., 1984

Figure 1.4

A.


Structure of α2
Tup1
a1
a1
Mcm1
Ssn6
a1
NH2
N-terminal domain
Linker
Homeodomain
COOH
Deg1

B.


Ssn6
Tup1
α2
α2
Tup1
Mcm
Mcm1
α2 Binding
Mcm1 Binding
α2 Binding

Chapter 2

A set of developmentally regulated genes are primed for rapid release from repression by Tup1/Ssn6-directed pre-acetylation of histone H3

Abstract

Cell type identity in the budding yeast *Saccharomyces cerevisiae* is determined by genetic regulators encoded at the *MAT* locus. In the α cell type, the *MATα* allele encodes the α2 protein, which represses the expression of a set of genes that are specifically expressed in the a cell type. α2 accomplishes repression of the a specific genes (asg) through the recruitment of the conserved Tup1/Ssn6 co-repressor complex, which acts to repress transcription through a variety of mechanisms. During a mating-type switch, α2 expression abruptly halts, thereby allowing the induction of asg. I examined the kinetics of asg expression and found that they are induced extremely rapidly upon loss of α2 from their promoters. The kinetics of induction could not be explained by pre-loading of RNA polymerase II or TBP to asg promoters prior to induction. However, I examined the acetylation state of asg promoters and found that they were all acetylated on lysine 9 of histone H3 even while repressed. Both acetylation of lysine 9 and asg induction kinetics were highly dependent on the Gcn5 component of the SAGA complex, indicating that this mark may prime asg for rapid activation. Furthermore, both acetylation of asg histones and SAGA-asg binding were highly dependent on the Tup1/Ssn6 complex, suggesting that the repressor complex itself signals the priming of asg for activation. Similar results were obtained for the rapidly induced *GRE2* gene. I propose that co-repressor directed acetylation of histones may be a widely used mechanism to

enable the rapid induction of repressed genes in response to changing environmental conditions.

Introduction

The budding yeast *Saccharomyces cerevisiae* can exist in two distinct haploid cell types referred to as a and α. Each type displays a unique set of mating behaviors. Both types are able to recognize and mate with cells of the opposite type to form a third cell type, which is diploid, but neither haploid cell can mate with a cell of the same type. The determining factor that commits individual cells to a particular fate is the allele present at a genetic locus known as *MAT* (Herskowitz, 1985; Nasmyth and Shore, 1987). This locus encodes a set of transcriptional regulators that direct the cell to express one genetic program or the other (Galgoczy et al*.,* 2004). For example, the *MAT*α allele encodes the α2 protein, which binds to a 32-base pair operator in the promoters of genes that are specifically expressed in the a cell type (a-specific genes or asg), and represses them (Johnson and Herskowitz, 1985).

Although yeast-mating type is genetically determined, the identity of an individual cell is not necessarily static. Yeast are able to switch their mating type by replacing the allele present at *MAT* with a cassette that carries the *MAT* allele of the opposite cell type (Strathern et al., 1979). The switching event begins when the HO endonuclease makes a double stranded cut in the *MAT* allele (Kostriken et al., 1983; Strathern et al., 1982). The cut is then repaired by inserting the new *MAT* information (Hicks et al., 1979). Changing mating-type in this way presents the cells with an interesting challenge: if the regulators that were produced before the switch remain in the cell, it would not be possible to establish the new phenotype properly. For example, if α2 were to persist

following a mating type switch, the cell becomes sterile (Laney and Hochstrasser, 2003). To prevent this from happening, each of the *MAT*-encoded regulators is rapidly degraded by the ubiquitin-proteasome system (Chen et al., 1993a; Hochstrasser and Varshavsky, 1990; Johnson, 1997; Nixon and Laney, unpublished observations).

In α cells, the expression of genes that are specific to the a cell type is repressed by the α2 regulator (Johnson and Herskowitz, 1985). α2 effects repression of asg by recruiting the general co-repressor complex Tup1/Ssn6 (Keleher et al., 1992; Komachi et al., 1994; Smith et al., 1995) to asg promoters, which then inactivate asg. Repression by Tup1/Ssn6 has been proposed to function through a number of different mechanisms including recruitment of histone deacetylases (HDACs) (Bone and Roth, 2001; Davie et al., 2003; Davie et al., 2002; Edmondson et al., 1996; Wu et al., 2001), interaction with the RNA polymerase II (Pol II) holoenzyme (Chen et al., 1993b; Green and Johnson, 2004; Gromoller and Lehming, 2000; Jiang and Stillman, 1992; Papamichos-Chronakis et al., 2000; Wahi and Johnson, 1995), exclusion or inhibition of transcriptional activators (Gavin et al., 2000; Gavin and Simpson, 1997) and by utilizing the Isw2-Itc1 complex to create repressive chromatin structure (Morohashi et al., 2006; Ruiz et al., 2003; Zhang and Reese, 2004). The various proposed mechanisms are not necessarily mutually exclusive, but nor are they all necessarily used at every Tup1/Ssn6 regulated promoter. In fact, genomic analysis has indicated that most Tup1/Ssn6-regulated genes use only a subset of the available mechanisms (Green and Johnson, 2004). Among the proposed

mechanisms, interaction with Pol II/mediator (Chen et al., 1993b; Gromoller and Lehming, 2000; Jiang and Stillman, 1992; Wahi and Johnson, 1995), recruitment of HDACs (Bone and Roth, 2001; Davie et al., 2003; Davie et al., 2002; Watson et al., 2000), and Isw2-dependent chromatin remodeling (Morohashi et al., 2006; Ruiz et al., 2003) have been specifically observed at α2-regulated promoters. The role of the recruitment of HDACs to asg is somewhat controversial, with some reports showing them to be important for asg repression (Davie et al., 2002; Edmondson et al., 1996; Watson et al., 2000) while another shows they are not involved (Green and Johnson, 2004).

While the mechanisms of asg repression have been studied extensively, very little is known about how asg activation takes place during an α-to-a mating type switch. Since persistent asg repression following a switch can lead to sterility (Laney and Hochstrasser, 2003), it is likely that cells are able to rapidly remodel asg promoters to an active state. Release from Tup1/Ssn6-mediated repression is typically a slow process, often taking several hours for genes to derepress completely (Geng and Laurent, 2004; Gligoris et al., 2007; Mennella et al., 2003; Tomar et al., 2008). However, there are known examples where Tup1/Ssn6 itself switches from being a repressor to being an activator (Papamichos-Chronakis et al., 2002; Proft and Struhl, 2002), and induction of the target genes can be quite rapid (Rep et al., 2001). In these cases, Tup1/Ssn6 participate in activation by helping to recruit the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex, a multi-subunit co-activator complex. The SAGA complex promotes the activation of many genes through multiple mechanisms (for review

see Baker and Grant, 2007). Perhaps the most well-studied mechanism is through the acetylation of histone tails by the Gcn5 subunit. Acetylation of histone tails on specific residues by SAGA is correlated with activation (Grant et al., 1999; Kuo et al., 1996; Kuo et al., 1998; Kurdistani et al., 2004; Pokholok et al., 2005). Here, I examined the activation and remodeling of asg following withdrawal of α2. I found that asg are activated extremely rapidly following the removal of α2 from their promoters, and that the kinetics of activation were dependent upon the SAGA complex. Surprisingly, I discovered that Gcn5 acetylates histone tails in the promoters of asg even when they are in the repressed state, and that acetylation was Tup1-dependent. Importantly, acetylation of repressed genes is not limited to asg. This suggests a novel mechanism for the rapid induction of repressed genes where the Tup1/Ssn6 repressor primes target genes for rapid activation by directing the SAGA-dependent pre-acetylation of histone tails.

Results

Tetracycline-regulated α2 effectively substitutes for endogenous α2

In order to study the changes in a-specific gene (asg) expression that occur during an α to a mating-type switch, I created a strain of α cells where the expression of α2 could be easily and quickly extinguished without the pleiotropic effects of a pharmacological protein synthesis inhibitor such as cycloheximide. Since repression of asg is relieved in homothallic strains when the α2 allele is removed during a mating-type switch (Strathern et al., 1982), I reasoned that rapid repression of α2 expression in α cells should serve as an accurate proxy for a switching event. To do this, I used the well-characterized tetracycline regulation system (Belli et al., 1998a; Belli et al., 1998b; Gari et al., 1997). A strain was built where the endogenous copy of *MATα2* was replaced with an antibiotic resistance marker, and a cassette expressing α2 under the control of the doxycycline (DOX)-repressible $tetO_2$ operator ($tetO_2$-α2) was integrated into the genome. Cells expressing $tetO_2$-α2 showed no obvious mating or sporulation defects, confirming that $tetO_2$-α2 effectively substitutes for endogenous α2. To determine if $tetO_2$-α2 allowed effective control of α2 expression, I examined α2 protein levels using quantitative immunoprecipitation/western blotting (figure 2.1A). When $tetO_2$-α2 cells were grown in the absence of DOX, α2 protein was expressed at ~1.6-fold higher levels than wild-type (wt) α cells. When cells were grown overnight in media containing 3 μg/ml DOX, α2 levels were nearly

undetectable. I concluded from these experiments that tetO$_2$-α2 cells provided us with a tool where α2 could be readily expressed at near-physiological levels and could be easily silenced by the addition of DOX.

I next determined if α2 expressed from the tetO$_2$ operator system was able to repress asg to the same extent as endogenously expressed α2. To determine this, total RNA was extracted from populations of asynchronous cells, and the presence of asg mRNA was examined by quantitative reverse-transcriptase PCR (qRT-PCR). When tetO$_2$-α2 cells were grown in the absence of DOX, the asg *STE6* and *STE2* were as efficiently repressed as they are in wt α cells (figure 2.1B top and bottom panels respectively). In the presence of DOX both *STE6* and *STE2* mRNA were present at levels indistinguishable from those in wt a cells. To ensure that DOX itself did not affect asg expression, I also examined the levels of *STE6* and *STE2* mRNA in wt a and α cells in the presence of DOX. In both cases the levels of mRNA in the presence of DOX were not significantly different than in the absence of DOX in either a or α cells. Similar results were obtained for the levels of *BAR1* and LacZ (expressed from the endogenous *MFA2* promoter) mRNA (figure 2.2C and D). I concluded from these experiments that the tetO$_2$-α2 system can be used as a physiologically relevant analogue for the control of asg expression by α2.

Distinct mechanisms contribute to the repression of different asg

In order to understand the changes that occur at asg during a mating-type switch, I first needed a better understanding of the conditions present at asg in

the repressed state. α2 repression of asg is mediated by the recruitment of the general co-repressors Tup1 and Ssn6 (Keleher et al., 1992; Komachi et al., 1994; Smith et al., 1995), which have been proposed to repress target genes through several different, non-mutually exclusive mechanisms (Bone and Roth, 2001; Davie et al., 2003; Davie et al., 2002; Green and Johnson, 2004; Gromoller and Lehming, 2000; Morohashi et al., 2006; Papamichos-Chronakis et al., 2000; Ruiz et al., 2003; Wu et al., 2001; Zhang and Reese, 2004). While much is known about the mechanisms that the Tup1/Ssn6 complex can use to repress transcription, studies of these mechanisms were conducted on several different classes of genes, and not all mechanisms are necessarily used at all types of genes. Thus, it is still relatively unclear how Tup1/Ssn6 represses asg.

To determine which mechanisms are involved in repressing asg, I examined the expression of several asg in $tetO_2$-α2 cells that carried combinations of *tup1Δ, ssn6Δ,* and *isw2Δ* alleles. Surprisingly, all of the asg tested did not yield the same results. In agreement with previous observations (Gavin et al., 2000), *STE6* and *STE2* showed only partial derepression in strains with *tup1Δ* or *ssn6Δ* single mutations, or in the strain carrying both deletions (figure 2.2A and B). Interestingly, both *BAR1* and *MFA2* were activated completely (figure 2.2C and D), suggesting that they are repressed by a distinct, completely Tup1/Ssn6-dependent mechanism. Single *isw2Δ* mutations showed only minor to moderate derepression of all of the asg, consistent with previous reports that Tup1/Ssn6-regulated genes show only slight to moderate

derepression in *isw2* mutants in spite of loss of nucleosome positioning (Morohashi et al., 2006; Ruiz et al., 2003; Zhang and Reese, 2004).

The partial derepression observed at *STE6* and *STE2* suggested the presence of a Tup1/Ssn6-independent-repression activity. I hypothesized that this activity might be carried out by Isw2 since it has been proposed that Isw2 scans the genome for target sites rather than being recruited by sequence-specific factors (Gelbart et al., 2005). To investigate this possibility, I examined expression of *STE6* and *STE2* in cells carrying *ssn6Δ tup1Δ isw2Δ* triple mutations. Interestingly, *STE6* and *STE2* did not show similar dependence on Isw2 in the absence of Tup1/Ssn6. The combination of these mutations led to complete derepression of *STE6* while it did not lead to greater derepression of *STE2* than any of the single mutants. It therefore appears that while both promoters require Isw2 for full repression, Isw2 function is dependent on Tup1/Ssn6 at *STE2*, but Isw2 function is Tup1/Ssn6 independent at *STE6*. Additionally, since single *isw2Δ* mutants show similar derepression of *STE2* as *tup1Δ* or *ssn6Δ* single mutants, it appears that the only mechanism that the Tup1/Ssn6 co-repressor utilizes to repress *STE2* is to identify it as an Isw2 target. This is in conflict with a previous report that *STE2* repression was largely dependent on Tup1-Srb7 interactions (Gromoller and Lehming, 2000). However, since *STE6* shows greater derepression when lacking both Tup1/Ssn6 and Isw2, it appears that they work through parallel pathways and at least one additional mechanism must contribute to *STE6* repression. I interpret these results to mean that *STE6* and *STE2* are repressed by distinct mechanisms.

Since the results indicated that Isw2 was not responsible for the apparent Tup1/Ssn6-independent repression of *STE2*, I hypothesized that it could be an effect of α2 itself. To examine this possibility, I compared the expression levels of the asg in the *tup1Δ, ssn6Δ,* and *isw2Δ* mutants to expression levels in the same strains after depleting α2 by the addition of DOX. Strikingly, I observed that *STE2* was completely derepressed in these strains regardless of the presence of Tup1/Ssn6 or Isw2. Thus, it appears that α2 has an as-yet unappreciated Tup1/Ssn6-independent repression activity at *STE2*. Even though simultaneous disruption of Tup1/Ssn6 and Isw2 caused complete derepression of *STE6*, I also observed that *STE6* showed increased derepression when α2 was depleted from cells carrying *tup1Δ, ssn6Δ,* or *isw2Δ* single mutations. It therefore seems that α2 displays a similar Tup1/Ssn6-independent repressive activity at *STE6*. Interestingly, *STE6* is not completely derepressed in these strains even when α2 is depleted, but it is completely derepressed when both Tup1/Ssn6 and Isw2 are absent. This may be due to changes in histone acetylation state in cells lacking Tup1/Ssn6 (figure 2.5A). It has been observed previously that α2 may be involved in recruiting Isw2 to *STE6*, as the catalytically inactive Isw2-K215R mutant shows enriched binding at *STE6* in wt α cells when compared to wt a cells or $\alpha 2^{-}$ α cells (Gelbart et al., 2005). This cannot account for the observed α2-dependent, Tup1/Ssn6-independent repression activity since the activity remains in cells carrying *isw2Δ* mutants (figure 2A).

Rapid induction of asg following the withdrawal of α2

While I was able to make significant observations concerning the mechanism of repression of several asg, I gained little information about the changes that take place at their promoters during an α to a switch. To gain further insight into the process of mating-type switching, I conducted a series of experiments where I made direct observations of events that take place during the activation of asg following the withdrawal of α2. Doxycycline was added to cell cultures and changes in promoter occupancy of several factors were measured over time using the chromatin immunoprecipitation (ChIP) assay. To be able to properly interpret the results, I first used quantitative immunoprecipitation/western blotting to determine how quickly α2 is cleared from cells following addition of DOX (figure 2.3A). Over the first 10 min of the assay, I observed little to no change in the cellular level of α2 protein. This lag period is likely a result of the time it takes doxycycline to enter cells and for existing *α2* mRNA to be degraded, and observations made during this period are not likely to be meaningful. Following the initial lag phase, α2 levels entered a phase of rapid decline until it approached the limit of detection of the assay at 30 min. During this phase, the levels of α2 declined at a similar rate to what has been observed previously (Chen et al., 1993a; Hochstrasser and Varshavsky, 1990; Johnson et al., 1998). The kinetics of α2 dissociation from the promoters of *STE6* and *STE2* were markedly different than the kinetics of protein degradation. Following the initial lag, α2 displayed biphasic dissociation kinetics. Over the following 10 min of the assay, α2 dissociated from promoters quite slowly, with roughly 60 percent of binding remaining at *STE6* and 40 percent remaining at *STE2*. α2 dissociation

then entered a more rapid phase, with little protein remaining bound after an additional 15 min. The difference in the kinetics between α2 degradation and dissociation from its target promoters may be the result of changes in accessibility of the protein to the ubiquitin-proteasome system when it is in its functionally-engaged form. α2-Tup1/Ssn6 interactions are known to mask the *Deg1* degradation signal in α2 (Laney et al., 2006), and a distinct set of factors are thought to specifically ubiquitinate DNA-bound α2 and signal its removal from its asg promoters (Wilcox and Laney, submitted; Yie and Hochstrasser, personal communication). It is noteworthy that the kinetics of α2 dissociation from its target genes presents a possible mechanism whereby the short-lived α2 repressor can bring about stable and robust repression of asg; in the event of a fleeting disruption in α2 expression due to an environmental stress, the protein could remain bound to its targets and continue to repress transcription for a time, thereby allowing a window for new α2 to be synthesized and proper protein levels to be restored.

I next examined the relationship between the loss of α2 from its target promoters and the kinetics of asg activation. To do so, I added DOX to cells and followed the accumulation of asg mRNA using qRT-PCR. I found that both *STE6* and *STE2* were activated with surprisingly rapid kinetics (figures 2.3B and C). Accumulation of both mRNAs began within 15-20 min of the addition of DOX (5-10 min after α2 loss). Importantly, activation kinetics closely mirrored the loss of α2 from the *STE6* and *STE2* promoters (compare figure 2.3A ChIP results with figures 2.3B and C), suggesting that there is very little time required for asg

promoters to switch from a repressed to an active state. Transcript levels of *STE6* and *STE2* reached maximal levels within 35 min of when α2 protein levels begin to drop (45 min. after the addition of DOX) and then declined over the following 45 min. This rapid activation is unprecedented for a gene being released from Tup1/Ssn6-based repression. For example, glucose-repressible genes and hypoxia-induced genes typically do not reach full activation until ~2 hours after being induced (Geng and Laurent, 2004; Gligoris et al., 2007; Mennella et al., 2003). DNA damage-induced genes can also take 1-2 hours to become fully active (Huang et al., 1998; Tomar et al., 2008). Osmotic-shock genes are among the most quickly induced, but display a 15 min lag before activation begins (Rep et al., 2001). Importantly, this lag is not the result of slow signal transduction or slow salt uptake since changes at the osmotic-regulated promoters can be observed within 3 min of the addition of salt (Proft and Struhl, 2002). The decline in asg mRNA levels after reaching full activation is not unexpected, as glucose-repressed, osmotic-shock, and DNA-damage induced genes have also been observed to reach maximal activation followed by a decrease in transcript levels after being released from Tup1/Ssn6-mediated repression (Geng and Laurent, 2004; Huang et al., 1998; Rep et al., 2001).

I next sought to elucidate the mechanism that enables the rapid activation of asg. I hypothesized that RNA polymerase II (Pol II) might be preloaded on asg promoters and able to transcribe asg immediately upon loss of α2 in a manner similar to what has been observed for the *Drosophila HSP70* gene (reviewed in Gilmour, 2009). To test this hypothesis I added DOX to cultures and followed the

association of Pol II with *STE6* over time using the ChIP assay. Contrary to my expectations, I observed that Pol II is recruited to asg coincident with the dissociation of α2 (compare figures 2.3A and D). I interpret these results to mean that Pol II can be quickly recruited to asg promoters following the removal of α2. I next hypothesized that the TATA-binding protein (TBP) might be pre-loaded onto asg promoters and assist in asg activation, even though previous observations indicated that it does not bind to repressed *MFA1* (Kuras and Struhl, 1999). To test this possibility, I constructed a strain of $tetO_2$-α2 cells carrying an HA-tagged version of TBP and examined its occupancy at the putative *STE2* TATA box in the absence or presence of DOX. Cells grown in the presence of DOX show an approximately 3-fold increase in the occupancy of TBP-HA relative to cells grown without drug (figure 2.3E). I interpret this result to mean that TBP does not associate with asg when they are in the repressed state.

Since it did not appear that asg activation kinetics were dependent on pre-loading of Pol II or TBP to their promoters, I next hypothesized that there would be a mechanism that quickly remodels asg promoters once α2 binding is lost, leading to the immediate removal of the Tup1/Ssn6 co-repressors. To examine this possibility, I conducted a series of DOX-chase experiments where I observed the changes in the binding of the co-repressor proteins following the loss of α2. To my surprise, I found that Tup1 dissociated from *STE6* only very slowly, with roughly 50% remaining bound to the *STE6* promoter after 1 hour (figure 2.3F) and roughly 40% remaining at *STE2* (figure 2.3G). I conducted a similar experiment in *ssn6Δ* cells expressing an HA-tagged version of Ssn6 from a

plasmid. I observed that Ssn6 did not display any significant dissociation from *STE6* or *STE2* over the course of the assay. It is therefore apparent that removal of Tup1/Ssn6 from asg promoters cannot be responsible for the rapid activation of asg. I also examined the dissociation of a myc-tagged version of Mcm1, a co-factor that enhances α2-asg promoter binding (Keleher et al., 1989), from the *STE6* promoter (figure 2.3F). Mcm1-myc dissociation from *STE6* closely mirrored that of Tup1. This result was not as surprising however, since Mcm1 also contributes to the activation of asg (Johnson and Herskowitz, 1985; Keleher et al., 1988)

Rapid activation of asg is SAGA-dependent

The surprising result that the Tup1/Ssn6 co-repressor complex remained bound to asg during their activation led me to hypothesize that the co-repressor complex might participate in asg activation. Other groups have demonstrated that the Tup1/Ssn6 co-repressor complex can assist in the activation of glucose-repressible and osmotic-shock induced genes (Papamichos-Chronakis et al., 2002; Proft and Struhl, 2002). In these cases, Tup1 and Ssn6 remain bound to their target genes during activation and help to recruit the SAGA complex. To determine if a similar mechanism might be utilized at asg, I examined the expression levels of *STE6* and *STE2* in $tetO_2$-α2 cells deleted for components of the of the SAGA complex. In cells deleted for the *h*istone *a*cetyl*t*ransferase (HAT) component of the SAGA complex, Gcn5, I observed that both asg failed to activate fully (figure 2.4A). Cells deleted for Spt7, a scaffolding subunit of the

SAGA complex necessary for its structural integrity (Grant et al., 1997), showed a similar 2-fold loss of asg expression. These results are consistent with the hypothesis that the SAGA complex is necessary for activation of asg. To observe the effects of SAGA mutants on the kinetics of asg activation I conducted another set of DOX-chase qRT-PCR experiments and compared the activation kinetics of the $tetO_2$-α2 SAGA mutant strains to the kinetics in the $tetO_2$-α2 strain. Both *STE6* and *STE2* activation kinetics were defective in the *gcn5Δ* strain (figure 2.4B and C). While $tetO_2$-α2 strains reached maximal expression within 45 min of the addition of DOX, the *gcn5Δ* strain did not approach these levels for at least one hour after addition. In the *spt7Δ* strain the kinetic defect was more severe, as *STE6* and *STE2* expression remained well below maximal levels at the end of the time course. To confirm this result for a third asg I took advantage of the *mfa2::LacZ* replacement in these strains and conducted similar experiments by using β-galactosidase activity as a reporter of *MFA2* activation. $tetO_2$-α2 cells showed similar activation of *MFA2* to those of *STE6* and *STE2* (figure 2.4D). The *gcn5Δ* strain showed a comparable impairment of expression kinetics to *STE6* and *STE2*, while the *spt7Δ* strain also showed a further defect in activation. In a previous report, it was shown that an additional factor, Cti6 was necessary for activation of *GAL1* (Papamichos-Chronakis et al., 2002). To determine if Cti6 is necessary for induction of asg, I created a *cti6Δ* mutation in the $tetO_2$-α2 strain and examined *STE6* mRNA levels in the presence of DOX (figure 2.4E). These experiments did not show any defect in *STE6* expression, suggesting that Cti6 does not play a significant role in

asg activation. I also examined the effects of the *cti6Δ* mutation on the kinetics of asg activation using the β-galactosidase assay and observed only a mild decline in the rate of activation, suggesting that Cti6 may play a minor role in promoting the kinetics of asg. This mild delay in activation could be a result of Cti6 helping to recruit SAGA as it does for glucose-regulated genes (Papamichos-Chronakis et al., 2002). Alternatively, it could be a secondary result. Cti6 has been shown to associate with the Rpd3-Sin3 complex (Gavin et al., 2002; Ho et al., 2002), and a *cti6Δ* mutation has been shown to both positively and negatively affect gene expression (Puig et al., 2004).

Tup1-dependent histone H3 lysine 9 acetylation of asg promoters in active and repressed states

Because the kinetics of asg activation were highly dependent on the SAGA complex, I next investigated the physical interactions of SAGA with asg promoters. SAGA is known to acetylate several lysine residues within the tails of histones, and acetylation is generally associated with active transcription (Grant et al., 1997; Kurdistani et al., 2004; Pokholok et al., 2005; Suka et al., 2001). In particular, the acetylation of histone H3 on lysine 9 and lysine 18 (AcH3K9 and AcH3K18) have been proposed to strongly promote transcription (Kurdistani et al., 2004). To investigate the acetylation state of asg, I conducted ChIP assays on wt α and a cells using an antibody that specifically recognizes histones acetylated on lysine 9. To account for changes in histone density between different expression states, acetyl-histone results were normalized to ChIP

measurements made using an antibody raised against the core of histone H3 that recognizes both acetylated and non-acetylated forms. Quite surprisingly, I observed no significant difference in the acetylation state of either *STE6* or *STE2* promoters in α and a cells (figure 2.5A). The results were Gcn5-dependent, confirming that acetylated histones were precipitated. Since asg activation kinetics are also Gcn5-dependent, I propose that Gcn5 promotes the rapid activation of asg by "pre-acetylating" repressed asg and thereby priming them for the rapid recruitment of the transcriptional machinery.

I next investigated whether the Tup1/Ssn6 co-repressor complex participates in the recruitment of the SAGA complex. I reasoned that if Tup1/Ssn6 participates in the activation of asg through the recruitment of SAGA, then *TUP1*-deleted cells should show a defect in acetylation. Consistent with this hypothesis, I observed that $tetO_2$-α2 cells carrying *tup1*Δ mutations displayed a large decrease in the levels of AcH3K9 at both *STE6* and *STE2* (figure 2.5A). Interestingly, activating the genes by the addition of DOX led to only minor changes in the levels of AcH3K9, suggesting that Tup1/Ssn6 is also necessary for the acetylation of histones in a cells. This result was unexpected, as it was previously believed that α2 is necessary to recruit Tup1/Ssn6 to asg since it makes direct interactions with both proteins (Komachi and Johnson, 1997; Komachi et al., 1994; Smith et al., 1995), and others have observed significant enrichment of Tup1-asg occupancy in α cells over a cells (Davie et al., 2002; Ducker and Simpson, 2000; Wu et al., 2001). I wished to confirm the dependence of Tup1-asg association on α2 myself, as several of these reports

disagreed significantly as to the extent of enrichment and the localization of Tup1 binding. I examined the asg promoter occupancy of Tup1 in α and a cells using the ChIP assay (figure 2.5B). I observed only two-fold enrichment of Tup1 binding at *STE6* in α cells as compared to a cells, and a more modest difference at *STE2*, but a four- to seven- fold enrichment in ChIP signal relative to *tup1Δ* cells. To confirm the specificity of these results, I also conducted ChIP using an irrelevant HA antibody, which yielded results similar to the *tup1Δ* cells. I conclude from these results that the association of Tup1 with asg in a cells is genuine, and α2 serves to enhance Tup1/Ssn6-asg promoter binding rather than recruit it. I also note that *tup1Δ* cells are unable to fully activate *STE6,* even in the absence of α2 (figure 2.2A). I hypothesize that this defect may be the result of the severe reduction in the acetylation levels of the *STE6* promoter due to the loss of Tup1.

Given the Tup1-dependence of histone acetylation and the presence of Tup1 at asg in a cells, I hypothesized that removal of Tup1 or Ssn6 would cause a defect the association of SAGA with asg. To test this hypothesis, I constructed a *gcn5Δ ssn6Δ* strain and rescued the *gcn5Δ* mutation with an HA-tagged version of *GCN5* carried on a plasmid. I used the ChIP assay to compare the association of HA-Gcn5 in the *gcn5Δ ssn6Δ* strain relative to a strain carrying a single *gcn5Δ* mutation and the HA-Gcn5 plasmid. I observed a two- to three-fold decrease in HA-Gcn5 association with asg in the strain lacking Ssn6, suggesting that the Tup1/Ssn6 complex physically recruits SAGA to asg (figure 2.5C).

Acetylation of asg H3 histones on lysines 14, 18, and 23

I next asked whether the acetylation of histones at repressed asg was specific to lysine 9, or if it also occurs at other lysines. Gcn5 is capable of acetylating several lysines in the tails of histone H3 including lysines 14, 18 and 23 (Suka et al., 2001). I obtained antibodies that specifically recognize histone H3 acetylated at each of these sites and used them to examine the acetylation of *STE6* and *STE2* promoters in the presence or absence of Gcn5 and under repressing or activating conditions. Unexpectedly, the results were quite different at *STE6* and *STE2*. *STE2* showed a nearly 3-fold enrichment of lysine 14 acetylation in a cells relative to α cells (figure 2.6B). Notably, the acetylation levels in α cells were only 2-fold higher than in *gcn5Δ* cells, suggesting that lysine 14 is a weak Gcn5 target in α cells. However, *STE6* showed only a modest enrichment in a cells, indicating that Gcn5 does acetylate lysine 14 at *STE6* in α cells, although less efficiently than in a cells. This result differs somewhat from previous results that showed a greater enrichment of acetylated histone H3 in a cells over α cells (Bone and Roth, 2001). I believe this discrepancy could be due to the fact that their study was conducted on a plasmid containing the *STE6* α2 operator, but none of the endogenous flanking sequences. Given that I have observed that Tup1 can associate with *STE6* in the absence of α2, it seems plausible that the flanking sequences may be important for Tup1 recruitment and therefore may affect the acetylation state. Additionally, this study, as well as an additional study that yielded similar results (Davie et al., 2002), was done using antibodies that recognize both acetylated lysine 9 and 14, while the antibody I

used was specific to lysine 14. At lysine 18, *STE2* showed a large difference between α and a cells (figure 2.6B). *STE6* also showed significant enrichment in a cells compared to α cells, although it was not as pronounced as at *STE2*. In spite of the enrichment of lysine 18 acetylation in a cells, α cells showed significant enrichment relative to *gcn5Δ* cells at both promoters. It therefore appears that while lysine 18 is hypoacetylated in α cells, it is still a target for Gcn5. I finally examined the acetylation state of histone H3 lysine 23 (figure 2.6C). At both *STE2* and *STE6*, acetylation was not significantly different in α and a cells. Thus, it appears that lysine 23 is acetylated at repressed promoters in a manner similar to lysine 9.

Pre-acetylation of histone H3 lysine 9 is common to all asg

I next asked whether the acetylation of *STE6* and *STE2* in repressed cells is particular to these promoters or if it is more generally applicable. To determine this I examined the acetylation state of each of the remaining asg. Because it appeared to be the most consistently acetylated residue, I chose to examine lysine 9. For each of the five remaining asg, AcH3K9 levels showed no significant difference between α and a cells (figure 2.7). I therefore conclude that the pre-acetylation of histone H3 on lysine 9 is a feature common to all α2-repressed genes.

Pre-acetylation of histone H3 lysine 9 is not restricted to asg

To determine if pre-acetylation of Tup1/Ssn6-repressed genes is a widely used mechanism to accelerate their induction, I examined the acetylation states of two additional Tup1/Ssn6-regulated promoters under both repressing and inducing conditions. I chose to study the *GAL1* and *GRE2* promoters because they exhibit very different kinetics of activation. *GAL1* expression does not begin for at least 30 min after being induced by a change in carbon source, and does not reach maximal levels for approximately 2 hours (Gligoris et al., 2007). *GRE2* shows much more rapid activation after being induced by osmotic shock. *GRE2* mRNA begins to be expressed 15 min after shock and reaches maximal levels within 45 min (Rep et al., 2001). The *GAL1* promoter showed 3-fold higher AcH3K9 levels when cells were grown in inducing conditions than when they were grown in repressing conditions (figure 2.8). However, activating *GRE2* expression led only to a 1.5-fold increase in AcH3K9 levels (figure 2.8). Thus, it appears that the rapidly induced *GRE2* is partly acetylated prior to induction, while the more slowly induced *GAL1* is poorly acetylated, consistent with my hypothesis that pre-acetylation of repressed promoters allows for their rapid induction. I interpret these results to mean that pre-acetylation of repressed genes is not limited to the asg and could be a widely used mechanism to allow the rapid induction of repressed genes.

Discussion

Mechanism of asg repression

Using tetO$_2$-α2 system I was able to make significant observations about the mechanisms governing repression of asg. The repression of *STE6* appears to be the most complicated of the asg tested. Deletion of *TUP1* or *SSN6* did cause derepression as expected. However, the loss of repression was only partial. Combined with the result that *isw2Δ* mutation enhanced derepression, it appears that full repression of *STE6* requires the cooperation of multiple mechanisms, with the chromatin remodeling activity of Isw2 being independent of Tup1/Ssn6. I did not further investigate the Tup1-dependent mechanism of repression, as recruitment of HDACs (Davie et al., 2002; Edmondson et al., 1996; Watson et al., 2000) and interactions with the Pol II holoenzyme (Green and Johnson, 2004; Gromoller and Lehming, 2000) have been reported previously. Strikingly, *STE2* appears to be regulated quite differently, as the only repressive function of Tup1/Ssn6 seems be the recruitment of Isw2 since *ssn6Δ tup1Δ isw2Δ* triple mutant was no more severe than *isw2Δ* or a *tup1Δ* single mutants. Deletion of *ISW2* seems to have little effect on the expression of either *BAR1* or *MFA2*, while deletion *TUP1* or *SSN6* led to full derepression. While similar results have been reported previously (Morohashi et al., 2006; Ruiz et al., 2003; Zhang and Reese, 2004), it is quite surprising that of the four asg I examined here, Tup1/Ssn6 appears to repress them in at least three distinct ways. This finding calls into question the interpretation of much of the data concerning the mechanism that Tup1/Ssn6 uses to repress asg promoters, as it

can no longer be assumed that results obtained for one asg can be regarded as representative of them all.

An additional significant observation that I made is that I appear to have uncovered a novel role for α2 in the repression of asg. I demonstrated that at both *STE6* and *STE2*, α2 itself contributes to asg repression independently of Tup1/Ssn6. I did not uncover the mechanism of this repression, but several possibilities present themselves. In the simplest model, α2 could recruit or aid in the recruitment of an additional repressive factor such as Isw2. Another possibility is that the binding of α2 to an asg promoter could sterically occlude an activator that would normally bind to a nearby DNA sequence. Ste12 is likely to be that activator. Ste12 is necessary for strong asg expression (Fields and Herskowitz, 1985). Similar to α2, Ste12 binds to asg promoters (Dolan et al., 1989). It is possible that both proteins cannot be bound to an asg promoter simultaneously, resulting in a competition between the two factors and a decrease in expression levels in the presence of α2. Investigation of this hypothesis is underway and I have obtained preliminary evidence indicating that α2 and Ste12 may compete for occupancy of the *STE6* promoter (figure 3.3C-E).

Pre-acetylation of histone H3 lysine 9 leads to rapid asg activation

I have demonstrated here that asg activate extremely rapidly following the silencing of α2 expression. In fact, a comparison of α2-asg dissociation kinetics with the kinetics of asg induction indicates that the time it takes for a promoter to switch from a repressed to an active state is so small as to be below the limits of

detection of the assays. The kinetics could not be explained by the pre-loading of components of the general transcription machinery such as RNA Pol II or TBP onto repressed promoters, nor could they be explained by a rapid remodeling event that leads to the eviction of the co-repressor complex. However, I did observe that asg are acetylated on lysine 9 of histone H3 even when they are in the repressed state. The deposition of this mark was dependent on the presence of Gcn5. Consistent with the hypothesis that this pre-acetylation mark is important in activation kinetics, removal of Gcn5 activity also leads to significant impairment of asg activation. Additionally, Tup1/Ssn6 appears to be necessary for recruitment of SAGA and for asg acetylation in both the active and repressed state. asg therefore represent another class of genes where the Tup1/Ssn6 co-repressor complex participates in both repression and activation. I also observed that the rapidly activated *GRE2* gene is acetylated nearly as strongly in the repressed state as it is in the active state. Because Tup1/Ssn6 also act as an activator at this gene (Proft and Struhl, 2002), I believe that the Tup1/Ssn6-directed pre-acetylation of repressed genes is not limited to the asg, and may in fact be a widely used mechanism to allow the rapid induction of repressed genes in response to changing environmental conditions.

The observations I have made here seem to be at odds with a previous report that recruitment of several HDACs to *STE6* is necessary for Tup1/Ssn6-mediated repression (Watson et al., 2000). These results seem incompatible with the idea that histone H3 is acetylated in the repressed state. I do not believe that these two models are necessarily mutually exclusive. I observed that both

STE6 and *STE2* histones are hypoacetylated at lysine 18, and to a lesser extent at lysine 14 in α cells (figure 2.6A and B). It remains a possibility that deacetylation of these residues is necessary for repression. This seems to be a plausible scenario given that of the various acetylated lysine residues, acetylated lysine 18 is the most strongly correlated with active genes (Kurdistani et al., 2004).

A model for the priming of asg promoters

The actual event that triggers asg-bound Tup1/Ssn6 to switch from a repressor to an activator remains unknown. However, it has been proposed that at glucose-repressed and salt-induced genes the critical event is the loss of physical interactions between Tup1/Ssn6 and the DNA-binding protein that recruits them (Papamichos-Chronakis et al., 2004; Proft et al., 2001). In light of this work, it seems possible that the activating signal is the dissociation of α2 from asg promoters. If this is the case, a model can be built to explain the mechanism of pre-acetylation of asg (figure 2.9). We have observed previously that in a population of cells as much as 80% of asg promoters are not bound by α2, and yet remain repressed (Wilcox and Laney, submitted). I believe that this observation indicates that α2-asg promoter interactions are highly dynamic, and that α2 associates and dissociates from its target sites readily. Under this model, when α2 dissociates from a repressed promoter, Tup/Ssn6 quickly switches from a repressing to an activating state. While in the activating state, Tup1/Ssn6 would then recruit SAGA and promote the acetylation of histone H3 on lysine 9

and perhaps on other residues as well. Thus, the activating process could begin even while α2 is still present in the cell. At some point during the activation process prior to the recruitment of TBP or Pol II, α2 would re-bind to its operator and reset the promoter to the repressed state. The promoter would therefore continually cycle between being repressed and activated without a transcript ever being produced. When α2 levels begin to decline following a genetic switching event, they would quickly fall below the levels necessary to rapidly rebind asg promoters, and activation would proceed. Given that at steady-state α2 is expressed well below its K_d (Wilcox and Laney, submitted), and that α2 has a half-life of less than 5 min, this could happen quite rapidly.

Physiological relevance of asg activation kinetics

The extreme kinetics of asg activation, coupled with the rapid turnover of the mating type regulators, suggest that there is a strong physiological need for phenotypic switching to happen rapidly. It has been observed previously that when α2 is made to persist following a mating-type switch, an improper genetic program is executed and cells become unable to mate (Laney and Hochstrasser, 2003). This raises the possibility that if asg are not induced quickly, their ability to mate may be delayed for a significant period of time. Even if this delay is relatively modest, it could present a significant barrier to mating activity. Cell type switching is a highly regulated process that occurs once per cell cycle in late G_1 (Nasmyth, 1983). If asg are not expressed completely before the cell commits to another switch, or if their expression is completed only a short time before the

next switch, there would be very little opportunity for mating. If α specific genes were similarly delayed in activation, cells would continue to switch with each cell cycle but would never mate and would be unable to access the diploid state.

Materials and Methods

Yeast strains, plasmids, and growth conditions

Strains used in this study are listed in Table 2.1. Gene disruptions were made using standard techniques (Guthrie and Fink, 1991). MHY608, MHY609, and KKY152 were obtained from Mark Hochstrasser. JY412 was constructed by crossing MHY608 to MHY609 to create JY99. pRS306-tTA-tetO$_2$-α2 was constructed by inserting an EcoRI-XhoI fragment from pCM224 (Belli et al., 1998a) into the MCS of pRS306 to make pRS306-tTA-tetO$_2$. α2 was amplified with BamHI ends and inserted into the BamHI site. An α spore was isolated from JY99 and transformed with StuI-digested pRS306-tTA-tetO$_2$-α2. The endogenous copy of α2 was then replaced with a hygromycin resistance cassette to make JY402. JY99 was transformed with EcoRV-digested pCM244 (Belli et al., 1998b) and an a-spore was isolated to make JY405. JY402 and JY405 were mated to make JY408. JY412 is a spore isolated from JY408. JY434, JY562, JY582, JY591, JY628, JY640, and JY814 were created by making the appropriate manipulations in JY408 and isolating the desired spores. JY472, JY474, and JY475 were made by crossing KKY152 to MHY609, isolating an a spore carrying *tup1Δ ssn6Δ mfa2Δ::LacZ*, mating it to JY412, and isolating spores with the indicated genotype. To make JY720, KKY152 was crossed to MHY609 and a *tup1Δ ssn6Δ mfa2Δ::LacZ* spore was isolated to make JY541. JY412 was crossed to JY541 to make JY718. *ISW2* was then replaced by a kanamycin resistance marker, and the desired spore was selected. JY830 was

made by crossing JY591 to KKY152 and selecting the appropriate spore. Cultures were grown in YPD medium (1% yeast extract 2% peptone 2% dextrose) unless otherwise noted. For all steady-state +DOX experiments cultures were grown overnight in 3 μg/ml doxycycline. pYX142-HA-GCN5 was provided by Dimitris Tzamarias and has been described previously (Papamichos-Chronakis et al., 2002) and YCp(23)SSN6-HA_3 was provided by Richard Zitomer and has been described previously (Mennella et al., 2003). Cultures were grown in SD-leucine media to select for the retention of pYX142-HA-GCN5.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed essentially as described (Strahl-Bolsinger et al., 1997) with slight modifications. Cells were grown overnight into log phase. 45 ml of cells (~5-50 ODs) were incubated at 30°C for 5 min in 1% formaldehyde and 20 mM sodium azide with agitation. Crosslinking was quenched by adding glycine to 125 mM and incubating 5 min at room temperature with occasional agitation. Cells were spun down and washed with ice-cold sterile water. Pellets were resuspended in 500 μl lysis buffer (50 mM HEPES-KOH ph7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 1 mM PMSF, 5 μg/ml pepstatin. 1 protease inhibitor cocktail tablet (Roche Cat. #11836153001) was added for every 5 ml of buffer). ~200μL acid-washed glass beads were added and samples were mixed in a turbo mixer on maximum setting for 30-40 min a 4°C. Lysates were isolated by puncturing the tubes with a red-hot needle and centrifuging at 2500 RPM for 3 min at 4°C.

Pellets were resuspended and sonicated in 10-second pulses five times each using a Branson 450 Sonifier at 1.5 power setting with 100% duty cycle. Samples were incubated on ice >2 min between pulses. Samples were then centrifuged at 13200 RPM for 5 min at 4^{o}C and the supernatants were moved to fresh tubes. Samples were spun again for 15 min and again the supernatants were moved to fresh tubes. Samples were pre-cleared by adding 30 μL protein A- or protein G-agarose beads (Repligen Cat. #IPA-300 and Roche Cat. #11719416001, respectively), as appropriate for the primary antibody type, and incubating at 4^{o}C on a rotamix. The beads were spun down and the lysates were placed in fresh tubes. Immunoprecipitations were performed on dilutions of the lysate. 1/20th of the volume used for immunoprecipitation was removed, diluted to 250 μL in TE/1%SDS and incubated at 65^{o}C overnight to reverse the crosslinks. This material was used as the “input” chromatin. Immunoprecipitations were done for 3-5 hours at 4^{o}C on a rotamix. 40 μL protein A- or protein G-agarose beads were added, as appropriate for the primary antibody type, and samples were incubated again on a rotamix at 4^{o}C for 1-3 hours. Beads were pelleted with a quick spin and washed twice with lysis buffer for 5 min at room temperature on a rotamix, once with high-salt (500 mM) lysis buffer, once with ChIP wash buffer (10 mM Tris-HCL pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and once with TE. Beads were then suspended in 100 μL ChIP elution buffer (50 mM Tris-HCL pH 8.0, 10 mM EDTA, 1%SDS) and incubated at 65^{o}C for 15 min. The supernatants were moved to fresh tubes and the beads were washed with 150 μL TE/0.67% SDS.

The beads were spun down again and the wash was pooled with the eluates. Samples were incubated at 65°C overnight to reverse crosslinks. 250 μL TE/20 μg/ml glycogen/400 μg/ml proteinase K was added to the samples and they were incubated at 37°C for >2 hours. LiCl was added to 440 μM and samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0). The aqueous phase was transferred to a new tube and then 1 ml ice-cold ethanol was added. Samples were incubated at -20°C for >2 hours or overnight and then centrifuged at 13200 RPM for 10 min at 4°C to pellet the chromatin. Pellets were washed with 75% ethanol and centrifuged again for 5 min. The ethanol was removed by aspiration and the pellets were dried. Pellets were resuspended in water and stored at -20°C.

Experiments were quantified by real-time PCR using an ABI 7300 Real Time PCR System and Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen Cat. #11744-500). To quantify the results, a dilution series of the input DNA was used to construct a standard curve and all results were measured as a fraction of the input DNA. To account for sample-to-sample variability, each sample was normalized to a fraction of its own input DNA. Primer pairs used are listed in Table 2.

Polyclonal Tup1 antiserum was provided by Alexander Johnson (figure 2.4) or by Sharon Dent (figure 2.6). Polyclonal anti-HA Y-11 antibodies were obtained from Santa Cruz (Cat. #sc-805) (figure 2.4A) and monoclonal anti-HA antibodies (clone 12CA5) (figure 2.4C) were obtained from Roche (Cat. #11666606001). Monoclonal anti-c-myc antibodies (clone 9E10) were obtained

from Roche (Cat. #MMS-15P). Monoclonal RNA polymerase II antibodies (CTD 4H8) were obtained from Upstate (Cat. #05-623). Polyclonal antibodies specific to acetyl-histone H3 lysine 9, 14, 18, and 23 were obtained from Millipore/Upstate (Cat. # 07-352, 07-353, 07-354, 07-355, respectively). Polyclonal core histone H3 antibodies were obtained from AbCam (Cat. #ab-1791).

Immunoprecipitation/Western Blotting

Total cellular protein was isolated using a slightly modified version of the NaOH-SDS lysis protocol (Kushnirov, 2000). Cells were grown overnight to log phase and 5-15 ODs of cells were pelleted by centrifugation and washed once with sterile water. Pellets were resuspended in 1.5 ml of 0.1 M NaOH and incubated at room temperature for 5 min. Cells were then pelleted by centrifugation and resuspended in 100 μL of SDS lysis buffer (1% SDS, 45 mM HEPES-NaOH pH 7.5, 15 mM DTT) and boiled for 5 min in a water bath. Samples were diluted with 1 ml triton lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Na-HEPES pH7.5, 5 mM EDTA) and centrifuged at maximum for 5 min at 4^{o}C. Supernatants were moved to fresh tubes. α2 serum was added and samples were incubated over night at 4^{o}C in a rotamix. The following morning 40 μL protein A-agarose beads were added and the samples were incubated 1-2 hours at 4^{o}C in a rotamix. Beads were pelleted by centrifugation and washed three times with triton lysis buffer. Beads were pelleted again and boiled for 5 min in 50 μL 2X LLB. Samples were then loaded onto 1.5 mm thick 15%

polyacrylamide gel and resolved by SDS-PAGE. Proteins were transferred to an Immobilon-FL membrane (Millipore Cat. #IPFL00010) at 20V for 2.5 hours at 4°C. Membranes were blocked in 2% milk for >4 hours and were probed overnight at 4°C with α2 serum diluted in 1% milk/TBS + 0.1% Tween-20 and with gentle agitation. Blots were washed three times for 5 min in TBS + 0.3% Tween-20 and twice with TBS + 0.1% Tween-20. Blots were then probed with quantum dots conjugated to goat anti-rabbit antibodies (Invitrogen Cat. #Q11421MP or Q11401MP) for 1 hour at room temperature with gentle agitation. Washes were repeated and the blots were scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare). Scans were quantified using ImageQuant software.

RNA Extraction and RT-PCR

Total cellular RNA was isolated using a modified version of a standard yeast genomic DNA isolation protocol (Hoffman and Winston, 1987). Cells were grown overnight into mid-log phase and 1 OD_{600} of cells was spun down for 10 seconds in a tabletop centrifuge. The medium was removed by aspiration and the cell pellet was rapidly frozen by immersion in a dry ice-ethanol bath. Cell pellets were resuspended in 200 μL DEPC-treated water and 200 μL DEPC-treated RNA extraction buffer was added (2% Triton X-100, 1% SDS, 250 mM NaCl, 10 mM Tris-HCl pH 8.0, 1mM EDTA). 200 μL phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) and ~200μL acid-washed glass beads were added and the samples were mixed in a turbo mixer at 4°C for 5 min. 200 μL DEPC-treated

water was added and samples were then centrifuged at 13200 RPM for 5 min at 4°C and transferred to new tubes. 600 μL isopropanol was added and the samples were incubated at -20°C for >30 min. or over night. The samples were then centrifuged for 12 min at 4°C. The supernatant was removed by aspiration and the pellets were resuspended in 34 μL DEPC-treated water. Samples were incubated at 37°C for 10 min to ensure that the pellet was completely dissolved. 4 μl 10X DNase buffer and 2 μl Turbo DNase were added (Ambion, Cat. #AM2238) and samples were incubated at 37°C for 30-35 min to digest genomic DNA. Samples were diluted with 350 μL DEPC-treated water and were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0). The aqueous phase was transferred to a fresh tube and sodium acetate was added to 100 mM. 1 ml of ethanol was then added and samples were incubated at -20°C for 2 hours or over night. Samples were then centrifuged at 13200 RPM for 15 min. at 4°C. The supernatant was removed by aspiration and the pellet was washed with 75% ethanol, centrifuged at 13200 RPM for 5 min at 4°C, and dried completely. Pellets were resuspended in 1 ml DEPC-treated water and stored at -20°C.

Results were quantified by reverse transcriptase real time PCR using an ABI 7300 Real Time PCR System and a Superscript III Platinum SYBR Green One-Step qRT-PCR kit with ROX (Invitrogen Cat. #11746-500). A standard curve was constructed from MHY608 for each experiment, and the results were obtained as a fraction of the positive control. To account for sample-to-sample variability, a standard curve was also made using *ACT1* primers, and each

sample was normalized to the amount of *ACT1* RNA in that sample. Primers used can be found in Table 3.

β-galactosidase Assays

Measurements of β-galactosidase activity were made using the Permeabilized Cell Assay as has been described previously (Guthrie and Fink, 1991). Briefly, cells were grown overnight into mid-log phase and 1 ml of culture was pelleted by centrifugation. Pellets were resuspended in 1ml Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0). ~60 μl chloroform and ~40 μl 0.1% SDS were added to each sample with a Pasteur pipette. Samples were mixed vigorously with a mini vortexer for 10-15 sec and incubated at 30°C for 3-5 min. 200 μl of 4 mg/ml ONPG was added to each sample. Samples were incubated at 30°C until they turned a slightly yellow color, and then 0.5 ml 1M Na_2CO_3 was added to terminate the reaction. Samples were centrifuged at 13200 RPM briefly, and the OD_{420} was measured in a spectrophotometer. Miller Units were calculated as follows: Miller Units= $1000(OD_{420}/((\text{reaction time in minutes})(\text{volume of culture pelleted})(OD_{600}$ of culture)).

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Table 2.1: Strains Used in this study

Strain	Relevant Genotype
MHY608	*MATa mfa2Δ::LacZ*
MHY609	*MATα mfa2Δ::LacZ*
JY412	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6*
JY434	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 MCM1::13myc-kanMX6*
JY472	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 ssn6Δ*
JY474	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 ssn6Δ tup1Δ*
JY475	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 tup1Δ*
JY550	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 GAL2*
JY562	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 isw2Δ::kanMX4*
JY582	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 cti6Δ::kanMX4*
JY591	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 gcn5Δ::kanMX4*
JY628	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 spt7Δ::kanMX4*
JY640	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 STE12::13myc*
JY720	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 tup1Δ ssn6Δ isw2Δ::kanMX4*
JY814	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 SPT15::3HA-kanMX6*
JY830	*MATa URA3::tetR-VP16::tetO$_2$-α2 ssn6Δ gcn5Δ::kanMX4*

Table 2.2: Primers Used in Real Time PCR

Primer Name	Sequence
5'AGA2	AATTTGTTTCAACCTGCCGG
3'AGA2	CATGTCAAATTTCAAACTGCGTG
5'ASG7	ACCGCATCGGGAAATTTACA
3'ASG7	CATGTTTCTTGTCGTCATTCTTTGA
5'BAR1	GGCTGCACTCATTCCGGTAC
3'BAR1	TCGCTATTATGTGACACTCGCC
GAL1 UAS For	ATATAAATGGAAAAGCTGCATAACCA
GAL1 UAS Rev	CTGAAAATGTTGAAAGTATTAGTTAAAGTGG
5pGRE2 Upstream Region	GGCCCTCACCTCTTTTGTACAA
3pGRE2 Upstream Region	CCGCGAGAAAATTCCGTATTC
5'MFA1	TTGGCCCATACCTTTATTCTTTG
3'MFA1	GCAACCACTGAATCTGT
5'MFA2 Upstream Region	TCCGTTAAGTGCATGCATAGGA
3'MFA2 Upstream Region	TGGCATCCATGTCATGTAAATTTC
5'STE2 TATAA	GGGTGGAATACTATTTAAGGAGTGCTA
3'STE2 TATAA	TAGCCAGAGCAGGTGAAAAGAAA
5'STE2 Upstream Region	AGAATTTAAGCAGGCCAACGTC
3'STE2 Upstream Region	TTGCCAGGCACAGGTCCTA
5' STE6 Upstream Region A	TTACACGCTGCTTCGCACAT
3' STE6 Upstream Region A	GCGGCACTTGAAAGCTCTCT

Table 2.3: Primers Used in Reverse Transcriptase Real Time PCR

Primer Name	Sequence
ACT1 RT-For	TGGATTCCGGTGATGGTGTT
ACT1 RT-Rev	TCAAAATGGCGTGAGGTAGAGA
AGA2 RT-PCR For	CCCCTCACCAACTTTAGAATCG
AGA2 RT-PCR Rev	CCCGTTGGCCAAAATAGTAGTC
ASG7 RT-For	TTCGCTGTTGCTATCACTGAAATT
ASG7 RT-For	CGGTGATACCCATAAAGGAAGAA
BAR1 RT-For	CCCGAGACAGAAGGCAGCTA
BAR1 RT-Rev	GACAGGAACACATCACCCAGAA
LacZ RT-PCR For	GGTGCAGCGCGATCGTA
LacZ RT-PCR Rev	CCGTGGCCTGATTCATTCC
5'STE2 RT-PCR	TCATCCTCGCATACAGTTTGGA
3'STE2 RT-PCR	CGTGGCCCACATTGATGATAAT
5'STE6 RT-PCR	ACATGTTTTGCGGACAGACG
3'STE6 RT-PCR	TCCTGTGCCTGATTCACCAA

Figure Legends

Figure 2.1 | Expressing α2 from a tetracycline-regulatable promoter effectively substitutes for endogenous expression. **A**, α2 protein levels in wt α and a cells, or in α cells expressing α2 from the tetracycline promoter ($tetO_2$-α2) in the absence or presence of 3 μM doxycycline (+DOX). α cell measurements were set to 1.0. **B**, mRNA levels of the asg *STE6* (top panel) and *STE2* (bottom panel) in wt a and α cells, or in $tetO_2$-α2 cells in the absence or presence of DOX. Measurements are normalized to *ACT1* mRNA and are expressed as arbitrary units. Error bars represent s.e.m. *n* =5-7 (α2 protein levels) or 3-11 (mRNA levels).

Figure 2.2 | Distinct mechanisms contribute to the repression of several asg. mRNA levels of the asg **A**, *STE6*, **B**, *STE2*, **C**, *BAR1*, and **D**, *LacZ*, expressed from the endogenous *MFA2* promoter, in wt a and α strains, or in $tetO_2$-α2 strains carrying *tup1Δ*, *ssn6Δ*, *ssn6Δ tup1Δ*, *isw2Δ*, or *ssn6Δ tup1Δ isw2Δ* mutations in the absence or presence of DOX. Error bars represent s.e.m. *n* =3-11.

Figure 2.3 | Rapid induction of asg following withdrawal of α2. **A**, Kinetics of degradation of α2 measured by quantitative IP-western blot (▲), and the kinetics of α2 dissociation from the promoters of *STE6* (♦) and *STE2* (×), measured by

ChIP following the addition of DOX. T0 measurements were set to 1.0. **B**, Accumulation of *STE6* mRNA following the addition of DOX in $tetO_2$-α2 cells. RT-PCR results were normalized as described in the legend to figure 2.1. **C**, As in **B**, but with *STE2* mRNA. **D**, Association of RNA Pol II with the promoter of *STE6* in $tetO_2$-α2 cells following the addition of DOX. Measurements at the 45 min time point were set to 1.0. **E**, Binding of TBP-3HA to the *STE2* TATA box in $tetO_2$-α2 cells in the absence or presence of DOX. **F**, Dissociation of Tup1 (♦), Ssn6-HA (▼), or Mcm1-13Myc (×) from the *STE6* promoter in $tetO_2$-α2 cells following the addition of DOX. **G**, Dissociation of Tup1 (♦) or Ssn6-3HA (▲) from the *STE2* promoter in $tetO_2$-α2 cells following addition of DOX. Error bars represent s.e.m. *n* =4 (IP-western), 3-6 (ChIP), or 2-4 (RT-PCR).

Figure 2.4 | Rapid induction of asg expression requires SAGA. **A**, *STE6* and *STE2* mRNA in wt a cells or in $tetO_2$-α2 cells carrying *gcn5Δ* or *spt7Δ* mutations in the absence or presence of DOX. **B**, Accumulation of *STE6* mRNA following the addition of DOX in $tetO_2$-α2 cells (♦) or $tetO_2$-α2 cells carrying *gcn5Δ* (▼) or *spt7Δ* (×) mutations. **C.** As in **B** but with *STE2* mRNA. **D**, Expression of β-galactosidase from the promoter of *MFA2* after the addition of DOX in $tetO_2$-α2 cells (♦) or $tetO_2$-α2 cells carrying *gcn5Δ* (▼), *spt7Δ* (×), or *cti6Δ* (▲) mutations. **E**, *STE6* mRNA levels in wt a cells, or in $tetO_2$-α2 cells carrying a *cti6Δ* mutation in the absence or presence of DOX. Error bars represent s.e.m. *n* =2-6.

Figure 2.5 | Tup1-dependant histone H3 lysine 9 acetylation of repressed and active asg. **A**, Acetylation of histone H3 on lysine 9 in the promoters of *STE6* and *STE2* in wt α and a cells, or in tetO$_2$-α2 cells carrying *gcn5Δ* or *tup1Δ* mutations in the absence or presence of DOX. Acetylation measurements were normalized to the amount of histone H3 present and are presented as arbitrary units. **B**, Association of Tup1 with the promoters of *STE6* and *STE2* in wt α and a cells, and in tetO$_2$-α2 cells carrying a *tup1Δ* mutation. Control experiments using antibodies for the irrelevant HA epitope were included. Measurements are presented in arbitrary units. **C**, Association of Gcn5-HA with the *STE6* or *STE2* promoter in tetO$_2$-α2 cells carrying a *gcn5Δ* mutation or in tetO$_2$-α2 a cells carrying *gcn5Δ ssn6Δ* mutations. Gcn5-HA was expressed from a plasmid. Error bars represent s.e.m. *n* =3-6.

Figure 2.6 | Acetylation of asg H3 histones on lysines 14, 18, and 23. Acetylation of histone H3 on **A**, lysine 14, **B**, lysine 18, and **C**, lysine 23 in the promoters of *STE6* (left panels) and *STE2* (right panels) in wt α or a cells, or in tetO$_2$-α2 strains carrying *gcn5Δ* mutations in the absence or presence of DOX. Measurements were normalized and are presented as in figure 5. Error bars represent s.e.m. *n* =2-12

Figure 2.7 | All asg are acetylated in the repressed state. Acetylation of histone H3 on lysine 9 in the promoters of *BAR1, MFA1, MFA2, AGA2,* and

ASG7 in wt α and a cells. Measurements were normalized and are presented as in figure 5. Error bars represent s.e.m. *n* =3-4

Figure 2.8 | Pre-acetylation of histone H3 is not limited asg. (left panel) Acetylation of histone H3 on lysine 9 in the promoter of *GAL1* under repressing (YPD) and inducing (YP + 3% raffinose + 3% galactose) conditions. (right panel) Acetylation of histone H3 on lysine 9 in the promoter of *GRE2* under repressing (YPD) and inducing conditions (YPD + 0.4 M NaCl, 20 min). Measurements are presented as in figure 5. Error bars represent s.e.m. *n* =3-5

Figure 2.9 | Model for pre-acetylation of asg. In the repressed state, α2 and Mcm1 bind to their operator and recruit Tup1/Ssn6 to repress asg. Because α2 is expressed below its K_d, it dissociates from asg leaving most of its sites unbound, but Tup1/Ssn6 remain. After α2 dissociates, Tup1/Ssn6 transition from a repressing state to an activating state, leading to the recruitment of SAGA and to the acetylation of histone H3 on lysines 9 and 23. Before activation can proceed to the recruitment of Ste12, TBP, or Pol II, α2 rebinds to its operator. The binding of α2 resets Tup1/Ssn6 to the repressive state. This may result in Tup1/Ssn6 recruiting an HDAC that removes the acetylation mark from histone H3, but I have not determined if this is the case. While α2 is depicted here as rebinding to asg following the recruitment of SAGA and the acetylation of histones, I believe that it may rebind during any point in the cycle and return Tup1/Ssn6 to its repressive state.

Figure 2.1

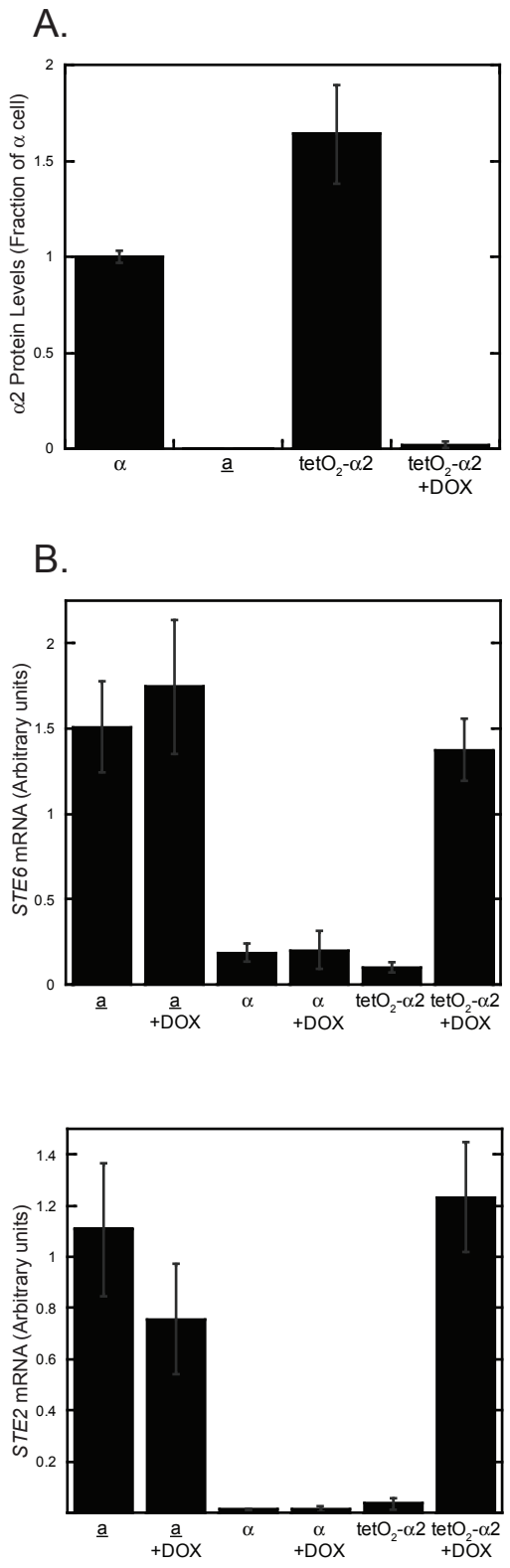

A.
α2 Protein Levels (Fraction of α cell)
2
1.5
1
0.5
0
α
a
tetO2-α2
tetO2-α2 +DOX
B.
STE6 mRNA (Arbitrary units)
2
1.5
1
0.5
0
a
a +DOX
α
α +DOX
tetO2-α2
tetO2-α2 +DOX
STE2 mRNA (Arbitrary units)
1.4
1.2
1
0.8
0.6
0.4
0.2
a
a +DOX
α
α +DOX
tetO2-α2
tetO2-α2 +DOX

Figure 2.2

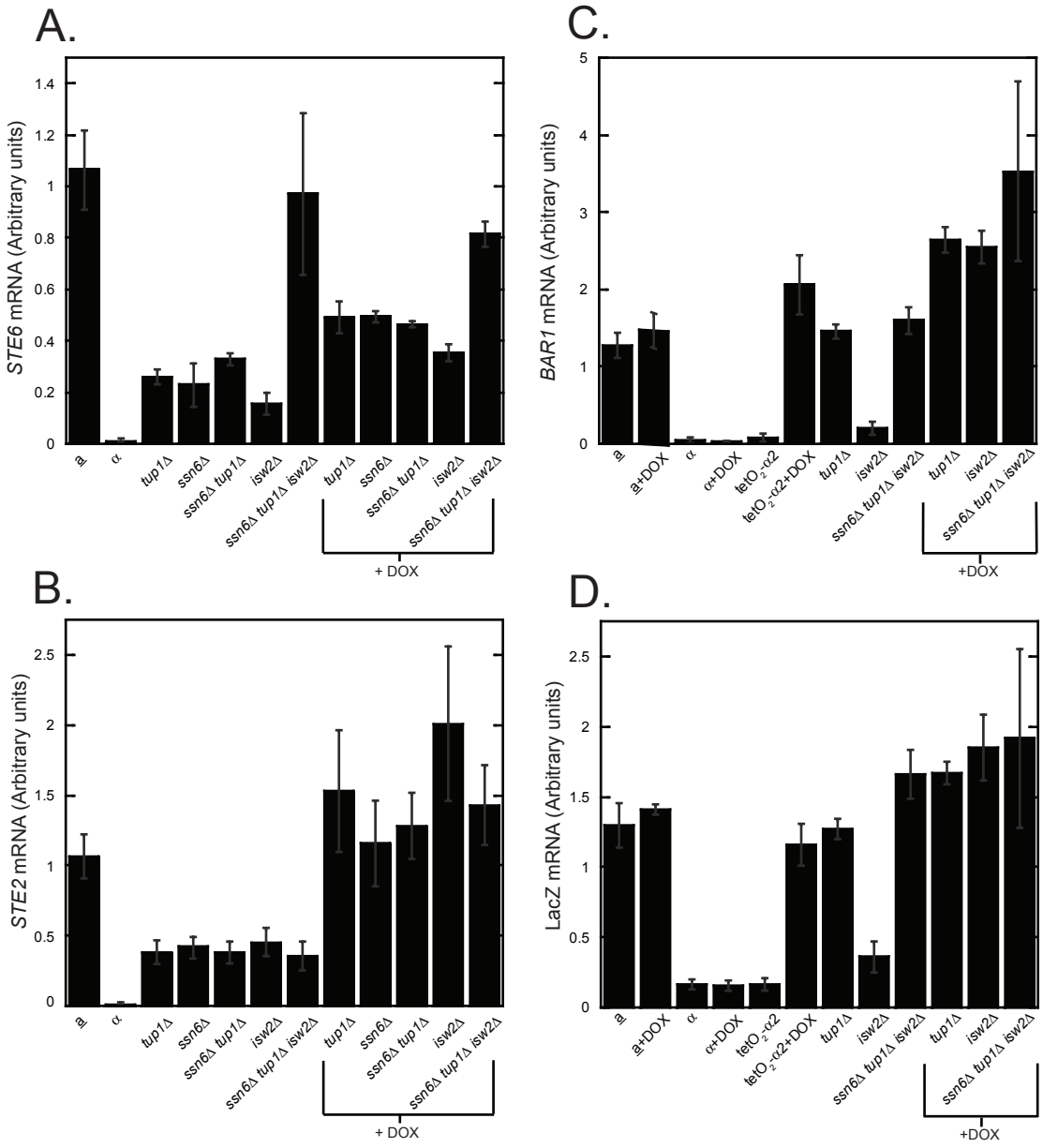

A.
STE6 mRNA (Arbitrary units)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
a
α
tup1Δ
ssn6Δ
ssn6Δ tup1Δ
isw2Δ
ssn6Δ tup1Δ isw2Δ
tup1Δ
ssn6Δ
ssn6Δ tup1Δ
isw2Δ
ssn6Δ tup1Δ isw2Δ
+ DOX
C.
BAR1 mRNA (Arbitrary units)
0
1
2
3
4
5
a
a+DOX
α
α+DOX
tetO2-α2
tetO2-α2+DOX
tup1Δ
isw2Δ
ssn6Δ tup1Δ isw2Δ
tup1Δ
isw2Δ
ssn6Δ tup1Δ isw2Δ
+DOX
B.
STE2 mRNA (Arbitrary units)
0
0.5
1
1.5
2
2.5
a
α
tup1Δ
ssn6Δ
ssn6Δ tup1Δ
isw2Δ
ssn6Δ tup1Δ isw2Δ
tup1Δ
ssn6Δ
ssn6Δ tup1Δ
isw2Δ
ssn6Δ tup1Δ isw2Δ
+ DOX
D.
LacZ mRNA (Arbitrary units)
0
0.5
1
1.5
2
2.5
a
a+DOX
α
α+DOX
tetO2-α2
tetO2-α2+DOX
tup1Δ
isw2Δ
ssn6Δ tup1Δ isw2Δ
tup1Δ
isw2Δ
ssn6Δ tup1Δ isw2Δ
+DOX

Figure 2.3

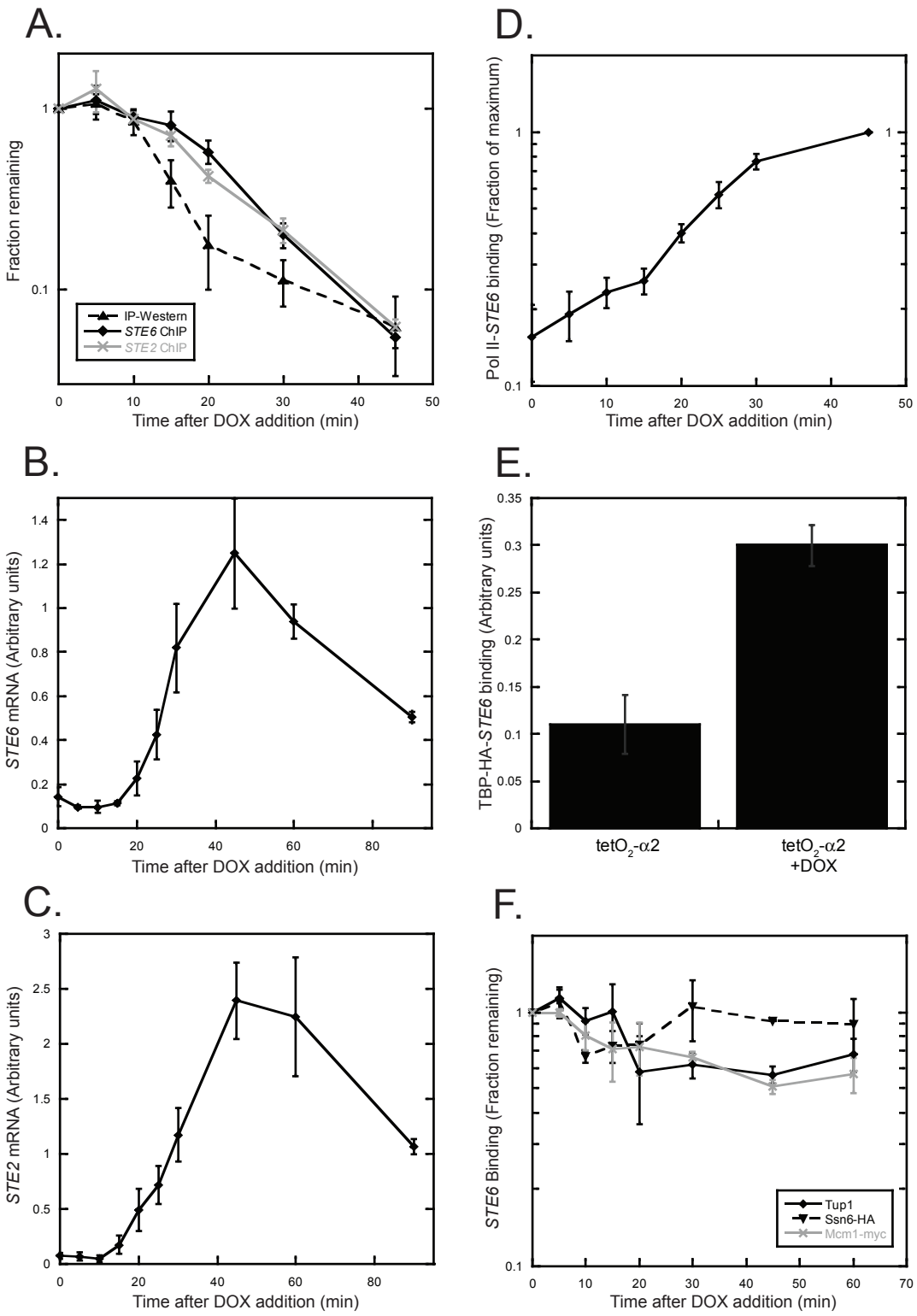

A.
Fraction remaining
IP-Western
STE6 ChIP
STE2 ChIP
Time after DOX addition (min)
D.
Pol II-STE6 binding (Fraction of maximum)
Time after DOX addition (min)
B.
STE6 mRNA (Arbitrary units)
Time after DOX addition (min)
E.
TBP-HA-STE6 binding (Arbitrary units)
tetO2-α2
tetO2-α2 +DOX
C.
STE2 mRNA (Arbitrary units)
Time after DOX addition (min)
F.
STE6 Binding (Fraction remaining)
Tup1
Ssn6-HA
Mcm1-myc
Time after DOX addition (min)

Figure 2.3 cont.

G.

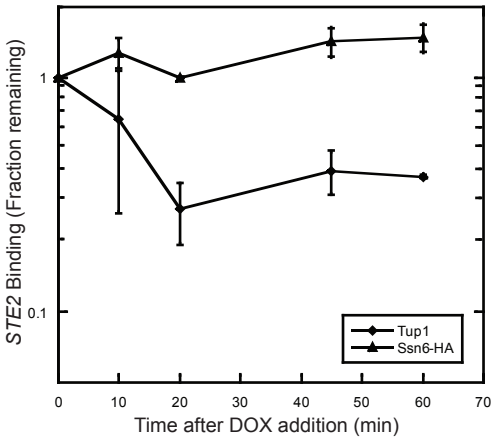


Figure 2.4

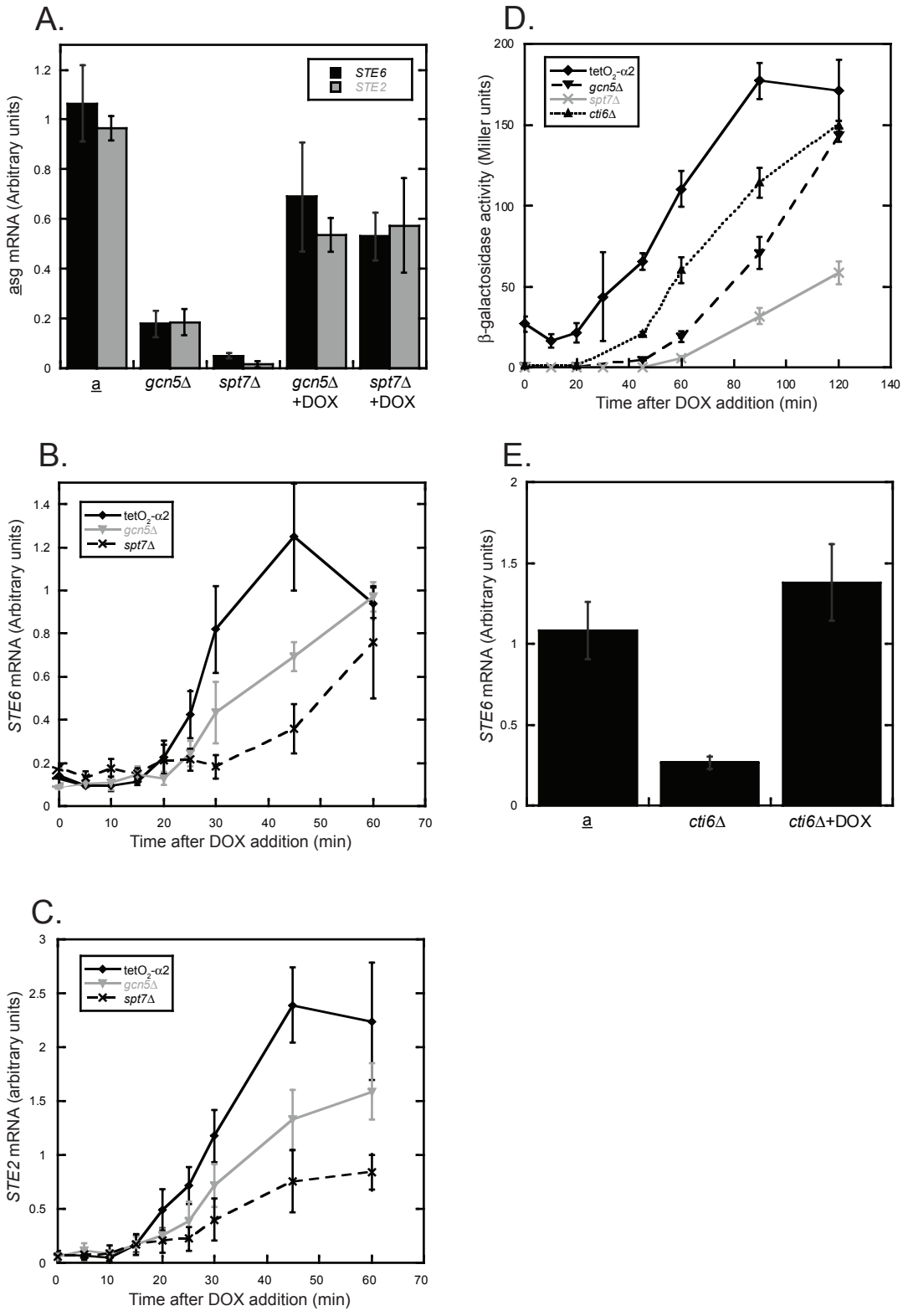

A.
asg mRNA (Arbitrary units)
STE6
STE2
a
gcn5Δ
spt7Δ
gcn5Δ +DOX
spt7Δ +DOX
D.
β-galactosidase activity (Miller units)
tetO2-α2
gcn5Δ
spt7Δ
cti6Δ
Time after DOX addition (min)
B.
STE6 mRNA (Arbitrary units)
tetO2-α2
gcn5Δ
spt7Δ
Time after DOX addition (min)
E.
STE6 mRNA (Arbitrary units)
a
cti6Δ
cti6Δ+DOX
C.
STE2 mRNA (arbitrary units)
tetO2-α2
gcn5Δ
spt7Δ
Time after DOX addition (min)

Figure 2.5

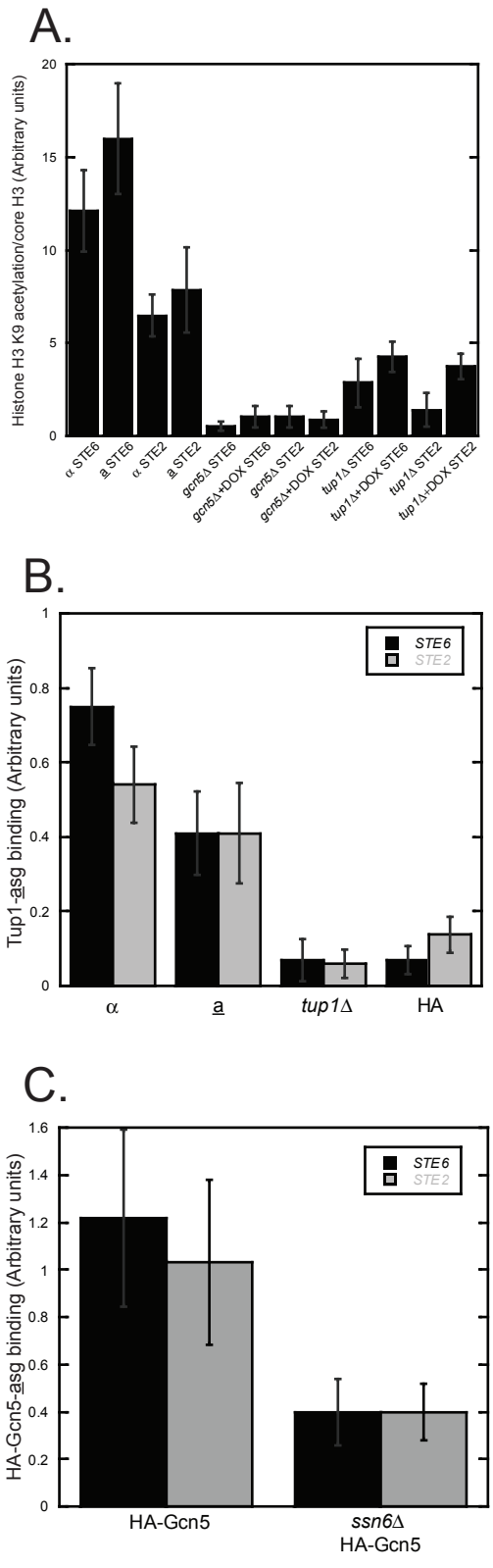

A.
Histone H3 K9 acetylation/core H3 (Arbitrary units)
20
15
10
5
0
α STE6
a STE6
α STE2
a STE2
gcn5Δ STE6
gcn5Δ+DOX STE6
gcn5Δ STE2
gcn5Δ+DOX STE2
tup1Δ STE6
tup1Δ+DOX STE6
tup1Δ STE2
tup1Δ+DOX STE2
B.
Tup1-asg binding (Arbitrary units)
1
0.8
0.6
0.4
0.2
0
STE6
STE2
α
a
tup1Δ
HA
C.
HA-Gcn5-asg binding (Arbitrary units)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
STE6
STE2
HA-Gcn5
ssn6Δ
HA-Gcn5

Figure 2.6

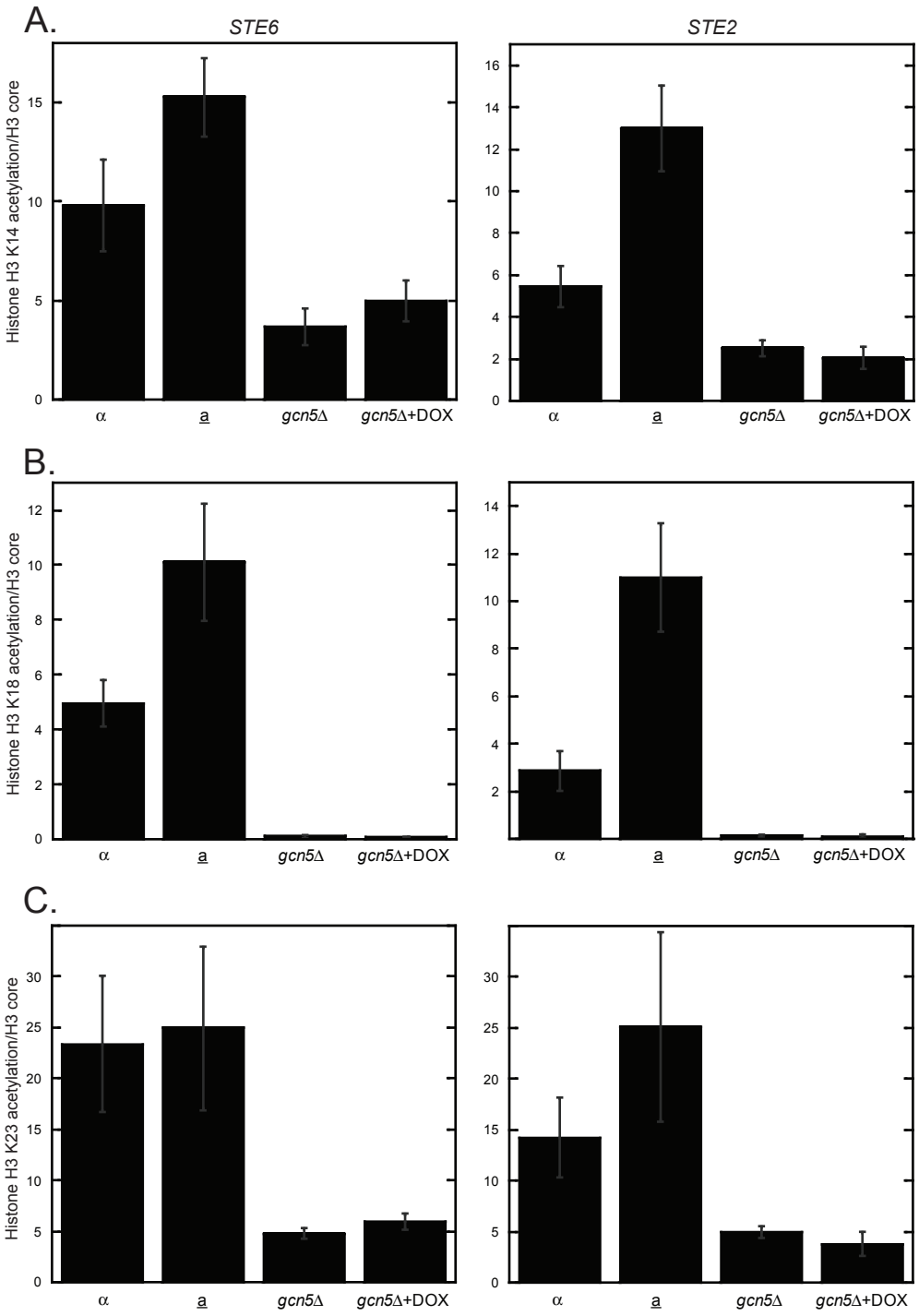

A.
STE6
STE2
Histone H3 K14 acetylation/H3 core
B.
Histone H3 K18 acetylation/H3 core
C.
Histone H3 K23 acetylation/H3 core
α
a
gcn5Δ
gcn5Δ+DOX

Figure 2.7

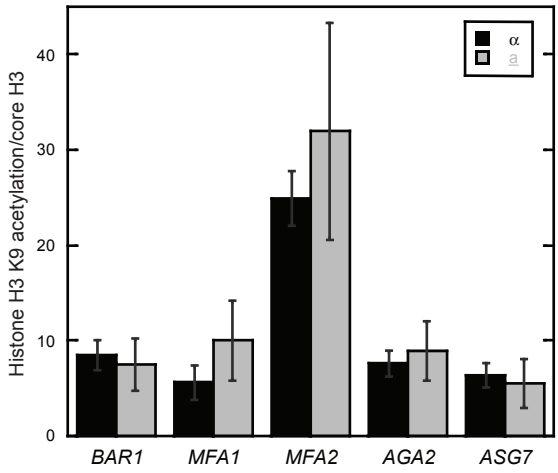

Histone H3 K9 acetylation/core H3
0
10
20
30
40
α
a
BAR1
MFA1
MFA2
AGA2
ASG7

Figure 2.8

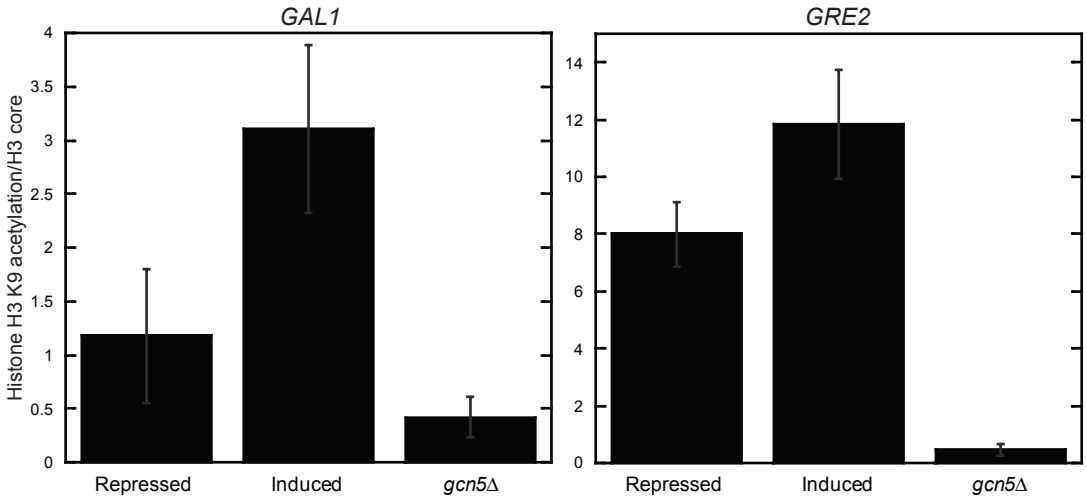
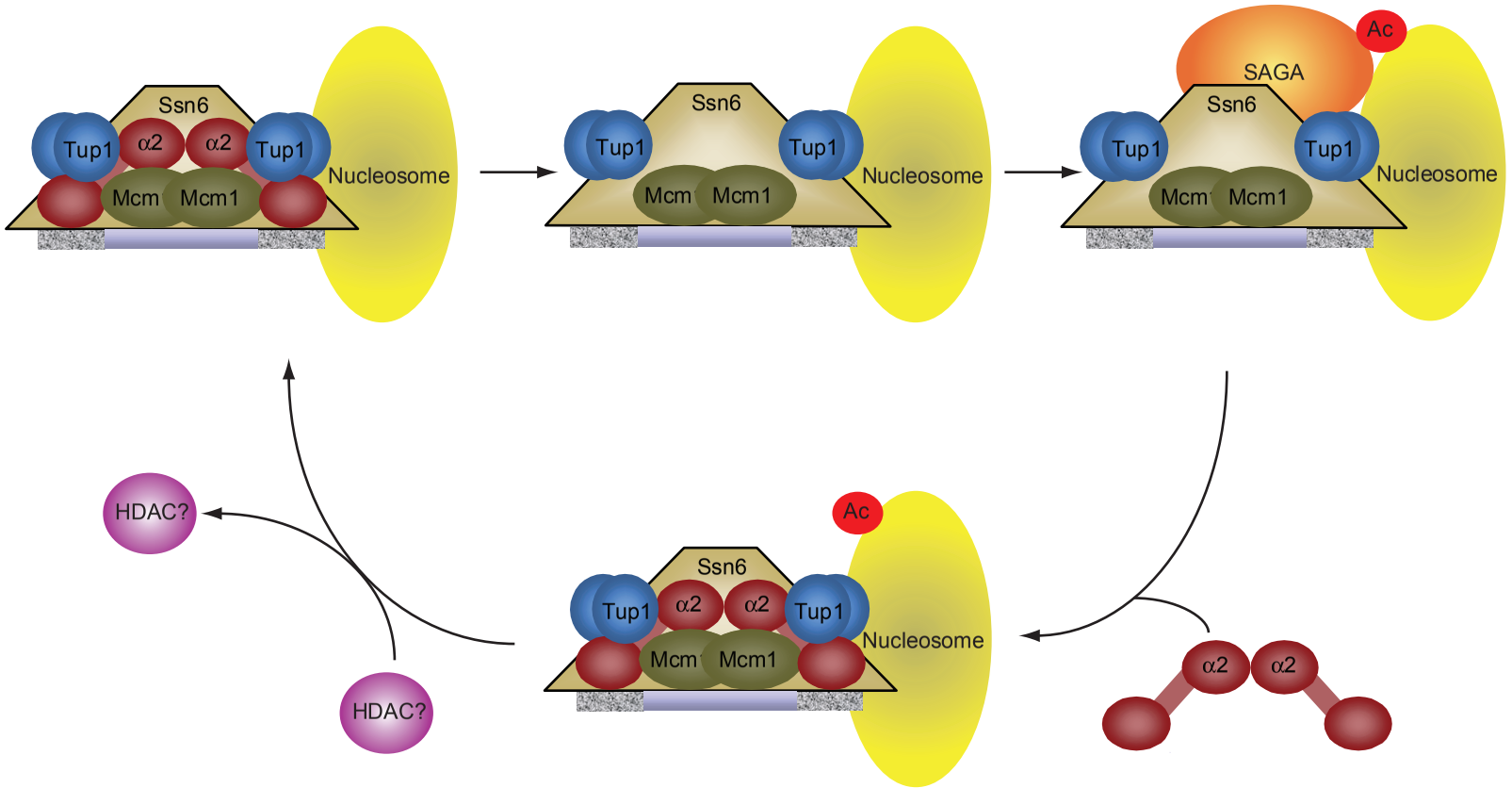

GAL1
GRE2
Histone H3 K9 acetylation/H3 core
0
0.5
1
1.5
2
2.5
3
3.5
4
0
2
4
6
8
10
12
14
Repressed
Induced
gcn5Δ
Repressed
Induced
gcn5Δ

Figure 2.9


Ssn6
Tup1
α2
α2
Tup1
Mcm
Mcm1
Nucleosome
Ssn6
Tup1
Tup1
Mcm
Mcm1
Nucleosome
Ac
SAGA
Ssn6
Tup1
Tup1
Mcm
Mcm1
Nucleosome
α2
α2
Ac
Ssn6
Tup1
α2
α2
Tup1
Mcm
Mcm1
Nucleosome
HDAC?
HDAC?

Chapter 3

Interactions of functionally engaged α2 overcome its inherent instability to allow stable repression of a-specific genes

Abstract

Mating type in yeast is genetically controlled by the allele present at the *MAT* locus, which encodes a set of master regulatory transcription factors that direct the cell to execute a specific genetic program. However, yeast are able to change their mating type by physically changing the information stored at *MAT*. To prevent the regulators from the previous cell type from disrupting the new phenotype, each of the mating-type regulators is constitutively targeted for rapid degradation in the proteasome. Interestingly, for one cell-type regulator, α2, rapid degradation might be expected to undermine its function. α2 represses the transcription of genes specific to the a cell type by recruiting the Tup1/Ssn6 co-repressor complex to their promoters. Paradoxically, in spite of its inherent instability, the repression engendered by α2 is both stable and robust. Recent evidence suggests that the functionally engaged sub-population of α2 may be shielded from turnover, but this pool has not been examined directly. I have investigated the behavior of this sub-population and found that interaction with its binding partner, Ssn6, significantly alters the dynamics of α2 interactions with its targets, allowing it to remain bound even while the majority of the protein is being degraded. This uncoupling of DNA-bound α2 from the labile pool of the protein was dependent on Isw2, suggesting that a condensed, repressive chromatin structure is able to shield functionally engaged α2 from destruction, thereby allowing it to form a stable co-repressor complex. Additionally, binding of the activator Ste12 promotes the dissociation of α2 from asg, consistent with the

hypothesis that chromatin structure protects α2 from being removed from its target genes.

Introduction

The budding yeast *Saccharomyces cerevisiae* is able to exist as one of three different cell types. Two of the types are haploids and have been termed a and α. Each type displays a specialized mating phenotype such that each is competent to mate with the other and give rise to a third type that is non-mating and diploid. The determining factor that directs a haploid cell to develop as one mating type or the other is the allele present at the *MAT* locus, which encodes a set of regulators that directs the cell to execute one genetic program or the other (Galgoczy et al., 2004; Herskowitz, 1985; Nasmyth and Shore, 1987). For example, in α cells, *MAT* encodes two proteins termed α1 and α2 (Strathern et al., 1981). α1 is an activator that is necessary for the expression of genes that are specific to the α cell type, while α2 is a repressor that inactivates expression of genes that are specifically expressed in the a cell type (a-specific genes or asg). Even though the expression profile of each type is genetically encoded, the mating-type of an individual cell is not necessarily static. Cells are able to switch mating-types by removing the allele present at *MAT* and replacing it with a cassette that contains the information of the opposite mating type (Strathern et al., 1979). The switching event begins when the product of the *HO* gene makes a double-stranded cut within *MAT* (Kostriken et al., 1983; Strathern et al., 1982). The cut is then repaired by inserting a new allele that contains the mating-type information of the other cell type (Hicks et al., 1979). As a result of switching the information contained at *MAT*, the cell will then express a new set of regulators and differentiate into a new cell type.

The ability of cells to switch presents them with an interesting problem. The mating-type regulators that were expressed before the switch would still be present, and would continue to direct the cell to develop into the previous cell type. At the same time, the new regulators would attempt to direct it to develop into the new cell type, and therefore a proper genetic program would not be executed. To prevent this condition from arising, each of the mating type regulators is targeted for rapid destruction in the proteasome, resulting in each regulator being cleared from the cell within minutes of a switching event (Chen et al., 1993; Hochstrasser and Varshavsky, 1990; Johnson, 1997; Nixon and Laney, unpublished observations). While this solution prevents regulators from two different cell types from ever existing in the same cell, it also creates an additional challenge that must be overcome. In α cells, the expression of asg is robustly and stably repressed, but it seems unlikely that the α2 repressor would be able to engender such stable repression when it itself is remarkably unstable. How this apparent paradox is resolved remains an unanswered question in yeast mating-type biology.

One possible way that the unstable α2 protein might be able to establish consistent repression would be for the sub-population of α2 that is bound to asg promoters to be protected from rapid degradation. This could be achieved if the degradation signals in α2 could be masked by interactions with other proteins. Degradation signal masking of α2 has been observed previously. When α2 forms a complex with the a1 protein in diploid cells it becomes quite stable, even though the machinery to degrade α2 remains intact (Johnson et al., 1998). If a

similar mechanism existed at asg in α cells, α2 would be able to repress asg without being constantly destroyed.

Functionally engaged α2 makes several interactions that could potentially mask its degradation signals. α2 represses asg by nucleating a co-repressor complex by forming direct interactions with three different proteins. α2 binds directly to Mcm1, a cofactor that enhances its DNA binding affinity (Keleher et al., 1988; Mead et al., 1996), as well as to Tup1 and Ssn6, two proteins that form a general repressor of transcription in yeast (Keleher et al., 1992; Komachi et al., 1994; Smith et al., 1995). Recent evidence suggests that the binding of Tup1 and Ssn6 might be responsible for stabilizing the DNA-bound form of α2 (Laney et al., 2006). When Tup1/Ssn6-α2 interactions were forced by vastly over-expressing Tup1 and Ssn6, the half-life of α2 was greatly extended. Stabilization of α2 could only be achieved if Tup1 and Ssn6 were over-expressed simultaneously. However, when the *Deg1* signal from the N-terminal region of α2 was fused to a normally stable protein, over-expression of Tup1 alone was able to achieve stabilization. This was not a surprising result, as the *Deg1* domain and the Tup1 binding domain in α2 overlap. It does, however, suggest that Ssn6 might be needed to mask other degradation signals in α2 to achieve stabilization.

While these observations are consistent with the hypothesis that Tup1 and Ssn6 might stabilize the DNA-bound pool of α2 by masking its degradation signals, they do not provide direct evidence because they did not specifically observe the sub-population of α2 that was bound to DNA as part of a co-

repressor complex. In order to specifically study the functionally engaged pool of α2, I have constructed a strain where the expression of α2 can be readily controlled, and used a time-resolved version of the ChIP assay to observe the dynamic interactions between α2 and its target DNA. I observed that α2 is able to stay bound to asg promoters after the unbound pool of protein has been degraded, suggesting that it is protected from proteolysis. The apparent protection was ablated in an α2 point mutant that has decreased affinity for binding to Ssn6. Surprisingly, the protection was also dependent on the presence of a functional Isw2-Itc1 chromatin-remodeling complex, suggesting that chromatin architecture may play an important role in protecting the DNA-bound pool of α2 from degradation. Consistent with a role for chromatin structure in α2-asg interactions, the binding of the asg activator Ste12 promoted rapid dissociation of α2 from its target genes, possibly by causing disorganization of chromatin structure.

Results

α2-Tup1/Ssn6 interactions affect α2-target gene binding dynamics

In order to study the dynamics of the functionally engaged pool of α2, I utilized a yeast strain where the sole copy of α2 was placed under the control of the easily regulatable tetracycline operator (described in detail in chapter 2). Characterization of the $tetO_2$-α2 strain indicated that it can be used as a physiologically relevant system for studying the dynamic behavior of α2 (figure 2.1). To observe the dynamics of interaction between α2 and its target operators, I extinguished α2 expression by adding doxycycline (DOX) to growing cultures, and observed the kinetics of its degradation and of its binding to asg promoters over time using quantitative immunoprecipitation/western blotting and ChIP assays. As discussed in chapter 2, after adding DOX to cultures there was an approximately 10 min lag period before the levels of α2 began to decline (figure 2.3A), likely due to the time necessary for the drug to enter the cell and for existing α2 mRNA to be cleared. After this time, total α2 protein levels declined with kinetics identical to those that have been observed previously (Chen et al., 1993; Hochstrasser and Varshavsky, 1990; Johnson et al., 1998). However, the kinetics of dissociation of α2 from its operators in the promoters of *STE6* and *STE2* were quite different than the turnover of the bulk population. Following the initial lag period, α2 dissociated from its target genes relatively slowly, with only a roughly 2-fold loss of binding over the next 10 min of the time course. Following this phase, dissociation became more rapid, and the ChIP signal approached

background levels within 45 min after the addition of DOX. If the binding of α2 to its promoters were regulated only by the abundance of α2 protein, it would be expected that the dissociation of α2 from DNA would have mirrored the kinetics of its degradation. The difference between the kinetics of turnover and dissociation could have been a result of α2 needing to be degraded below its K_d before loss of binding would result. However, we have observed that the kinetics of dissociation remain the same even if α2 is vastly over-expressed, and we have calculated that physiological levels of α2 are well below its K_d (Wilcox and Laney, submitted). This suggests that dissociation kinetics are not primarily determined by turnover rate, and suggests that α2-DNA interactions are under additional levels of control. The bi-phasic nature of the dissociation curve suggested that the kinetics of α2-DNA interactions are controlled by at least two processes; one process that controls the early, slower dissociation phase, and another that governs the rapid phase. It is unlikely that either phase is governed completely by ubiquitin-dependent proteolysis, since the kinetics do not match the kinetics of degradation. Even during the rapid phase, α2 dissociated with a half-time of approximately ~7½ min. While this is quite rapid, it does not match the ~4½ min half-life that I observed for turnover.

Recent data has demonstrated that degradation signals in α2 can be masked by over-expression of the co-repressor components Tup1 and Ssn6, thereby greatly extending the half-life of the protein (Laney et al., 2006). Interestingly, stabilization of full-length α2 could only be achieved by over-expression of both Tup1 and Ssn6, while degradation of normally stable proteins

that had been fused to *Deg1* could be achieved by over-expression of Tup1 alone. Thus, it seems that Tup1 binding is sufficient to mask recognition of the *Deg1* signal. This is likely explained by the fact that the Tup1 binding domain in α2 and *Deg1* overlap (Hochstrasser and Varshavsky, 1990; Johnson et al., 1998; Komachi et al., 1994). While this does explain how Tup1 might protect α2 from degradation, it also raises the question of why over-expression of Ssn6 in addition to Tup1 is necessary to stabilize full-length α2. The simplest explanation is that Ssn6 binding masks an additional degron in α2. This possibility seems plausible, since it is known that there is at least one other degradation signal in α2 (Hochstrasser and Varshavsky, 1990). To address this question, I examined the effects of Ssn6 binding to α2 on the dynamics of α2-DNA interactions.

To determine the role of Ssn6 in α2-target gene interactions, I first followed the dissociation of α2 from *STE6* in an *ssn6Δ* strain. Both an *ssn6Δ* strain and an *ssn6Δ tup1Δ* strain showed significantly decreased dissociation kinetics relative to control cells (figure 3.1A). However, upon investigation of the half-life of the total population of α2 by IP/western blotting, I observed that its degradation had been significantly impaired by the loss of Ssn6 (figure 3.1B). I did not repeat the IP/western to confirm this result, but instead I took advantage of the R713A point mutation, a known α2 mutant that has significantly reduced binding affinity for Ssn6, but does not affect its DNA-binding affinity (Smith and Johnson, 2000) or turnover rate (figure 3.1B). I introduced this mutation into the copy of α2 controlled by the $tetO_2$ operator and examined the dissociation of α2 from *STE6* following the addition of DOX (figure 3.1C). Dissociation kinetics in

this strain were significantly altered. The slow dissociation between 10 and 20 min post DOX addition was eliminated, and instead the fast dissociation phase began immediately. It therefore seems that the association of Ssn6 with α2 is necessary for the creation of the slow phase, and it may do so by protecting α2 from ubiquitination and removal from the DNA. To confirm that R173A-α2 was dissociating from DNA more rapidly than wt α2, I also examined the steady-state binding of R173A-α2 to the *STE6* promoter and compared it to wt α2 binding (figure 3.1D). I found that wt α2 bound to *STE6* at five-fold higher levels than R173A-α2. This difference in binding cannot be a result of the mutation affecting DNA biding directly, since it has previously been shown that the R173A mutation does not alter α2-asg promoter binding *in vitro* (Smith and Johnson, 2000). I therefore interpret this data to mean that the decrease in steady-state binding of R173A-α2 to *STE6* is a result of its increased dissociation rate.

I next reasoned that if disrupting the interaction between α2 and Ssn6 could result in the loss of the slow dissociation phase and decreased steady-state binding to *STE6*, then enhancing the interaction should have the opposite effect. I tested this by expressing *SSN6* from the *GPD* or *GAL1* promoter in addition to the endogenous copy of *SSN6*, and observed the effects on α2-DNA binding dynamics. Expressing *SSN6* from the *GPD* promoter altered the dissociation of α2 from the *STE6* operator (figure 3.1E). Kinetics were impaired following the addition of DOX to the media, and it appeared that the slow dissociation phase may have been extended. Expressing *SSN6* from the *GAL1* promoter resulted in a similar, but more severe response. I also examined the

steady-state level of α2 binding to *STE6* in the *SSN6* over-expressing strains. While expression of *SSN6* from the *GPD* promoter did not seem to affect the steady-state binding of α2 to its operator, expressing it from the *GAL1* promoter did cause a nearly 2-fold increase in binding relative to $tetO_2$-α2 cells (figure 3.1D). These results are consistent with a role of Ssn6 in protecting α2 from removal from DNA when it is in its functionally engaged form.

I also attempted to ascertain what effect Tup1 has on the stability of promoter-bound α2. I first investigated this by following the dissociation of α2 from *STE6* in a *tup1Δ* strain. After the addition of DOX, the dissociation kinetics in the *tup1Δ* strain closely resembled those of the $tetO_2$-α2 strain (figure 3.1A). Over the first 30 min of the assay, the kinetics were identical. At later time points, there was a minor difference in dissociation, as loss plateaued at ~15% of binding remaining. This may be a secondary effect of the pleiotropic nature of the *tup1Δ* mutation. To attempt to observe the effects of Tup1 on α2-DNA binding dynamics without the complication of secondary effects from loss of Tup1 activity, I next examined the dissociation of α2 carrying the L10S mutation, which greatly impairs the association of Tup1 with α2 (Komachi and Johnson, 1997; Komachi et al., 1994). I found that L10S-α2 did not dissociate from *STE6* at an appreciable rate (figure 3.2A). However, this result is not meaningful, as I found that the mutation also severely hindered the degradation of the bulk population of α2 as measured by western blotting (figure 3.2B). I also attempted to examine the dynamics of α2-DNA binding in a version of α2 that carried both the L10S and the R173A mutations, however I found that it also did not dissociate from

STE6 at a measurable rate due to a similar inhibition of bulk α2 turnover (figure 3.2A and B). Finally, I examined α2 dissociation kinetics in a strain expressing *GAL1*-driven *TUP1* in addition to the endogenously expressed *TUP1*. While results of this experiment were somewhat erratic, I did observe a reproducible decline in dissociation rate that was reminiscent of the kinetics in the *SSN6* over-expressing strains (compare figure 3.2C to 3.1E), suggesting that enhancing α2-Tup1 interactions can inhibit the removal of α2 from its target operator.

Isw2 activity slows the dissociation of α2 from asg

While it appeared that interaction with Ssn6 led to a change in the dissociation rate of α2 from its target site, it was not apparent if this was a direct result of α2-Ssn6 interactions, or if it was a secondary effect of changes in the local chromatin structure that result from Tup1/Ssn6-based repression. A functional co-repressor complex has a profound effect on the local chromatin structure at asg, causing nucleosomes to become phased into a repressive structure, with the first nucleosome positioned just 13 bp from the α2 operator (Ganter et al., 1993; Roth et al., 1990; Shimizu et al., 1991). To investigate the possibility that changes in chromatin architecture affect α2-DNA binding dynamics, I examined the kinetics of α2 dissociation from *STE6* in cells carrying an *isw2Δ* mutation. The α2/Tup1/Ssn6 complex requires a functional Isw2-Itc1 complex to position nucleosomes at asg (Morohashi et al., 2006; Ruiz et al., 2003). Disruption of this complex leads to moderate derepression of *STE6* (figure 2.2A), but has never been demonstrated to affect the DNA-bound co-

repressor complex. The *isw2Δ* strain displayed a similar change in α2-asg dissociation to the R173A strain (figure 3.3A). The initial lag phase was lost, and the rapid dissociation phase began immediately, although the kinetics of the rapid phase were somewhat impaired relative to $tetO_2$-α2 cells. To confirm this result, I also examined the steady-state binding of α2 to *STE6* (figure 3.3B), and found that *isw2Δ* displayed a 3-fold reduction in α2-DNA binding. As was the case for the kinetic experiments, this result resembles the result observed for the R173A-α2 ChIP experiment, although it was somewhat less severe. I interpret this result to mean that much of the change that I observed in R173A-α2-target gene interactions relative to wt α2 interactions was due at least partly to failure of the co-repressor complex to establish a repressive chromatin structure. This result was unexpected, as it has never been reported that a repressive chromatin structure has any effect on α2 binding properties. It is also counter-intuitive, since nucleosomes do not cover the α2 binding site at repressed promoters. However, it is noteworthy that the rapid α2-DNA dissociation phase begins coincident with the activation of asg (figure 2.3B and C), which is presumably when the remodeling of chromatin structure into an open state has been completed.

Ste12, but not general activators, promotes α2 dissociation from asg

The surprising discovery that chromatin structure can affect α2-DNA binding kinetics led us to ask if other mutations that affect asg expression might have an effect on α2-target gene interactions. To examine this, I chose to

investigate the effect of Ste12, an activator that binds asg promoters and is necessary for their expression (Dolan et al., 1989; Fields and Herskowitz, 1985), on α2-target gene binding kinetics. I conducted the DOX-chase ChIP experiment in *ste12Δ* cells, and observed a mild decrease in the dissociation rate of α2 from both *STE6* and *STE2* (figure 3.3C and D). This effect may represent an extension of the slow dissociation phase, but it is difficult to make a strong conclusion because the change in kinetics was minor and there was significant error in these measurements. To further investigate its role in α2-target gene dynamics, I constructed a strain carrying a C-terminally myc-tagged version of Ste12, and measured its association with *STE6* following repression of α2 expression (figure 3.3E). I observed no binding of Ste12-myc to *STE6* for at least the first 20 min of the assay, and then it rapidly bound to *STE6* and reached maximal binding by 30 min. The kinetics of Ste12-myc binding to *STE6* suggested that Ste12-myc was able to associate with asg rapidly upon the loss of α2, as Ste12-myc association with asg was almost perfectly coincident with the dissociation of α2. There are two possible ways that I believe this data can be interpreted. The first is that α2 and Ste12 could compete for binding to asg promoters, and that in α cells there is sufficient α2 present to out-compete Ste12. Once α2 levels begin to decline, Ste12 may be able to effectively compete for target promoter binding, resulting in a concomitant increase in Ste12 binding as α2 binding decreases. A Ste12/α2 competition could also explain why the *ste12Δ* mutation appeared to have slower kinetics of α2 dissociation from asg. If Ste12 is not present, then α2 may be able to rebind to asg, resulting in a slower

apparent dissociation rate, as measured by DOX-chase ChIP assays. An alternate hypothesis could be that Ste12 affects α2-target gene dynamics by altering chromatin structure. Activation of asg leads to disordering of positioned nucleosomes (Gavin et al., 2000), and as an activator of asg Ste12 should promote this disordering. Therefore, in *ste12Δ* cells, which are unable to activate asg expression properly (Fields and Herskowitz, 1985), nucleosome positioning may remain in a relatively repressive state, resulting in impaired dissociation of α2 from its targets. Furthermore, it appears that Ste12 associates with *STE6* coincident with its activation (compare figure 2.3B with 3.3E), and at approximately the same time that α2 enters the fast dissociation phase, further raising the possibility that Ste12 promotes rapid dissociation of α2 from its targets by opening chromatin structure and allowing access of factors that destabilize α2-target gene interactions.

To determine whether the Ste12 effect was specific or a general effect of loss of an asg activator, I analyzed the effects of deletion of other activators on α2-target gene dynamics. I chose to investigate the dynamics of α2-target gene interactions in $tetO_2$-α2 *gcn5Δ*, $tetO_2$-α2 *spt7Δ*, and $tetO_2$-α2 *cti6Δ* strains. I selected Gcn5 and Spt7 because they have profound effects on the kinetics of asg activation (figure 2.4B, C, and D). I have also observed that Cti6 has a mild effect on the kinetics of *MFA2* activation (figure 2.4D), so I investigated it as well. However, in preliminary experiments I was unable to see any significant difference in the dissociation of α2 from *STE6* in these strains (figure 3.4A), and I therefore did not continue to investigate these strains thoroughly. I also

attempted to investigate the role of the Swi/Snf complex in α2-target gene dynamics by building a *swi2Δ* strain. Unfortunately, this strain showed severe growth defects and a severe reduction of α2 expression levels (figure 3.4C). I attempted to use the DOX-chase ChIP assay in this strain, and α2 appeared to dissociate significantly more quickly than in $tetO_2$-α2 cells (figure 3.4D), but the results were erratic and difficult to interpret because of the change in α2 expression.

Our lab has recently discovered that removal of α2 from *STE6* depends on the ubiquitin-directed chaperone Cdc48 (Wilcox and Laney, submitted). This prompted us to ask whether other molecular chaperones may be involved in α2-asg dissociation. I chose to examine Sba1, the yeast homologue of a mammalian chaperone that has been implicated in disassembly of transcriptional regulatory complexes (Fang et al., 1998; Freeman and Yamamoto, 2002). I found that disruption of *SBA1* had little effect on the dynamics of α2-target gene interactions (figure 3.4A). Surprisingly though, I did find that the α2-asg binding levels were reduced in *sba1Δ* strains (figure 3.4B). This was unexpected, as deletion of a chaperone involved in removal of transcription factors would likely increase promoter occupancy. This may be an indirect effect of the deletion of *SBA1*. An alternate possibility is that Sba1 may help to remove α2 from any low-affinity, non-functional sites that it may bind to. There are at least 50 such sites scattered throughout the genome (Zhong et al., 1999), and if α2 binds these sites and does not dissociate quickly, the pool of α2 available for asg binding may be diminished. I investigated the non-specific binding of α2 in *sba1Δ* strains using

cycloheximide-chase ChIP experiments. I observed the binding of α2 to the promoters of *ACT1* and *DOA3*, neither of which are α2 targets (figure 3.5A). I found that there was a detectable level of binding at both of these promoters, and at both promoters binding declined after 15 min. This indicates that Sba1 may be involved in removal of α2 from non-specific binding sites. I must note however, that these experiments were done using a different ChIP protocol than the other experiments presented here (see Materials and Methods), and I have found it to be less reliable than our typical protocol. Additionally, the values presented in this experiment have not been normalized.

I also attempted to determine the ATP-dependence of the dissociation of α2 from DNA, as this would provide further evidence that Isw2 does affect α2 binding dynamics. To do so, I depleted cells of ATP by adding sodium azide, which inhibits cytochrome c oxidase (Bennett et al., 1996), or CCCP, which uncouples mitochondria by increasing conductance of ions across their membranes (Hopfer et al., 1968), to growing cells, and then used the time-resolved ChIP assay. Both experiments yielded qualitatively similar results; a fraction of α2 binding appeared to be labile, while a fraction appeared to remain bound over the course of the assay (figure 3.5B), although the relative size of the fractions appeared quite different depending on which ATP-depleting agent was used. I must again note that these results were obtained with the less effective protocol and are normalized to the quantity of a non-specific gene immunoprecipitated rather than to the input DNAs.

Discussion

Uncoupling of α2-DNA binding dynamics from the degradation of α2

Using time-resolved ChIP assays, I have demonstrated here that the binding dynamics between α2 and its target operators are not primarily determined by the proteasome-dependent degradation of α2. The kinetics of loss of α2 binding from an asg promoter were markedly different than the kinetics of its destruction, with protein remaining bound to the promoter of *STE6* even while the overall population of α2 protein had been degraded to undetectable levels. Since α2 is expressed well below its K_d, and its binding sites are not saturated under normal expression levels (Wilcox and Laney, submitted), the disparity in kinetics cannot be explained by re-binding of α2 to the *STE6* operator. Rather, the functionally engaged pool of α2 seems to be protected, at least in part, from proteasome-dependent degradation. This protection is likely due in part to degradation signal masking of α2 by interaction with Tup1/Ssn6, as this has been demonstrated to occur to the bulk population of the protein under conditions of exceptionally high Tup1 and Ssn6 expression. Here, I have specifically examined the DNA-bound pool of α2 and found that increased levels of its binding partners Tup1 or Ssn6 lead to slower loss of α2 from its targets, while impairment of α2-Ssn6 interactions caused it to dissociate more quickly. This behavior is consistent with the hypothesis that its degradation signals are masked by interaction with its cofactors. The observation that deletion of *TUP1* had little effect on α2 dynamics, while over-expressing *TUP1* slowed dissociation

is somewhat puzzling, as in one case it appeared that Tup1 is not required for α2 dynamics, while the other suggested that it may be sufficient to impair dissociation. A possible explanation might be that Ssn6 is more important in determining kinetics, and so loss of Tup1 does not have a significant effect. However, greatly over-expressing Tup1 may cause an increase in Ssn6 recruitment to asg since Ssn6 and Tup1 make physical interactions (Williams et al., 1991).

The influence of Isw2 on α2-target gene binding dynamics

Quite surprisingly, I found that the uncoupling of α2-DNA binding kinetics from its degradation kinetics was largely dependent on the presence of Isw2, a Tup1/Ssn6 interacting protein that assists in the formation of repressive chromatin structures (Morohashi et al., 2006; Ruiz et al., 2003; Zhang and Reese, 2004). This result was not expected, as no link has ever been demonstrated between nucleosome positioning and the dynamics of co-repressor-DNA interactions. Furthermore, the nucleosome position at asg has been extensively investigated by footprinting analysis, and the asg operator has never been observed to be protected by a nucleosome (Ganter et al., 1993; Roth et al., 1990; Shimizu et al., 1991). However, since Isw2 does not make any known physical interactions with α2, the assembly of repressive chromatin structure is implicated by this result. Even though nucleosomes do not protect the α2 binding site, the creation of an Isw2-dependent higher-order chromatin structure might affect α2 dynamics. Other observations are consistent with the

role of chromatin structure in protection of α2. It has been observed previously that asg promoter activation by Mcm1 causes the local chromatin structure to become disordered (Gavin et al., 2000). I note that the change in α2 dissociation kinetics from the slow phase to the rapid phase occurs just as asg begin to be transcribed (figure 2.3A-D). Since chromatin is becoming disorganized by transcriptional activation at this time, it is possible that the change from slow to fast dissociation is due to the chromatin structure being remodeled and thereby allowing additional factors to act on α2 more efficiently.

The role of Ssn6 in α2-target gene binding dynamics

The observation that α2-DNA binding dynamics are altered by loss of Isw2 activity does not necessarily rule out the hypothesis that Ssn6 binding to α2 directly affects its stability. In both the kinetic and the steady-state experiments, the *isw2Δ* deletion had a milder effect on α2-target gene interactions than did the R173A mutation, which affects its interaction with Ssn6. This could be the result of Ssn6 having both a direct effect on α2 stability, and an indirect one that is mediated by Isw2 and chromatin structure. Over-expressing *SSN6* also led to increased stabilization of the DNA-bound pool of α2 relative to the unbound pool. This could be interpreted two ways. Firstly, raising Ssn6 levels could have resulted in increased α2-Ssn6 binding, leading to increased masking of a degradation signal. Secondly, increased Ssn6 binding could have attracted Isw2 more strongly, resulting in a more stable chromatin structure than is normally present at asg, and causing increased occlusion of the degradation machinery

from access to the DNA-bound α2. Further experimentation will be necessary to resolve the relative importance of these two possible mechanisms. Most importantly, to determine if the results of the R173A mutation and the results of the *isw2Δ* mutation represent two distinct mechanisms to stabilize functionally engaged α2, the two mutations could be combined in the same strain. If they work through separate pathways, then the effect of α2-DNA interaction should be more severe than either mutation alone. Alternatively, if they work through the same pathway and Ssn6 only serves to stabilize α2 by recruiting Isw2, then the two mutations should be no more severe than the R173A mutation alone.

The role of Ste12 in promoting the dissociation of α2 from its target sites

I have observed here that α2 interactions with its targets may be influenced by the presence of Ste12, as loss of Ste12 seemed to cause a small decrease in the rate of α2 dissociation from its target genes. However, it is not yet clear why Ste12 would have such an effect. The observation that Ste12 cannot bind to asg in the presence of α2, but that it rapidly binds immediately upon the dissociation of α2, suggests that the two proteins may complete for asg binding, but that α2 is expressed at significant enough levels to completely prevent Ste12 from binding in α cells. Interestingly, because there is no detectable kinetic delay between the dissociation of α2 and association of Ste12, α2 must therefore be expressed at levels that are just high enough to occlude Ste12, but low enough to allow Ste12 to bind asg as soon as there is a small decline in α2 protein levels. This fine-tuning of α2 expression levels provides

another potential mechanism that allows for the rapid activation of asg upon a mating-type switch, as expressing higher levels of α2 would take more time to degrade it to a level where Ste12 could effectively compete for binding, and activation would therefore be delayed. Consistent with this hypothesis, I have observed that increasing α2 levels does lead to delayed asg activation kinetics (see appendix figure A.1A).

An alternate hypothesis to explain the effect of Ste12 on α2 binding dynamics could be that because Ste12 is an activator, it promotes the disorganization of chromatin, and therefore allows better access to factors that remove α2 from DNA. In this manner, Ste12 would serve as the functional antagonist of Isw2, which seems to stabilize asg-target gene interactions by creating an ordered, repressive chromatin structure. It has been shown previously that asg expression leads to disordering of nucleosomes around asg (Gavin et al., 2000), but this study did not investigate the role of Ste12 in determining chromatin structure. However, since Ste12 binding happens just as asg promoters are becoming active, and the rapid phase of α2-asg dissociation begins at this same time, it does seem plausible that Ste12 binding promotes open chromatin structure, resulting in the increased α2 dissociation rate. This model is not necessarily mutually exclusive with the competition model. It is possible that α2 and Ste12 compete for asg binding, but that the competition does not affect dissociation rate, while remodeling the chromatin structure does. Alternatively, both mechanisms might contribute to the kinetics of α2-asg dissociation. Further investigation will be necessary to dissect the relative

importance of these two models.

Materials and Methods

Yeast strains and plasmids

All manipulations were made using standard techniques (Guthrie and Fink, 1991). The $tetO_2$-α2 cells used in this chapter were described in chapter 2. All yeast were grown in YPD medium except $tetO_2$-α2 P_{GPD}-SSN6, which was grown in SD-Leu media to maintain the p425-P_{GPD}-SSN6 plasmid, and the P_{GAL}-*SSN6* and P_{GAL}-*TUP1* expressing strains, which were grown in YP + 3% raffinose + 3% galactose to induce expression. To make JY533, JY536, and JY546, the endogenous copy of *MAT*α 2 was replaced with a hygromycin resistance cassette. *α2* was then amplified using primers that incorporated the desired mutations and BamHI restriction sites. This fragment was then cloned into the BamHI site in pRS306-tTA-$tetO_2$. JY600, JY635 and JY639 were made by replacing the endogenous copies of *SBA1, STE12,* or *SWI2* in JY408 (see chapter 2 methods) with a kanamycin resistance marker and selecting spores with the desired genotype. JY572 was made by digesting pRS306-P_{GAL1}-SSN6 with BstEII and integrating it into the *LEU2* locus in JY550 (see chapter 2 methods). JY573 was made by digesting pTRP-TUP1-ΔNsiI with XbaI and integrating it into the endogenous *TRP1* locus in JY550. Construction of all other strains was described in chapter 2 Materials and Methods.

Chromatin Immunoprecipitation

DOX-chase ChIP assays were performed exactly as described in chapter 2 unless otherwise noted. For cycloheximide (Chx)-chase ChIP assays, Chx was

added to 200 μg/ml at T0. For the sodium azide chase, Na-azide was added to 20 mM at T0. For the CCCP chase, CCCP was added to 40 μM. For the experiments shown in figure 3.5, the DNA purification step of the ChIP protocol was modified. After digestion with proteinase K, samples were diluted five-fold into buffer PB from a Quiagen PCR purification kit and were passed through a Quiaquick DNA binding column from that kit. The column was washed with buffer PE as per the manufacturer's instructions. DNA was then eluted with TE, and diluted with H_2O. For the experiments shown in figure 3.5B, the results were normalized to the quantity of the non-specific gene *DOA3* or *ACT1* DNA that had been precipitated rather than to the input DNA. The antibody used was the same rabbit polyclonal α2 antisera as in chapter 2. All experiments were quantified using real-time PCR as described in chapter 2 using 5' and 3' STE6 upstream region A primers.

Immunoprecipitations and western blotting

All IP/western experiments were conducted exactly as described in chapter 2. The only exception to this is the DOX-chase of $tetO_2$-L10S-α2, which was done by straight western blot. Cells were grown into mid-log phase and were lysed using a slightly modified NaOH-SDS lysis protocol (Kushnirov, 2000). Cells were pelleted by a quick centrifugation at maximum speed and were washed with sterile water. They were then resuspended in 1.5 ml of 0.1 M NaCl, and incubated at room temperature for 5 min. The cells were then pelleted again, and the NaOH was removed by aspiration. Samples were then boiled for

5 min in 50 μl 2XLLB. Membranes were pelleted by centrifugation at maximum speed for 1 min and then the extracts were loaded directly onto a 1.5 mm thick 15% polyacrylamide gel. The gel was run overnight and then transferred at 20V for 2.5 hours, blocked >4 hours in 2% milk, and probed overnight with 1:1000 affinity purified α2 polyclonal antibody. The washes, secondary antibody incubation, visualization, and quantitation were all done as described in chapter 2.

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Table 3.1: Strains Used in this study

Strain	Relevant Genotype
MHY608	*MATa mfa2Δ::LacZ*
MHY609	*MATα mfa2Δ::LacZ*
JY412	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6*
JY472	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 ssn6Δ tup1Δ*
JY474	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 ssn6Δ*
JY475	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 tup1Δ*
JY533	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-R173A-α2 matα2Δ::hphMX6*
JY536	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-L10S-α2 matα2Δ::hphMX6*
JY546	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-L10S-R173A-α2 matα2Δ::hphMX6*
JY562	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 isw2Δ::kanMX4*
JY572	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 GAL2 LEU2::pRS305-GAL1-SSN6*
JY573	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 GAL2 TRP1::pTRP1-TUP1*
JY582	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 cti6Δ::kanMX4*
JY591	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 gcn5Δ::kanMX4*
JY600	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 sba1Δ::kanMX4*
JY628	*Matα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 spt7Δ::kanMX4*
JY635	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 LEU2::tetR'-SSN6 matα2Δ::hphMX6 ste12Δ::kanMX4*
JY639	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 swi2Δ::kanMX4*
JY640	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 STE12::13myc*
246.1.1	*MATα*

Table 3.2: Approximate α2-*STE6* dissociation half-times (min)

Genotype	First phase	Second phase
$tetO_2$-α2	15	7-8
tup1Δ	15	7-8
R173A-α2	9	NA
P_{GPD}-SSN6	17	NA
P_{GAL1}-SSN6	28	11
P_{GAL1}-TUP1	31	NA
isw2Δ	8	NA
ste12Δ	15	NA
cti6Δ	7	6
gcn5Δ	9	3-4
spt7Δ	15-16	9-10
sba1Δ	8	NA

Figure Legends

Figure 3.1 | Interaction with Ssn6 alters α2-DNA binding dynamics. **A**, Dissociation of α2 from the *STE6* operator in $tetO_2$-α2 cells (♦), $tetO_2$-α2 *tup1Δ* cells (▲), $tetO_2$-α2 *ssn6Δ* cells (▼), or in $tetO_2$-α2 *tup1Δ ssn6Δ* cells (×). **B**, α2 protein levels following the addition of DOX in $tetO_2$-α2 cells (♦), $tetO_2$-α2 *ssn6Δ* cells (▲), or in $tetO_2$-R173A-α2 cells (▼). **C**, Dissociation of α2 from the *STE6* operator in $tetO_2$-α2 cells (♦), or $tetO_2$-R173A-α2 cells (▲). **D**, Binding of α2 to its operator in the promoter of *STE6* in $tetO_2$-α2 or $tetO_2$-R173A-α2 cells, or in $tetO_2$-α2 cells expressing *SSN6* from the *GPD* or the *GAL1* promoter. **E**, Dissociation of α2 from the *STE6* operator in $tetO_2$-α2 cells (♦), or in $tetO_2$-α2 cells expressing *SSN6* from the *GPD* (▲) or the *GAL1* (▼) promoter. Error bars represent s.e.m. *n* =1-9

Figure 3.2 | Interaction with Tup1 may affect α2-DNA binding dynamics. **A**, Dissociation of α2 from the *STE6* operator in $tetO_2$-α2 cells (♦), $tetO_2$-L10S-α2 cells (▲), or $tetO_2$-L10S-R173A-α2 cells (▼). **B**, α2 protein levels following the addition of DOX in $tetO_2$-α2 cells (♦), $tetO_2$-L10S-α2 cells (▲), or $tetO_2$-L10S-R173A-α2 cells (▼). **C**, Dissociation of α2 from the *STE6* operator in $tetO_2$-α2 cells (♦), or in $tetO_2$-α2 cells expressing *TUP1* from the *GAL1* promoter (▲). Error bars represent s.e.m. *n* =1-4

Figure 3.3 | Effects of Isw2 and Ste12 on α2-DNA binding dynamics. **A**, Kinetics dissociation of α2 from its operator in the promoter of *STE6* in $tetO_2$-α2 cells (♦), or in $tetO_2$-α2 *isw2Δ* cells (▲). **B**, Binding of α2 to its operator in the promoter of *STE6* in $tetO_2$-α2 or in $tetO_2$-α2 *isw2Δ* cells. **C**, Kinetics dissociation of α2 from its operator in the promoter of *STE6* in $tetO_2$-α2 cells (♦), or in $tetO_2$-α2 *ste12Δ* cells (▲). **D**, Kinetics of dissociation of α2 from its operator in the promoter of *STE2* in $tetO_2$-α2 cells (♦), or in $tetO_2$-α2 *ste12Δ* cells (▲). **E**, Association of Ste12-myc with the promoter of *STE6* following the addition of DOX in $tetO_2$-α2 cells. Error bars represent s.e.m. *n* =1-8

Figure 3.4 | SAGA, Cti6, and Sba1 do not affect α2 dissociation from asg. **A**, Dissociation of α2 from the *STE6* operator in $tetO_2$-α2 cells (♦), $tetO_2$-α2 *spt7Δ* cells (▲), $tetO_2$-α2 *gcn5Δ* cells (▼), $tetO_2$-α2 *cti6Δ* cells (×), or in $tetO_2$-α2 *sba1Δ* cells (◯). **B**, Binding of α2 to its operator in the promoter of *STE6* in $tetO_2$-α2 or in $tetO_2$-α2 *sba1Δ* cells. **C**, Two DOX-chase IP/western blots of α2 in $tetO_2$-α2 *swi2Δ* cells. a, wt a cells, α, wt α cells, ND, no DOX. **D**, Dissociation of α2 from the *STE6* operator in $tetO_2$-α2 cells (♦), or $tetO_2$-α2 *swi2Δ* cells (▲). Error bars represent s.e.m. *n* =1-3

Figure 3.5 | Sba1 and ATP depletion affect α2-asg binding dynamics. **A**, Dissociation of α2 from the promoters of *ACT1* (♦) or *DOA3* (▲) following the

addition of cycloheximide to *sba1Δ* cells. **B**, Dissociation of α2 from the promoter of *STE6* following the addition of sodium azide (♦) or CCCP (▲) to wt α cells. Error bars represent s.e.m. *n* =1-2.

Figure 3.1

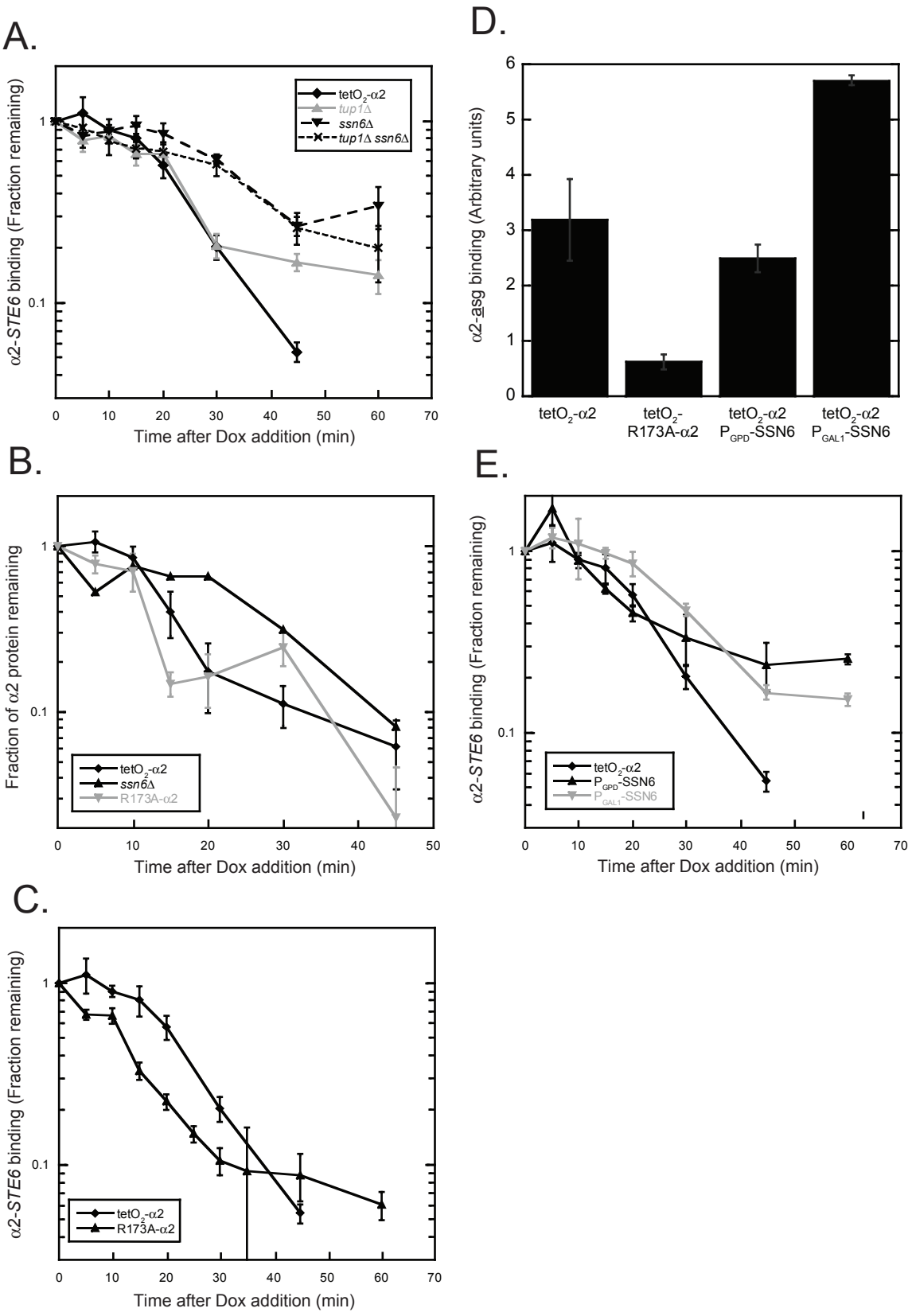
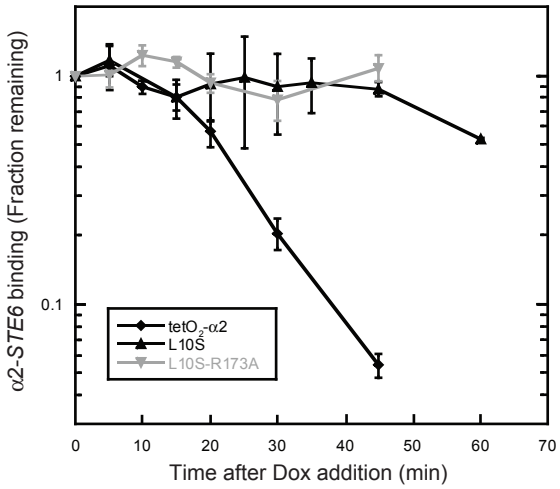
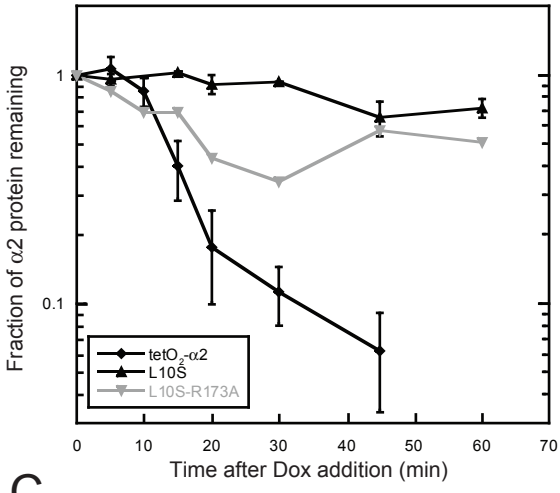

A.
α2-STE6 binding (Fraction remaining)
Time after Dox addition (min)
tetO2-α2
tup1Δ
ssn6Δ
tup1Δ ssn6Δ
B.
Fraction of α2 protein remaining
Time after Dox addition (min)
tetO2-α2
ssn6Δ
R173A-α2
C.
α2-STE6 binding (Fraction remaining)
Time after Dox addition (min)
tetO2-α2
R173A-α2
D.
α2-asg binding (Arbitrary units)
tetO2-α2
tetO2-R173A-α2
tetO2-α2 PGPD-SSN6
tetO2-α2 PGAL1-SSN6
E.
α2-STE6 binding (Fraction remaining)
Time after Dox addition (min)
tetO2-α2
PGPD-SSN6
PGAL1-SSN6

Figure 3.2

A.



B.



C.

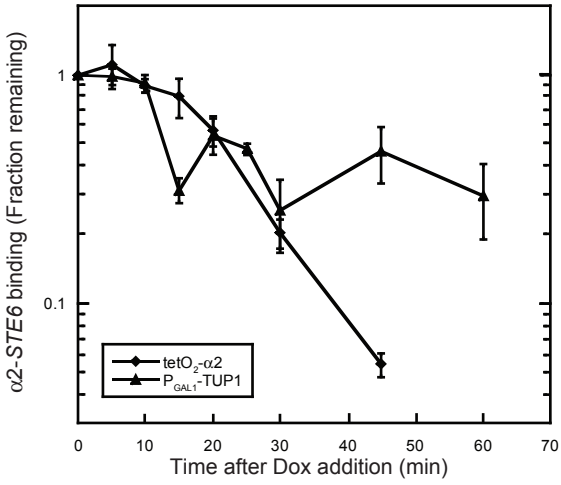


Figure 3.3

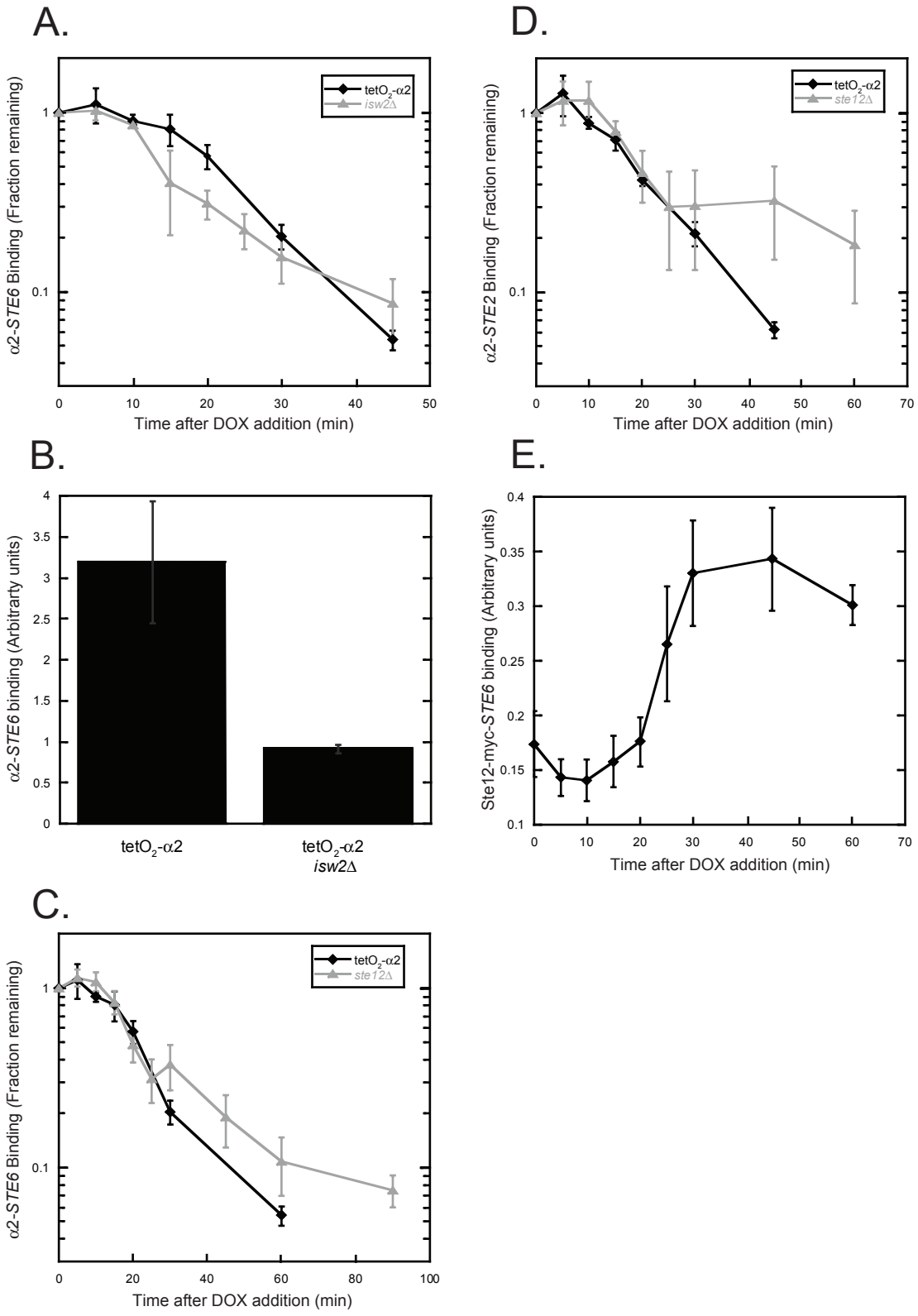

A.
tetO2-α2
isw2Δ
α2-STE6 Binding (Fraction remaining)
Time after DOX addition (min)
D.
tetO2-α2
ste12Δ
α2-STE2 Binding (Fraction remaining)
Time after DOX addition (min)
B.
α2-STE6 binding (Arbitrarty units)
tetO2-α2
tetO2-α2
isw2Δ
E.
Ste12-myc-STE6 binding (Arbitrary units)
Time after DOX addition (min)
C.
tetO2-α2
ste12Δ
α2-STE6 Binding (Fraction remaining)
Time after DOX addition (min)

Figure 3.4

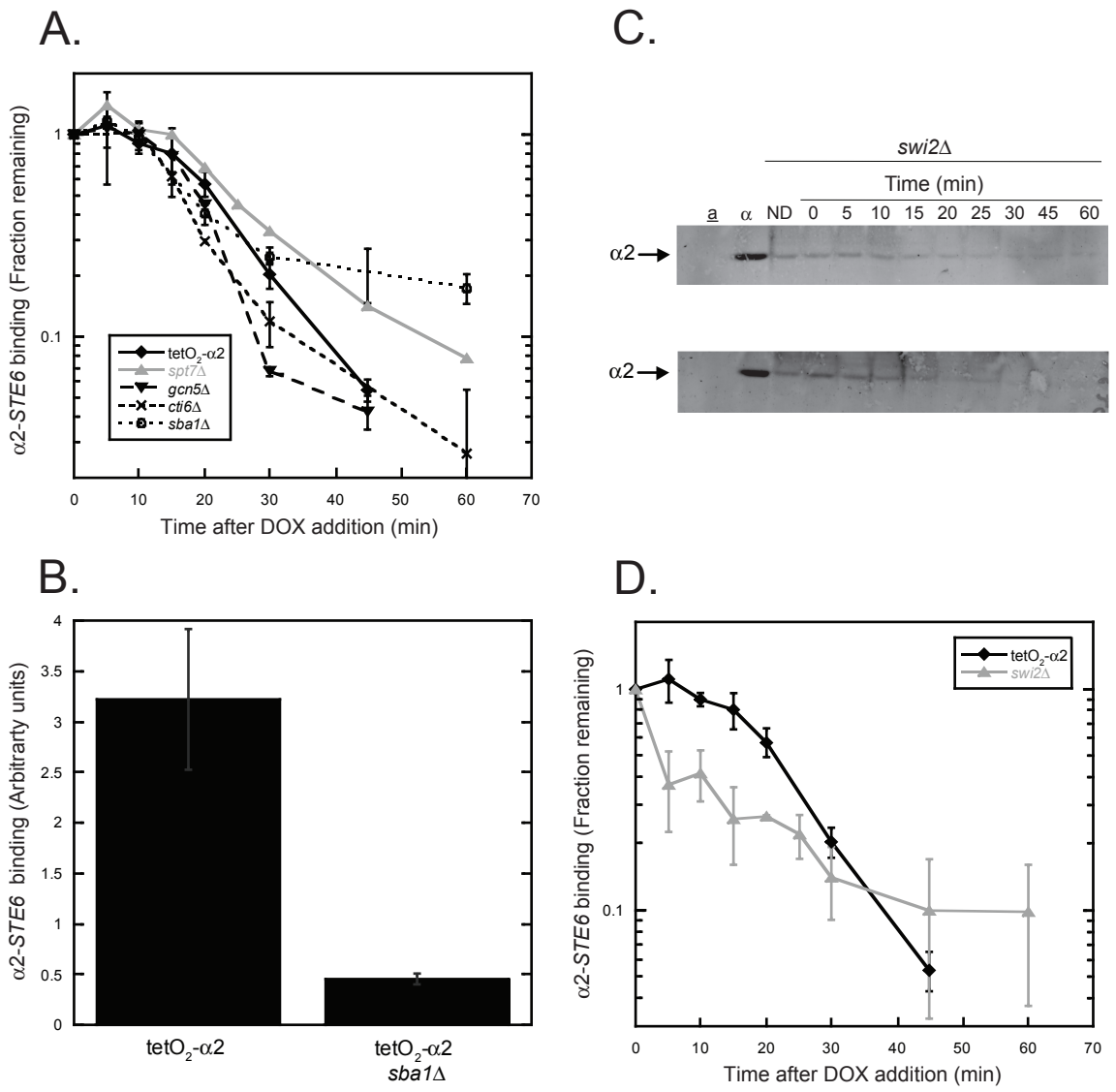
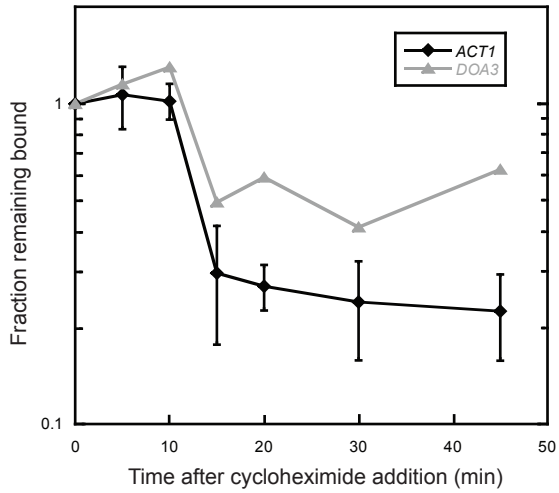
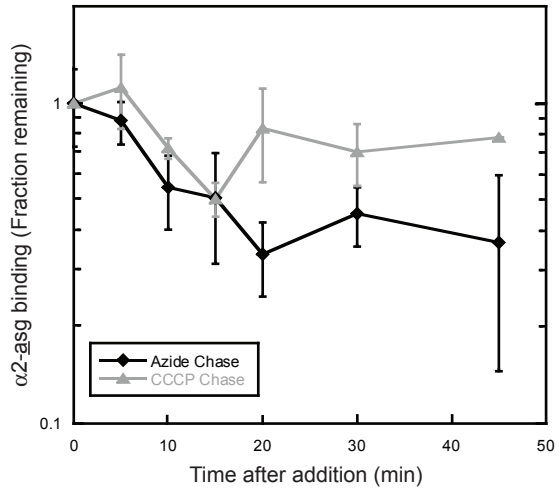

A.
α2-STE6 binding (Fraction remaining)
Time after DOX addition (min)
tetO2-α2
spt7Δ
gcn5Δ
ctf6Δ
sba1Δ
C.
swi2Δ
Time (min)
a α ND 0 5 10 15 20 25 30 45 60
α2
α2
B.
α2-STE6 binding (Arbitrarty units)
tetO2-α2
tetO2-α2
sba1Δ
D.
α2-STE6 binding (Fraction remaining)
tetO2-α2
swi2Δ
Time after DOX addition (min)

Figure 3.5

A.


ACT1
DOA3
Fraction remaining bound
1
0.1
0
10
20
30
40
50
Time after cycloheximide addition (min)

B.


α2-asg binding (Fraction remaining)
1
0.1
Azide Chase
CCCP Chase
0
10
20
30
40
50
Time after addition (min)

Chapter 4

Discussion and future directions

Discussion: The interactions of α2 and the control of yeast mating-type determination

Interactions with sequence-specific DNA-binding proteins direct Tup1/Ssn6 activity: implications for α2-mediated control of mating type

As discussed in chapter 1, interactions between sequence-specific DNA-binding proteins and Tup1/Ssn6 have a direct impact on the specific mechanisms that Tup1/Ssn6 will use to repress or activate target promoters. This is evidenced by the fact that different mechanisms are used in the repression of different subsets of repressed promoters, and that mutating the various TPR domains in Ssn6 cause differing amounts of derepression of different genes (Tzamarias and Struhl, 1995). Also, it appears that the loss of interaction between Tup1/Ssn6 and specific DNA-binding proteins is the trigger that signals Tup1/Ssn6 to transition from a repressor to an activator (Papamichos-Chronakis et al., 2004; Proft et al., 2001; Proft and Struhl, 2002), further highlighting the role of specific interactions in influencing Tup1/Ssn6 activity. Based on the information discussed in chapter 1, interactions between Tup1/Ssn6 and DNA-binding proteins can be categorized into three distinct patterns. Comparison between these patterns and the interactions I have observed between α2 and Tup1/Ssn6 may help us understand the mechanisms that underlie asg repression.

The most obvious similarities between the interactions of known sequence-specific binding factors with Tup1/Ssn6 and the observations I have made here occur at glucose-regulated and salt stress-regulated promoters. At

both of these target types, the DNA-binding proteins, Mig1 and Sko1 respectively, recruit Tup1/Ssn6 to bring about repression. When either promoter type is activated, the DNA-binding protein becomes phosphorylated and is rapidly shuttled out of the nucleus (De Vit et al., 1997; Pascual-Ahuir et al., 2001), but the functionally-engaged subpopulation of the protein remains bound to the target gene (Papamichos-Chronakis et al., 2004; Proft et al., 2001; Proft and Struhl, 2002). The DNA-bound proteins do appear to be phosphorylated as well, and phosphorylation greatly reduces their ability to physically interact with Tup1/Ssn6. Also, in spite of the disruption of the physical interactions with the DNA-binding proteins, Tup1/Ssn6 remain bound both types of promoters, and participates in activation by attracting the SAGA complex (Papamichos-Chronakis et al., 2002; Proft and Struhl, 2002). Similar to these cases, I have observed here that Tup1/Ssn6 remain bound to the promoters of asg and also assist in their activation by attracting the SAGA complex. Furthermore, it appears from our data that pre-acetylation of repressed genes takes place at salt stress-induced genes as well as at asg. This does not appear to be the case at *GAL1*, possibly due to the action of Gal80, an additional repressor that also functions at some glucose-regulated genes (reviewed in Johnston, 1987). Thus, it appears that α2 regulates asg through the same or a very similar mechanism to those used by Mig1 and Sko1 to regulate their target genes. However, there is one key difference between these regulators and the behavior of α2. Following a mating-type switch, α2 is not known to be phosphorylated, it is degraded rather than relocated to the cytoplasm, and it does not remain bound to asg promoters.

This may represent a fundamental difference between α2-Tup1/Ssn6 interactions and Mig1- or Sko1-Tup1/Ssn6 interactions, but it is not necessarily the case. The loss of α2 from asg promoters may simply represent another way that DNA binding protein-Tup1/Ssn6 interactions are broken, and the transition of Tup1/Ssn6 from an activator to a repressor may therefore be accomplished through a similar mechanism at asg as at glucose- or salt stress-regulated genes. Also, while α2 is degraded rather than relocated to the cytoplasm, both of these processes physically remove the DNA-binding protein from the nucleus. While a reason for sequestering Mig1 and Sko1 in the cytoplasm has not been demonstrated, a strong possibility is that this serves to prevent the rebinding of either factor to their target sites and inappropriately re-establishing repression. Degradation of α2 also accomplishes this end. The use of degradation rather than localization may be consistent with the different functions of the regulators. Once a mating-type switch takes place, asg no longer need to be repressed. In the case of glucose- or salt stress-regulated genes, environmental changes could happen at any time, so it may be beneficial for the repressors to remain intact in order for repression to be able to be rapidly re-established.

The hypoxia-regulated genes represent the second category of Tup1/Ssn6-regulated promoters. These genes differ from the previous group in that the DNA-binding protein Rox1 does not remain bound during induction, but the Tup1/Ssn6 complex does (Mennella et al., 2003). Also, it does not appear that Tup1/Ssn6 acts as an activator at these promoters (Mennella et al., 2003), although this has been challenged (Papamichos-Chronakis et al., 2002). The

behavior of Rox1 is similar to that of α2 during induction: after the onset of hypoxia, Rox1 is degraded, although not as quickly as α2 (Mennella et al., 2003). This similarity may help to explain the Tup1/Ssn6-independent activity of α2 that I have observed here. Repression of hypoxia-regulated genes can be maintained by exclusion of TBP even if Tup1/Ssn6 is unable to establish a repressive chromatin structure (Mennella et al., 2003). Exclusion of TBP could be an effect of another Tup1/Ssn6-dependent mechanism, but the fact that I have demonstrated that α2 has a Tup1/Ssn6 independent repression activity raises the possibility that both Rox1 and α2 may be able to partially repress transcription without the influence of the co-repressor complex. The degradation of these repressors could be necessary to completely activate their respective target genes. This is likely to be the case if Rox1 or α2 prevent the binding of an activator. I believe that this may be possible because I have observed that α2-asg binding prevents the binding of the activator Ste12. If TBP binding to these promoters requires an activator, Rox1 or α2 could prevent TBP binding indirectly by preventing the activator from binding.

The DNA damage-regulated genes represent the final class of Tup1/Ssn6 regulated promoters. Neither Crt1 nor Tup1/Ssn6 has been demonstrated to remain bound to these target genes under inducing conditions, and therefore the regulation of this gene set bears little resemblance to the regulation of asg. Additionally, Crt1 has been implicated in the activation of these genes since it has been shown to bind directly to Swi/Snf and TFIID (Zhang and Reese, 2005). Also, there appears to be a great deal of redundancy in the repression of Crt1

target genes, as individual mutations in mediator, Isw2, or HDACs have little effect on *RNR3* or *HUG1* expression (Zhang and Reese, 2004). At asg, mutation of *ISW2* can cause significant derepression (figure 2.2), and mutation of *SRB7* causes near complete derepression of *STE2* (Gromoller and Lehming, 2000), suggesting a lower level of redundancy. Therefore, while both asg and DNA-damage inducible genes are Tup1/Ssn6 regulated, the behavior of the DNA-binding proteins and of the co-repressor complex do not display a great deal of similarity, and it is difficult to gain any further insight into asg repression by studying this group of genes.

The role of α2 binding dynamics in the repression of asg

The similarity between Mig1, Sko1, and α2 implies that the event that directs Tup1/Ssn6 to switch from repressing asg to activating them is the loss of physical interaction between Tup1/Ssn6 and α2. If this is the case, then it has implications for the role of α2 dynamics in regulating asg expression. Our lab has demonstrated that as much as 80% of asg promoters are not bound by α2 at any given time, and yet repression is not compromised (Wilcox and Laney, submitted). Therefore, if the loss of α2 binding leads to Tup1/Ssn6 activation, as much as 80% of the Tup1/Ssn6 bound to asg promoters could be functioning as an activator at any given time. This scenario raises the question of how asg remain unexpressed in α cells. One possible way this apparent paradox may be resolved is if α2-asg binding is dynamic, and α2 binds to asg temporarily, establishes repression, dissociates, and then quickly rebinds. If this were the

case, the time that Tup1/Ssn6 has to behave as an activator would be fleeting, as the rebinding of α2 would quickly convert the complex back to a repressive state before the activation process could be completed. Interestingly, this constant cycling of Tup1/Ssn6 from an activating to a repressing state could explain how pre-acetylation of asg is accomplished. Tup1/Ssn6 may remain in the active state long enough to attract SAGA and for Gcn5 to acetylate histone H3 on lysine 9, but not long enough for the activation process to proceed past this point and produce mRNA (this model is discussed in more detail in chapter 2).

An interesting corollary to this dynamic binding model arises from our observation that Tup1/Ssn6 plays a significant role in determining α2-asg binding dynamics. I have demonstrated that over-expression of either Tup1 or Ssn6 appears to prolong the binding of α2 to asg, while disrupting the interaction between α2 and Ssn6 appears to shorten it (figure 3.1C-D and 3.2C). I can conclude from this that Tup1/Ssn6 is, at least in part, responsible for creating the biphasic α2 dissociation curve I have observed at *STE6* and *STE2*. The effect on binding appears to be at least partly indirect, as the activity of Isw2 is necessary for biphasic dissociation to be established. It would therefore appear that Tup1/Ssn6 repressive activity directs itself to preferentially act as a repressor at asg by causing α2 to remain bound longer than it would in the absence of the co-repressor. Extending α2 binding in this manner may also enhance its apparent Tup1/Ssn6-independent repression activity, since prolonging α2 occupancy at asg would also add to the time that it would be present and able to

occlude Ste12 from binding. Taken together, each of these observation suggests the existence of a positive feedback loop on asg repression, where α2-asg binding recruits Tup1/Ssn6 to establish repression, and the presence of Tup1/Ssn6 causes more persistent α2 binding, which then enhances repression by directing Tup1/Ssn6 to remain in a repressive state for a greater fraction of time than it would if α2 dissociated more quickly. The loop would be broken when α2 levels decline following a switch, preventing it from rebinding quickly enough to maintain Tup1/Ssn6 in a repressive state.

α2 as a major determinant of developmental state

I have seen here that the many dynamic interactions of the α2 protein allow this single transcriptional regulator to act as one of the primary determinants of developmental state in yeast. Its unique set of biochemical properties allows it to act as a repressor of developmentally regulated genes by interacting with and recruiting a co-repressor complex (Keleher et al., 1992; Komachi et al., 1994; Smith et al., 1995), while at the same time allowing it to act as a molecular switch for changing developmental state by being rapidly degraded (Hochstrasser and Varshavsky, 1990; Laney and Hochstrasser, 2003). Finally, as I have demonstrated here, its biochemical properties also allow it to act to pre-program the cell for differentiation into a new cell type by priming promoters for rapid activation by directing them to be partially acetylated while they are still in a repressed sate. I believe that the biochemical properties that allow α2 to act as a key determinant of cell-type identity in yeast make it ideal for

study as a model system for the behavior of master regulators of development and cell type in higher eukaryotes. At the heart of its ability to enable switches in developmental state is its inherent instability: once α2 synthesis stops following a genetic switch, cells must quickly rid themselves of the regulator to assume a new phenotypic state (Hochstrasser and Varshavsky, 1990; Laney and Hochstrasser, 2003). Even though yeast change mating type by replacement of the allele at *MAT* with a cassette that carries the mating-type information of the new cell type (Strathern et al., 1979), and the physical removal of genes coding for regulators is not likely to be a widely used mechanism in the developmental biology of metazoans, the induction of a phenotypic switch by degradation of a negative transcriptional regulator has been observed across a wide array of organisms (for reviews see Bowerman and Kurz, 2006; Inoue and Imamura, 2008; Ooi and Wood, 2007; Schwechheimer and Schwager, 2004; Stone and Callis, 2007), and thus the biochemical properties of α2 may be representative of a fundamental paradigm of cellular differentiation and development.

Several examples of the destruction of transcriptional regulators acting as key developmental determinants have been characterized in various organisms. For instance, in *Arabidopsis thaliana*, the hormone auxin stimulates the E3 complex SCF^{TIR1} to ubiquitinate and signal the destruction of the AUX/IAA family of transcriptional repressors, thereby allowing the induction of auxin responsive genes (Schwechheimer and Schwager, 2004; Stone and Callis, 2007). In another example, in response to TGF-β signaling, a family of proteins known as Smads binds to and affects the activity of both transcriptional activators and

repressors, and this signaling can be ablated by the Smurf family of E3 proteins that direct the ubiquitination and degradation of the Smads (Inoue and Imamura, 2008). Similarly, in *C. elegans*, degradation of several factors is necessary for determination of cell fate early in development (Bowerman and Kurz, 2006; Lin, 2003). Regulation of neuronal development provides perhaps the best mechanistic analogue for the regulation of development by α2. A set of neuron-specific genes are regulated by *r*epressive *e*lement-1 *s*ilencing *t*ranscription factor (REST), which represses their transcription by recruiting a multitude of cofactors, including several HDACs/chromatin remodeling factors, and by interacting with RNA polymerase II (Ooi and Wood, 2007). α2/Tup1/Ssn6 co-repressor complex has been reported to use similar mechanisms to repress asg (Bone and Roth, 2001; Chen et al., 1993; Davie et al., 2002; Green and Johnson, 2004; Gromoller and Lehming, 2000; Jiang and Stillman, 1992; Roth et al., 1990; Roth et al., 1992; Wahi and Johnson, 1995). When neuronal stem cells are induced to differentiate into neurons, the E3 $SCF^{\beta\text{-}TRCP}$ signals the induction of neuron-specific gene expression by ubiquitinating REST and targeting it for destruction in the proteasome (Guardavaccaro et al., 2008; Westbrook et al., 2008). This system is extremely reminiscent of the activation of asg during a mating-type switch, and highlights the utility of studying the molecular mechanisms of α2 regulation as a model for regulators of differentiation in higher eukaryotes.

The results presented here demonstrate a novel mechanism that regulates the switching of a cell's phenotype to a new differentiated state. In

addition to identifying and repressing a set of developmentally regulated genes, the α2/Tup1/Ssn6 co-repressor complex also primes the promoters of these genes for extremely rapid activation by directing them to be acetylated on a specific histone H3 residue. This prepares the a-specific genetic program for expression in a cells, even while they are still being repressed in an α cells, and could be therefore be said to be "preprogramming" the cells with the genetic program that they will express after they undergo a genetic switch. Since the expression of this new program is partly triggered by the degradation of the master regulator α2, and induction of cellular differentiation by degradation of a transcriptional repressor is a common mechanism in many organisms, it is possible that preprogramming a cell with the gene expression profile that will be utilized in a later cell type could be a widely used mechanism in development. This certainly seems possible, since the pre-acetylation of *GRE2* demonstrates that this mechanism is not limited to one set of repressed promoters in yeast. Priming sets of genes for rapid activation in this way may be important for the proper development of higher eukaryotes. For example, in *Drosophilla*, development of photoreceptors is temporally coordinated by the passage of the morphogenetic furrow, which has been hypothesized to coordinate eye development by providing morphogens at a specific time during development (reviewed in Brennan and Moses, 2000). If the cells in the eye do not execute the proper genetic program that allows them to respond to these morphogens in a timely manner, they might fail to differentiate properly. It is possible that

priming of developmentally regulated promoters may help prevent situations such as this from arising.

Future directions

Histone acetylation at asg

My observation of pre-acetylation of asg in α cells has presented additional questions concerning the regulation of asg. Most importantly, while I have demonstrated that the Tup1/Ssn6 co-repressor directs the pre-acetylation of histone H3 on lysine 9 by recruiting the SAGA complex, I have not determined the mechanism that it uses to do so. I have hypothesized in this chapter and in chapter 2 that this may be brought about by dynamic binding of α2 causing Tup1/Ssn6 to constantly oscillate between a repressing and an activating state, but I have not investigated if this is the case. The most straightforward way to investigate this possibility would be to greatly increase the levels of α2 protein in the cell. We have observed previously that over-expressing α2 causes it to occupy a significantly larger fraction of asg promoters than it does under endogenous expression conditions (Wilcox and Laney, submitted). According to our model, increasing the amount of α2-asg binding should force Tup1/Ssn6 to exist in the repressing state for an increased fraction of the time, and this may cause a decline in the acetylation of asg histones. I have done a preliminary set of experiments where I expressed α2 from the *GAL1* promoter in $tetO_2$-α2 cells, but I observed no difference in AcH3K9 levels between cells where expression of P_{GAL1}-α2 had been induced versus when it had not been induced (see appendix

figure A.1B). While this does not agree with our hypothesis, it does not necessarily disprove it either. In order for this experiment to show a difference in acetylation levels, there would have to be an HDAC recruited to asg to remove the acetylation mark. While HDACs have been demonstrated to interact with Tup1 and to be important in the repression of asg (Bone and Roth, 2001; Davie et al., 2003; Davie et al., 2002; Watson et al., 2000; Wu et al., 2001), it has never been shown that an HDAC is recruited to asg that will specifically deacetylate lysine 9. Also, if the kinetics of HDAC recruitment or activity were relatively slow, there may be insufficient time for the lysine 9 acetylation to be removed. I have designed an experiment to study my model that takes this limitation into account. Rather than over-expressing α2 in otherwise wild-type cells, I will do so in cells where *GCN5* expression can be controlled. I will over-express α2 in cells that do not express *GCN5*, and therefore have un-acetylated histones. I will then induce the *GCN5* expression. If increased α2-asg occupancy does inhibit the ability of Tup1/Ssn6 to direct histone H3 acetylation on lysine 9, then acetylation should be lowered or delayed in the α2 over-expressing strains relative to cells expressing endogenous levels of α2, and I should be able to detect these changes using the ChIP assay.

Another question that has arisen from the results I have obtained here concerns how histones can be pre-acetylated when it has been observed previously that HDACs are required for asg repression (Watson et al., 2000). This issue may be resolved if the deacetylation of histone H3 on lysine 14 or 18 is necessary for repression, while deacetylation of lysine 9 is not. To investigate

this possibility, cells deleted for several HDACs could be constructed, and the effect on the acetylation of lysines 14 and 18 could be measured by the ChIP assay. If HDACs do act at asg, then I should observe an increase in acetylation of each of these residues in α cells. A similar experiment has been conducted by deleting *HDA1* and examining the acetylation of histone H3 on lysine 18 at *MFA2* (Green and Johnson, 2004). It was observed in this strain, acetylation did increase by four-fold over wt in α cells. Unexpectedly, the *hda1Δ* mutation did not result in derepression of *MFA2* in α cells. This result may indicate that acetylation of lysine 18 is not important at asg, but it is also possible that there are redundant mechanisms that keep asg repressed in *hda1Δ* cells, as has been observed previously for *RNR3* (Zhang and Reese, 2004). Also, the results of this experiment were not normalized to H3 occupancy at *MFA2*, and it remains possible that much of the increase in acetylation may actually be due to an increase in histone H3 occupancy at *MFA2*. Additionally, the acetylation of lysines 14 and 23 were not investigated. It remains unknown if their acetylation depends on other HDACs, as has been reported for *STE6* (Watson et al., 2000).

Regulation of Tup1 activity at asg

Another question that has arisen from the work I have presented here concerns the interaction of Tup1 with asg. It was previously believed that the binding of Tup1 to asg was completely dependent on α2 binding. However, I have observed here that Tup1 is able to associate with asg in a cells. This suggests the existence of another DNA-binding factor that recruits Tup1 to asg in

a cells. One possible way to investigate how Tup1 is recruited to asg in a cells would be to identify all of the proteins that bind to asg promoters in a cells. This could be done by isolating asg promoter-protein complexes and identifying the bound proteins using mass spectroscopy (MS). Once all of the proteins that bind to asg are identified, I can select candidate proteins and investigate whether or not they are necessary for Tup1 binding by deleting each candidate and examining Tup1-asg binding by ChIP. This experiment has the added benefit that it may identify additional asg-binding factors that might be interesting for further study. It would be particularly interesting to determine if any additional factors are present at asg in α cells that may contribute to the kinetics of their activation.

Several different approaches for isolating proteins bound to asg may be required for this experiment to be fruitful. The simplest way to conduct this experiment would be to use a modified version of the ChIP assay in α cells using α2 antibodies. The formaldehyde crosslinking step of the ChIP procedure should ensure that protein complexes remain intact during the precipitation step. The crosslinks could then be reversed, and the precipitated proteins could then be submitted for MS analysis. This experiment has the obvious limitation that some proteins that bind to asg in a cells might not do so in α cells. Also, it may be difficult to precipitate sufficient quantities of protein for analysis by MS. An alternate way to approach this experiment could be rather than precipitating asg in a ChIP assay, a cell extracts could be passed through a column containing purified α2 rather than precipitated. α2 should retain asg promoters and their

associated proteins by binding to its target sites. The complex could then be eluted, and the resulting sample could be analyzed by MS.

asg activation

I have observed here that the α2/Tup1/Ssn6 co-repressor complex assists in the activation of asg during a mating-type switch by attracting the SAGA complex, which then pre-acetylates the histones bound at asg. While this does potentially explain how asg may be activated rapidly, there are many other events that must occur for asg promoters to become active following loss of α2 binding. Studies of the activation of many genes have revealed that activation can be a complex process, involving an ordered recruitment of sequentially acting factors that each contribute to activation (reviewed in Biddick and Young, 2009). The factors involved in activation include gene specific activators as well as multiple general complexes, including several HAT complexes (for example SAGA, NuA4), chromatin remodeling complexes (Swi/Snf, RSC), TFIID, the mediator complex, and others. The requirements for each of these factors differs greatly from one gene to the next, and the factors involved in asg activation have not been studied in great detail. Obviously, since it appears to partially acetylate asg while they are still in the repressed state, SAGA may be the first activator to act at asg. However, because interaction between Tup1 and Srb7 have been demonstrated to be necessary for asg repression (Gromoller and Lehming, 2000), it is possible that mediator binds to repressed asg, and that it can participate in activation immediately upon loss of Tup1/Ssn6 directed repression.

In fact, this could serve as a second mechanism that assists in establishing the rapid kinetics of asg activation. I have also observed that the activator Ste12 binds to *STE6* immediately upon dissociation of α2 (figure 3.3E). Ste12 may bind asg and immediately stimulate transcription by interacting with pre-bound mediator. It is therefore likely that mediator acts at an early point in asg activation.

I have designed experiments to determine the order of co-activators binding to asg. While I would like to use the DOX-chase ChIP assay to observe the timing of association of factors with asg promoters, the activation of asg occurs on such a rapid timescale that I am unable to resolve the timing of the association of each activator in this way. For example, Ste12, which may be among the first proteins to bind asg, and Pol II, which should be the last, have indistinguishable binding curves in the DOX-chase ChIP assay (compare figure 2.3D to figure 3.3E). Because of this, I must determine the order of binding based on epistasis experiments. For example, our preliminary data shows that a *gcn5Δ* mutation prevents the binding of Swi/Snf to *STE6*, suggesting that SAGA activity is necessary for Swi/Snf recruitment, and that it must therefore act before Swi/Snf. Using this technique, I can delete several known activators and observe their effects on the binding of other proteins, and deduce the order of addition of activators to asg by determining dependency relationships.

The effects of Tup1/Ssn6 complex on α2-asg dynamics

I have observed here that the Tup1/Ssn6 co-repressor complex plays a large role in the establishment of α2-asg binding dynamics. The presence of both Tup1 and Ssn6 appear to promote stable interactions between α2 and its target sites. While previous data suggested that this may be a result of protection of the DNA-bound pool of α2 from degradation by masking degradation signals (Laney et al., 2006), I have observed here that the asg binding dynamics are also dependent on Isw2, suggesting a role for chromatin structure in α2-target gene binding. Since both the R173A-α2 mutation and *isw2Δ* mutation had similar effects on α2 binding dynamics, it is not possible to determine if the effect of the R173A-α2 mutation altered dynamics directly by impairing α2-Ssn6 interactions, or indirectly, since failing to recruit Ssn6 would be expected to cause failure of Isw2 to act at asg. I can address this issue using a simple epistasis experiment. An *isw2Δ* mutation could be introduced into the $tetO_2$-R713A-α2 strain, and the dissociation of α2 from asg promoters could then be measured using the DOX-chase ChIP assay. If this strain shows more rapid dissociation of α2 from its targets, then it is likely the R173A mutation and the *isw2Δ* mutation act through two independent mechanisms to affect α2 dynamics. If they show no more severe change in dynamics than either mutation alone, then the mutations are likely to work through the same pathway. In either case, the effect of Isw2 on α2 dynamics still implicates chromatin structure as a determinant of α2-target gene dynamics. To investigate this possibility directly, I can use a technique that disrupts the organization of chromatin without mutating α2, Isw2, or histones. Recent evidence has demonstrated that insertion of

certain sequences into the promoters of Tup1/Ssn6-repressed genes causes a disruption of nucleosome positioning and repression that resembles the disruption seen in *isw2Δ* mutants (Morohashi et al., 2006). Disruption of α2-asg binding dynamics in a strain carrying one of these insertions would confirm the role of nucleosome positioning in establishing α2-asg binding dynamics.

The possibility that nucleosome positioning may affect α2-DNA binding dynamics raises another interesting set of questions. If chromatin organization does prevent α2 from dissociating from asg promoters, it is likely that it does so by restricting the accessibility of a factor that helps to remove α2 from DNA. The Slx5/Slx8 E3 complex and the ubiquitin-selective chaperone Cdc48 have recently been identified in the ubiquitination of DNA-bound α2 and in its removal from DNA, respectively (Wilcox and Laney, submitted; Yie and Hochstrasser, personal communication). These proteins are therefore excellent candidates for factors that may be inhibited from interacting with α2 by the presence of a repressive chromatin structure. Studying the binding of these factors to asg using ChIP experiments in wt α cells or in *isw2Δ* cells could reveal if their access to DNA-bound α2 is limited by the presence of ordered nucleosomes.

The role of Ste12 in α2 dynamics and repression

I have observed here that α2-asg binding prevents the association of Ste12 with asg promoters, suggesting that the two proteins may compete for asg binding. The preliminary observation that a *ste12Δ* mutation slows the dissociation of α2 from asg promoters is also consistent with this hypothesis,

because Ste12 binding may accelerate the apparent rate of α2 dissociation from asg by preventing it from rebinding after it dissociates. A competition between Ste12 and α2 could explain the α2-dependent, Tup1/Ssn6-independent repression activity I have observed at *STE6* and *STE2* (figure 2.2A and B). If α2 and Ste12 do complete for asg binding, I should be able to impair α2 binding by over-expressing or hyper-activating Ste12. Similarly, over-expression of α2 should impair Ste12 binding. However, this is a less straightforward experiment to do because Ste12 binding is difficult to measure quantitatively by ChIP, as I only observed a two-fold change in ChIP signal in my DOX-chase experiments.

An alternate explanation for the change in α2 binding dynamics in *ste12Δ* mutants could be that Ste12 affects chromatin structure. Certainly, this would not be completely unexpected, since activators often recruit chromatin remodeling complexes (Biddick and Young, 2009). To determine if Ste12 affects α2-asg dynamics by altering chromatin structure, DOX-chase ChIP experiments could be done in an *isw2Δ ste12Δ* strain. This strain would not be capable of positioning nucleosomes. If Ste12 alters α2 dynamics by affecting chromatin structure, the α2 dissociation curve in this strain should appear as it does in a strain that only carries the *isw2Δ* mutation. If the loss of Ste12 does have an additional effect on α2 binding, then it is more likely to be due to α2 and Ste12 competing for asg binding.

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Appendix

Increased α2 expression does not cause loss of histone H3K9 pre-acetylation at asg in α cells

The results that I presented in chapter 2 demonstrated that asg are activated extremely rapidly upon withdrawal of α2, and that they are primed for activation by being partially acetylated while they are in the repressed state. While I found this priming to be dependent on the Gcn5 subunit of the SAGA complex, and on the Tup1/Ssn6 complex, I did not determine the mechanism that directs the acetylation of repressed asg. As discussed in chapters 2 and 4, I have hypothesized that the pre-acetylation of asg may be a result of α2 dynamically associating with and dissociating from asg promoters. When α2 dissociates, it could allow Tup1/Ssn6 to change from acting as a repressor to acting as an activator, only to have repression be re-established when α2 rebinds its operator prior to the actual synthesis of mRNA. A simple test of this hypothesis would be to raise α2 protein levels and then observe the kinetics of asg activation and the acetylation state of the promoter. I did this by integrating a P_{GAL1}-α2-3HA construct into $tetO_2$-α2 strains. To observe the effect of this construct on asg activation, I grew cells in YP + raffinose + galactose media to induce expression of α2, and then did a DOX + glucose chase β-galactosidase experiment (figure A.1A). While $tetO_2$-α2 cells began to express β-gal within 20 minutes, and reached maximal expression levels within two hours, $tetO_2$-α2 P_{GAL1}-α2 cells did not begin to express until 90 min after the addition of DOX and glucose, and did not reach maximal expression by the end of the time course. The expression levels appeared to plateau after three hours in $tetO_2$-α2 P_{GAL1}-

α2-3HA cells. I did not determine if this plateau is genuine or if it is an effect of the cells beginning to enter stationary phase at this point in the time course. Importantly, the delay in the kinetics of activation is consistent with the hypothesis that raising α2 levels, which leads to an increase in α2-asg binding (Wilcox and Laney, submitted), could cause asg to remain in the repressed state longer and take longer to activate. This is not conclusive evidence however, as higher α2 protein levels would also require a longer time to degrade below the concentration where it could rebind to its promoters quickly enough to maintain asg in repressed states, and this would also increase the expected time for asg to derepress as well.

I next examined the H3K9 acetylation state in α2 over-expressing strains directly using the ChIP assay. I found that AcH3K9 levels were identical in $tetO_2$-α2 P_{GAL1}-α2-3HA cells whether they were grown under inducing or non-inducing conditions (figure A.1B). This would seem to suggest that α2 protein levels do not affect acetylation of histones at asg. However, this does not necessarily disprove the model. For over-expression of α2 to cause a decrease in acetylation levels, there would need to be an HDAC recruited to asg that can quickly remove the acetylation mark, and the role of HDACs in specifically deacetylating H3K9 at asg has not been determined. I am conducting further experiments to determine if α2 binding dynamics direct the pre-acetylation of asg (specific experiments are discussed in chapter 4).

Effects of co-activator mutants on asg expression

I have observed here that the SAGA complex is necessary for the rapid activation of asg, and at least part of its function seems to be the pre-acetylation of asg histones. Interestingly, the *spt7Δ* mutation had a more severe defect in activation kinetics than the *gcn5Δ* mutation (figure 2.3B, C, and D). This suggests that the SAGA complex has additional activities that contribute to asg activation. To begin to determine what these activities may be, I have conducted preliminary experiments in *spt3Δ* mutant cells. Spt3 is a component of the SAGA complex that has been shown to both positively and negatively regulate the deposition of TBP on specific promoters (Belotserkovskaya et al., 2000; Dudley et al., 1999; Yu et al., 2003). Using qRT-PCR experiments, I observed that deletion of *SPT3* led to a strong loss of repression of *STE2* (figure A.2A), suggesting that Spt3 plays an inhibitory role in the binding of TBP to *STE6*. Interestingly, *spt3Δ* cells also showed increased expression under activating conditions, which suggests that Spt3 has an inhibitory role even under inducing conditions.

Because I found that Isw2 played a role in both repression of asg (figure 2.2A-D), and in the dynamics of α2-asg binding (figure 3.3A and B), I chose to investigate the role the Swi/Snf complex, the functional antagonist of Isw2 (Peterson, 2002; Tomar et al., 2009), in the expression of asg. I examined expression of *STE2* in *swi2Δ* cells. Under repressing conditions, I observed a small amount of derepression of *STE2* (figure A.2A). While this result was unexpected, Swi2 has been observed to act as a repressor previously (Holstege et al., 1998; Martens and Winston, 2002; Sudarsanam et al., 2000), and Swi/Snf

might therefore have an unappreciated role in asg repression. Upon the addition of DOX, *STE2* expression was indistinguishable from a cells. This would seem to indicate that while Swi/Snf may have a role in asg repression, it is dispensable for activation. Interestingly, *swi2Δ* cells showed a defect in the activation of *MFA2* (figure A.2B). This provides another example of different asg being regulated by distinct mechanisms. Because it appeared to affect α2-asg binding dynamics (figure 3.3C and D), I examined the need for Ste12 in the basal activation of asg as well. I found that *STE6* expression was minimal in a *ste12Δ* strain, and that even under inducing conditions, *ste12Δ* cells were unable to activate *STE6* expression. These results are similar to what has been observed previously (Fields and Herskowitz, 1985).

I also examined the effects of several co-activator mutations on the kinetics of asg activation using the β-galactosidase assay as a reporter for *MFA2* expression. DOX was added to growing cultures at T0 and β-gal activity was monitored over time. While $tetO_2$-α2 cells activated quite rapidly, with β-gal activity being detectable within 20 min of DOX addition, in *swi2Δ* cells expression did not begin for one hour after DOX addition and accumulated very slowly (figure A.2B). Surprisingly, expression in the *swi2Δ* strain never rose above roughly 30% of the expression of $tetO_2$-α2 cells, in contrast to the *STE2* qRT-PCR data. This indicates that there may be different activator requirements at *MFA2* and *STE2*. As was the case for *STE6*, β-gal was never expressed at significant levels in *ste12Δ* cells, again confirming the requirement of Ste12 for asg activation. I also examined the kinetics of β-gal activity in *sba1Δ* cells and

unexpectedly found that even though it had only a marginal effect on the kinetics of activation, the *sba1Δ* mutation caused a 1½ -fold decrease in overall expression. It is possible that this is an indirect effect, but this cannot be determined without further investigation.

Materials and Methods

Yeast strains, plasmids, and growth conditions

Methods used in the creation of strains and plasmids were described in chapters 2 and 3. JY685 was made by replacing *SPT3* with a kanMX4 cassette in JY408 and selecting spores with the desired genotype. JY621 was made by crossing JY550 to a wt a cell to make JY619. JY619 was then transformed with linearized p304-P_{GAL1}-α2-3HA. Expression of the construct was confirmed by western blotting, and a spore with the desired genotype was isolated. All strains were grown in YPD except during the induction of *GAL1* promoter-driven α2, which was grown in YP + 3% raffinose + 3% galactose. For the DOX/glucose chase experiments DOX was added to 3 μM and glucose was added to 2% at T0.

Chromatin Immunoprecipitation

All ChIP experiments were conducted, normalized, and reported as described in chapter 2.

Expression analysis

qRT-PCR and β-galactosidase assays were done as described in chapter 2.

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Table A.1: Strains Used in this study

Strain	Relevant Genotype
MHY608	*MAT**a** mfa2Δ::LacZ*
MHY609	*MATα mfa2Δ::LacZ*
JY412	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6*
JY550	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 GAL2*
JY591	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 gcn5Δ::kanMX4*
JY600	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 sba1Δ::kanMX4*
JY621	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 TRP1::P$_{GAL1}$-α2-3HA*
JY635	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 LEU2::tetR'-ssn6 matα2Δ::hphMX6 ste12Δ::kanMX4*
JY639	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 swi2Δ::kanMX4*
JY685	*MATα mfa2Δ::LacZ URA3::tetR-VP16::tetO$_2$-α2 matα2Δ::hphMX6 spt3Δ::kanMX4*

Figure Legends

Figure A.1 | Increased α2 protein levels delay asg activation, but do not affect asg AcH3K9 levels.

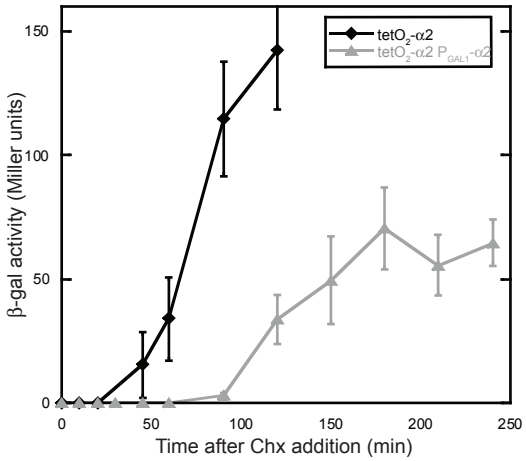
A, β-galactosidase expression from the endogenous *MFA2* promoter following the repression of α2 in $tetO_2$-α2 cells (♦) or $tetO_2$-α2 P_{GAL1}-α2 cells (▲). **B**, Acetylation of Histone H3 lysine 9 at *STE6* in $tetO_2$-α2 P_{GAL1}-α2 under inducing or non-inducing conditions. Results are normalized and presented as described in figure 2.5.

Figure A.2 | Effects of co-activator mutations of asg expression.

A, *STE2* mRNA levels in wt a or α cells, and in $tetO_2$-α2 cells carrying *spt3Δ* or *swi2Δ* mutations in the presence or absence of DOX, or *STE6* mRNA in wt a cells and $tetO_2$-α2 cells carrying a *ste12Δ* mutation in the absence or presence of DOX. **B**, Expression of β-galactosidase from the endogenous *MFA2* promoter following the addition of DOX to $tetO_2$-α2 cells (♦) or to $tetO_2$-α2 cells carrying *swi2Δ* (▲), *ste12Δ* (▼), or *sba1Δ* (×) mutations.

Figure A.1

A.


tetO2-α2
tetO2-α2 PGAL1-α2
β-gal activity (Miller units)
0
50
100
150
0
50
100
150
200
250
Time after Chx addition (min)

B.

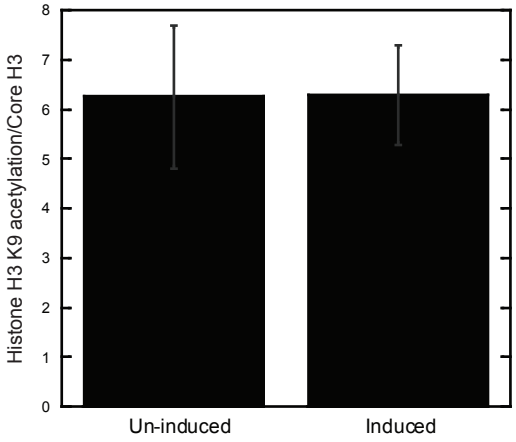
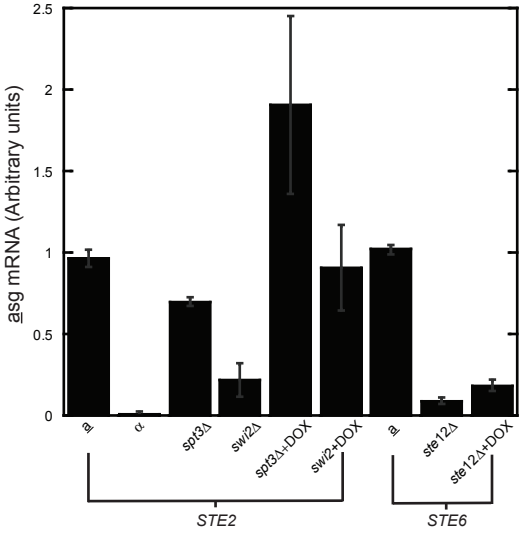
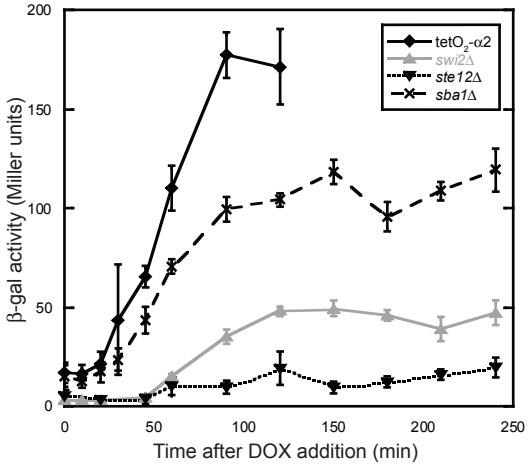

Histone H3 K9 acetylation/Core H3
0
1
2
3
4
5
6
7
8
Un-induced
Induced

Figure A.2

A.


asg mRNA (Arbitrary units)
2.5
2
1.5
1
0.5
0
a
α
spt3Δ
swi2Δ
spt3Δ+DOX
swi2+DOX
a
ste12Δ
ste12Δ+DOX
STE2
STE6

B.


β-gal activity (Miller units)
200
150
100
50
0
0
50
100
150
200
250
Time after DOX addition (min)
tetO2-α2
swi2Δ
ste12Δ
sba1Δ