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Maintenance of Open Chromatin States by Histone H3 Eviction and H2A.Z

by

Laura Marie Lombardi

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jasper Rine, Chair

Professor Michael Botchan

Professor Barbara Meyer

Professor Robert Fischer

Spring 2011

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by Laura Marie Lombardi

Abstract

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Professor Jasper Rine, Chair

The size constraints of the nucleus necessitate condensation of eukaryotic DNA into chromatin. The fundamental subunit of chromatin is the nucleosome, ~147 bp of DNA wound about the histone octamer. Each octamer typically contains two copies each of the canonical histones H2A, H2B, H3 and H4. However, packaging DNA limits its availability to enzymes necessary for the maintenance and expression of our heritable material. More precisely, all chromatin-dependent processes—transcription, replication, recombination, and repair—are affected by the position and occupancy of nucleosomes. Given the transcriptional challenges inherent to DNA packaging, this dissertation documents studies aimed at addressing this fundamental question: How does a cell modify chromatin to achieve proper gene expression? To this end, I pursued functional studies in *S. cerevisiae* of a potential chromatin modifier, Yta7, and a novel chromatin modification, H2A.Z acetylation.

My studies on Yta7, a conserved bromodomain-containing protein with AAA-ATPase homology, identified this protein as a novel regulator of histone H3 eviction or degradation. Cells lacking Yta7 exhibited both increased levels of chromatin-incorporated histone H3 and decreased nucleosome spacing. Importantly, this modulation of H3 levels occurred post-transcriptionally. The *yta7Δ* mutant's transcriptional defects were partially suppressed by decreased dosage of histones H3 and H4, indicating the transcriptional impact of this increased nucleosome density. Additionally, Yta7 associated with inducible genes only upon transcriptional activation, with prominent enrichment within open reading frames. Yta7 and its ATPase function were required for the proper induction of these genes. Further, loss of local Yta7 activity resulted in a 5' to 3' gradient of H3 accumulation within a large open reading frame upon transcriptional activation, indicating a direct requirement for Yta7's regulation of H3 levels at that gene. In support of a direct mechanism of histone eviction or degradation by Yta7, Yta7 directly interacts with histone H3 *in vitro*. Further, over-expressing Yta7 resulted in a ~65% decrease in levels of chromatin-bound H3, as assayed by chromatin immunoprecipitation. Taken together, my studies support a model in which Yta7 utilizes the energy released upon ATP hydrolysis to evict and/or facilitate the degradation of histone H3. As bulk chromatin from cells without Yta7 exhibited increased nucleosome density and decreased dosage of either H3 or H4 suppresses the growth defect of the *yta7Δ* mutant, Yta7 presumably evicts or degrades an H3/H4 dimer or tetramer. Thus, these studies identified a protein that limited the extent of DNA packaging, thereby facilitating RNA polymerase activity upon transcription.

Restricting nucleosome density represents one mechanism for enabling transcriptional activation. Another possible mechanism is modifying the nucleosomes themselves, by covalently modifying the incorporated histones or changing which histones are incorporated. Although nucleosomes typically contain two copies of each canonical histone, histone variants, such as H2A.Z and H3.3, can be substituted at specific genomic locations for their cognate canonical histone. The histone H2A variant H2A.Z is conserved and essential in all multicellular eukaryotes assayed. Yeast cells lacking H2A.Z exhibit a broad range of chromatin-based phenotypes, including defective gene induction, genomic instability, and spreading of the Sir-silencing complex from heterochromatin into euchromatic domains. However, the importance of its N-terminal tail acetylations to these functions remained unclear. Therefore, I undertook studies to determine the genome-wide requirements for H2A.Z acetylation, assess the role of individual acetylation sites and identify which proteins might interpret these modifications.

My work on H2A.Z acetylation indicated that the transcriptome of cells lacking H2A.Z acetylation exhibited fewer expression defects than cells lacking H2A.Z. In contrast to proposed roles in transcriptional activation, cells lacking H2A.Z acetylation exhibited a bias toward up-regulation of genes. Genes that were down-regulated in these cells, however, were highly enriched for telomere-adjacent genes, consistent with Sir silencing antagonism or altered telomeric structure. In keeping with more recent work, my data supported a model of acetylation-site equivalence and additive activity of H2A.Z acetylation. Additionally, this work identified the double bromodomain-containing TFIID-associated Bdf1 as interacting with H2A.Z in an acetylation-dependent manner *in vivo*. As Bdf1 is required to inhibit Sir-complex spreading from the telomeres, this work provides insight into the potential mechanism of Bdf1's Sir complex antagonism. Further work will have to be performed to determine if the down-regulation of telomere-adjacent genes in cells that cannot acetylate H2A.Z is Sir-dependent and whether these genes' requirement for acetylated H2A.Z is a direct one. However, these studies on H2A.Z acetylation are consistent with a model in which H2A.Z acetylation prevents chromatin condensation by the Sir proteins, an alternative mechanism for maintaining proper gene expression.

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Chapter 1

An Introduction to Chromatin Structure, Modification, and Dynamics

Organisms have evolved complex mechanisms to survive environmental changes. Whether the stress is nutritional, genotoxic, or the imperative of a developmental program, precise transcriptional and post-transcriptional responses are required. However, transcriptional responses must operate within the context of chromatin, the condensed proteinaceous structures into which eukaryotic DNA is packaged. This dissertation documents studies aimed at addressing this fundamental question: How does a cell modify chromatin to achieve proper gene expression?

Understanding the nature of this problem requires an introduction to chromatin structure and the myriad ways organisms have evolved to operate within it. Thus, my discussion begins with a description of the most fundamental unit of chromatin structure: the nucleosome.

I. Chromatin's Fundamental Unit: The Nucleosome

Given the size constraints of the nucleus, DNA must be compacted. Further, this compaction must also neutralize the charge repulsion inherent to a high concentration of negatively charged DNA polymer. Charge neutralization and compaction are accomplished at the most fundamental level of chromatin structure by association of DNA with histone proteins. Histones are small, positively charged proteins of four canonical types: H2A, H2B, H3 and H4. ~147bp of DNA wrapped around a histone octamer forms the fundamental unit of chromatin, the nucleosome (Luger et al. 1997; Richmond and Davey 2003). The nucleosome is assembled from two H3/H4 dimers which form a stable tetramer wrapped by the central ~80bp of DNA and then two H2A/H2B dimers, one above and one below the plane formed by the tetramer (Fig. 1.1) (Das et al. 2010). As predicted from the order of assembly, the H2A/H2B dimers are more easily dissociated from the nucleosome than the H3/H4 tetramer (Oohara and Wada 1987; Yager et al. 1989). Nucleosomes are separated by a stretch of linker DNA, whose length varies depending on the cell-type and the species (~10 – 90 bp) (Bavykin et al. 1990). More complex eukaryotes possess the linker histone, histone H1, which binds to linker DNA and facilitates the formation of higher-order chromatin structures (Happel and Doenecke 2009).

Modifying nucleosome properties

Nucleosomes cover the majority of an organism's genome. However, nucleosomes exhibit considerable structural diversity based upon the incorporation of histone variants and covalent modifications of histones.

Of the four canonical core histone types—H2A, H2B, H3 and H4, histones H2A and H3 exhibit the greatest numbers of variants. Canonical histones and variants are distinguished by their sequence, their period of expression, and their abundance. Canonical histones are generally encoded together in multi-copy gene clusters and expressed exclusively in S-phase. Expression of variants, however, is replication independent, and occurs throughout the cell cycle. Additionally, variants are encoded by genes present in only one or two copies and are generally less abundant (Talbert and Henikoff 2010). The ability of variants to be incorporated into chromatin outside of S-phase allows for functional specialization. Histone H3 variants include the centromere-specific variant, CENP-A, and H3.3, which is deposited at sites of active transcription. Incorporation of histone variants can alter the structure and stability of the nucleosome (Park and Luger 2008). The H2A variant H2A.Z, for example, destabilizes H3.3-

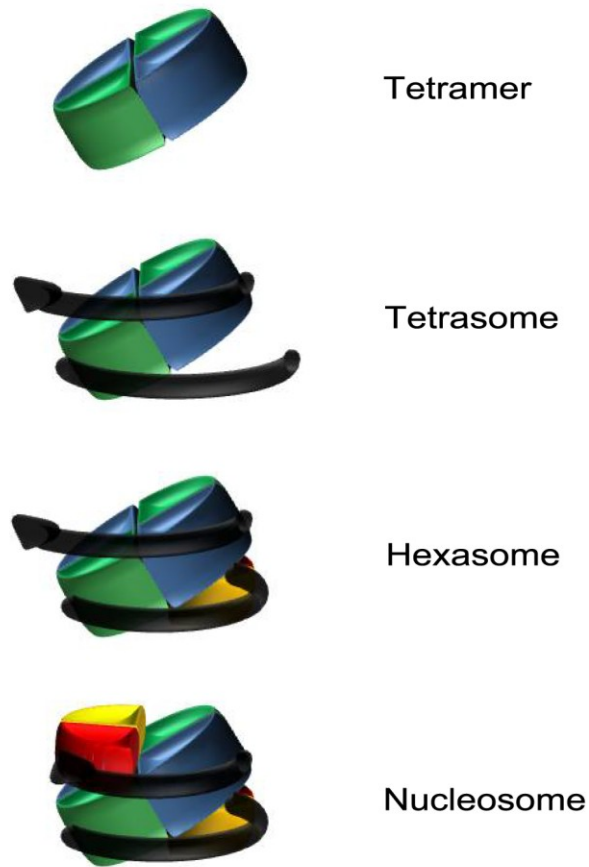


Figure 1.1. The modular nature of the nucleosome.

The H3/H4 tetramer organizes the central ~80 bp of DNA to form the tetrasome. H2A/H2B dimers are then incorporated on each side, resulting in the complete nucleosome. H3/H4 are represented in blue and green; H2A/H2B in yellow and red.

containing nucleosomes (Jin and Felsenfeld 2007). Given that H2A.Z-containing nucleosomes are enriched at poised promoters, the decreased stability H2A.Z imparts to nucleosomes is thought to promote the nucleosome loss required for transcriptional activation (Abbott et al. 2001).

In addition to changing the histone content of nucleosomes, nucleosomes are diversified by covalent modifications of histones. Histones can be decorated by many different chemical modifications, including acetylation, methylation, phosphorylation, ubiquitination, citrullination, ADP-ribosylation, o-GlcNAcylation, etc. (Campos and Reinberg 2009; Sakabe et al. 2010). These modifications most frequently occur on the histones' poorly structured N-terminal tails which extend from the surface of the nucleosome, but a few also occur within the protein core. These posttranslational modifications can alter the biophysical properties of the nucleosome or act as binding surfaces for chromatin regulators. Biophysically, modifications can interfere with histone-histone or histone-DNA contacts within and between nucleosomes. Acetylation of H4 K16, for example, disrupts a salt bridge with an H2A molecule within an adjacent nucleosome, disabling higher-order structures (Campos and Reinberg 2009). The protrusion of the histone tails and their high density of modifiable amino acids also makes histone tails superb recruitment platforms. For example, lysines within histone tails are frequently acetylated in euchromatin, which is transcriptionally active chromatin, and act to target necessary proteins. Specifically, acetylated lysines are bound by bromodomains within various proteins involved in transcriptional initiation and elongation (Yang 2004).

Establishing chromatin states

Thus, nucleosome diversity is achieved by variant incorporation and differential histone modifications. How diversity is originally established is defined to a large extent by the DNA sequence elements nearby. The binding of site-specific DNA binding proteins to their cognate sequences helps to recruit chromatin regulators. The establishment of an active chromatin state is well-illustrated by the steps in transcriptional induction of the *GAL* genes, the products of which are required to metabolize galactose. In medium containing galactose, Gal4 bound to an upstream activating sequence is able to recruit the coactivator SAGA, which is a histone acetyltransferase (HAT). The acetyl-lysine marks put on by SAGA recruit bromodomain-containing chromatin remodelers (described below), such as SWI/SNF, that facilitate loading of RNA polymerase II (PolII) (Weake and Workman 2010). Thus, a cascade of interactions is triggered by a particular DNA sequence. Repressive chromatin structures can be similarly established. In the case of Sir-mediated silencing in budding yeast, a silencer sequence is bound by site-specific DNA binding proteins, which recruit the histone deacetylase Sir2 in partnership with Sir4. Sir2 acts on adjacent nucleosomes providing hypoacetylated H3 and H4 tails, which are binding platforms for the rest of the silencing complex, Sir3 and Sir4 (Rusche et al. 2003). The Sir complex thereby compacts the underlying chromatin and inhibits transcription within the region.

II. Attaining Access to DNA

Although nucleosomes help solve the problem of DNA packaging, the nucleosome solution creates its own problem: decreased access for all DNA-dependent processes, such as transcription, replication and DNA repair.

Chromatin remodelers

Nucleosomes occupy the majority of DNA within a genome. Spacing of nucleosomes is variable within a genome due to the presence or absence of the linker histone H1 and its variants, the occurrence of DNA sequences which disfavor nucleosome binding, and the activity of chromatin remodelers (Jiang and Pugh 2009; Radman-Livaja and Rando 2010). Chromatin remodelers are a diverse set of enzymes that utilize the energy released upon ATP hydrolysis to manipulate nucleosomes and help provide the necessary access to DNA. Different remodelers can 1) slide nucleosomes along DNA (ISWI family), 2) evict nucleosomes or dimers from a nucleosome (SWI/SNF family), 3) replace dimers in an incorporated nucleosome (SWR1 Complex), and 4) change the conformation of a nucleosome (Clapier and Cairns 2009). Nucleosome sliding or eviction is often required to expose a DNA sequence element for binding. However, nucleosome sliding is also utilized to tightly space nucleosomes for repressive chromatin structures. The activity of histone dimer exchange is important for replacing canonical histone dimers with dimers containing histone variants. For example, the SWR1 Complex replaces an H2A/H2B dimer with an H2A.Z/H2B dimer (Mizuguchi et al. 2004). Finally, there are proteins that assist in remodeling activities, by altering nucleosome conformation and facilitating dimer eviction and exchange, but do not hydrolyze ATP. Therefore, these proteins are not technically considered remodelers, but they form a special class of histone chaperones.

Facilitating access without ATP hydrolysis: histone chaperones and exchange

Although originally applied specifically to proteins that bound soluble histone dimers and facilitated their import into the nucleus, “histone chaperone” has become a blanket term for proteins that bind histones and participate in nucleosome assembly or disassembly in an ATP-independent fashion (Avvakumov et al. 2011). Histone chaperones earned their name because their binding of highly basic histones, generally via a large acidic domain, can prevent non-specific histone-DNA interactions (Das et al. 2010). Most chaperones exhibit a clear binding preference for H2A/H2B or H3/H4. Further, there are chaperones specific for histone variant-containing dimers, such as Daxx in complex eukaryotes, which specifically binds H3.3/H4 dimers (Avvakumov et al. 2011). Additionally, chaperones can be classified based on their differing contribution to certain chromatin processes. Chaperones required for DNA replication assemble nucleosomes in the wake of DNA polymerase, but, in the case of Asf1, may also assist in splitting parental H3/H4 tetramers for reincorporation into newly synthesized DNA (Probst et al. 2009). In contrast, chaperones involved in transcription facilitate dimer exchange, dimer eviction and reassembly of nucleosomes behind RNA polymerase.

Of the three known chaperones of all H2A-type/H2B dimers, FACT, Nap1 and Chz1, only the FACT complex directly participates in nucleosome assembly during DNA replication or repair. On the other hand, Nap1 and Chz1 import the dimers into the nucleus and assist FACT during transcription (Avvakumov et al. 2011). In budding yeast, which contains only the H3.3 form of histone H3, there are a multitude of chaperones known for H3/H4: Asf1 (hCIA-I), CAF-1 complex, HIR complex, Rtt106, Vps75 (hTAF-I β), and Spt6. All of these H3/H4 chaperones, with the exception of Spt6, were originally identified for their role in DNA replication or repair. Thus, of the six, only Spt6 is thought to be specific for transcription (Avvakumov et al. 2011). However, the functional distinctions among H3/H4 chaperones remain blurry because, until

recently, a broader requirement for H3/H4 chaperones in transcription was not apparent. Further, many H3/H4 chaperones function together. Asf1, for example, is considered an “upstream chaperone,” because it is unable to perform nucleosome assembly *in vitro* unless in complex with the CAF-1 or HIR complex (De Koning et al. 2007). Thus, Asf1 is thought to provide the requisite H3/H4 dimers to downstream chaperones which can assemble nucleosomes.

Thus, nucleosome dynamics persist outside of S-phase due to the activity of chromatin remodelers, histone chaperones, and enzymes such as PolII. Because the nucleosome consists of a central H3/H4 tetramer flanked by two H2A/H2B dimers, it was predicted that H2A/H2B dimers would be subject to greater rates of loss from the nucleosome. Earlier findings on replication-independent histone exchange *in vivo* appeared to confirm this prediction, with some studies noting a 20-fold greater rate of H2A/H2B exchange than H3/H4 exchange (Jackson and Chalkley 1985; Kimura and Cook 2001; Thiriet and Hayes 2005). However, these studies assayed the rates of H3 exchange by determining the rate of canonical H3 incorporation into chromatin and, thus, were blind to the incorporation of H3 variants or canonical H3 loss from chromatin. In contrast, experiments originally performed in *Drosophila* demonstrated that the H3 variant H3.3 is incorporated into transcriptionally active chromatin and regulatory regions outside of S-phase (Ahmad and Henikoff 2002; Mito et al. 2007; Deal et al. 2010). This role of H3.3 has now been observed in vertebrates as well (Chow et al. 2005; Jin et al. 2009; Sutcliffe et al. 2009). In budding yeast, which contains only the centromere-specific and H3.3 forms of H3, many recent studies have demonstrated replication-independent exchange of H3 and H4 (Linger and Tyler 2006; Dion et al. 2007; Jamaï et al. 2007; Rufiange et al. 2007). Only one study assayed H2B and H3 exchange in parallel; they found broader H2B exchange, however, the results suggested that 30-50% of chromatin-bound H3 undergoes exchange in an hour in the absence of replication (Jamaï et al. 2007).

All relevant *S. cerevisiae* studies found high H3 exchange in intergenic regions, regardless of the transcriptional status of adjacent genes. However, transcriptionally active promoters exhibited higher rates of exchange than inactive promoters. Although approximately one-fourth of the intergenic rate, H3 exchange also occurred in open reading frames (ORFs), and the rate of exchange was positively correlated with PolII density (Dion et al. 2007; Jamaï et al. 2007; Rufiange et al. 2007). How this exchange might be achieved is discussed below.

An example of attained access: transcription

The first step in the process of fruitful transcription is RNA polymerase gaining promoter access. Access is achieved via the activity of chromatin remodelers, such as SWI/SNF and RSC, which can slide or evict nucleosomes to expose binding sites of site-specific or general transcriptional activators (Cairns 2009). Additionally, the activity of the SWR1 complex which exchanges H2A/H2B dimers for H2A.Z/H2B dimers is thought to poise nucleosomes adjacent to the transcriptional start site for ease of loss (Mizuguchi et al. 2004; Zhang et al. 2005). However, ORF nucleosomes are well-positioned and nucleosomes pose a strong barrier to PolII transcription *in vivo* and *in vitro* (Izban and Luse 1992; Kireeva et al. 2005; Hodges et al. 2009; Churchman and Weissman 2011). Thus, an assembly of accessory factors is required for transcription.

The first such factor to be isolated was the FACT (FACilitates Chromatin Transcription) complex, biochemically isolated as necessary for PolII-mediated transcription on a chromatinized template *in vitro* (Orphanides et al. 1998). As aforementioned, the FACT complex is considered an H2A/H2B chaperone. It induces an alternative conformation of the nucleosome upon binding, which is thought to promote the dissociation of an H2A/H2B dimer, either passively or by passage of PolII (Belotserkovskaya et al. 2003; Xin et al. 2009). Interestingly, in certain *in vitro* conditions PolII transcription through a nucleosome is capable of dislodging an H2A/H2B dimer, leaving behind a hexasome (Kireeva et al. 2002; Xin et al. 2009). Thus, FACT may assist this eviction *in vivo*. Additionally, the FACT complex is thought to reassemble nucleosomes in the wake of PolII, based on assembly activity *in vitro* (Belotserkovskaya et al. 2003) and transcriptional initiation occurring from cryptic start sites in ORFs in the *spt16* mutant (Mason and Struhl 2003). Thus, FACT is thought to help recycle the original histones by moving H2A/H2B dimers originally in front of PolII to the rear of PolII. Consistent with this model of activity, FACT is found throughout active ORFs (Mason and Struhl 2003). The H3/H4 counterpart of FACT is thought to be the transcription-specific H3/H4 chaperone, Spt6. In the absence of Spt6, there is a global decrease in ORF-bound nucleosomes, consistent with a requirement for Spt6 in nucleosome reassembly after transcription (Kaplan et al. 2003; Ivanovska et al. 2011).

The recycling of ORF-bound histones by FACT and Spt6 upon transcription may serve as a way of conserving posttranslational histone marks, but, it does not explain the H3 exchange observed in ORFs of transcriptionally active genes. Asf1 in complex with the HIR complex has been shown to be important for replication-independent deposition of H3.3 in *Drosophila* (Tagami et al. 2004) and H3 in budding yeast (Green et al. 2005). However, it is not known how transcription-coupled eviction of H3 occurs. One model is that nucleosome loss can occur when PolII is engaged at high densities on a template due the creation of the unstable hexasome and the inability of the nucleosome to reform before a following PolII molecule evicts the hexasome (Kulaeva et al. 2010). Alternatively, H3/H4 eviction might require the active participation of H3/H4 regulators. Supplying credence to an accessory model, Asf1 is thought to facilitate eviction, possibly by tetramer splitting (Schwabish and Struhl 2006). However, it is difficult to tease out regulators of H3/H4 eviction because decreased transcription results in decreased eviction. Cells without Asf1, for example, have decreased levels of PolII at multiple ORFs which would also result in decreased eviction, but this study argues that H3 is preferentially retained compared to H2B (Schwabish and Struhl 2006).

III. A Novel Regulator of Chromatin Structure: Yta7

Now that the complexities of operating within a chromatinized genome are apparent, I can introduce a chromatin-associated protein that motivated many of the experiments described herein, Yta7. I chose Yta7 as a subject of study for many reasons. The two main reasons were the following: 1) the protein contains the unique domain combination of a putative bromodomain and a putative AAA-ATPase, possibly resulting in acetyl-lysine-targeted enzymatic activity, and 2) the *YTA7* gene of *Saccharomyces* is highly conserved from yeast to humans (Fig. 1.2). High conservation provided the potential for findings relevant to human health.

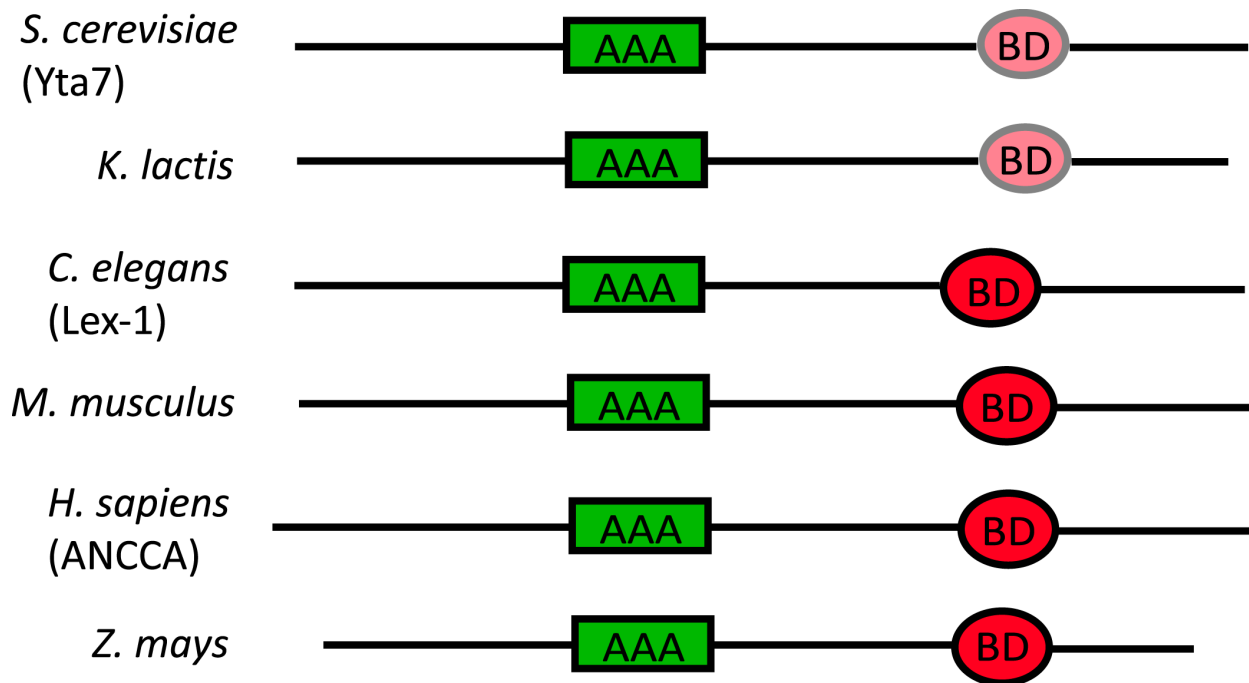


Figure 1.2. The combination of a AAA-ATPase domain coupled to a bromodomain is well-conserved.

Yta7 orthologs in indicated species are presented. The total length and domain spacing are conserved, as is the acidity of the N-terminus. The lighter shading of the bromodomains (BD) in the fungal orthologs represents their deviation from the canonical bromodomain sequence.

At the onset of this study little was known of *YTA7* or its orthologs, except for two studies in *S. cerevisiae* indicating *yta7* mutants are defective for restricting Sir-protein silencing from spreading in *cis* (Jambunathan et al. 2005; Tackett et al. 2005). Since then, the human ortholog, ANCCA (ATPase Nuclear Coactivator Cancer-Associated), has been identified as a coactivator of both estrogen and androgen receptors, with increased expression of ANCCA being associated with breast and prostate cancers with poor prognoses (Zou et al. 2009; Kalashnikova et al. 2010). Interestingly, ANCCA's ATPase activity is required for its coactivator function (Zou et al. 2007; Revenko et al. 2010), consistent with my findings discussed in chapter 2. Additionally, biochemistry performed on *S. cerevisiae*'s Yta7 demonstrated that Yta7 contains two histone-interaction domains: a noncanonical bromodomain and an acidic N-terminal region. The bromodomain directly interacts with histone H3 *in vitro* (Gradolatto et al. 2009).

Working independently, I sought to determine the domain requirements for Yta7 function, global transcriptional requirements for Yta7, and the directness of these requirements. My results allowed me to determine that Yta7 is a novel modulator of chromatin-bound H3, helping to limit nucleosome density. This likely occurs via Yta7-mediated eviction or degradation of H3/H4 dimers, as Yta7 function is dependent on its ATPase domain. Further, Yta7 is required at inducible genes for proper expression, with H3 accumulating in its absence. Therefore, we posit a model in which Yta7 is recruited to transcriptionally active genes, which exhibit high replication-independent turnover rates, to help evict H3/H4 in front of PolII or degrade the evicted histones. This model is discussed in detail in Chapter 2, as is Yta7's functional collaboration with the histone variant H2A.Z.

IV. Approach and Contents

S. cerevisiae, a budding yeast, is arguably the most genetically accessible eukaryote. Aside from the wealth of genomic resources and data available, budding yeast is further advantageous when studying the importance of histone modifications and dosage, as there are only two copies of each canonical histone. Therefore, supplying a cell with only the mutant form of a given histone is easily accomplished. Additionally, *S. cerevisiae* is one of the few organisms that does not require H2A.Z to live (Zlatanova and Thakar 2008). Thus, studies of the variant's function are much more tractable in yeast.

I capitalized on these traits of budding yeast to determine the sensitivity of *yta7Δ* mutants to histone H3 and H4 dosage, which was pivotal in prompting my investigation of Yta7's role in limiting nucleosome density, described in Chapter 2. Also addressed in Chapter 2 is the functional collaboration of Yta7 and H2A.Z. These studies would not have been possible in a more complex eukaryote. Additionally, in Chapter 3, I discuss experiments I was able to perform examining the role of H2A.Z's acetylation and how this modification is functionally translated by Bromodomain Factor 1 (Bdf1).

Chapter 2

Direct Regulation of Nucleosome Density by the Conserved AAA-ATPase Yta7

I. Abstract:

Yta7 is a highly conserved bromodomain-containing protein with AAA-ATPase homology originally implicated in heterochromatin boundary function in *S. cerevisiae*. Although increased activity of the human ortholog has been implicated in malignant breast tumors, Yta7's precise mode of action is unknown. Yeast cells without Yta7 exhibited substantial transcriptional similarity to cells lacking the histone variant H2A.Z, suggesting a role for Yta7 in gene induction. Indeed, Yta7's ATPase function was required for galactose- and sporulation-induced transcription. Further, Yta7 associated with the *GAL* gene cluster only upon transcriptional induction, with prominent enrichment within open reading frames. Intriguingly, the *yta7Δ* mutant's transcriptional defects were partially suppressed by decreased dosage of histones H3 and H4. Consistent with this suppression, cells lacking Yta7 exhibited both increased levels of chromatin-incorporated histone H3 and decreased nucleosome spacing. Importantly, this modulation of H3 levels occurred post-transcriptionally. Loss of local Yta7 activity resulted in H3 accumulation at the 3' end of a large open reading frame upon transcriptional activation. Further, over-expressing Yta7 resulted in decreased chromatin-bound H3. As Yta7 binds histone H3 *in vitro*, these results suggested a direct role for Yta7 in H3 eviction or degradation.

II. Introduction:

The size constraints of the nucleus necessitate condensation of eukaryotic DNA into chromatin. The fundamental subunit of chromatin is the nucleosome, ~147 bp of DNA wound about the histone octamer (Luger et al. 1997). Each octamer typically contains two copies each of the canonical histones H2A, H2B, H3 and H4. However, histone variants, such as H2A.Z, macro H2A, H3.3, CENP-A, and others, are substituted at specific genomic locations for their cognate canonical histone (Campos and Reinberg 2009; Talbert and Henikoff 2010). All chromatin-dependent processes—transcription, replication, recombination, and repair—are affected by the position and occupancy of nucleosomes. With respect to transcription, nucleosomes constrain access of site-specific and general transcription factors to their DNA template (Workman and Kingston 1998).

Access of transcription factors to DNA is facilitated by chromatin remodelers, which can reveal transcription factor binding sites by nucleosome sliding (Cairns 2009), and by the incorporation of histone variants, such as H2A.Z and H3.3, that can destabilize nucleosomes (Jin and Felsenfeld 2007; Kumar and Wigge 2010). Once RNA PolII is engaged on the DNA template, nucleosomes must be navigated during transcriptional elongation. The clearing of H2A/H2B dimers from transcribed regions is facilitated by the FACT complex (Belotserkovskaya et al. 2003; Xin et al. 2009), originally identified as necessary for PolII transcription on a chromatinized template *in vitro* (Orphanides et al. 1998). The H3/H4 tetramer was long believed to stay associated with DNA during PolII-mediated transcription (Kireeva et al. 2002; Thiriet and Hayes 2005). However, recent studies clearly indicate that replication-independent replacement of canonical H3 with H3.3 occurs during transcription (Ahmad and Henikoff 2002; Chow et al. 2005; Jin et al. 2009; Sutcliffe et al. 2009). In budding yeast, which contains only the centromere-specific and H3.3 forms of H3, replication-independent H3 exchange in ORFs is positively correlated with PolII occupancy (Dion et al. 2007; Jamai et al. 2007; Rufiange et al. 2007). Although histone chaperones responsible for replication-

independent deposition of H3.3 have been identified, how eviction of H3 occurs is presently unclear. *In vitro*, high densities of PolII can evict the entire histone octamer (Kulaeva et al. 2010), but, *in vivo*, other factors appear to be required for eviction, such as the H3/H4 chaperone Asf1 (Adkins and Tyler 2004; Schwabish and Struhl 2006; Rufiange et al. 2007). Whether Asf1 acts alone in this capacity, or with other proteins, is unknown.

The *YTA7* gene of *Saccharomyces* is conserved from yeast to humans and encodes the only bromodomain-containing AAA-ATPase in yeast. The human ortholog, ANCCA (ATPase Nuclear Coactivator Cancer-Associated), is a coactivator of both estrogen and androgen receptors, and increased expression of ANCCA is associated with breast and prostate cancers with poor prognoses (Zou et al. 2009; Kalashnikova et al. 2010). However, Yta7's precise mode of action is unclear and, in *S. cerevisiae*, there are conflicting claims regarding Yta7's function. *yta7* mutants were originally isolated as being defective for restricting Sir-protein silencing from spreading (Jambunathan et al. 2005; Tackett et al. 2005). Subsequently, Yta7 has been described either as a repressor (Gradolatto et al. 2008) or an activator (Fillingham et al. 2009) of histone gene expression.

The conserved domain structure of Yta7 is suggestive of function. Yta7 appears to contain two histone-interaction domains: a noncanonical bromodomain and an acidic N-terminal region. Although bromodomains typically bind acetylated lysines, *in vitro* analyses suggest that Yta7's bromodomain preferentially interacts with the unacetylated and unmethylated N-terminal tail of histone H3 (Gradolatto et al. 2008; Gradolatto et al. 2009). Additionally, Yta7 contains a putative AAA-ATPase domain of the NSF/Cdc48/Pex family, members of which are classically hexameric and involved in unfolding or manipulating proteins (Erzberger and Berger 2006). Indeed, the human ortholog, ANCCA, assembles into oligomers, and its ATPase activity is required for its coactivator function (Zou et al. 2007; Revenko et al. 2010). Thus, its domain structure implied that Yta7 might function enzymatically on histones or nucleosomes. The goal of this work was to test these implications and uncover what Yta7's molecular function might be. The experiments described here led to the view that Yta7 participates in the eviction and/or degradation of histone H3.

III. Materials and Methods:

Yeast strains and media

All yeast strains were derived from W303-1a, except those used in Fig. 2.2A, D (listed in Table 2.1). One-step integration of knockout cassettes and C-terminal epitope tags was performed as described (Longtine et al. 1998; Goldstein and McCusker 1999; Puig et al. 2001) (see Table 2.2 for primer sequences). All gene disruptions were confirmed by 5'- and 3'-junction PCR. Strains containing integrated *pGAL1::FMP27* and *pGAL1::YTA7* were constructed by insertion as described (Longtine et al. 1998). All epitope-tagging was confirmed by immunoblotting as described below. Yta7-TAP provided full Yta7 function, as assayed by heterochromatin boundary function.

The *YTA7* clone was generated by PCR amplification off genomic DNA with primers adding 40 bp of homology to HindIII and EcoRI-digested pRS316. This product was co-transformed with doubly-digested pRS316. The complementing clone (pJR2860) contained ~400 bp upstream and

Table 2.1. Strains used in Chapter 2.

Strain	Genotype	Source
W303-1a	<i>MATa ade2-1; can1-100; his3-11; leu2-3,112; trp1-1; ura3-1</i> (JRY3009)	R. Rothstein
JRY7754	<i>MATa htz1Δ::HIS3MX</i>	Kobor et al. 2004
JRY7972	<i>MATa HTZ1-FLAG::KanMX</i>	Babiarz et al. 2006
JRY8689	<i>MATa HTZ1-FLAG::KanMX YTA7-TAP::TRP1</i>	This study
JRY9199	<i>MATa HTZ1-FLAG::KanMX yta7Δ::natMX</i>	This study
JRY9200	<i>MATa yta7Δ::natMX</i>	This study
JRY9201	<i>MATa; pRS316</i>	This study
JRY9202	<i>MATa yta7Δ::natMX; pYTA7/pRS316</i>	This study
JRY9203	<i>MATa yta7Δ::natMX; pyta7K460A/pRS316</i>	This study
JRY9204	<i>MATa yta7Δ::natMX; pyta7E519Q/pRS316</i>	This study
JRY9205 & 9206	<i>MATa (hht1-hhf1)Δ::HygMX</i>	This study
JRY9207 & 9208	<i>MATa yta7Δ::natMX (hht1-hhf1)Δ::HygMX</i>	This study
JRY9209 & 9210	<i>MATa HIS3MX-pGAL1::FMP27 RPB3-3HA::TRP1</i>	This study
JRY9211 & 9212	<i>MATa HIS3MX-pGAL1::FMP27 RPB3-3HA::TRP1 yta7Δ::natMX</i>	This study
JRY9213	<i>MATa HTZ1-FLAG::KanMX HIS3MX-pGAL1::FMP27</i>	This study
JRY9214	<i>MATa HTZ1-FLAG::KanMX HIS3MX-pGAL1::FMP27 YTA7-TAP::TRP1</i>	This study
JRY9215	<i>MATa YTA7-TAP::TRP1</i>	This study
JRY9216 & 9217	<i>MATa HIS3MX-pGAL1::YTA7-TAP::TRP1 HTZ1-FLAG::KanMX</i>	This study
JRY9218	<i>MATa HTZ1-FLAG::KanMX swr1Δ::HIS3MX</i>	This study
JRY9219 & 9220	<i>MATa HTZ1-FLAG::KanMX swr1Δ::HIS3MX yta7Δ::natMX</i>	This study
JRY9221 & 9222	<i>MATa YTA7-TAP::TRP1 swr1Δ::HIS3MX</i>	This study

For sporulation analysis

SK-1	<i>MATa/α ho::LYS2 lys2 ura3</i> (JRY2692)	N. Kleckner
JRY9223	<i>MATa/α ho::LYS2 lys2 ura3 yta7Δ::natMX</i>	This study

For Fig. 2.2D derived from DDY277 & DDY282 (Jambunathan et al. 2005); all *ADE2 his3 leu2 lys2Δ trp1 ura3*

JRY9224	<i>MATa HMRIΔ; pRS316</i>	This study
JRY9225	<i>MATa HMRIΔ-E-Boundary-a1; pRS316</i>	This study
JRY9226	<i>MATa HMRIΔ-E-Boundary-a1 yta7Δ::KanMX; pRS316</i>	This study
JRY9227 & 9228	<i>MATa HMRIΔ-E-Boundary-a1 yta7Δ::KanMX; pYTA7/pRS316</i>	This study
JRY9229	<i>MATa HMRIΔ-E-Boundary-a1 yta7Δ::KanMX; pyta7K460A/pRS316</i>	This study
JRY9230	<i>MATa HMRIΔ-E-Boundary-a1 yta7Δ::KanMX; pyta7E519Q/pRS316</i>	This study

Table 2.2. Primers used in Chapter 2.

<i>yta7</i> KO	TCTCTTCTCGCTATTTATTACAAATTGGTGCAGAAAGAACCGGATCCCCGGGTAAATTAA AACTACATTTAAGAATTATATAAACATTATGGACTCCTGCCGATGAATTCGAGCTCGTTT
<i>YTA7</i> Cloning	TGGCGGCCGCTCTAGAACTAGTGGATCCCCGGGCTGCAG TGCTGAGTTTGAGATGTTTT CTGGGTACCGGGCCCCCTCGAGGTCGACGGTATCGATCGGCTATTGATGGTACGGATC
<i>yta7K460A</i>	CCTCCTGGTACGGGTGCAACACTTATGGCAAG CTTGCCATAAGTGTTCACCCGTACCAGGAGG
<i>yta7E519Q</i>	CTTCCATCATTTTTTTTGGATCAAAATCGACGGATTAGCGCC GGCGCTAATCCGTCGATTTGATCAAAAAAATGATGGAAG
<i>YTA7-TAP</i>	AACAGGAAGTGCATGAAATAATAAAATTTTTATCTGAATCCATGGAAAAGAGAAG AACTACATTTAAGAATTATATAAACATTATGGACTCCTGCTACGACTCACTATAGGG
<i>(hht1-hhf1)</i> KO	ATATTTGCTTGTGTACCGTTTTCTTAGAATTAGCTAAACGGATCCCCGGGTAAATTAA TTTTGTTCGTTTTTTACTAAACTGATGACAATCAACAAACGATGAATTCGAGCTCGTTT
<i>pGAL1::FMP27</i>	AAGAACGGTAGCTATTACAAAGGACAGTAAAGCAAACAGGAATTCGAGCTCGTTTAAAC ATATAAGCCACTTGTACAGTAGAACATTAATCGGAAACATCATTTTGAGATCCGGGTTTT
<i>RPB3-HA</i>	AATGGGTAATACTGGATCAGGAGGGTATGATAATGCTTGGCGGATCCCCGGGTAAATTAA GTTCACTTGTTTTTTTCTCTATTACGCCCACTTGAGAAGAATTCGAGCTCGTTTAAAC
<i>pGAL1::YTA7</i>	CCCATTGTTGTTTTCGACAGTCATAAAAGAAGTTACCACGAATTCGAGCTCGTTTAAAC CTTCAACGTCGCTACCGCGTCTATTCTTAAATTCGTGCCATTTTGAGATCCGGGTTTT
<i>SPO1</i>	GAC AAC TAT CCA AAC GGC TCC AAG CCA GTG GGT AAC ATA CTG CTA ACT
<i>ZIP1</i>	AAGTGTCTAAGGTGGATCATATAAG GAA GGG TTG GTT AAC TCT AGG TC
<i>GIP1</i>	CAC ATT TCG CGG CCT TAC TAA TA TGAGGTACTCCGATCCTTTATACA
<i>PXA1</i>	TTGGCTTAACCTACCTAGATGCCAT CGT TGT CAA TCG ACG TGA TTG C
<i>FOX2</i>	ATA TCG ATC CCA CGC TAG CCA A TAC AGC GGC GTT ATC CAA TAC A
<i>ACT1</i>	TGTCCTTGTA CTCTCCGGT CCGGCCAAATCGATTCTCAA (described in Babiarz et al. 2006)
<i>SPO11</i>	CCT ATG GGA TTA GCA TAG CCC TA TGTTGGCAGTCAGAGATGCTATC
<i>YSW1</i>	ACCTGCTGTAGTGCCTAAACA GGC AGA TCT CTC GTA GAA CCT
<i>GAL1 +1400</i>	TGGATCATATGGTTCCCGTTTGAC GGT ACT TGA CCT TGT AGA ACT CAT TG
<i>GAL10 +1865</i>	CTACAAA GCCAACGGTC TTAGGC CTTGCTTCGTAACCAGCAGACAA
<i>PRP8</i>	GAGTGTGGCTAAATTTCTTAAGAGG TCGAATACTCTCAGGCATCATTTCT (described in Babiarz et al. 2006)
<i>GAL10 +535</i>	CCT TAG GGC CTA CTA ATC CGT A AGAGGGATGTGCGCCAATTG

Table 2.2. Primers used in Chapter 2 (Continued).

<i>GAL10</i> +114	GGT TAC AGG TGG TGC TGG ATA C GAT GCT TGG TCA AGA CCT CTA A
<i>GAL1</i> Prom	AAGTACGGATTAGAAGCCGCC GGCCAGGTTACTGCCAATTT
<i>GAL1</i> +97	CTGAGTTCAATTCTAGCGCAAAGG CTCTACCAGGCGATCTAGCAACAA
<i>GAL1</i> +756	CTCTGTTTGCGGTGAGGAAGATCA CAGCTGTAGTGACTTCTACCACTC
<i>GAL7</i> -326	AGCACGAGCC GCGGAGTTCA T GATCACGGTCAACAGTTGTCCGAG
<i>GAL7</i> +217	GGTTAGG TCAACAGGAG GCTG AGATTGCACTTTAAGCAGCCTATT
<i>SUC2</i> -317	ATACG CGTAGCGTTA ATCGACC TAC TAT AGA GCC CTT AGG CAA CAT
<i>SUC2</i> +69	CACTAACGTATATGATGCTTTTGCA TTT CAT CGT ACC ACA ACC CAT T
<i>SUC2</i> +564	AGCTGCCAACTCCACTCAATTC TTG GCA AAT GCA GAT TCT AGC TT
<i>ADY3</i>	AAT TAC TGA CGA GTT AGC TGG AAA TGCGATTGCACATAGGTAAGGTC
<i>HHT</i>	GGT GGT GTT AAG AAG CCT CA CTT GAG CGA TTT CTC TGA CC
<i>HHF</i>	AGC TAG AAG AGG TGG TGT CA TCTTTCTCTTGGCGTGTTTCG
<i>HTA1</i>	ACGTTACCATTGCCCAAGG GTTTAGTTTCCTTCCGCCTTC (described in Fillingham et al. 2009)
<i>GAL10</i> +1092	CAG ACC GAG TTG CAG GTT GAA GAC CAACAATGCTGGCGCCCAAATTG
ORF-Free Chr I	GTT TAT AGC GGG CAT TAT GCG TAG ATC AG GTT CCT CTA GAA TTT TTC CAC TCG CAC ATT C (described in Mason and Struhl 2005)
<i>pGAL1::FMP27</i>	CTGGGGTAATTAATCAGCGAAGCGATG CACTTGTACAGTAGAACATTAATCGGAAAC (described in Mason and Struhl 2005)
<i>FMP27</i> (.5kb)	CGCAATTAGTCAACAACGATATCACGATTG CCTACTTGAAGTCCATCCTTCAGAGG (described in Mason and Struhl 2005)
<i>FMP27</i> (2kb)	CATATCATCCACCCTAGGTGCTAGGTCGG GAGCTGACCAGACCTAACCATAGTAGCGTG (described in Mason and Struhl 2005)
<i>FMP27</i> (4kb)	AGATATTACTCGTTGTTTCGTGCCCAG TCCCAAAACCCTAGTTTAACAGAAGG (described in Jimeno-Gonzalez et al. 2010)
<i>FMP27</i> (6kb)	CGTACTGTTGAAATGGAACGAGGACGC ATCGCTTCCATACTCGTTGTATCATCAGTC (described in Mason and Struhl 2005)
<i>FMP27</i> (8kb)	GAGGGTCACA GATCTATTAC TTGCC GTTGTGAGTTGCTTCAGTGGTGAAGTG (described in Mason and Struhl 2005)

~300 bp downstream intergenic sequence (see Table 2.2). Site-directed mutagenesis of pJR2860 was performed to generate the point mutants *yta7K460A* (pJR2861) and *yta7E519Q* (pJR2862).

Transformants of SK1 were generated by electroporation. Briefly, mid-log phase cells were incubated in TE with 100 mM lithium acetate for 45min at 30°C. DTT was added to 25 mM and the cells left at 30°C for another 15 min. Cells were then washed with water and once with cold 1 M sorbitol. Cells were electroporated at 1.5 kV, 25 μ F, 200 Ω and immediately resuspended in cold 1 M sorbitol. Cells were plated on selective medium containing 1 M sorbitol.

Transformations were performed in diploids. Heterozygous diploids were sporulated and homozygous diploids produced by single-cell matings. Synchronous meiotic entry was achieved as previously described (Cao et al. 1990).

For galactose induction experiments, cells were grown to OD₆₀₀ 0.6 in YPRaffinose (2%) at which point pre-warmed 20% galactose was added to a final concentration of 2%. Unless otherwise indicated, all “+ Gal” data points represent 1hr post-induction.

Microarrays

Cultures were grown at 30°C to OD₆₀₀ 0.8 - 1. RNA was extracted with hot acid phenol as described (Collart and Oliviero 2001). A denaturing gel was run to assess the quality of the RNA. For each experiment, 20 ug of total RNA from each strain and 20 ug of total RNA from a pooled reference was primed with 5 ug/ul oligo dT & 5 ug/ul pdN₁₅ for ten minutes at 70°C, followed by ten minutes on ice. Roche Transcriptor was utilized for reverse transcription with a 3:2 ratio of amino-allyl-dUTP:dTTP. Reactions were performed at 42°C for 12 hr. Residual RNA was then hydrolyzed with 250 mM NaOH for 15 min at 65°C. cDNA was isolated from this mixture via Qiagen MinElute Reaction Cleanup Kit. After dye coupling (Amersham Cy3 and Cy5) for 2 hr at RT, the cDNA was purified with Qiagen MinElute Reaction Cleanup Kit and quantified with a Nanodrop spectrophotometer (Thermo Scientific). Reference cDNA was pooled and equal amounts were mixed with each experimental sample. Hybridizations were performed for 12 – 14 hr at 65°C using the MAUI Hybridization System (BioMicro Systems, Salt Lake City, UT).

Custom microarrays were fabricated as described (DeRisi et al. 1997) at the University of California, San Francisco, Center for Advanced Technology (UCSF CAT) with Operon’s Aros V1.1 and YBOX (Yeast Brown lab Oligo eXtension) 70-mer probes. Specific protocols for array printing, post-processing, and washing are at <http://cat.ucsf.edu>.

After washing, the arrays were scanned on a Genepix 4000B scanner (Molecular Devices Axon, Sunnyvale, CA) and the images analyzed using Genepix Pro 6.1 software. After removing poor quality spots, the data were corrected using TiGR MIDAS v2.20 LOWESS normalization. Analysis was performed using TiGR MeV version 3.1 (Saeed et al. 2006). Significantly affected genes were determined using the SAM (Significance Analysis of Microarrays) statistical package (Tusher et al. 2001) with a 1.5 median false gene cut-off.

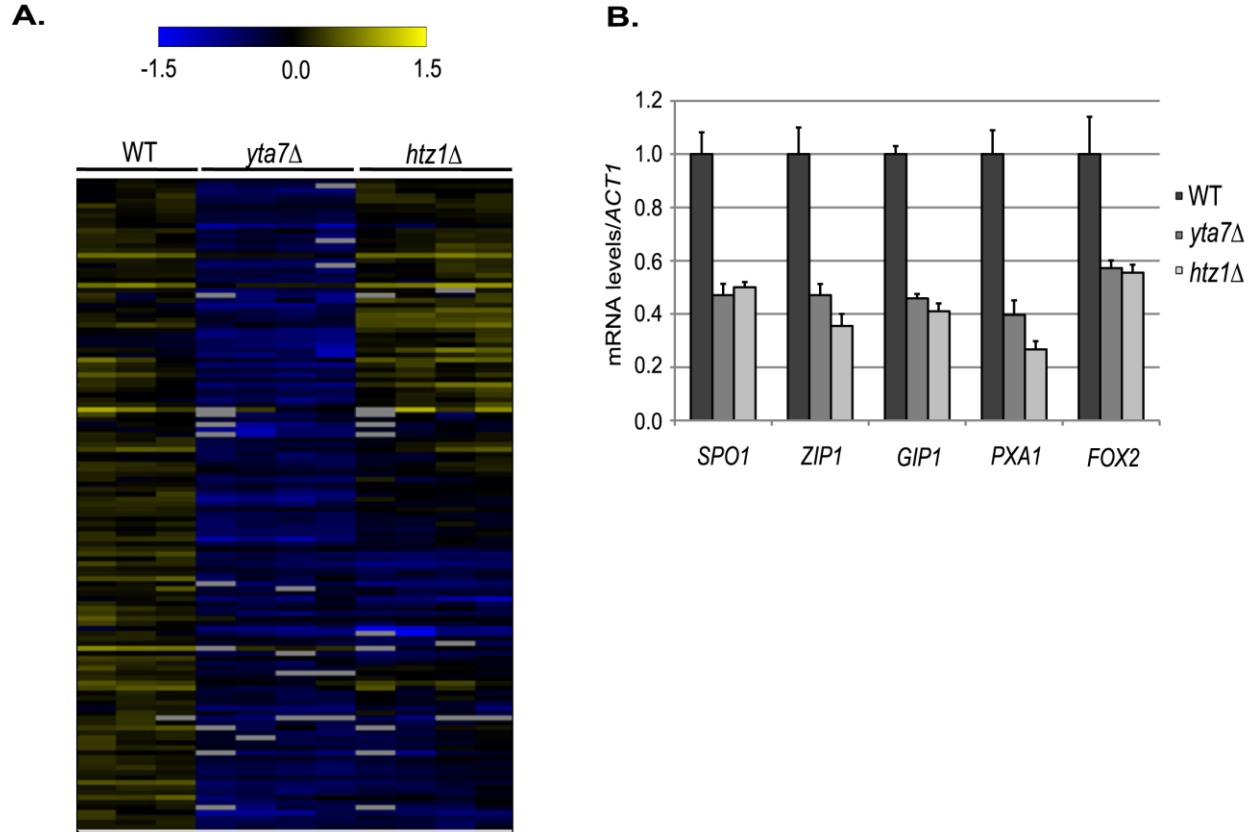


Figure 2.1. Significant co-regulation by Yta7 and H2A.Z.

(A) Hierarchical clustering analysis of genes significantly downregulated in the *yta7Δ* mutant indicated that the majority were also downregulated in cells lacking H2A.Z (*htz1Δ*). Both genes and samples were clustered according to complete linkage, with the distance metric of Pearson correlation. Values are $\log_2(\text{strain/pooled reference})$, where each column is a biological replicate. Gray rectangles indicate missing data values for that experiment. (B) RNA analysis of inducible genes in non-inducing conditions. The level of each transcript was normalized to *ACT1* mRNA levels and adjusted to a wild-type level set equal to 1. In this and all other figures, the error bars represent the standard error of the mean of at least three biological replicates.

RNA isolation and qRT-PCR

RNA isolation for all experiments except Figs. 2.2C, 2.4B, 2.7C, and 2.8A was performed using the hot-phenol method (Collart and Oliviero 2001). Total RNA was digested with DNase I (Roche) and purified using the RNeasy Minelute kit (Qiagen). RNA for Figs. 2.2C, 2.4B, 2.7C, and 2.8A was isolated directly from whole-cell extract using RNeasy Mini kit (Qiagen) with on-column DNase I (Qiagen) digestion. cDNA was synthesized using the Super-Script III First-Strand Synthesis System (Invitrogen). Oligo(dT) priming was used for all experiments, except for Figs. 2.4B and 2.8A, for which random hexamer priming was utilized. Quantitative PCR on cDNA was performed using an MX3000P qPCR machine (Agilent) and the Dynamo HS SYBR Green qPCR kit (NEB). Amplification values for all primer sets were normalized to actin (*ACT1*) cDNA amplification values. Samples were analyzed in technical triplicate for three or four independent RNA preparations.

ChIP analysis

All cells were cross-linked with 1% formaldehyde at OD₆₀₀ 0.6 – 0.9. For Yta7-TAP ChIP experiments, cells were cross-linked for 45 minutes at 30°C. For H3 and Rpb3-3HA ChIP, cells were cross-linked for 20 minutes at room temperature. Cross-linking was quenched by addition of glycine to a final concentration of 300 mM. Cells were then washed twice with TBS and lysed with 0.5 mm zirconia beads in FA lysis buffer (Aparicio et al. 2005) using the MP Fastprep-24. For Yta7-TAP ChIP, chromatin was isolated at 74,000g for 36 minutes then washed for 1 hr at 4°C. Sonication yielded an average sheared DNA size of 400-500 bp.

For H3 and Rpb3-3HA ChIP, chromatin was isolated as described (Aparicio et al. 2005). For the Yta7-TAP IPs, 30 ul of IgG Sepharose (GE Healthcare) was incubated for 1.5 hr at 4°C with sonicated chromatin from 150 ml of culture at ~0.6 OD₆₀₀. For H3 IPs, 1.5 ug of rabbit α -H3 (Abcam ab1971) was incubated overnight at 4°C with 25 ul ProteinA Sepharose (GE Healthcare) and sonicated chromatin from 15 OD₆₀₀ units of cells. For Rpb3-3HA IPs, 25 ul monoclonal anti-HA-agarose (Sigma) was incubated overnight at 4°C with sonicated chromatin from 30 OD₆₀₀ units of cells. Resin washing, IP elution, and DNA purification were performed as described (Aparicio et al. 2005). Quantitative PCR was performed on precipitated DNA fragments as described above. The negative control primer set for Yta7-TAP ChIP amplifies a region within the *PRP8* ORF and was chosen as an internal control, because it corresponded to a locus with an IP/IN signal consistently indistinguishable from the no-tag control. Yta7-TAP ChIP values are presented as IPx/IP_{PRP8}, except for Fig. 2.8B, for which values were normalized over the positive control region, the *HHT1* promoter. Because a negative control region is not possible for H3, presented values are IP/IN.

Micrococcal Nuclease Digestion

Digests were performed largely as described (Lam et al. 2008) with the following modifications: 100 ml of cells at ~1.0 OD₆₀₀ were harvested. Cells were washed once in Pre-spheroplast Buffer (20 mM KPO₄, pH 7.0, 1 M sorbitol, 10 mM DTT), and then incubated in Spheroplast Buffer (20 mM KPO₄, pH 7.0, 1.1 M sorbitol, 0.5 mM CaCl₂, 0.5 mM PMSF, 1.2 mg/ml Zymolyase 100T) for 30 minutes at 30°C. Extent of spheroplasting was assessed by absorbance readings of dilutions into 1% SDS. Micrococcal nuclease digests were performed in parallel for 10 minutes

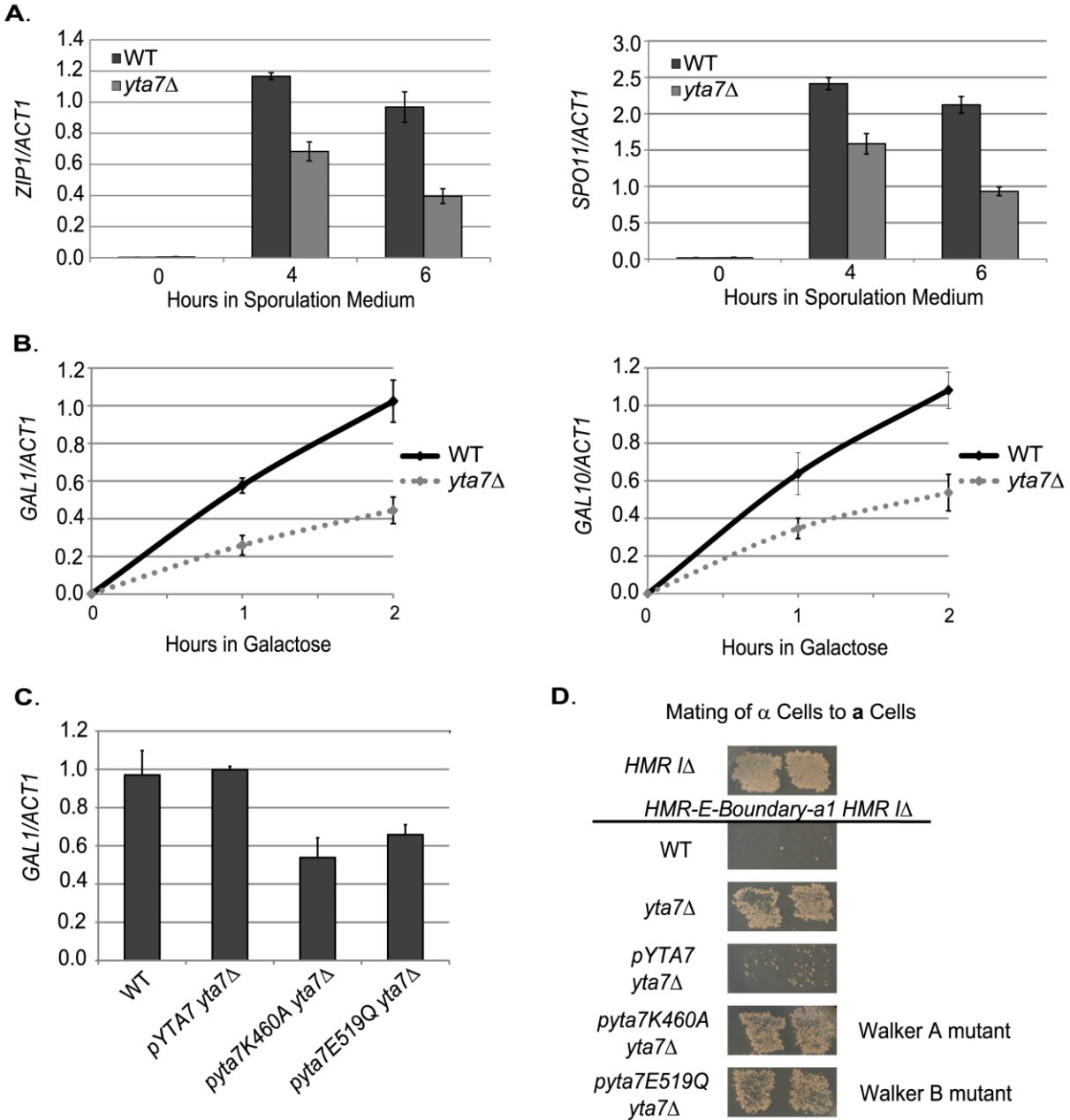


Figure 2.2. Inducible genes required Yta7 and its AAA-ATPase domain for proper expression.

((A) RNA analysis of SK1 wild type and *yta7Δ* homozygotes. Cells were pre-grown in YPAcetate, represented by the $t = 0$ time point, and then resuspended in sporulation medium for times indicated. (B) RNA analysis of galactose induction. Cells with grown in YPRaffinose (2%, non-inducing) to OD_{600} 0.6, at which point cells were harvested for the $t = 0$ time point. Pre-warmed galactose was then added to a final concentration of 2%. (C) RNA analysis as above except cells were harvested after 1 hr with galactose. (D) Mating test of α cells. Wild type is unable to mate if the "Boundary," 1kb of DNA normally to the right of *HMR*, is inserted between the *HMR-E* silencer and *a1*, denoted *HMR-E-Boundary-a1*. This mating defect is because Sir proteins cannot traverse this "Boundary" to silence *a1*. When the *yta7Δ* mutation is introduced into this background, mating is partially restored (Jambunathan et al. 2005), indicating that the "Boundary" is partially defective.

at 37°C. Reactions were promptly stopped by addition of EDTA and SDS to final concentrations of 25 mM and 0.5%, respectively. Isolated DNA was quantified by Nanodrop and 25 ug of each sample electrophoretically separated on a 1.5% agarose gel. Nucleosome repeat lengths were calculated as described (Godde and Widom 1992).

Protein Analysis

15 ml of cells at OD₆₀₀ ~1.0 were collected. Yeast whole-cell extracts were prepared by resuspending cells in 20% trichloroacetic acid (TCA) and performing mechanical lysis with 0.5 mm zirconia beads. Supernatant was removed and beads were washed three times with 5% TCA. The supernatants after each wash were pooled, and precipitated proteins were collected by centrifugation. Pellets were air dried and resuspended in 75 ul 3X SDS sample buffer and 37.5 ul 1 M Tris base. Samples were boiled and spun. The supernatant was then evaluated by standard SDS-PAGE and immunoblotting. Detection and quantitation was performed with the Li-cor Odyssey imaging system. Antibodies used for immunoblotting: rabbit anti-H3 antibody (Abcam ab1791), mouse anti-HA (Sigma H 9658), rabbit monoclonal anti-calmodulin binding protein (Millipore #05-932), and—as a loading control—mouse anti-Phosphoglycerate kinase antibody (Invitrogen #459250).

IV. Results:

Inducible genes required Yta7 for proper induction

To clarify the function of this chromatin-interacting protein, the genome-wide transcriptional requirements for Yta7 were determined in *S. cerevisiae*. In rich medium, 3% of transcripts were significantly misregulated in the *yta7Δ* mutant, of which 64% were down-regulated (Significance Analysis of Microarrays (SAM), 1.5 median false gene cut-off). Misregulated transcripts were enriched for those whose expression is inducible relative to constitutively expressed genes. To assess if this rich-medium expression signature might represent a general role in induction, we assayed cells lacking the histone variant H2A.Z, which is required for optimal induction of multiple inducible genes (Santisteban et al. 2000; Zhang et al. 2005; Gevry et al. 2009; Wan et al. 2009; Halley et al. 2010). Although cells lacking H2A.Z (*htz1Δ*) exhibited broader transcriptional misregulation (7% of transcripts) than the *yta7Δ* mutant, the majority of transcripts downregulated in the *yta7Δ* mutant behaved similarly in the *htz1Δ* mutant (Fig. 2.1A). Specifically, in non-inducing conditions, many inducible genes required Yta7 and H2A.Z for their normal level of basal transcription (Fig. 2.1B). These results suggested that Yta7 might be required for optimal gene induction.

To test Yta7's potential role in gene induction, two distinct classes of inducible genes were assayed: early meiotic genes and galactose-inducible genes. Early meiotic genes were selected because the set of genes downregulated in the *yta7Δ* expression profile was enriched for meiotic genes (GO Slim analysis). Galactose-inducible genes were selected because they are a thoroughly studied paradigm of induction. The induction of meiotic genes was evaluated in the *S. cerevisiae* strain SK1 due to its highly efficient and synchronous meiosis and sporulation (Primig et al. 2000). *yta7Δ* homozygotes failed to reach wild-type levels of gene expression for the early meiotic genes *SPO11* and *ZIP1* (Fig. 2.2A). This defect in achieving maximal induction was also

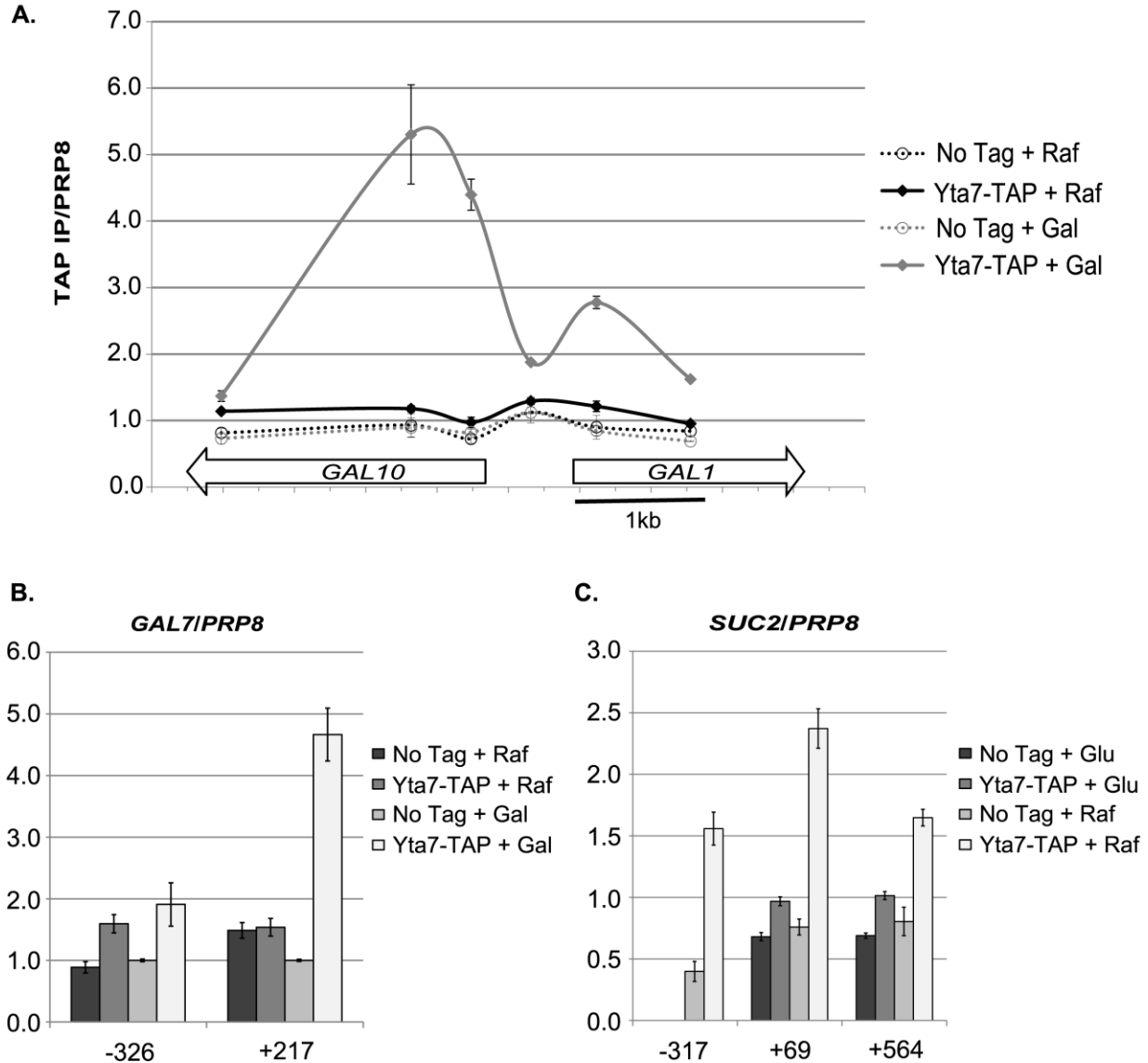


Figure 2.3. Yta7 localized to inducible genes upon activation.

(A) ChIP analysis of Yta7-TAP across *GAL10-GAL1* in inducing and non-inducing conditions. The two conditions represent YPRaffinose (2%, non-inducing) and the same cultures 1h after addition of galactose (+ Gal). Values were obtained with qPCR and represent the IP signal over the IP signal for the negative internal control *PRP8*. (B) ChIP analysis of Yta7-TAP, as above, at *GAL7*. Numbering in this and subsequent figures refers to base pairs from the start of the open reading frame for the gene indicated. (C) Yta7-TAP enrichment at *SUC2* in YPD (2%) versus YPRaffinose (2%), in which *SUC2* is induced.

observed for the mid-sporulation genes *GIP1* and *YSW1* (data not shown). Moreover, Yta7 was also required for proper induction of the galactose-inducible genes *GAL1* and *GAL10* (Fig. 2.2B).

Yta7's AAA-ATPase domain was required for its function

To investigate the importance of Yta7's putative AAA-ATPase domain to its function, *GAL1* induction was assayed in strains containing mutations in the Walker A (K460A) or Walker B (E519Q) motifs of Yta7's AAA-ATPase domain. Both mutants were defective for *GAL1* induction, indicating that residues essential for ATP binding and hydrolysis (Hanson and Whiteheart 2005) were required for Yta7's role in induction (Fig. 2.2C). As *YTA7* was identified through its contribution to the boundary between silenced chromatin at the *HMR* locus and active genes nearby (Jambunathan et al. 2005; Tackett et al. 2005), we further assayed these ATPase mutants for boundary function. Point mutants in either the Walker A or Walker B motif of Yta7's AAA-ATPase domain were indistinguishable from the *yta7Δ* mutant (Fig. 2.2D). Thus, the results from two independent assays clearly demonstrated that Yta7's AAA-ATPase was essential for its function.

Yta7 localized to inducible genes upon activation

To test if this role in induction was due to direct action of Yta7 at the *GAL* gene cluster, chromatin immunoprecipitation (ChIP) was performed on Yta7-TAP in inducing and non-inducing conditions. Yta7 became enriched at the *GAL1*, *GAL7*, and *GAL10* loci specifically upon galactose addition (Fig. 2.3A,B). Interestingly, the peak of Yta7's localization occurred in the 5'-end of the *GAL1* and *GAL10* ORFs, with intermediate levels near the promoter and no detectable binding in the 3'-end. This preference for the 5'-end of the ORF was further observed upon tiling of the long inducible gene *pGAL1::FMP27* (see below). Yta7's localization at *GAL7* also exhibited preferential ORF enrichment (Fig. 2.3B). Further, consistent with the induction-dependent localization of Yta7 observed at the *GAL* gene cluster, Yta7 localized to *SUC2* only upon induction (Fig. 2.3C). Taken together with the defective *GAL* gene induction in the *yta7Δ* mutant, these data indicated that Yta7 was directly required for *GAL* gene induction and that Yta7's transcription-coupled function was exerted primarily in the 5'-end of ORFs.

In accordance with Yta7's enrichment in ORFs, Yta7 interacts *in vivo* with Chd1, a helicase thought to regulate the transition between transcriptional initiation and elongation (Simic et al. 2003), and Spt16, the largest subunit of the FACT complex which facilitates elongation by destabilizing nucleosomes (Tackett et al. 2005; Formosa 2008; Lambert et al. 2009; Lambert et al. 2010). As Yta7's bromodomain can directly interact with histone H3 *in vitro* (Gradolatto et al. 2008), it was possible that H3 modification—particularly acetylation—targeted Yta7 to the 5' region of ORFs. Interestingly, however, *in vivo* and *in vitro* analyses of Yta7's interaction with histone H3 have thus far failed to indicate any preferred modification state of H3 (Gradolatto et al. 2008; Gradolatto et al. 2009). Thus, Yta7's interaction with histone H3 appeared insufficient to explain Yta7's targeting. In principle, if H3 were not recruiting Yta7, then Yta7's interaction with H3 might reflect Yta7 activity exerted after localization. Thus, given Yta7's requirement for its AAA-ATPase domain (Fig. 2.2C,D), we hypothesized that Yta7 might act on H3, rather than being recruited by it.

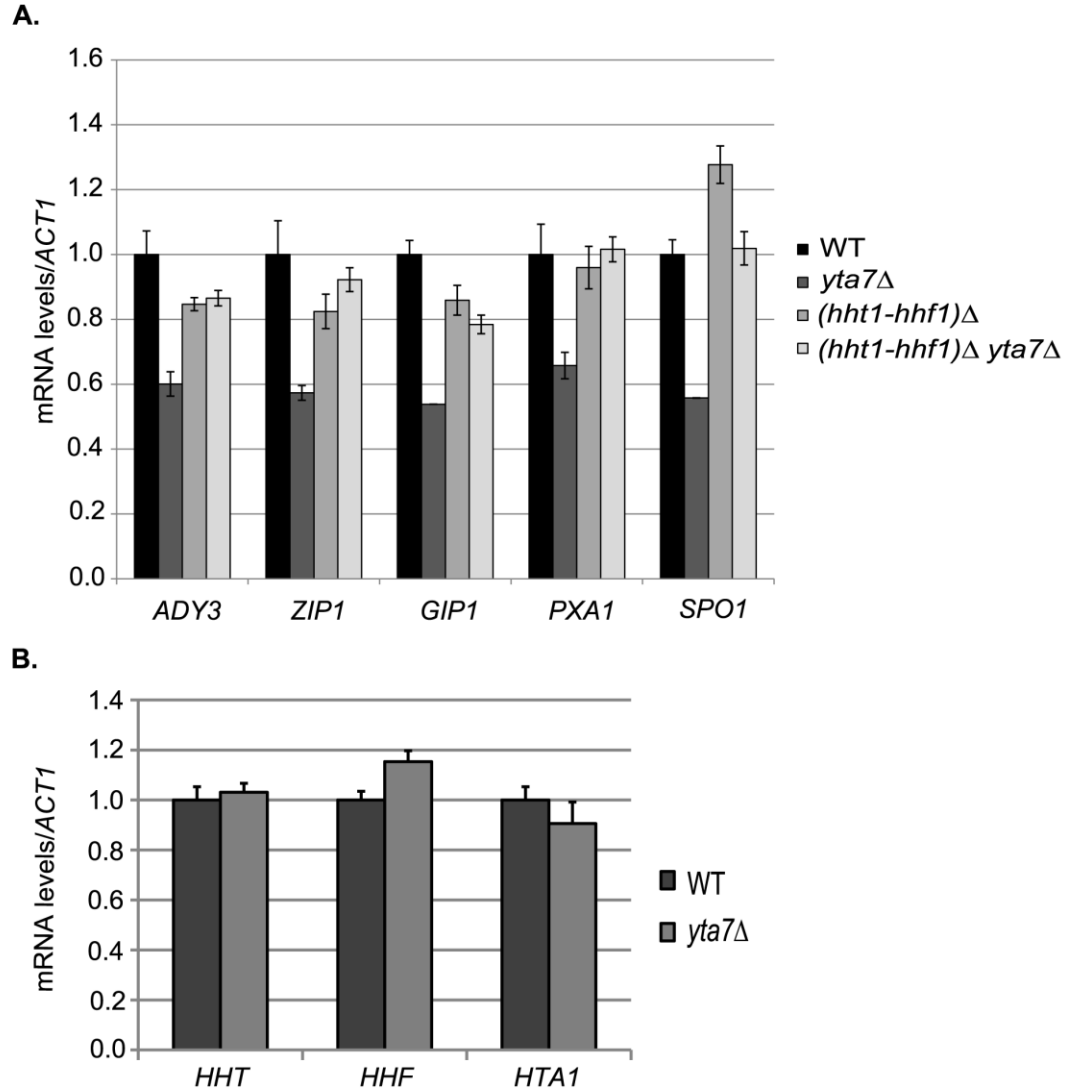


Figure 2.4. Phenotypic suppression with decreased dosage of histones H3 and H4.

A) qRT-PCR analysis of transcript levels in YPD. The level of each transcript was normalized to *ACT1* mRNA levels and adjusted to a wild-type level set equal to 1. B) Little or no changes to histone transcript levels in the *yta7*Δ mutant. RNA analysis was performed on asynchronously growing cells. *HHT* and *HHF* primer sets detect total H3 and H4 transcripts, respectively, whereas *HTA1* primers are specific to the H2A transcripts from the "copy 1" locus.

Supporting the hypothesis that Yta7 might act upon H3, *yta7Δ* mutants are sensitive to the dosage of histones H3 and H4 (Collins et al. 2007; Costanzo et al. 2010). Specifically, *yta7Δ* mutants grow better when a copy of the gene encoding either H3 or H4 is deleted. This growth defect suppression is not observed upon decreased dosage of the genes encoding H2A or H2B. Formally, it would appear that cells without Yta7 experience a specific “excess” of histones H3 and H4. Following this reasoning, we observed that many of the *yta7Δ* mutant’s transcriptional defects were at least partially suppressed when one of the two copies of the H3 and H4 genes were removed (*hht1-hhf1*)Δ (Fig. 2.4A). Thus, the loss of Yta7 might result either in over-expression of H3 and H4 or a cellular state sensitive to normal levels of H3 and H4 expression.

In *S. cerevisiae* two copies of each canonical histone gene are organized into four paired loci (*HHT1-HHF1*, *HHT2-HHF2*, *HTA1-HTB1*, and *HTA2-HTB2*). Three of the four pairs are thought to be similarly regulated, with the exception of *HTA2-HTB2*, which is not subject to Hir-mediated repression (Osley and Lycan 1987; Xu et al. 1992). In asynchronously growing cells, Yta7 is highly enriched at all four canonical histone gene pairs. For three of the gene pairs, Yta7 is preferentially enriched in the ORFs, but at *HTA1-HTB1*, it is enriched in the promoter (Gradolatto et al. 2008). However, there are conflicting data on whether Yta7’s role at these loci is activating or repressive (Gradolatto et al. 2008; Fillingham et al. 2009). In principle, suppression of the *yta7Δ* mutant’s phenotype by decreased dosage of H3 and H4 might reflect excess histone gene expression caused by the loss of Yta7-mediated repression. However, there were no significant differences in the levels of histone gene transcripts in the *yta7Δ* mutant relative to wild type (Fig. 2.4B). Therefore, we explored post-transcriptional explanations for how histone dosage intersected with Yta7’s function. Specifically, we tested whether there was more H3 (and presumably H4) incorporated into chromatin in the *yta7Δ* mutant relative to wild type.

Loss of Yta7 resulted in increased nucleosome density

To test the prediction of increased levels of histones H3 and H4 in chromatin in the *yta7Δ* mutant, H3 ChIP was performed for the *GAL* gene cluster in inducing and non-inducing conditions. Consistent with decreased rates of transcription, there was a 2-3X increased H3 ChIP signal at the *GAL* genes in the *yta7Δ* mutant. An ORF-Free control region (Mason and Struhl 2005) also exhibited an increase in H3 signal in the absence of Yta7, although to a lesser extent (Fig. 2.5A). This increased H3 signal was also observed at repressed *GAL10* and at the highly transcribed *ACT1* locus (Fig. 2.5B). To determine 1) if this increase in H3 “ChIP-ability” in the *yta7Δ* mutant represented an actual increase in nucleosome occupancy and 2) what the global effects on chromatin structure might be, we performed micrococcal nuclease digests on bulk chromatin. Digested chromatin from the *yta7Δ* mutant displayed faster migrating mono-, di-, and tri-nucleosome products than wild type for various concentrations of micrococcal nuclease (Fig. 2.5C). This effect was most obvious on the di- and tri-nucleosome mobilities, rather than on mono-nucleosomes, as would be expected if relative mobility were dominated by the inter-nucleosome distance. Thus, this digestion profile was consistent with increased nucleosome density and a shorter DNA linker between nucleosomes. Indeed, the *yta7Δ* mutant exhibited a 9 ± 2 bp decrease in nucleosome repeat length. As the average linker length in *S. cerevisiae* is ~18bp (Mavrich et al. 2008), the average linker length in the *yta7Δ* mutant appeared to approach the naturally occurring minimum of 7-9bp (Godde and Widom 1992; Lantermann et al. 2010).

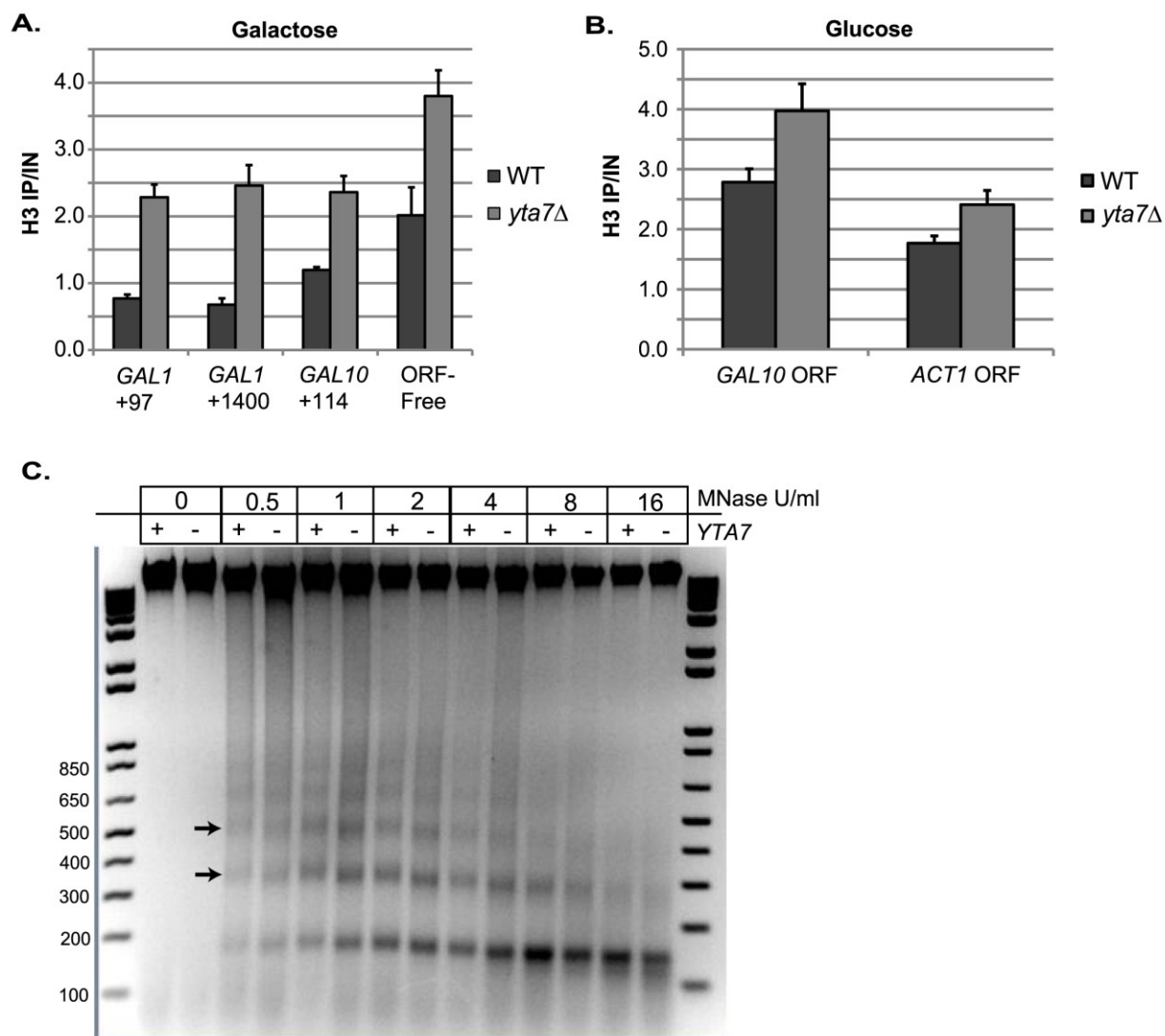


Figure 2.5. Loss of Yta7 resulted in increased nucleosome density.

(A) H3 ChIP performed on cells pre-grown in YPRaffinose (2%, non-inducing) then grown with galactose for 2h. DNA values were quantified by qPCR and are presented as IP/Input for each primer set. (B) H3 ChIP performed on cells growing in YPD. (C) Micrococcal nuclease digestion performed on spheroplasts. Digestions were performed in parallel for 10 minutes each at 37°C. Isolated DNA was quantified and equal amounts electrophoretically separated on a 1.5% agarose gel.

To get a higher resolution look at what happens to H3 occupancy during induction in the *yta7Δ* mutant, the *GAL1* promoter was inserted in front of an 8kb ORF to generate *pGAL1::FMP27*. This gene fusion is commonly employed in *S. cerevisiae* transcriptional studies to provide enhanced spatial resolution of transcriptional events, as this ORF is 5-fold the length of the *GAL1* ORF, allowing for clear distinction of 5'- versus 3'-ORF effects (Mason and Struhl 2005). Upon induction, Yta7 became strongly enriched at the 5'-end of the ORF (Fig. 2.6A), consistent with our data above. Given Yta7's direct effect on *FMP27* during induction, we then assayed H3 occupancy upon induction in the presence and absence of Yta7. Cells without Yta7 displayed increased levels of H3 throughout the locus. The increased H3 levels were consistent with the 50% decrease in *FMP27* mRNA levels observed upon induction in the *yta7Δ* mutant. Further, relative to wild-type cells, induced *pGAL1::FMP27* in the *yta7Δ* mutant exhibited progressively more H3 the farther downstream from the promoter probed (Fig. 2.6B). This resulted in a 50% greater H3 ChIP signal at 8kb than 500bp into the gene.

In principle, given the inverse correlation between transcription levels and histone occupancy, the gradient of increasing H3 signal toward the 3'-end of *FMP27* in the *yta7Δ* mutant could be due to decreased transcription along the course of the gene. However, PolIII localization in the *yta7Δ* mutant appeared uniformly reduced throughout the locus, rather than a gradient (Fig. 2.6B). Thus, as no additional decrease in PolIII occupancy was observed at the 3'-end, the 3'-ORF H3 accumulation was not due to decreased PolIII transit. Instead, these data implied that Yta7 function at the 5'-end of the ORF prevented H3 accumulation throughout the ORF, with an increasing gradient of H3 peaking at the 3'-end of *FMP27*. Therefore, we tested Yta7's ability to limit the amount of chromatin-bound H3.

H3 levels varied inversely with Yta7 levels

Levels of chromatin-incorporated histone H3 were evaluated by ChIP in cells over-expressing Yta7 (*pGAL1::YTA7*) to assess Yta7's capacity to influence chromatin-bound H3. These cells exhibited less than half the H3 signal of wild type in the regions probed (Fig. 2.7A), and also exhibited a ~30% decrease in total H3 protein levels (Fig. 2.7B). However, H3 transcript levels were not decreased in these strains (Fig. 2.7C), indicating that the decrease in H3 occurred through a post-transcriptional mechanism. On the contrary, cells over-expressing Yta7 displayed a moderate yet significant increase in H3 transcript levels ($p = 0.03$, 2-tailed t-test). This increase could reflect a direct activating role of Yta7 at the *HHT1* and *HHT2* loci or an autoregulatory response to decreased H3 protein levels. In contrast, these same strains grown in medium repressing *pGAL1::YTA7* phenocopied the *yta7Δ* mutant, displaying increased levels of incorporated H3 at the regions assayed (Fig. 2.7A). Further, in repressive conditions, *pGAL1::YTA7* strains displayed increased levels of total H3 (Fig. 2.7B). Thus, the level of histone H3, but not its transcript, was inversely correlated with the level of Yta7.

Yta7 and H2A.Z collaborated for GAL gene induction

Microarray analysis of cells grown in rich medium indicated that 25% of genes downregulated in the *yta7Δ* mutant were also downregulated in cells lacking H2A.Z. Because H2A.Z is also required for proper *GAL* gene induction (Santisteban et al. 2000; Adam et al. 2001; Halley et al. 2010), we assessed the possibility of functional overlap between Yta7 and H2A.Z at the *GAL* gene cluster. Specifically, the extent of *GAL* gene induction was assayed in

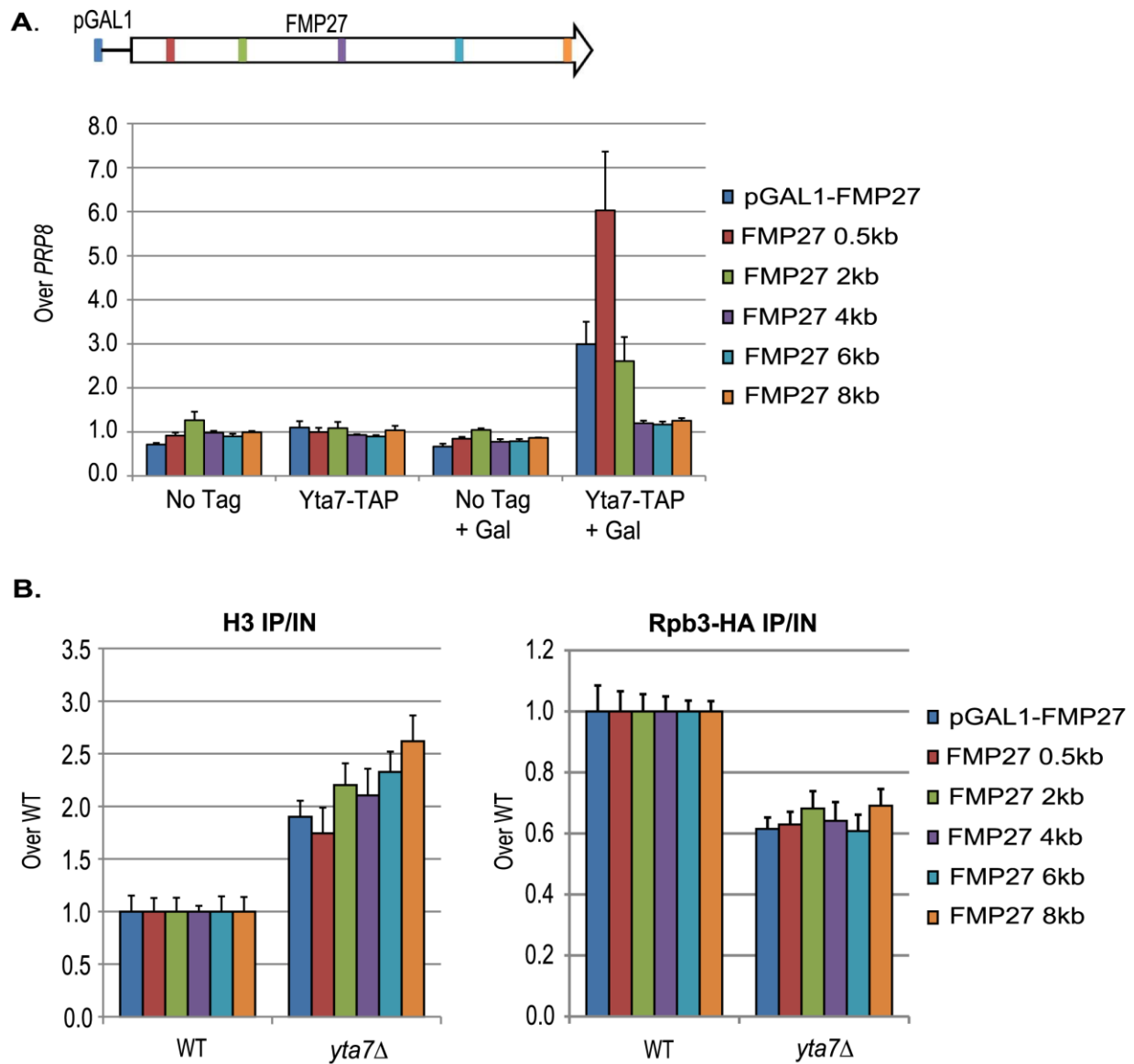


Figure 2.6. Impaired H3 removal upon transcriptional activation.

(A) ChIP analysis of Yta7-TAP across the inducible *FMP27* allele in inducing and non-inducing conditions. The two conditions represent YPRaffinose (2%, non-inducing) and the same cultures 1h after addition of galactose. Values were obtained with qPCR and represent the IP signal over the IP signal for the negative internal control *PRP8*. (B). H3 and Rpb3-HA ChIP performed in parallel on the same sonicated chromatin samples. Represented values are IP/Input, adjusted to a wild-type level set equal to 1 for each primer set. Raw IP/Input values for Rpb3-HA ChIP in both wild type and the *yta7Δ* mutant were relatively uniform ($\pm 15\%$) across the locus. Distances are with respect to the start of the open reading frame.

cells lacking both Yta7 and Swr1, the catalytic subunit of the H2A.Z deposition complex. The *swr1Δ* mutant was used in lieu of the *htz1Δ* mutant, as the *htz1Δ* mutant exhibits phenotypes due both to the loss of H2A.Z and to problematic Swr1 activity in the absence of H2A.Z (Halley et al. 2010; Morillo-Huesca et al. 2010). Strikingly, each single mutant and the *swr1Δ yta7Δ* double mutant displayed the same decrease in *GAL1* transcript levels (Fig. 2.8A). This result strongly suggested that Yta7 and H2A.Z functioned together to ensure proper *GAL* gene induction. Yta7 levels were independent of Swr1 function (Fig. 2.8C), arguing against various indirect models for how Yta7 function depended on Swr1 and H2A.Z.

Among the possible ways that H2A.Z and Yta7 might collaborate for proper *GAL* gene induction would be for H2A.Z to recruit Yta7. Indeed, H2A.Z is enriched at the *GAL* gene cluster prior to induction (Adam et al. 2001), lending temporal plausibility to this hypothesis. As predicted by this hypothesis, Yta7 localization at *GAL10* and *GAL7* upon induction decreased by 50% in the *swr1Δ* mutant (Fig. 2.8B, $p = .006$, 2-tailed t-test). Thus, proper H2A.Z localization played an important role in recruiting Yta7 to the *GAL* gene cluster.

V. Discussion:

Motivated by the conservation of a AAA-ATPase domain coupled to a bromodomain and the importance of Yta7's human ortholog, ANCCA, to human health, we investigated how Yta7 might utilize ATP hydrolysis upon histone interaction. This work established that Yta7 acted to limit nucleosome density, localizing to sites undergoing high rates of replication-independent H3 exchange, where its ATPase domain was required to facilitate gene induction.

Yta7's ATPase-dependent role in gene induction

Transcriptional analysis of cells lacking Yta7 demonstrated a broad misregulation of inducible genes in non-inducing conditions. Many non-induced genes exhibited decreased basal expression levels, a property shared with cells lacking the histone variant H2A.Z. As cells lacking H2A.Z exhibit induction defects for many genes, we tested the role of Yta7 in gene induction. Yta7's ATPase function was required for the proper induction of meiosis- and galactose-induced genes. Further, this induction defect was likely a direct effect of lacking Yta7 activity, as Yta7 was recruited to these genes upon induction. Additionally, Yta7 may be broadly required for gene induction, as many other inducible genes were misregulated in the *yta7Δ* mutant grown in rich medium.

Interestingly, many inducible genes in non-inducing conditions are particularly sensitive to the dose of histones H3 and H4, becoming inappropriately up-regulated upon decreased dosage (Wyrick et al. 1999) or further downregulated upon increased H3/H4 dosage (Singh et al. 2010). Increased amounts of histones H3 and H4 could contribute to increased nucleosome occupancy, which inhibits RNA PolII transcription (Hodges et al. 2009; Churchman and Weissman 2011; Wang et al. 2011). Thus, the suppression of the *yta7Δ* mutant's transcriptional defect at many loci by decreased histone H3 and H4 dosage prompted us to determine if cells lacking Yta7 contained an overabundance of nucleosomes.

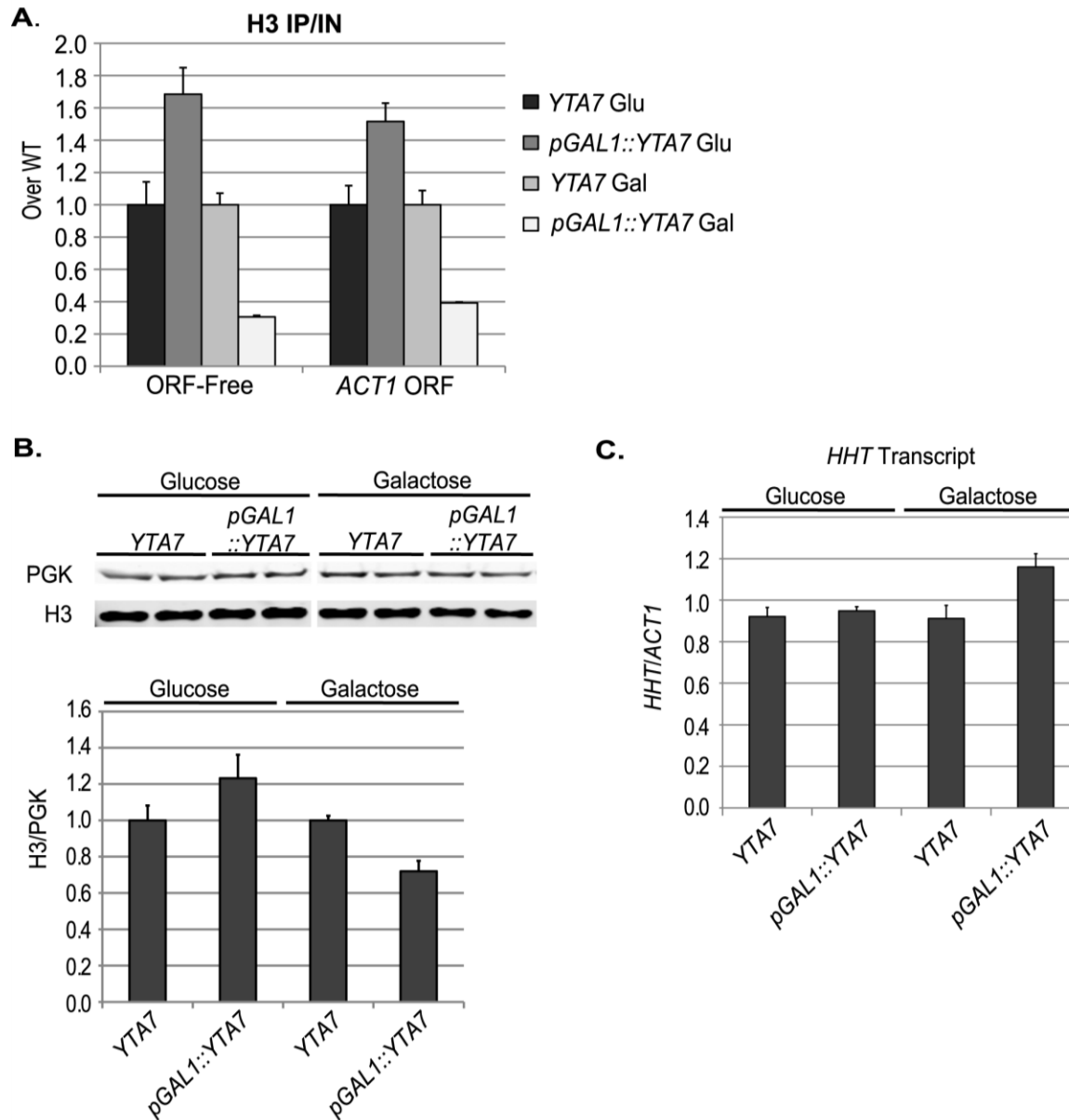


Figure 2.7. Yta7 decreased histone H3 levels post-transcriptionally.

(A) H3 ChIP performed on cells not expressing Yta7 (Glucose) or over-expressing Yta7 (Galactose). Cells were grown in YPD or YPGalactose overnight, then diluted into fresh medium of the same type and grown for 6h until reaching mid-log growth. Values are IP/Input normalized to wild-type levels in each medium. (B) Representative immunoblot of histone H3 and phosphoglycerate kinase (PGK), as a loading control, on extracts of the above cells, taken immediately prior to cross-linking. All bands are from the same blot with a sizing ladder lane cropped from the middle. Duplicate lanes for a given genotype represent independent preps. Quantitation is based on immunoblotting of four separate sample preparations. Values are the ratio of H3 band pixels over the PGK band pixels and are adjusted to the ratio for wild type in each medium type. (C) qRT-PCR analysis of H3 transcript levels on the same cultures analyzed in A and B. *HHT* measures H3 transcript from both the *HHT1* and *HHT2* locus.

Molecular basis of H3 and H4 dosage suppression of yta7Δ

Bulk chromatin of the *yta7Δ* mutant displayed increased nucleosome occupancy, as observed with micrococcal nuclease analysis. This increase was also evident by H3 ChIP at selected loci. Given that histones H3 and H4 exist in the nucleus only as a heterodimer or tetramer, and that *yta7Δ* mutants grow better when a copy of the gene encoding either H3 or H4 is deleted (Collins et al. 2007; Costanzo et al. 2010), we reasoned that histones H3 and H4 would behave similarly in the *yta7Δ* mutant. Thus, we focused our studies on histone H3, which Yta7 directly binds.

In contrast to the *yta7Δ* mutant, chromatin in cells over-expressing Yta7 was significantly depleted of histone H3. Total levels of histone H3 were also decreased, although, H3 transcript levels were not. Thus, our data demonstrated that Yta7 modulated levels of histone H3 protein, independently of perturbations in H3 transcript levels. As Yta7 is a chromatin-associated protein that can directly bind H3 (Gradolatto et al. 2008; Gradolatto et al. 2009), Yta7 presumably acted directly on H3 to modulate its levels. The pronounced depletion of chromatin-incorporated H3 upon over-expression of Yta7 and the increased nucleosome occupancy in the absence of Yta7 indicate that Yta7 acts to restrict the amount of H3 in chromatin. Further, at the inducible *FMP27*, to which Yta7 localized upon induction, Yta7 was required upon induction to prevent the accumulation of H3 throughout the ORF, with an increasing gradient of H3 peaking at the 3'-end of *FMP27*. Taken together, the global and site-specific data strongly implied that Yta7 helps evict or degrade histone H3. Given the tight biochemical association of H3 and H4 and the observed increase in nucleosome density in the *yta7Δ* mutant, this is presumably also true for H4. Further, Yta7's requirement for its AAA-ATPase domain suggests that ATP hydrolysis could provide the requisite energy for eviction or degradation.

In support of Yta7 directly catalyzing eviction or degradation of histones H3 and H4 is the partial suppression of *yta7Δ* by decreased H3 and H4 dosage. This suppression is reminiscent of observations on mutations in *SNF2*, which encodes the catalytic subunit of the SWI/SNF chromatin-remodeling complex responsible for evicting H2A/H2B dimers in regulatory regions. Suppression of gene expression defects in *snf2* mutants is achieved by decreasing the dosage of its substrate, histones H2A and H2B (Hirschhorn et al. 1992). Further, Yta7 localized to induced genes, which are expected to have high rates of H3 and H4 turnover in their ORFs. Our data were consistent with previous evidence that Yta7 is enriched at some of the most highly transcribed genes: *FBA1*, *TEF1*, *PMA1* (Holstege et al. 1998; Gradolatto et al. 2008). Thus, Yta7's localization at highly transcribed genes fits with our proposal that Yta7 functions to either facilitate H3 eviction or degrade evicted H3. Intergenic regions have the highest replication-independent H3 turnover rates, and this turnover is largely independent of transcription rate (Dion et al. 2007; Rufiange et al. 2007). Therefore, it is possible that Yta7 also functions at some intergenic regions, as implied by our data for the ORF-free region analyzed in Figs. 2.5A, 2.7A.

The ability to regulate levels of chromatin-associated H3 could be utilized for processes other than transcription. Thus, Yta7 may also exert S-phase specific activity, such as eviction of H3 and H4 at the replication fork or degradation of excess soluble H3 and H4 after replication completion. Indeed, Yta7 associates with Rad53 (Smolka et al. 2005; Breikreutz et al. 2010), a

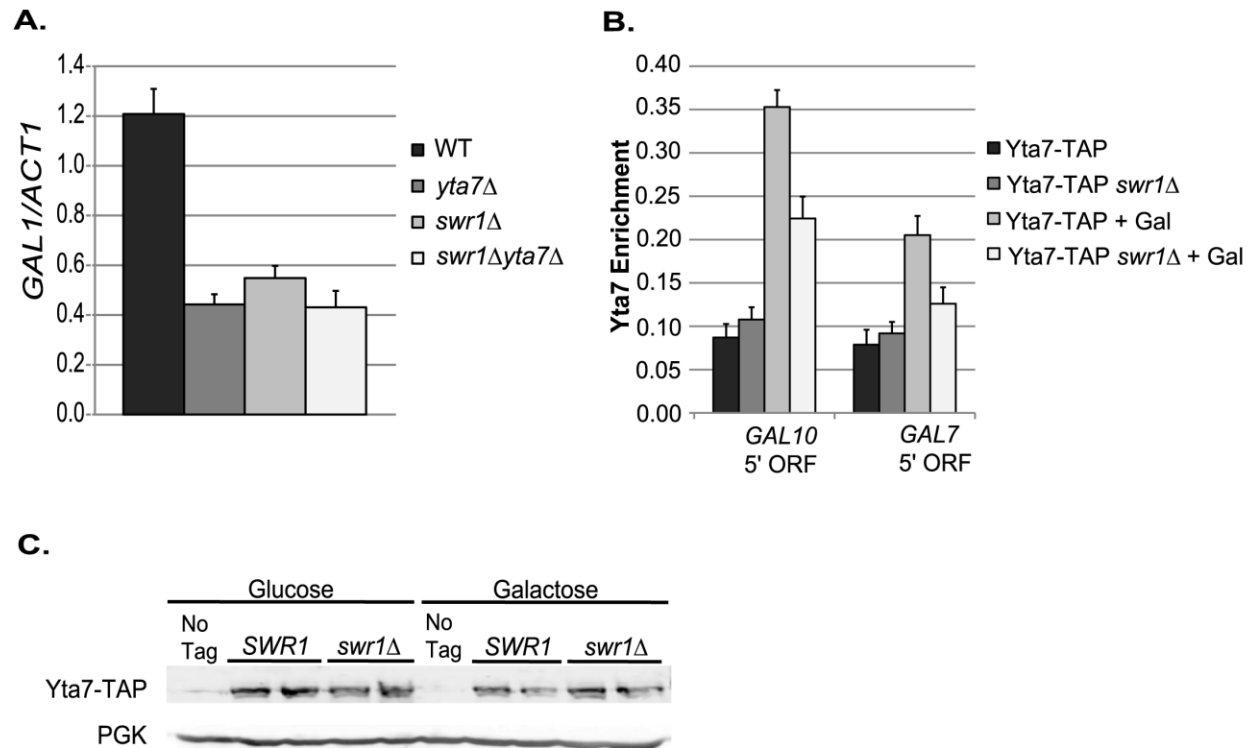


Figure 2.8. Yta7 and H2A.Z functioned together for proper *GAL* gene induction.

(A) qRT-PCR analysis of *GAL1* transcript levels after 1h with galactose. Cells were pre-grown in YPRaffinose (2%, non-inducing). (B) Yta7-TAP ChIP analysis of cells in YPRaffinose (2%) and the same cultures 1h after addition of galactose. Values were obtained with qPCR and represent the IP signal over the IP signal for the positive internal control *HHT1* promoter. (C) Immunoblot of Yta7-TAP and phosphoglycerate kinase (PGK). Cells were grown in YPD or YPGalactose overnight, then diluted into fresh medium of the same type and grown for 6h until reaching mid-log growth. Duplicate lanes for a given genotype represent independent preps.

kinase that facilitates the ubiquitylation and degradation of excess soluble histones (Singh et al. 2009). Strikingly, like *yta7Δ* mutants, the growth defect of *rad53* mutants is suppressed by decreasing the dosage of H3 and H4 (Gunjan and Verreault 2003). Further, cell-cycle specific activities of Yta7 may be reflected by its phosphorylation by Cdk1 (Ubersax et al. 2003). Thus, our data are consistent with Yta7 potentially playing a critical role in the Rad53 pathway of histone degradation.

Collaboration with H2A.Z

The functional overlap of Yta7 and H2A.Z displayed by the array analysis prompted us to investigate their potential collaboration in gene induction. We demonstrated that Yta7 and H2A.Z functioned together for proper *GAL* gene induction. Additionally, loss of H2A.Z deposition into chromatin resulted in decreased Yta7 localization at the *GAL* gene cluster upon induction. However, H2A.Z localization at *GAL* gene cluster was unaffected in the *yta7Δ* mutant (data not shown). Thus, Yta7 localization was at least partially H2A.Z dependent. Upon induction, Yta7 may be recruited directly by H2A.Z, or indirectly via the FACT complex. The 5'-ORF bias in Yta7's localization is consistent with the +1 nucleosome being the most H2A.Z-enriched nucleosome in budding yeast (Albert et al. 2007).

H2A.Z's contribution to Yta7 targeting could explain how H2A.Z localization is strongly correlated with high replication-independent H3 turnover rates (Dion et al. 2007). Further, H2A.Z and Yta7 have both been previously implicated in "boundary" function in *S. cerevisiae* (Meneghini et al. 2003; Jambunathan et al. 2005; Tackett et al. 2005; Babiarz et al. 2006). The boundary mechanism is unclear, but perhaps H2A.Z-mediated recruitment of Yta7 results in H3 eviction or nucleosome clearing, which would be sufficient to inhibit spreading of the Sir silencing complex (Bi et al. 2004). Alternatively, Yta7 could compete with Sir3 for H3 binding or even evict Sir3-bound H3.

The role of Yta7 demonstrated here for gene induction in *S. cerevisiae* is striking given that the human ortholog, ANCCA, functions in induction of estradiol- and androgen-induced genes (Zou et al. 2007; Zou et al. 2009). Interestingly, H2A.Z is also critical for ER α -dependent transcription (Gevry et al. 2009), suggesting conservation of the observed co-regulation by H2A.Z and Yta7.

Chapter 3

The Functional Impact of H2A.Z Acetylation and its Interpretation by Bdf1

I. Abstract:

The histone H2A variant H2A.Z is conserved and essential in all complex eukaryotes assayed. However, the function of its N-terminal tail acetylations remains unclear. In yeast, H2A.Z acetylation is enriched at highly transcribed genes, yet its requirement at these genes has not been assessed. Additionally, the importance of H2A.Z's acetylation to H2A.Z's anti-Sir silencing activity is debated. In this study, I sought to clarify the genome-wide requirements for H2A.Z acetylation, assess the role of individual acetylation sites and determine which proteins might interpret these modifications. The transcriptome of cells lacking H2A.Z acetylation exhibited fewer expression defects than cells lacking H2A.Z. In contrast to proposed roles in transcriptional activation, cells lacking H2A.Z acetylation exhibited a bias toward up-regulation of genes. Genes that were down-regulated in these cells, however, were highly enriched for telomere-adjacent genes, consistent with Sir silencing antagonism or altered telomeric structure. In keeping with more recent work, my data supported a model of acetylation-site equivalence and additive activity of H2A.Z acetylation. Additionally, this work identified the double bromodomain-containing TFIID-associated Bdf1 as interacting with H2A.Z in an acetylation-dependent manner *in vivo*. As Bdf1 is required to inhibit Sir complex spreading from the telomeres, this work provides insight into the mechanism of Bdf1's Sir complex antagonism.

II. Introduction:

As discussed in previous chapters, nucleosomes can be modified by the incorporation of histone variants, such as H2A.Z and H3.3, and further differentiated by covalent modifications of histones (Campos and Reinberg 2009; Talbert and Henikoff 2010). In this chapter, I address the intersection of these two types of nucleosome modification: covalent modification of a histone variant. Specifically, this chapter describes work aimed at determining the function of H2A.Z N-terminal tail acetylations and isolating proteins that specifically recognize these modifications.

H2A.Z is well conserved and essential in all multicellular eukaryotes assayed (Zlatanova and Thakar 2008). Yeast cells lacking H2A.Z exhibit a broad range of chromatin-based phenotypes, including defective gene induction (Santisteban et al. 2000; Zhang et al. 2005; Wan et al. 2009; Halley et al. 2010), genomic instability (Krogan et al. 2004; Keogh et al. 2006), and spreading of the Sir-silencing complex from heterochromatin into euchromatic domains (Meneghini et al. 2003; Jambunathan et al. 2005; Babiarz et al. 2006; Zhou et al. 2010). H2A.Z's role in gene induction is conserved in vertebrates (Gevry et al. 2009), as is its role in chromosome segregation (Rangasamy et al. 2004). Additionally, insulators exhibit H2A.Z enrichment in vertebrates (Jin et al. 2009), implying conservation of H2A.Z function in chromatin domain delimitation.

In yeast, ~60% of promoters contain H2A.Z (Guillemette et al. 2005; Raisner et al. 2005). The SWR1 complex deposits H2A.Z into chromatin by replacing H2A/H2B dimers in nucleosomes with H2A.Z/H2B dimers (Mizuguchi et al. 2004). The predominant position of H2A.Z in promoters is the so-called +1 nucleosome, either slightly overlapping the transcription start site (TSS) or positioned just downstream of the TSS (Raisner et al. 2005; Albert et al. 2007). Thus, the +1 nucleosome tends to straddle the beginning of the open-reading frame (ORF). At some genes, another H2A.Z-containing nucleosome can occur upstream of the +1 nucleosome after a nucleosome-free region of ~150 bp at the -1 position (Raisner et al. 2005;

Albert et al. 2007). H2A.Z-containing nucleosomes are also well-represented at the +2 and +3 positions within ORFs (Albert et al. 2007). Like all histones, H2A.Z's occupancy is anti-correlated with transcription rates (Guillemette et al. 2005; Li et al. 2005; Zhang et al. 2005).

The effects of H2A.Z on nucleosome stability have been controversial, but some of the conflicting results might be resolved by the recent discovery that H2A.Z's effects on stability depend on the type of histone H3 present in the nucleosome. Specifically, H2A.Z destabilizes H3.3-containing nucleosomes (Jin and Felsenfeld 2007). This finding is consistent with results from budding yeast indicating that H2A.Z-containing nucleosomes are more salt-labile than canonical nucleosomes (Zhang et al. 2005), as budding yeast contains the H3.3 form of H3. Given that H2A.Z-containing nucleosomes are enriched at promoters poised for induction and are required for proper induction of many genes, the decreased stability H2A.Z-containing nucleosomes is thought to promote the nucleosome loss required for transcriptional activation (Abbott et al. 2001). Interestingly, nucleosomes containing H2A.Z are also thought to be more thermo-labile than canonical nucleosomes, facilitating temperature-sensitive responses in *Arabidopsis* (Kumar and Wigge 2010).

Like the canonical histones, H2A.Z's N-terminal tail is acetylated. There are four lysines on H2A.Z's N-terminal tail and all four are acetylated *in vivo*: K3, K8, K10, and K14R (Babiarz et al. 2006; Millar et al. 2006). The K14-Ac is the most abundant acetylation, present on ~ 40% of H2A.Z molecules (Millar et al. 2006). The histone acetyltransferase (HAT) NuA4, which acetylates the N-terminal tails of H4 and H2A, is required *in vivo* for H2A.Z acetylation and can acetylate H2A.Z *in vitro* (Babiarz et al. 2006; Keogh et al. 2006; Millar et al. 2006). Additionally, cells lacking Gcn5, the HAT of the SAGA co-activator complex, have decreased levels of H2A.Z acetylation *in vivo*, but it is unclear if this is a direct effect (Babiarz et al. 2006; Millar et al. 2006). Although highly transcribed genes contain low levels of H2A.Z, the H2A.Z remaining is enriched for K14-Ac (Millar et al. 2006). Thus, H2A.Z K14-Ac behaves like a classical transcription-associated histone acetylation, with its genomic localization well-correlated with N-terminal tail acetylations of canonical histones (Millar et al. 2006). However, the requirement for H2A.Z acetylation in transcriptional activation has not been tested.

Given that the Sir silencing complex contains the histone deacetylase Sir2 and requires hypoacetylated histone H3 and H4 tails for binding and compaction (Rusche et al. 2003), it was possible that H2A.Z's acetylation might be crucial to H2A.Z's antagonism of Sir silencing. Indeed, HATs and proteins containing the acetyl-lysine binding module, the bromodomain, can inhibit Sir complex activity (Ladurner et al. 2003; Jambunathan et al. 2005). However, tests of the ability of H2A.Z acetylation to inhibit Sir silencing yielded conflicting results. Specifically, cells lacking H2A.Z exhibit Sir-dependent repression of genes adjacent to the silenced *HMR* locus, but cells unable to acetylate H2A.Z do not (Babiarz et al. 2006; Millar et al. 2006). On the other hand, cells unable to acetylate H2A.Z do display Sir-dependent repression of genes adjacent to telomere IXR (Babiarz et al. 2006). However, it is unclear if this is broadly true, as H2A.Z K14-Ac, the most abundant site, is not required for the expression of a reporter gene 1 kb from telomere VR (Keogh et al. 2006). Further, H2A.Z K14-Ac is depleted within 50 kb of telomeres, decreasing steadily toward the telomeres (Millar et al. 2006).

The goals of the work described herein were to determine the functional significance of H2A.Z acetylation, ascertain any functional distinctions between acetylation sites, and isolate proteins that specifically interact with acetylated H2A.Z. This allowed me to assess any role of H2A.Z acetylation in transcriptional activation and determine if H2A.Z acetylation was broadly required for telomere-proximal gene expression.

III. Materials and Methods:

Yeast strains and media

All yeast strains were derived from W303-1a (listed in Table 3.1). One-step integration of knockout cassettes and C-terminal epitope tags was performed as described (Longtine et al. 1998; Goldstein and McCusker 1999; Puig et al. 2001) (see Table 3.2 for primer sequences). All gene disruptions were confirmed by 5'- and 3'-junction PCR. All epitope-tagging was confirmed by immunoblotting as described below.

Microarrays

Cultures were grown at 30°C to OD₆₀₀ 0.8 - 1. RNA was extracted with hot acid phenol as described (Collart and Oliviero 2001). A denaturing gel was used to assess the quality of the RNA. For each experiment, 20 ug of total RNA from each strain and 20 ug of total RNA from a pooled reference was primed with 5 ug/ul oligo dT & 5 ug/ul pdN₁₅ for ten minutes at 70°C, followed by ten minutes on ice. Roche Transcriptor was utilized for reverse transcription with a 3:2 ratio of amino-allyl-dUTP:dTTP. Reactions were performed at 42°C for 12 hr. Residual RNA was then hydrolyzed with 250 mM NaOH for 15 min at 65°C. cDNA was isolated from this mixture via Qiagen MinElute Reaction Cleanup Kit. After dye coupling (Amersham Cy3 and Cy5) for 2 hr at RT, the cDNA was purified with Qiagen MinElute Reaction Cleanup Kit and quantified with a Nanodrop spectrophotometer (Thermo Scientific). Reference cDNA was pooled and equal amounts were mixed with each experimental sample. Hybridizations were performed for 12 – 14 hr at 65°C using the MAUI Hybridization System (BioMicro Systems, Salt Lake City, UT).

Custom microarrays were fabricated as described (DeRisi et al. 1997) at the University of California, San Francisco, Center for Advanced Technology (UCSF CAT) with Operon's Aros V1.1 and YBOX (Yeast Brown lab Oligo eXtension) 70-mer probes. Specific protocols for array printing, post-processing, and washing are at <http://cat.ucsf.edu>.

After washing, the arrays were scanned on a Genepix 4000B scanner (Molecular Devices Axon, Sunnyvale, CA) and the images analyzed using Genepix Pro 6.1 software. After removing poor quality spots, the data were corrected using TiGR MIDAS v2.20 LOWESS normalization. Analysis was performed using TiGR MeV version 3.1 (Saeed et al. 2006). Significantly affected genes were determined using the SAM (Significance Analysis of Microarrays) statistical package (Tusher et al. 2001) with a 1.5 median false gene cut-off.

Spatial analysis was performed on significantly affected genes by calculating the distance of the 5'-end of the gene to the closest X-core element. Relative enrichments displayed in Fig. 3.1A

Table 3.1. Strains used in Chapter 3.

Strain	Genotype	Source
W303-1a	<i>MATα ade2-1; can1-100; his3-11; leu2-3,112; trp1-1; ura3-1</i> (JRY3009)	R. Rothstein
JRY7754	<i>MATα htz1Δ::HIS3MX</i>	Kobor et al. 2004
JRY7972	<i>MATα HTZ1-FLAG::KanMX</i>	Babiarz et al. 2006
JRY7983	<i>MATα htz1K3,8,10,14R-3FLAG::kan</i>	Babiarz et al. 2006
JRY8685	<i>MATα BDF1-TAP::TRP1 HTZ1-FLAG::KanMX</i>	J. Babiarz
JRY8686	<i>MATα BDF1-TAP::TRP1 htz1K3,8,10,14R-FLAG::KanMX</i>	J. Babiarz
JRY8689	<i>MATα HTZ1-FLAG::KanMX YTA7-TAP::TRP1</i>	J. Babiarz
JRY8690	<i>MATα htz1K3,8,10,14R-FLAG::KanMX YTA7-TAP::TRP1</i>	J. Babiarz
JRY9199	<i>MATα HTZ1-FLAG::KanMX yta7Δ::natMX</i>	This study
JRY9231	<i>MATα htz1K14R-3FLAG::kan</i>	This study
JRY9232	<i>MATα htz1K3,8,10,14R-FLAG::KanMX yta7Δ::natMX</i>	This study
JRY9233	<i>MATα htz1K3R-3FLAG::kan</i>	This study
JRY9234	<i>MATα htz1K8R-3FLAG::kan</i>	This study
JRY9235	<i>MATα htz1K10R-3FLAG::kan</i>	This study
JRY9236	<i>MATα htz1K10,14R-3FLAG::kan</i>	This study
JRY9237	<i>MATα BDF1-TAP::TRP1 htz1K3R-3FLAG::kan</i>	This study
JRY9238	<i>MATα BDF1-TAP::TRP1 htz1K8R-3FLAG::kan</i>	This study
JRY9239	<i>MATα BDF1-TAP::TRP1 htz1K10R-3FLAG::kan</i>	This study
JRY9240	<i>MATα BDF1-TAP::TRP1 htz1K14R-3FLAG::kan</i>	This study
JRY9241	<i>MATα YTA7-TAP::HIS3MX hhf1-hht1Δ::HygMX; hhf2-hht2Δ::NatMX; pHHF2-HHT2/316</i> (pJR2657)	J. Babiarz
JRY9242	<i>MATα YTA7-TAP::HIS3MX hhf1-hht1Δ::HygMX; hhf2-hht2Δ::NatMX; phhf2-K5,8,12R-HHT2/314</i> (pJR2760)	J. Babiarz

Table 3.2. Primers used in Chapter 3.

<i>yta7</i> KO	TCTCTTCTCGCTATTTATTCACAATTGGTGCAGAAAGAACCGGATCCCCGGGTAAATTA AACTACATTTAAGAATTATATAAACATTATGGACTCCTGCCGATGAATTCGAGCTCGTTT
<i>YTA7-TAP</i>	AACAGGAACTGTCGATGAAATAATAAAATTTTATCTGAATCCATGGAAAAGAGAAG AACTACATTTAAGAATTATATAAACATTATGGACTCCTGCTACGACTCACTATAGGG
<i>BDF1-TAP</i>	CTCATCTTCAGATGACGATGTTAGCAGCGAAAAGTGAAGAAGAGTCCATGGAAAAGAGAAG TTCTTCTCAGTCGTTGAAGATAATCAAATTCAAAATTCAGTACGACTCACTATAGGG
<i>YIR042C</i>	TTTAAATGGGCCGTCAGGAGGGCA AAACCATTGAGTGTCGCGAGTGTC
<i>ACT1</i>	TGTCCTTGACTCTTCCGGT CCGGCCAAATCGATTCTCAA (described in Babiarz et al. 2006)

and 3.3 were determined for each mutant as a percentage of the total number of genes down- or up-regulated. Significance within each interval was ascertained by performing a chi-square test on observed versus expected values for a given mutant. The expected value for a given mutant within a given interval was calculated as follows: # genes in interval genome-wide * (affected genes/total genes). Significance for the number of HZADs contained within a gene set was calculated using the hypergeometric distribution calculator found at <http://www.alewand.de/stattab/tabdiske.htm>.

RNA isolation and qRT-PCR

RNA isolation was performed using the hot-phenol method (Collart and Oliviero 2001). Total RNA was digested with DNase I (Roche) and purified using the RNeasy Minelute kit (Qiagen). cDNA was synthesized using the Super-Script III First-Strand Synthesis System (Invitrogen) with oligo(dT) priming. Quantitative PCR on cDNA was performed using an MX3000P qPCR machine (Agilent) and the Dynamo HS SYBR Green qPCR kit (NEB). Amplification values for all primer sets were normalized to actin (*ACT1*) cDNA amplification values.

Immunoprecipitation and Protein Analysis

For Bdf1-TAP IPs, cells were grown to mid-log phase, and 100 OD₆₀₀ units were harvested by centrifugation. Cells were resuspended in 50 mM Tris-Cl at pH 7.8, 50 mM NaCl, 1.5 mM MgAc, 100 mM Na Butyrate, 5 mM Nicotinamide, and Complete Protease inhibitor cocktail [Roche]). Acid-washed glass beads were added, and the cells were disrupted mechanically using a bead beater (BioSpec Products) for 5 min. CaCl₂ was added to a final concentration of 1 mM, and chromosomal DNA was digested using 6 U of micrococcal nuclease (Sigma) for 10 min at 37°C. Insoluble material was removed by centrifugation. The supernatant was removed and incubated with 30 ul of IgG Sepharose (GE Healthcare) for 1.5 hr at 4°C. Beads were then pelleted and washed three times with 0.6 ml of the same buffer as above. After washing, the supernatant was removed and 50 ul 3X SDS sample buffer was added. The Yta7-TAP IP in Fig. 3.5C was performed as above.

Due to low H4 antibody affinity, the Yta7-TAP IP probing H4 association was modified. 2 L of cells were grown to ~3.0 OD₆₀₀. Cells were harvested, mechanically lysed on dry ice in a coffee grinder, and resuspended as above. Cleared supernatant was applied to 140 ul of IgG Sepharose and treated as above, except elution was performed with 280 ul 3X SDS sample buffer.

Samples were then boiled and spun. The supernatant was evaluated by standard SDS-PAGE and immunoblotting. Detection was performed with the Li-cor Odyssey imaging system. Antibodies used for immunoblotting: rabbit anti-FLAG antibody (Sigma) for protein A-containing TAP tag detection, mouse anti-FLAG (Sigma), and rabbit anti-H4 (Abcam ab10158).

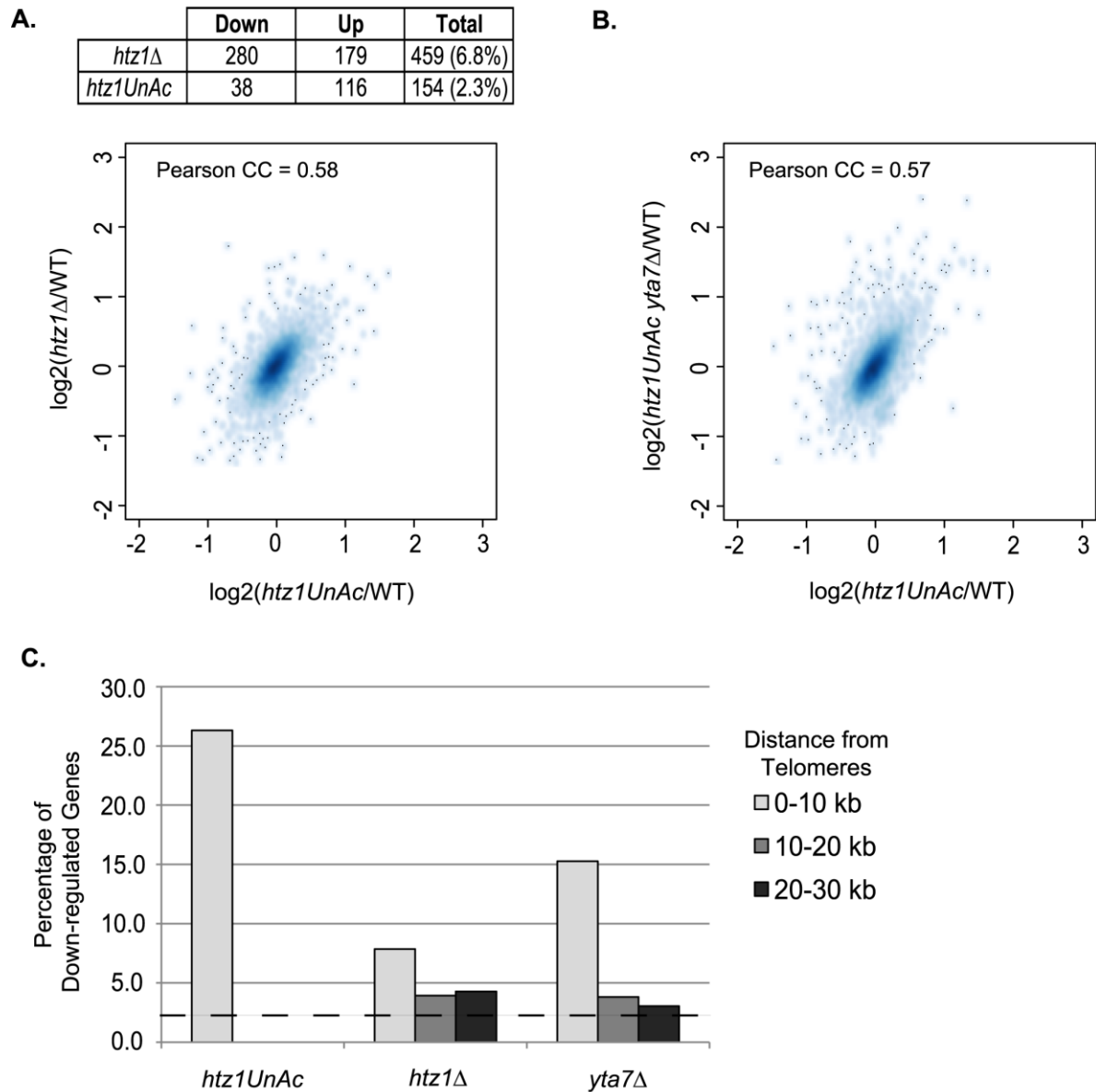


Figure 3.1. Loss of H2A.Z acetylation largely resulted in transcriptional up-regulation, but down-regulated genes were enriched adjacent to telomeres.

A) Table listing the number of significantly affected genes in the *htz1UnAc* (*htz1K3,8,10,14R*) and *htz1Δ* mutants (Significance Analysis of Microarrays (SAM), 1.5 median false gene cut-off). Correlation plot of $\log_2(htz1UnAc/WT)$ versus $\log_2(htz1Δ/WT)$, each the average of four independent microarray experiments. Pearson CC = Pearson correlation coefficient. B) Correlation plot as above, but between $\log_2(htz1UnAc/WT)$ and $\log_2(htz1UnAc yta7Δ/WT)$. C) Spatial analysis of significantly down-regulated genes in the three mutants listed. The distance from the most interior X-core element of the closest telomere was calculated for each significantly down-regulated gene. The number of genes that fell within the distance bins for each mutant are presented as a percentage of the total number of down-regulated genes in that mutant. The dashed line indicates the percentage of genes found in each 10 kb bin out of the genomic total.

IV. Results:

H2A.Z acetylation was required for telomere-adjacent gene expression, but its loss favored up-regulation

To understand the genome-wide role of H2A.Z acetylation in transcriptional regulation, transcriptome analysis was performed in cells containing only H2A.Z that could not be acetylated. Specifically, the four lysines (K3, K8, K10, and K14) on its N-terminal tail that are acetylated (Babiarz et al. 2006; Millar et al. 2006) were mutated to arginine to prevent acetylation, yet maintain the basic charge. This quadruple mutant is referred to throughout this chapter as *htz1UnAc*. To compare the function of H2A.Z acetylation to any potentially acetylation-independent roles of H2A.Z, the null mutant (*htz1Δ*) was assayed in parallel. Acetylation-independent roles of H2A.Z were anticipated based on the phenotypic distinctions between cells lacking H2A.Z (*htz1Δ*), or deficient in H2A.Z incorporation into chromatin (*swr1Δ*), and cells unable to acetylate H2A.Z (*htz1UnAc*). *htz1Δ* and *swr1Δ* mutants exhibit slow growth and sensitivity to various genotoxic stresses, whereas the *htz1UnAc* mutant does not (Babiarz et al. 2006; Millar et al. 2006). Additionally, as H2A.Z acetylation is necessary for antagonism of Sir silencing at one telomeric region (IXR) (Babiarz et al. 2006), we wanted to assay in parallel the effects of losing a different factor implicated in anti-silencing activity and assess any functional overlap. To this end, strains lacking Yta7 and strains lacking both Yta7 and H2A.Z acetylation were also assessed.

Cells unable to acetylate H2A.Z exhibited misregulation of ~2% of the genome, compared to ~7% affected in cells lacking H2A.Z (Fig. 3.1A). Further, cells lacking H2A.Z acetylation exhibited a misregulation profile qualitatively distinct from the *htz1Δ* mutant, because ~3 times more genes were up-regulated than down-regulated. This profile was in contrast to the *htz1Δ* mutant, which exhibited a bias toward down-regulation (Fig. 3.1A). However, overall, the *htz1UnAc* and *htz1Δ* mutant profiles correlated well (Fig. 3.1A), as strong as the correlation between *htz1UnAc* and *htz1UnAc yta7Δ* profiles (Fig. 3.1B). Although the *htz1UnAc* transcriptome demonstrated a bias toward up-regulation, the set of genes that were down-regulated contained a high density of telomere-adjacent genes. Specifically, ~25% of genes down-regulated in the *htz1UnAc* mutant were within 10 kb from a telomeric X-core element (Fig. 3.1C). The *htz1Δ* and *yta7Δ* mutants also exhibited a significant enrichment of telomere-proximal genes in their sets of down-regulated genes (Fig. 3.1C, Table 3.3). Thus, all mutants assayed were important for maintaining the expression of telomere-proximal genes.

Regulation of telomere-proximal genes by H2A.Z involved acetylation-dependent and -independent functions

In-depth analysis of telomere-proximal gene regulation in these mutant transcriptomes yielded three distinct classes of H2A.Z-dependent genes: those specifically requiring H2A.Z acetylation (Fig. 3.2A), those requiring both H2A.Z acetylation and Yta7 (Fig. 3.2B), and those requiring Yta7 and acetylation-independent H2A.Z function (Fig. 3.2C). Genes exhibiting a statistically significant transcriptional defect in all mutants assayed were limited, but the expression of the *htz1UnAc yta7Δ* double mutant at these genes indicated that H2A.Z acetylation

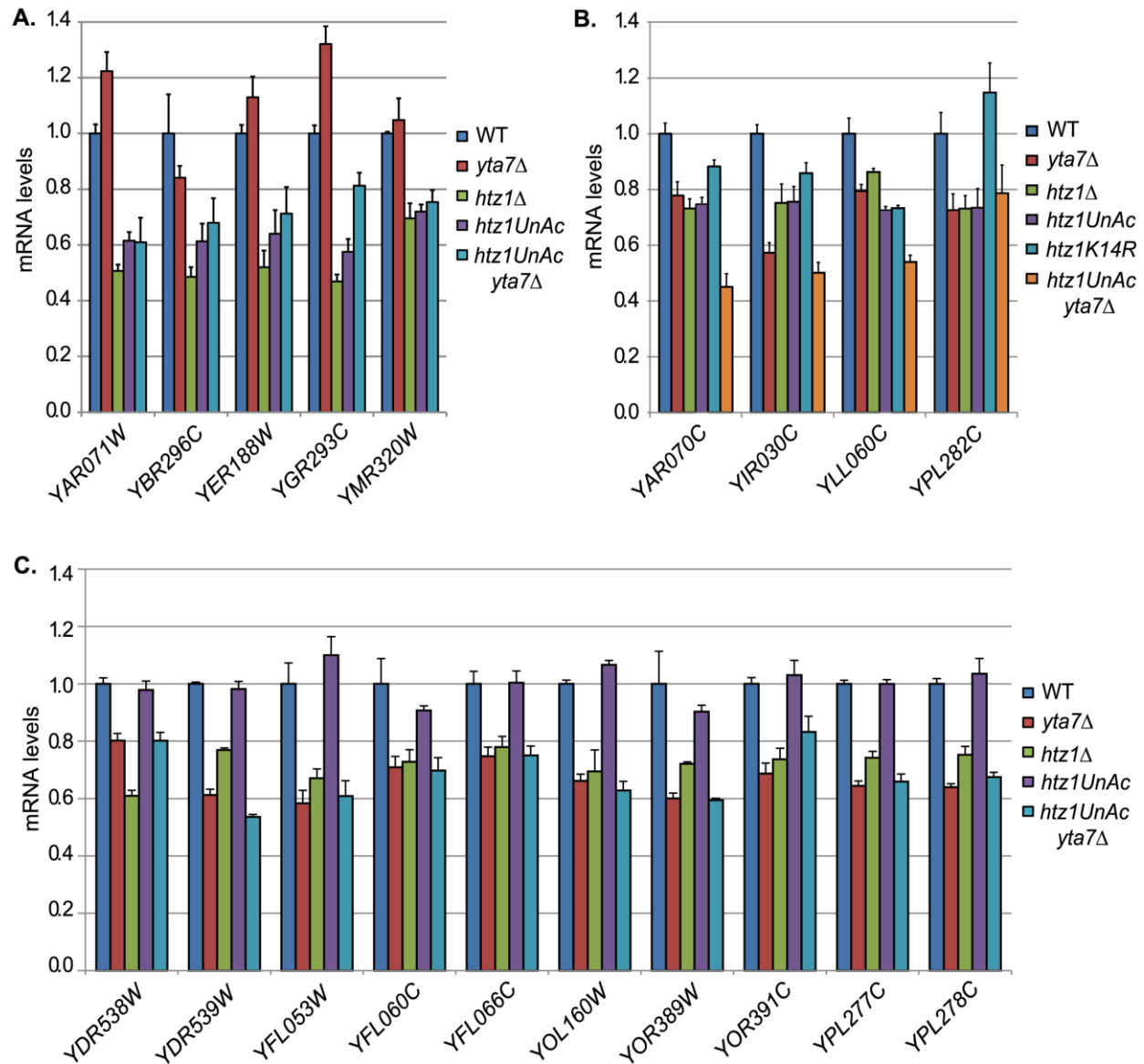


Figure 3.2. H2A.Z acetylation-dependent and -independent transcription of telomere-proximal genes.

A) mRNA levels of telomere-proximal genes that exhibited a specific requirement for H2A.Z acetylation. Transcript levels were determined by four independent microarray experiments. The wild-type level for each transcript was set equal to 1. The error bars in this and all subsequent figures represent the standard error of the mean (SEM). B) mRNA levels, as above, but for telomere-proximal genes that exhibited dependence on both H2A.Z acetylation and Yta7. At these genes, transcript levels were significantly ($p < 0.05$) different from wild-type levels for all mutants except *htz1K14R*. C) Transcript levels for telomere-proximal genes which required Yta7 or H2A.Z for proper expression, but did not appear to require H2A.Z acetylation. At these genes, transcript levels in the *yta7Δ* and *htz1Δ* mutants were significantly ($p < 0.05$) different from levels in wild type and the *htz1UnAc* mutant. All genes in this figure are within 30 kb from the telomeric X-core element.

and Yta7 could contribute additively or non-additively to gene expression (Fig. 3.2B). Thus, depending on the telomere-proximal gene, Yta7 and H2A.Z acetylation appeared to contribute independently or work together to ensure proper expression. The most abundant class consisted of genes that were significantly down-regulated in the *htz1Δ* and *yta7Δ* mutants compared to wild type and the *htz1UnAc* mutant (Fig. 3.2C). It is possible that the microarray experiments were not sensitive enough to detect a defect in the *htz1UnAc* mutant, but the *htz1Δ* mutant clearly had broader transcriptional effects genome-wide than the *htz1UnAc* mutant, implicating acetylation-independent roles of H2A.Z. Thus, at face value, this third class of H2AZ-dependent genes appeared dependent on Yta7 function but independent of H2A.Z acetylation.

Loss of H2A.Z and its acetylation resulted in up-regulation of genes 10 – 20 kb from the telomere

Surprisingly, spatial analysis of significantly affected genes in all of the mutant strains indicated that both *htz1UnAc* and *htz1Δ* mutants contained an enrichment of telomere-proximal genes in their up-regulated gene sets. Although, the *htz1UnAc* mutant displayed significant enrichment of genes within 10 kb of the telomere in its set of down-regulated genes, this mutant also contained significant enrichment of genes 10 -20 kb from the telomere in its up-regulated gene set (Fig. 3.3, Table 3.3). On the other hand, the *htz1Δ* mutant displayed significant enrichment of genes 10 -20 kb from the telomere in those down-regulated, as expected, but also those up-regulated. This is in stark contrast to the *yta7Δ* mutant, which exhibited significant telomere-proximal enrichment only for down-regulated genes (Fig. 3.3, Table 3.3). These data suggested that H2A.Z and its acetylation played an unanticipated role in balancing telomere-proximal gene expression, maintaining expression within 10 kb of the telomere and dampening expression 10 – 20 kb from the telomere. Further, this distinction implied the existence of differentially regulated spatial domains at different distances from the telomere.

The additive effect of H2A.Z acetylation sites on the repression of telomere-adjacent genes

To assess the functional significance of the most abundant H2A.Z acetylation, K14-Ac (Millar et al. 2006), the transcriptomes of an *htz1K14R* and the *htz1UnAc* mutant were compared. Surprisingly, the *htz1K14R* mutant significantly affected only ~0.2% of the genome compared to the ~2% of the *htz1UnAc* mutant (Fig. 3.4A), indicating that H2A.Z K14-Ac was largely dispensable in the conditions assayed. H2A.Z K14-Ac was specifically required for proper expression at the genes adjacent to telomere IXR and, intriguingly, at the poorly-described “non-transcribed spacer” regions (*NTS1-2* and *NTS2-1*) of the rDNA repeats (Fig 3.4A). To expand this analysis, all *htz1* single acetylation mutants were assayed for expression levels of the telomere IXR-adjacent gene *YIR042C*. Strikingly, each *htz1* single acetylation mutant displayed a partial decrease in transcript levels compared to the *htz1UnAc* mutant (Fig. 3.4B), consistent with an additive model of roughly equivalent acetylation sites. This pattern is seen for histone H4 acetylation in *S. cerevisiae*, where inability to acetylate K5, K8, or K12 results in equivalently small defects and loss of any two sites results in larger defects, with the greatest defect in the triple mutant (Dion et al. 2005).

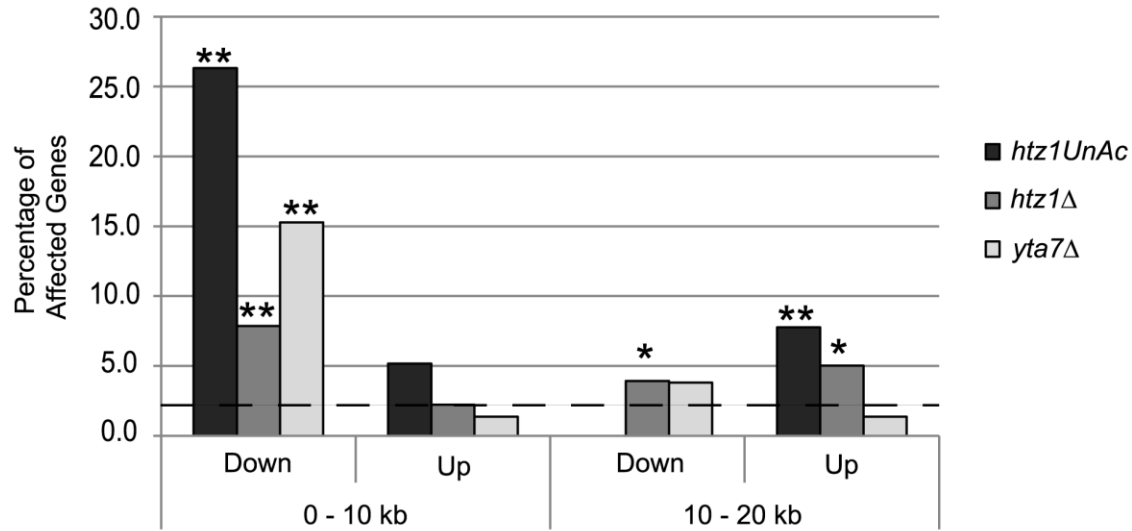


Figure 3.3. Cells without H2A.Z or its acetylation exhibited up-regulation of genes beyond a telomere-adjacent domain of down-regulation.

Spatial analysis of significantly affected genes in the three mutants listed. The distance from the most internal X-core element of the closest telomere was calculated for each significantly affected gene. The number of genes that fell within the distance bins for each mutant are presented as a percentage of the total number of down- or up-regulated genes in that mutant. The dashed line indicates the percentage of genes found in each 10 kb bin out of the genomic total. ** signifies p-values < 0.0001. Significance was determined by chi-square analysis.

Table 3.3. Significant misregulation of telomere-proximal genes.

		p-value	
<i>htz1UnAc</i>	0-10kb	Down	2.09E-20
		Up	N.S.
	10-20kb	Down	N.S.
		Up*	2.58E-05
	20-30kb	Down	N.S.
		Up	N.S.
<i>htz1Δ</i>	0-10kb	Down	6.72E-10
		Up	N.S.
	10-20kb	Down	0.05
		Up*	6.83E-03
	20-30kb	Down	0.02
		Up*	1.38E-02
<i>yta7Δ</i>	0-10kb	Down	3.30E-20
		Up	N.S.
	10-20kb	Down	N.S.
		Up	N.S.
	20-30kb	Down	N.S.
		Up	N.S.

p-values were calculated using the chi-square test.

*denotes significant enrichment in up-regulated gene sets

N. S. = not significant; $p > 0.05$

Bdf1's interaction with H2A.Z was acetylation-dependent in vivo

In principle, functional equivalence of N-terminal tail acetylation sites might suggest a charge neutralization mechanism of acetylation function, whereby each acetylation has a comparable effect on electrostatic interactions. However, acetylated-lysines do not just neutralize charge but act to recruit bromodomain-containing proteins. Thus, functional equivalence of acetylation sites might alternatively reflect promiscuous binding on the part of bromodomain-containing proteins.

Given the amino acid sequence similarity between histones H4 and H2A.Z and the ability of the NuA4 histone acetyltransferase to acetylate both (Babiarz et al. 2006; Keogh et al. 2006; Millar et al. 2006), we assessed the ability of the H4-Ac interactor, Bdf1 (Bromodomain Factor 1), to also interact with H2A.Z in an acetylation-dependent manner (Ladurner et al. 2003; Hassan et al. 2006). Specifically, the interaction between Bdf1 and H2A.Z was assessed in wild type and in cells unable to acetylate H2A.Z. Bdf1 immunoprecipitated from wild-type cells exhibited a strong association with H2A.Z. In contrast, in cells unable to acetylate H2A.Z, Bdf1 co-immunoprecipitated with only background levels of H2A.Z, indicating that Bdf1's *in vivo* interaction with H2A.Z was strongly acetylation-dependent (Fig. 3.5A). For acetylated positions on histone H4's N-terminal tail Bdf1 lacks a preferred acetylated lysine, directly interacting the most tightly with the tetra-acetylated N-terminal tail of H4 (Ladurner et al. 2003; Hassan et al. 2006). Likewise, Bdf1's association with H2A.Z *in vivo* was not dependent on a single acetylation site (Fig. 3.5B). Further, if the function of H2A.Z acetylation were largely to recruit Bdf1 or similar factors, the apparently additive binding of Bdf1 is consistent with the additive nature of the transcriptional defects observed for *htz1* acetylation mutants.

Due to the collaboration of Yta7 and H2A.Z at telomere-proximal regions and more broadly, as discussed in chapter 2, but limited evidence for functional overlap with H2A.Z acetylation, we tested whether Yta7's *in vivo* interaction with H2A.Z exhibited any acetylation dependence. Yta7 co-immunoprecipitated with H2A.Z from wild-type cells, but from cells that could not acetylate H2A.Z, Yta7's association with H2A.Z was reduced (Fig. 3.5C). However, Yta7's interaction with histone H4 appeared to exhibit a greater acetylation dependence than with H2A.Z. Thus, Yta7 function may combine the inputs of both H4 and H2A.Z acetylation.

V. Discussion:

Distinctions between the functional requirements for H2A.Z and its acetylation

Transcriptome analysis of cells able to acetylate H2A.Z indicated that H2A.Z acetylation was not as broadly required as H2A.Z itself. As aforementioned, this more restricted phenotype was anticipated based on the lack of any observable growth phenotype of the *htz1UnAc* mutant compared to the notably reduced vigor of the *htz1Δ* mutant. One caveat to concluding that H2A.Z has functional significance independent of its acetylation is the recent determination that the *htz1Δ* mutant exhibits phenotypes due both to the loss of H2A.Z and to problematic Swr1 activity in the absence of H2A.Z (Halley et al. 2010; Morillo-Huesca et al. 2010). Specifically, it is thought that the SWR1 complex continues to remove H2A/H2B dimers from nucleosomes in the *htz1Δ* mutant, although unable to replace that dimer with a H2A.Z/H2B dimer. Thus, the

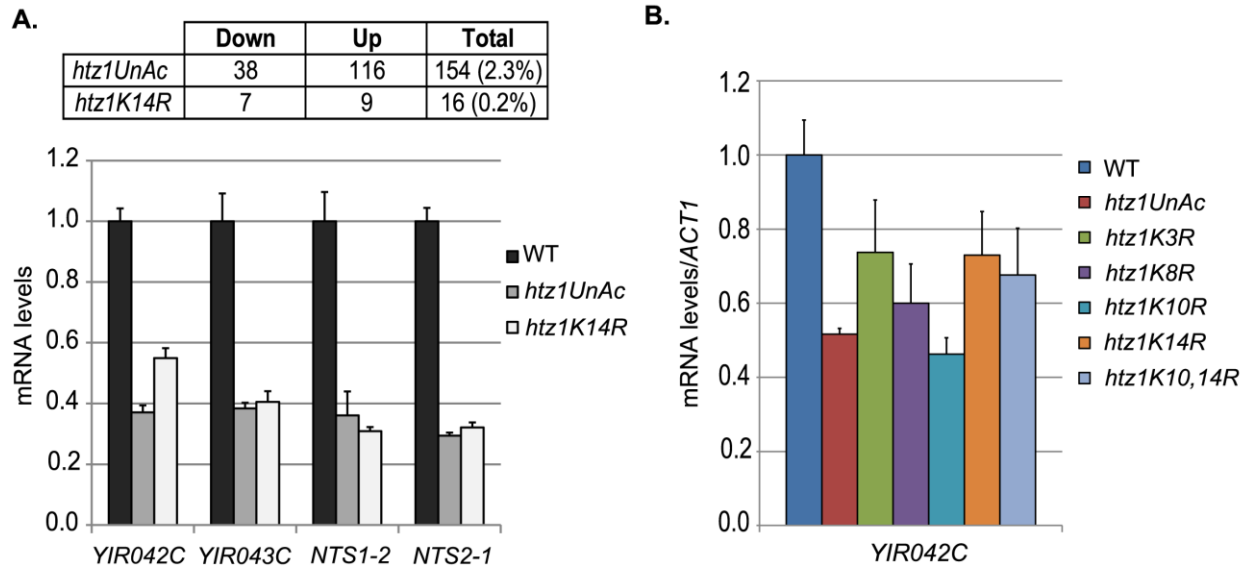


Figure 3.4. H2A.Z K14 acetylation was largely dispensable.

A) Table listing the number of significantly affected genes in the *htz1K14R* mutant compared to the *htz1UnAc* (*htz1K3,8,10,14R*) mutant (Significance Analysis of Microarrays (SAM), 1.5 median false gene cut-off). mRNA levels of the indicated loci, as determined by four independent microarray experiments. *NTS1-2* and *NTS2-1* are present in each rDNA repeat. NTS stands for "non-transcribed spacer." The wild-type level of each transcript was set equal to 1. B) qRT-PCR analysis of *htz1* single acetylation mutants. *YIR042C* transcripts were normalized over *ACT1* transcript levels and the wild-type ratio set equal to 1. The data presented are the average of two biological replicates.

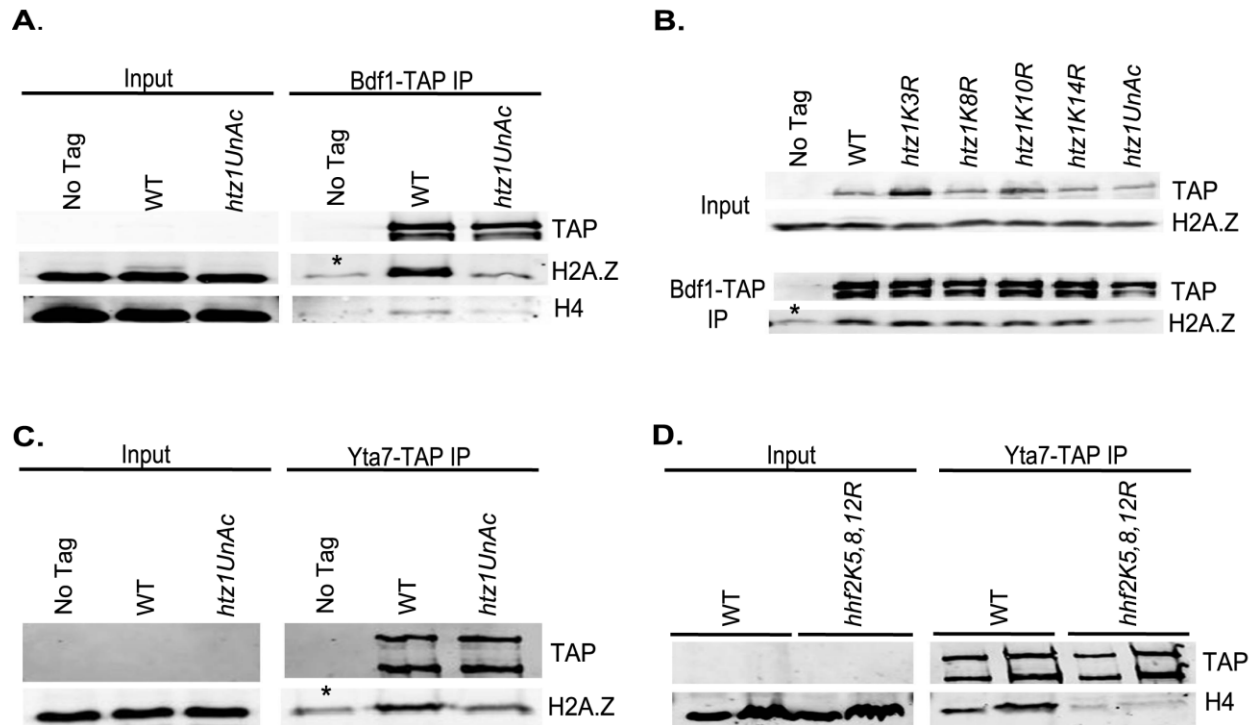


Figure 3.5. Bdf1 interacted with H2A.Z *in vivo* in an acetylation-dependent manner.

A) Immunoprecipitation of Bdf1-TAP in strains harboring *HTZ1-FLAG* or *htz1UnAc-FLAG*. Co-immunoprecipitating H2A.Z and H4 were detected with antibodies against FLAG and histone H4. *As can be observed in this and subsequent immunoblots in the "No Tag" lane, H2A.Z-FLAG exhibited non-specific interaction with the resin used for the anti-TAP immunoprecipitation. B) Immunoprecipitation of Bdf1-TAP as above in strains containing only *htz1UnAc-FLAG* or single *htz1* acetylation mutants. C) Immunoprecipitation of Yta7-TAP in strains harboring *HTZ1-FLAG* or *htz1UnAc-FLAG*. D) Yta7-TAP was immunoprecipitated from strains containing wild-type histone H4 or only H4 that could not be acetylated at lysines 5, 8, and 12 (*hhf2K5,8,12R*). For each strain, two different sample volumes were loaded.

htz1Δ swr1Δ double mutant is less sensitive to genotoxic drugs than the *htz1Δ* mutant (Halley et al. 2010; Morillo-Huesca et al. 2010). However, the *swr1Δ* mutant itself exhibits slow growth and drug sensitivities (Kobor et al. 2004; Mizuguchi et al. 2004; Halley et al. 2010), unlike the *htz1UnAc* mutant (Babiarz et al. 2006; Millar et al. 2006), indicating acetylation-independent roles for H2A.Z. Thus, the ideal strain to compare to the *htz1UnAc* mutant would have been an *htz1Δ swr1Δ* mutant, which lacks H2A.Z but does not exhibit any transcriptional artifacts due to problematic SWR1 complex activity. Determination of the transcriptional differences between the *htz1Δ swr1Δ* and *htz1UnAc* mutants would have allowed the separation of acetylation-dependent and -independent functions of H2A.Z.

The *swr1Δ* and *htz1Δ swr1Δ* mutants exhibit ~50 -80% the number of down-regulated genes as the *htz1Δ* mutant, but still display a bias toward down-regulation (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004; Morillo-Huesca et al. 2010). Thus, the 3-fold bias toward up-regulation in the *htz1UnAc* mutant was surprising, especially given the enrichment of H2A.Z K14-Ac at highly transcribed genes (Millar et al. 2006) and the requirement for H2A.Z acetylation for induction of *GAL* and oleate-responsive genes (Wan et al. 2009; Halley et al. 2010). Hence, the second caveat to extrapolating the function of H2A.Z acetylation from the quadruple acetylation mutant is that the function of one acetylation could mask the function of another. For example, one site could facilitate transcriptional repression, although there exists little evidence for this mechanism of gene repression. The possibility of aneuploidy was also considered, but the up-regulated genes in the *htz1UnAc* mutant were not clustered, eliminating that possibility. Given the role of H2A.Z acetylation in gene induction, it is also possible that steady-state measurements of transcripts from cells grown in rich medium are insensitive to a more extensive role of H2A.Z acetylation in transcriptional activation.

H2A.Z acetylation was broadly required for telomere-adjacent gene expression

Genes that were significantly down-regulated in the *htz1UnAc* mutant were enriched (25%) for telomere-adjacent genes, consistent with the Sir-mediated repression that occurs in the *htz1UnAc* mutant at telomere IXR (Babiarz et al. 2006). All these genes fell within 10 kb of the most internal X-core element of the telomere, indicating short-range effects. Thus, this analysis expanded the number of telomere-adjacent regions which required H2A.Z acetylation for proper expression from one to nine out of the 32 telomeres in *S. cerevisiae*: IR, IVL, VR, VIIR, IXR, XIIL, XIIIR, XVL, and XVII. As this analysis was not performed in parallel with an *htz1UnAc sir2Δ* double mutant, we could not conclude whether this down-regulation was due to Sir-dependent silencing, but our data were consistent with this model. Given the enrichment of H2A.Z throughout the telomeric repeats, X-core elements, and Y' elements of the telomeric region (Albert et al. 2007), it is possible that structural perturbations of the telomeric region occur in the *htz1UnAc* and *htz1Δ* mutants, resulting in increased recombination or telomeric shortening. Due to their repetitive nature, telomeric regions are inherently recombinogenic (Louis et al. 1994). Modification of Sir complex recruitment sites or telomeric shortening could potentially alter the domain of Sir silencing.

The possibility of Sir-mediated repression is also relevant to the enrichment of telomere-adjacent genes showing down-regulation in the *yta7Δ* mutant, given Sir silencing of the genes adjacent to *HMR* in the *yta7Δ* mutant (Jambunathan et al. 2005; Tackett et al. 2005; Zhou et al.

2009). As discussed in chapter 2, however, telomere-proximal gene expression is highly sensitive to histone H3 and H4 dosage (Wyrick et al. 1999; Singh et al. 2010), well beyond the range of Sir complex activity (Wyrick et al. 1999). As the effective dose of H3 and H4 is increased in the *yta7Δ* mutant, the observed down-regulation of telomere-proximal genes in cells without Yta7 might reflect the impact of higher nucleosome density. According to the seminal H2A.Z work in budding yeast, Meneghini et al., 2003, there are 18 clusters of 3-4 genes that require H2A.Z for expression, termed H2A.Z activated domains (HZADs). 15 of the 18 HZADs are within 30 kb of the telomere. In total, the HZADs consist of 69 genes, 46% of which are subject to Sir-dependent silencing in the absence of H2A.Z (Meneghini et al. 2003). 25% genes in HZADs are also subject to repression by the histone deacetylase Hda1, which maintains large domains of repression 5 – 20 kb from the telomeric X-core element (Robyr et al. 2002). Interestingly, 16 of the genes down-regulated in the *yta7Δ* mutant, are within HZADs ($p = 1 \times 10^{-13}$, hypergeometric distribution). ~30% of these genes are subject to Sir silencing, according to Meneghini et al., 2003, indicating a slight bias toward Sir-independent transcriptional defects in the *yta7Δ* mutant. Instead, 45% of the genes within HZADs that are down-regulated in the *yta7Δ* mutant are genes that are regulated by Hda1, indicating a ~2-fold enrichment. Thus, I posit a model in which increased nucleosome density in the *yta7Δ* mutant facilitates repression of different types depending on the telomere-proximal region, Sir-mediated repression closest to the telomere and Hda1-mediated repression more internally.

Although the mechanism of down-regulation of telomere-proximal genes in the *htz1UnAc*, *htz1Δ*, *yta7Δ* mutants is unclear from this study, it is clear that distinct classes of regulation existed for H2A.Z-activated genes. As expected if H2A.Z acetylation were crucial to telomere-proximal gene expression, there were genes which exhibited equal sensitivity to the loss of H2A.Z and its acetylation. However, there were also genes which required both H2A.Z acetylation and Yta7, although this overlap was limited both at telomere-proximal genes and genome-wide. Further, there were telomere-proximal genes whose expression appeared independent of H2A.Z acetylation, but dependent on Yta7 and H2A.Z. For this last class, it is formally possible that microarrays were not sensitive enough to detect an acetylation-dependent defect. However, independent qRT-PCR analysis indicated no or only a slight requirement for H2A.Z acetylation at a sampling of these genes (data not shown), confirming a quantitative difference between the loss of Yta7 or H2A.Z and the loss of H2A.Z acetylation.

Evidence for distinct telomere-proximal domains of regulation

A surprising discovery of this study was the enrichment of telomere-proximal genes in the set of significantly up-regulated genes in the *htz1UnAc* mutant. Specifically, cells lacking H2A.Z acetylation exhibited down-regulation of genes within 10 kb from the telomere, but up-regulation of genes 10 - 20 kb from the telomere. Strikingly, analysis indicated that up-regulated genes in the *htz1UnAc* mutant included nine genes from telomere-proximal HZADs ($p = 2 \times 10^{-6}$, hypergeometric distribution). This represents a significant enrichment compared to HZAD genes present in the up-regulated gene set of the *htz1Δ* mutant ($p = 0.08$, hypergeometric distribution). However, the *htz1Δ* mutant also exhibited a significant amount of up-regulated genes 10 – 20 kb from the telomere ($p = 0.007$, chi square). These data suggested a model in which telomere-proximal regions are divided by structural or regulatory features that are modified by H2A.Z and its acetylation.

Lack of acetylation-site specificity

Strikingly, preventing the acetylation of K14, the mark present on ~40% of H2A.Z molecules (Millar et al. 2006), resulted in the misregulation of only a handful of genes, one-tenth of those misregulated in the *htz1UnAc* mutant. Genes adjacent to telomere IXR did display significant down-regulation in the *htz1K14R* mutant, equivalent to that of the *htz1UnAc* mutant. Thus, although H2A.Z K14-Ac is enriched at highly transcribed genes (Millar et al. 2006), there was no indication of a functional consequence of the loss of this acetylation site at highly transcribed genes. The down-regulation of telomere-adjacent genes in this mutant was also surprising, given the depletion of this modification within 50kb of the telomere (Millar et al. 2006). The enrichment of H2A.Z K14-Ac at *YIR042C* and *YIR043C* falls in the bottom ~20% and ~10%, respectively, of intergenic levels (Millar et al. 2006). At face value, this would suggest an indirect mechanism of telomere-adjacent gene protection by H2A.Z K14-Ac, but it is formally possible that this modification exerts greater effects at low abundance in telomere-adjacent regions, potentially through the recognition by a telomere-specific factor.

That the *htz1K14R* mutant exhibited only one-tenth of the number of significantly affected genes as the *htz1UnAc* mutant suggested either that the most abundant modification was not the most important or that the function of H2A.Z K14-Ac could be substituted by acetylation of the other three sites. Effects of the other single acetylation mutants on *YIR042C* expression indicated a partial defect with respect to the *htz1UnAc* mutant, with the exception of the *htz1K10R* mutant. As this experiment was only performed in duplicate at one gene, the significance of the *htz1K10R* mutant result is unclear. However, taken together with the lack of a preferred H2A.Z site of acetylation by Bdf1, my data are consistent with functional equivalence of the modifications and a dependency on the number of acetylations, rather than the nature of those acetylations. This additive contribution of acetylation is the case for the first three acetylation sites, K5, K8, and K12R, on the H4 N-terminal tail in *S. cerevisiae* (Dion et al. 2005). Indeed, a recent genetic interactions study of the single *htz1* acetylation mutants indicated no distinguishing features between the mutants (Mehta et al. 2010). Analysis of *GALI* induction, which is defective in the *htz1UnAc* mutant, by myself and others also failed to distinguish between the acetylation sites, supporting a quantitative contribution by each of H2A.Z's acetylations (data not shown, (Halley et al. 2010).

Evidence for interpretation of H2A.Z acetylation by Bdf1

Bdf1's strong acetylation-dependent interaction with H2A.Z *in vivo* was surprising, given that yeast cells lacking both Bdf1 and H2A.Z acetylation are dead (personal communication--Josh Babiarz). The simplest interpretation of this genetic result, given that Bdf1 directly interacts with acetylated H4 (Ladurner et al. 2003; Hassan et al. 2006) and that yeast cells lacking both H4 acetylation and H2A.Z acetylation are inviable (Babiarz et al. 2006), is that Bdf1 is responsible for interpreting H4 acetylation, while H2A.Z acetylation exerts an independent function. However, my work suggests this model is overly simplistic. In fact, when it comes to the capacity of bromodomain-containing proteins, new discoveries are still being made. Bdf1 contains two different bromodomains, both of which are capable of directly interacting with acetylated H4, each exhibiting a preference for tetra-acetylated H4 (Hassan et al. 2006). Preference of a single bromodomain for a tetra-acetylated substrate over any singly acetylated

peptide suggested the capacity of a single bromodomain to bind multiple acetyl-lysine moieties in a single binding pocket. Bdf1 is a member of the BET family of bromodomain-containing proteins, all of which contain two tandem bromodomains. It was recently discovered for the BET family member Brdt in mice that a single bromodomain cooperatively binds two adjacent acetyl-lysines on one H4 tail (Moriniere et al. 2009). This discovery suggests that the double bromodomains of Bdf1 may bind four different acetyl-lysine moieties and helps to explain the perceived promiscuity of bromodomain binding.

Thus, I propose a model in which Bdf1 can interpret both H4 and H2A.Z acetylations. Direct binding studies and functional analysis will have to be performed to determine if this is the case, but the ability to recognize both H4 and H2A.Z acetylation appears consistent with the observed acetylation dependence of Yta7's interaction with both H4 and H2A.Z. Further, Bdf1's interaction with acetylated H2A.Z is consistent with the requirement for Bdf1 to prevent Sir-complex spreading in from the telomeres (Ladurner et al. 2003). Additionally, in yeast Bdf1 forms a stable dinucleosome complex, a single molecule simultaneously binding the +1 and +2 nucleosomes of actively transcribed genes (Koerber et al. 2009), potentially allowing recognition of two independent histone tails. Further, as Bdf1 is a substoichiometric member of the SWR1 complex (Krogan et al. 2003; Kobor et al. 2004), interpretation of H2A.Z acetylation by Bdf1 raises the interesting possibility of a feed-forward mechanism of maintaining acetylated H2A.Z at highly transcribed genes.

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