The role of the histone variant H2A.Z/Htz1p on TBP recruitment, chromatin
dynamics and regulated expression at oleate-responsive genes

Yakun Wan,1 Ramsey Saleem,1 Alexander Ratushny,1 Oriol Roda,1
Jennifer Smith,1 Chan-Hsien Lin,1,2 Jung-Hsien Chiang,1,2 John Aitchison1*

1. Institute for Systems Biology, Seattle, WA 98103
2. Department of Computer Science and Information Engineering,
National Cheng Kung University, Tainan, Taiwan

*Corresponding author
Institute for Systems Biology
1441 N 34th St. Seattle, WA 98103
Phone: (206) 732-1344
Fax: (206) 299-6574
E-mail: jaitchison@systemsbiology.org

Word count for Materials and Methods: 874 words
Word count for the Introduction, Results and Discussion: 4324 words
Running Title: Control of gene induction by Htz1p
Abstract

The histone variant H2A.Z (Htz1p) has been implicated in transcriptional regulation in numerous organisms, including *Saccharomyces cerevisiae*. Genome-wide transcriptome profiling and chromatin immunoprecipitation studies identified a role for Htz1p in the rapid and robust activation of many oleate-responsive genes encoding peroxisomal proteins, and in particular, *POT1, POX1, FOX2* and *CTA1*. Swr1p, Gcn5p and Chz1p dependent association of Htz1p into these promoters in their repressed states appears to establish an epigenetic marker for rapid and strong expression of these highly inducible promoters. Isw2p also plays a role in establishing the nucleosome state of these promoters, and associates stably in the absence of Htz1p. Analysis of the nucleosome dynamics and Htz1p association with these promoters suggests a complex mechanism in which Htz1p-containing nucleosomes at fatty acid-responsive promoters are disassembled upon initial exposure to oleic acid leading to the loss of Htz1p from the promoter. These nucleosomes reassemble at later stages of gene expression. While these new nucleosomes do not incorporate Htz1p, the initial presence of Htz1p appears to mark the promoter for sustained gene expression and the recruitment of TBP.
Introduction

The organization of DNA into chromatin provides cells with a key regulatory mechanism for gene expression by limiting access of the genome to the transcriptional machinery. The nucleosome represents a basic structural unit of chromatin and post-translational modifications of histones serve as signals to define active, repressed or inert chromatin states. In addition, chromatin states and gene expression can be influenced by the dynamics of histones and their nonallelic variants. Indeed, exchange of canonical histones for histone variants appears to be a key mechanism by which the transcriptional machinery overcomes the restricted access imposed by nucleosome positioning (1). Of the many classes of histone variants discovered, the Z variant of H2A is perhaps the best characterized. H2A.Z differs from the canonical H2A histone in both the length and sequence of the C-terminus (37), and is conserved from yeast to mammals (15). Early studies with H2A.Z in Tetrahymena showed that H2A.Z incorporation is linked with transcriptionally active chromatin (35). The Saccharomyces cerevisiae orthologue of H2A.Z is called Htz1p and is encoded by HTZ1. Although HTZ1 is not essential gene under standard laboratory growth conditions, Htz1p is implicated in transcriptional regulation. Global chromatin studies have revealed that Htz1p preferentially associates with the two nucleosomes flanking the nucleosome free region of promoters (12, 18, 26, 41), and this association is inversely proportional to transcription rates (3, 18).

Studies of the role of Htz1p in transcriptional regulation at specific promoters, such as those of GAL1 and PHO5 (1, 30) indicate that the presence of Htz1p at promoters is dynamic; Htz1p is bound in their repressed states, but dissociates during the activation process. Accordingly, it is proposed that nucleosomes containing Htz1p are poised to undergo nucleosome displacement allowing for rapid transcriptional responses (41).

The yeast S. cerevisiae is an excellent model for understanding the mechanisms of cellular responses to induced perturbations. Upon exposure of yeast to fatty acids, such as oleate, cells respond by dramatically altering their gene expression patterns, inducing genes required for peroxisomal β-oxidation and peroxisome biogenesis (32). Genetic screens to identify proteins specifically required for efficient fatty acid metabolism in S. cerevisiae (34) identified metabolic enzymes, proteins required for biogenesis of the organelle, signaling proteins and transcriptional regulators, and chromatin modifiers.
Among this latter class of proteins, this approach identified genes encoding Htz1p, RNA Polymerase II, Mediator subunits and components of chromatin remodeling complexes. We thus seek to understand the nature of how chromatin is regulated and remodeled in response to exposure to fatty acids and the specific role Htz1p plays in these regulation/remodeling processes.

In this report, transcriptomes of WT and \textit{htz1}Δ strains were compared during exposure to oleic acid. While loss of Htz1p reduced the expression of many genes, genes involved in the fatty acid response were particularly sensitive. A model is proposed, in which Htz1p-containing nucleosomes at fatty acid-responsive promoters are disassembled upon initial exposure to oleic acid leading to the loss of Htz1p from the promoter. These nucleosomes reassemble, at later stages of gene expression. While these nucleosomes do not incorporate Htz1p, the initial presence of Htz1p appears to mark the promoter for sustained gene expression and the recruitment of TBP.
Materials and Methods

Strains and growth conditions.

All yeast strains used in this study are indicated in Table 1. Haploid strains with myc-tagged genes were made by genomically tagging target genes with the sequence encoding 13 copies of the c-myc epitope from pFA6a-13MYC (20) by homologous recombination into BY4742 (wild type) using a previously described PCR-based procedure (2). Strains were verified by PCR analysis of the tagged gene loci and Western blot analysis of the fusion proteins. Examination of growth characteristics of each strain suggests that the chimeras did not alter protein function. For all experiments, control strains were otherwise isogenic to test strains. Strains were cultured at 30°C in the following media: YPD (1% yeast extract, 2% peptone, 2% glucose), SCIM (0.17% yeast nitrogen base without amino acids and ammonium sulfate (YNB-aa-as), 0.5% yeast extract, 0.5% peptone, 0.079% complete supplement mixture, 0.5% ammonium sulfate) containing 0.5% Tween 40 (w/v) and 0.2% (w/v) oleate.

RNA preparation and microarray analysis.

Yeast cultures were grown at 30°C to a density of ~1 × 10^7 cells/ml. Cells were collected and immediately frozen in liquid nitrogen. Total RNA was isolated by hot acid phenol extraction. Total RNA was treated with RNase-free DNase I and purified with a Qiagen RNeasy kit. Microarray labeling and hybridization reactions were performed as previously described (7). Two color microarrays, comparing RNA from experimental conditions (wild-type (WT) and htz1Δ cells grown in oleate (SCIM) for 6 h) to RNA from control WT cells grown in glucose-containing medium (YPD), were performed using Agilent whole-genome *S. cerevisiae* arrays. All experiments were performed with duplicate experimental and duplicate technical replicates of each condition and the Log_{10} of the average mRNA abundance ratios are reported. Differentially expressed genes were identified by maximum-likelihood analysis (λ≥100) (14, 32) and significantly affected genes in the mutants were identified by a change in expression of two-fold or more compared to the expression in the relevant WT strains.
For quantitative reverse transcription-PCR, total RNA was directly reverse transcribed using the First Strand cDNA Synthesis Kit from Fermentas (Catalog: K1611). cDNAs were treated by RNase H and diluted 1:100 for quantitative PCR. RT-PCR was done using a 7900 HT Fast Real-time PCR systems and DyNAmo™ Flash SYBR Green qPCR Kit (NEB, F-415L) with gene-specific oligonucleotides. mRNA levels were normalized relative to ACT1 mRNA levels from three independent RT-PCR analyses. Primers for RT-qPCRs are available on request.

**ChIP and Real Time PCR.**

For each chromatin immunoprecipitation (ChIP) experiment, yeast strains were first grown in glucose medium (YPD) to a density of $\sim 1 \times 10^7$ cells/ml, and then transferred to oleate medium (SCIM) for indicated times. ChIP experiments were performed as described by (33) with the following modifications: For HA-Htz1p ChIP, cells were cross-linked with 1% formaldehyde for 45 min at room temperature. 2 µg of anti-HA antibody (12CA5) was prebound with to 50 µl of pan-mouse IgG Dynabeads (Dynal Biotech) and then incubated with 1mg (protein) of supernatant from the sheared chromatin overnight at 4°C. TBP (Spt15p-Myc) ChIP was performed as described by (33). Cells were cross-linked with 1% formaldehyde for 2 hours at room temperature. 2µl of anti-Myc antibody (9E11; Abcam) was pre-bound to 50 µl of pan-mouse IgG Dynabeads and then incubated with 1mg (protein) of supernatant from sheared chromatin overnight at 4°C.

All ChIP experiments were performed in triplicate. The purified ChIP samples were used in quantitative PCR (qPCR) analysis. Real-Time qPCR was performed by using an iCycler instrument (ABI 7900) and DyNAmo™ Flash SYBR Green qPCR Kit. The average of three independent replicates is reported as relative amplification of each target of interest compared to a normalization control amplicon, within the non-promoter IGRi YMR325W. Primer sequences are available on request. Occupancy level was determined by dividing the relative abundance of an experimental target by the relative abundance of a control target. This ratio represents the enrichment of ChIP DNA over the input DNA for a specific target versus the control target.
FACS analysis.

Procedures were performed as previously described (27). Fluorescence intensities of individual cells were measured using a FACS Calibur flow cytometer (BD Biosciences). Data analysis was performed using WinMDI 2.8 (available from http://FACS.scripps.edu/).

Nucleosome scanning assay (NuSA).

Two hundred ml of cells at OD$_{600}$ of 1.0 in either glucose or after transfer to oleate-containing media for the indicated time were treated with 1% formaldehyde for 20 min, followed by 5 min incubation in 125 mM glycine. Cell permeabilization, micrococcal nuclease digestion, protein degradation and DNA purification steps were performed as described in (38). DNA samples were then treated with RNase A and analyzed in a 2% agarose gel to quantify nucleosomal content. The bands corresponding to mononucleosomal DNA were extracted using a Qiagen gel extraction kit. Q-PCR analysis on digested DNA was performed. Q-PCR primers are available on request and cover the promoter regions of POT1, CTA1, POX1 and FOX2 with overlapping amplicons averaging 100 bp in size. To define nucleosome occupancy, the protection value of each amplicon was normalized to CEN3 values as described (6). The N+1 nucleosome refers to the first nucleosome downstream of the transcription start site which is located at open reading frame regions. The N-1 nucleosome refers to the first nucleosome upstream of the transcription start site which is located at promoter regions.
Results

Htz1p is required for transcriptional activation of a subset of oleic acid-responsive genes.

Transcriptome profiling was used to obtain a global understanding of how Htz1p contributes to gene expression in response to external stimuli. To do so, we focused on gene induction upon shift from glucose to oleic acid growth conditions. This condition was chosen because we and others have previously shown that this transition leads to dramatic alterations in gene expression patterns (16, 32, 33), genes involved in fatty acid metabolism are significantly induced under these conditions, and because it has been shown that \textit{S. cerevisiae}\textit{htz1}\text{\textDelta} strains have a specific growth defect when grown on fatty acids (19, 34). In accordance with previous genome-wide analyses of oleate responses (16, 32, 33), a large portion of the genome responds to the transition (Fig 1A; column 1). Reflecting the non-fermentative metabolism of oleate by the coordinated activities of peroxisomes and mitochondria, the most significantly enriched classes of induced genes include genes linked to mitochondrial respiration and peroxisomal lipid metabolism (GO terms oxidative phosphorylation, electron transport chain and aerobic respiration, hypergeometric \(p<10^{-10}\); components of the mitochondrial respiratory chain - \(p<10^{-11}\); fatty acid oxidation and peroxisome organization and biogenesis related - \(p<10^{-6}\), and the peroxisomal compartment - \(p<10^{-12}\)). By comparison, there were many genes that were relatively unresponsive in \textit{htz1}\text{\textDelta} cells (Fig 1A, column 2; Fig 1B). This included genes that were both poorly induced and genes that were poorly repressed in \textit{htz1}\text{\textDelta} cells compared to WT (Fig 1A). Among the genes induced upon oleate exposure, 292 were expressed at least two-fold less in \textit{htz1}\text{\textDelta} cells than in WT cells (Fig 1B). Interestingly, these poorly induced genes were most enriched for those annotated with peroxisomal functions and components; but were not enriched for annotations of mitochondrial components or aspects of mitochondrial respiration (fatty acid and lipid oxidation - \(p\approx4.0\times10^{-12}\); and peroxisomes - \(p\approx9\times10^{-20}\)) (Fig. 1C). Indeed, 26 genes (of 57 total) encoding peroxisomal proteins showed significantly reduced transcription in an \textit{htz1}\text{\textDelta} background (Fig. 1C). These data suggest that Htz1p is required for the regulated expression of a large number of genes upon transition from one state to another. In the
case of transition to oleate, genes linked to peroxisomal fatty acid oxidation are normally highly induced and their expression is the most significantly affected in the absence Htz1p.

**HTZ1 is required for normal peroxisomal beta-oxidation.**

The finding that normally highly induced genes linked to fatty-acid oxidation are poorly expressed in *htz1Δ* cells is consistent with the finding that cells lacking *HTZ1* show a specific impairment of fatty acid metabolism (19, 34). Like mutants defective in peroxisomal function (e.g. *pex3Δ*), *htz1Δ* cells exhibit a growth defect on fatty acid-containing medium (YPBO), but not on glucose (YPD) containing media (Fig 2A), nor on other non-fermentable carbon sources such as glycerol (YPG) or acetate (YPA) requiring mitochondrial function (34). As expected, the WT cells grew normally on different carbon sources.

To examine the effect of Htz1p on the organelle itself, we examined peroxisomes by fluorescence microscopy. WT and *htz1Δ* cells expressing peroxisomal thiolase Pot1p, tagged by genomic integration with GFP, were incubated in oleate medium and observed over a time course of induction by direct fluorescence confocal microscopy (Fig. 2B). In glucose-containing medium peroxisomes were barely detectable. However, upon shift to oleic acid, WT cells induced the expression and import of Pot1p-GFP as indicated by the accumulation of punctate fluorescent structures (29). However, there was a dramatic delay in the appearance of punctate GFP fluorescence in *htz1Δ* cells compared with WT cells induced over the same time period. Together these data suggest that peroxisome biogenesis per se is not defective in *htz1Δ* cells. Rather the defect in the ability to metabolize oleate effectively is a result of relatively poor expression of genes required for (peroxisomal) fatty acid metabolism.

**Transcriptional response of POT1, POX1, FOX2 and CTA1.**

To further examine the molecular defects associated with the loss of Htz1p, we focused on 4 strongly induced peroxisomal matrix enzymes, encoded by *POT1, FOX2, POX1* and *CTA1* (Fig. 3A, red). These genes are normally repressed on glucose, and strongly induced on oleic acid (32). Quantitative RT-PCR of these mRNAs demonstrated
that in the absence of Htz1p, each of these genes was repressed as in WT cells, but their induction was impaired upon transition to oleate medium (Fig. 3A). Interestingly, the expression of each of these genes appeared to be most significantly affected at the later time points after transition to oleate (compare 4 and 6 hours of induction to 0.5 and 1 hour of induction). These data suggest that loss of Htz1p did not dramatically alter the initial response, but was important for the sustained expression of these four genes.

Having demonstrated a role for Htz1p in the normal regulation of PON1, FOX2, POX1 and CTA1 expression in the presence of oleic acid, we next sought to determine if Htz1p binds the cognate promoters of these genes using chromatin immunoprecipitation (ChIP) of a strain expressing an HA-tagged version of Htz1p. Cells were grown in either repressed (glucose) or activated (oleate) conditions. Htz1p-HA was immunoprecipitated with anti-HA antibody and isolated DNA was analyzed by PCR. This analysis revealed that Htz1p was bound to each of the four promoters (POT1, FOX2, POX1 and CTA1) in their repressed states (Fig. 3B). These data are consistent with genome wide characterization of levels of Htz1p association with these promoters (41). The association of Htz1p with these promoters was dynamic; when cells were shifted to oleic acid activating conditions, Htz1p levels on the POT1, POX1, and FOX2 promoters were dramatically reduced. Dissociation from the CTA1 promoter was not observed. These data suggest that loss of Htz1p from promoters is coincident with gene activation, but that dissociation is not required for the induction of all genes.

Swr1p, Chz1p and Gcn5p - dependent association of Htz1p to promoters.

Swr1p, Chz1p and Gcn5p have been implicated in modulating Htz1p association at promoter regions. Swr1p is part of the SWR1-C multisubunit protein complex, necessary for Htz1p deposition at repressed promoters (24). Chz1p, was recently identified as a histone chaperone that preferentially interacts with Htz1p (21) and Gcn5p is the histone acetyltransferase subunit of the SAGA complex (36). To investigate whether these factors affect Htz1p binding to the oleate-responsive promoters and subsequent expression, the association of Htz1p with POT1, POX1, FOX2 and CTA1 promoters was investigated in cells lacking these proteins under conditions of repression (2% glucose) and expression of these genes was monitored upon oleate induction (Fig.
4. Similar to its role at the well-studied GAL1 promoter, Swr1p is required for Htz1p binding to oleate responsive promoters suggesting a common role for Swr1p at disparate, highly inducible promoters. Likewise, Gcn5p was required for efficient Htz1p binding. This suggests that Gcn5p, which plays a role as a coactivator of transcription through histone acetylation (11), controls the binding or stability of Htz1p at repressed promoters. This may also be via histone acetylation. In the absence of Chz1p, Htz1p occupancy at each of the four promoters was was decreased. As expected the amount of Htz1p on each of these promoters in mutant strains remained low upon switch to oleate (data not shown).

Microarray analyses in gcn5Δ, swr1Δ, and chz1Δ mutants support a model in which initial Htz1p association with the promoter is required for subsequent full induction. The expression levels of POT1, FOX2, POX1 and CT1 were significantly reduced in mutant strains compared to WT upon oleate induction. Moreover, all 26 genes encoding peroxisomal proteins that showed transcriptional defects in htz1Δ cells (Fig. 1C) were similarly reduced in their expression at least two-fold in gcn5Δ, swr1Δ, and chz1Δ mutants compared to WT (Fig. 4B). Together, these data suggest that factors functionally associated with Htz1p, such as the chromatin remodeling component Swr1p, histone acetyltransferase Gcn5p and chaperone Chz1p, regulate the deposition or maintenance of Htz1p at repressed promoters, which in turn, facilitates rapid activation of transcription.

Acetylation of Htz1p is required for efficient transcriptional induction.

Acetylation of Htz1p is known to occur at sites of active transcription (23). The finding that Gcn5p is required for expression of oleate responsive genes suggests that acetylation on Htz1p is required for the oleate response. To address this question, plasmids expressing either one of two acetylation mutants of Htz1p (pCM314 (Htz1p-K14A) or pCM330 (Htz1p-K14R)) were introduced into htz1Δ cells and expression was monitored by FACS, confocal microscopy and quantitative RT-PCR. FACS and confocal microscopy demonstrated that Pot1p-GFP fluorescence in cells carrying pCM305 (WT HTZ1) was stronger than that in those cells carrying empty plasmid (pRS416), or acetylation mutants (pCM330, or pCM314) during a time course of oleate incubation but
the peroxisomes were morphologically normal (data not shown). mRNA levels of \textit{POT1}, \textit{CTA1}, \textit{FOX2} and \textit{POX1}, determined by quantitative RT-PCR were consistent with the GFP reporter analysis (Fig 5A). The K14A acetylation mutant of Htz1p showed a defect in the normal induction of \textit{POT1}, \textit{CTA1}, \textit{FOX2} and \textit{POX1}. In addition, cells expressing Htz1p K14A also exhibited a growth defect on fatty-acid containing media, but not on glucose containing media. This growth defect was less pronounced than the null mutant of \textit{HTZ1} (data not shown). The association of Htz1p-K14A with these oleate responsive promoters at two time points (0 h and 6 h), also revealed that Htz1p-K14A association was diminished under glucose conditions (Fig. 5B). Significant differences in association of Htz1p-K14R on these promoters were not observed during 6 h of oleate induction. These data indicate that acetylation of Htz1p is required for association with oleate responsive promoters during repressed conditions and the acetylation of Htz1p is not required for the dissociation of Htz1p from oleate responsive promoters during oleate induction (Fig. 5B). These data collectively indicate that acetylation of Htz1p is important for expression of fatty acid responsive genes and normal peroxisomal matrix protein assembly.

**TBP is not efficiently recruited to oleate inducible promoters in the absence of Htz1p.**

We next directly analyzed \textit{in vivo} binding of the transcriptional machinery to repressed and activated promoters in both WT and \textit{htz1}Δ strains (Fig. 6A). As expected, the binding of TBP to the \textit{POT1}, \textit{POX1}, and \textit{CTA1} promoters increased with gene expression in oleate in WT cells. The abundance of TBP did not significantly increase on the \textit{FOX2} promoter following oleic acid induction but was present at higher initial levels than the other three other promoters studied. Nonetheless at all four promoters in \textit{htz1}Δ cells, TBP binding was significantly reduced compared to WT cells. The reduced levels of TBP binding were not attributable to decreased cellular levels of TBP. Western blot analysis of both WT and \textit{htz1}Δ cells demonstrated that TBP levels were equivalent between the strains and did not significantly change during oleate induction (Fig 5B). These results suggest a positive function for the Htz1p-containing nucleosomes in the
recruitment of TBP to the repressed promoters during the process of transcriptional activation.

Htz1 regulates nucleosome-promoter association during activation.

A nucleosome scanning assay (NuSA) was used to investigate the role of Htz1p in modulating chromatin structure by measuring nucleosome occupancy and location within oleate responsive promoters during activation (POT1, POX1, FOX2 and CTA1) (Fig. 7). Mononucleosome-associated DNA was isolated from yeast cells before and after oleate induction and quantitative real time PCR (qPCR) was used to measure dynamic nucleosome occupancy during activation in WT and htz1Δ cells. The precise positions of the nucleosomes were determined by qPCR corresponding to their known positions (17). Overall the gross nucleosome position pattern at each of the four promoters under repressed conditions was the same in WT and htz1Δ cells. The major nucleosome changes were observed at position N-1 in each promoter. These nucleosomes appeared to begin disassembly from each promoter at the earliest time point measured (5 min) and continued through to the 30 min time point. After this initial disassembly, nucleosomes were detected to have begun reassembly after 1 h of induction (Fig. 7). These reassembled nucleosomes likely do not contain Htz1p. As shown in Fig. 3B, Htz1p is progressively lost from these promoters during the 6 h period of induction. Notably, the nucleosomes of each promoter appeared to be more protected at the later time points (6 h) in htz1Δ cells compared to WT. This was most evident at the N-1 position of the promoters of POX1 and CTA1 (and at the N-2 position of POX1). These data suggest that upon oleate treatment the nucleosome proximal to initiation site in each promoter disassembles leading to Htz1p loss and initial transcriptional activation. During prolonged expression, nucleosomes reassemble, but these reassembled nucleosomes do not contain Htz1p.

Interplay between Htz1p and chromatin remodeling factor Isw2p.

While Htz1p is proposed to contribute to nucleosome disassembly during induction, the results presented above indicate that the overall chromatin structure at the promoters was not extensively perturbed in htz1Δ cells. To gain insight into the potential
additional mechanisms at play during the transcriptional induction, we considered additional chromatin bound proteins. One such protein is Isw2p. Isw2p is an ATP-dependent chromatin remodeling factor that has previously been shown to be required for maintenance of chromatin structure at the \textit{POT1} promoter (8, 9, 39). We therefore investigated Isw2p function at the \textit{POT1}, \textit{POXI}, \textit{FOX2} and \textit{CTA1} promoters in WT and \textit{htz1Δ} cells.

Nucleosome protection assays in \textit{isw2Δ} cells led to significant changes in the nucleosome structure in all four promoters (Fig 8A). These data indicate that Isw2p plays a role in chromatin structure of these four promoters and are consistent with previous work on the \textit{POT1} promoter (8, 9, 39).

Next we used ChIP to assay the ability of Isw2p to associate with the four oleate responsive promoters. Previous work has shown that in WT cells Isw2p does not stably associate with these promoters, which suggests that under normal conditions, Isw2p contributes to the nucleosome structure at these Htz1p-containing \textit{POT1}, \textit{POXI}, \textit{FOX2} and \textit{CTA1} promoters through transient interactions (9). Similarly, we found very low levels of Isw2p association with these promoters in WT cells, in glucose and after oleate induction. However, substantial amounts of Isw2p were observed in association with each of these promoters in the \textit{htz1Δ} cells. Isw2p remained associated with these promoters during their activation, suggesting a role in establishing chromatin dynamics in the absence of Htz1p (Fig. 8B).
Discussion

Exposure of yeast cells to oleate results in large scale reorganization of gene expression regulatory networks and provides an excellent experimental system for understanding the mechanisms of gene expression at both the molecular and network levels (28, 33). The resulting changes in gene expression are widespread, representing the reorganization of regulatory networks governing numerous categories of gene function. For example genes involved in protein translation and glycolysis are repressed, reflecting the shift in growth rates and metabolism (16, 32, 33). Likewise, genes linked to mitochondrial respiration and peroxisomal fatty acid metabolism is induced, reflecting the cells shift to non-fermentative β-oxidation as an energy source (16, 32, 33). The ability of yeast cells to adapt to this shift is dependent on the HTZ1 gene encoding the histone variant Htz1p/H2A.Z (19, 32). The data presented here demonstrate that Htz1p plays a critical role in this transition by contributing to the recruitment of TBP to oleate responsive genes leading to rapid and robust expression of highly inducible genes.

Transcriptome profiling studies presented here demonstrate that expression of genes that are normally highly responsive to oleate is impaired in the absence of Htz1p. Because, under these conditions, many of the most strongly induced genes are required for peroxisomal β-oxidation and peroxisome proliferation, lack of Htz1p renders cells unable to respond efficiently to the transition and metabolize the fatty acids.

In order to elucidate the step-wise molecular function of Htz1p in the transcriptional regulation of these genes, we generated and compared various time course datasets to analyze chromatin states before and after the switch to oleic acid. We used chromatin immunoprecipitations to assay the dynamic association of Htz1p at the promoters of four model genes (POT1, POX1, FOX2, and CTA1) encoding peroxisomal matrix enzymes, the expression of which was perturbed by deletions of HTZ1. Htz1p has been proposed to preferentially bind repressed promoters facilitating the rapid activation of the associated genes (41). Consistent with the current models, we demonstrate that Htz1p tends to be bound to these promoters in their repressed states (glucose) and disassociates from these promoters once the cells are exposed to oleate; however, this association and dissociation pattern occurs at levels that are promoter specific. The
methods employed here did not reveal significant dissociation of Htz1p from the CTA1 promoter. The data suggest that Htz1p levels on the CTA1 promoter are lower (~2-fold over control regions) than the other promoters examined. Thus, Htz1p does not appear to dissociate from the CTA1 promoter following oleic acid induction. The mechanisms underlying promoter specific effects of Htz1p and other epigenetic factors remain fertile ground for future study.

In addition, data presented here support previous studies in both yeast and mammalian cells that demonstrate that Htz1p is deposited at promoters by the chromatin remodeling protein Swr1p (24, 40). Similarly, as in other transcriptional responses (23), Gcn5p/Esa1p mediated acetylation at Lys14 of Htz1p is required for efficient transcriptional activation. Moreover, Gcn5p/Esa1p mediated acetylation at Lys14 of Htz1p is required for efficient association of Htz1p at some oleate responsive promoters. The significantly decreased association of the Htz1p-K14R mutant was observed under repressive conditions (i.e. glucose), and this decreased binding of Htz1p was also observed in cells lacking the enzyme (Gcn5p) responsible for Htz1p acetylation. Htz1p acetylation mutant cells also displayed defects in peroxisome proliferation and growth on oleic acid, similar to an HTZ1 null mutant. These data demonstrate that acetylation of Htz1p, mediated by Gcn5p, is required for association with oleate responsive promoters during repressed conditions and for normal transcriptional induction contributed by Htz1p.

Among the known effectors of Htz1p, Chz1p is relatively less well characterized. Luk et al (21) identified a role for Chz1p as a nuclear chaperone for Htz1p though the functional relationship between Chz1p and Htz1p with respect to transcriptional regulation remained uncharacterized. Here, we report that Chz1p, like Swr1p and Gcn5p, is also involved in the deposition of Htz1p at repressed promoters.

With respect to the role of Htz1p in TBP recruitment, studies of the GAL promoters have drawn different conclusions. In a recent study, TBP recruitment to the GAL1 promoter in htz1Δ strains was indistinguishable from that of WT cells (10). However, earlier studies showed Htz1p-dependent enrichment of TBP to GAL1 and GAL10 promoters during a time course of galactose induction (1). In the case of the fatty-acid inducible promoters tested here, absence of Htz1p led to significant reduction in the
recruitment of TBP during oleate induction. We did not observe increased enrichment of
TBP at FOX2 promoter during oleate induction. The dynamics of TBP binding appear to
be promoter specific. The relative abundance of TBP at the FOX2 promoter prior to
induction by oleic acid (compared to later time points) suggests that activation of FOX2
does not require additional TBP binding and that the gene exists in a state poised for its
activation upon receiving the correct signals (i.e. oleic acid). Comprehensive
investigation of the dynamic and quantitative role of Htz1p in the recruitment of factors
such as mediator and TBP to different promoters throughout the genome remains for
future studies.

Our data suggest that activation of repressed genes leads to a dynamic
reorganization of chromatin structure. Specifically, upon oleate treatment the nucleosome
proximal to initiation site in each promoter disassembles. This coincides with the ejection
of Htz1p from the promoter. These data are in agreement with previous studies that
indicate nucleosome disassembly from promoters during activation provides access to the
transcriptional machinery (25). While Htz1p is proposed to contribute to nucleosome
disassembly during induction, surprisingly, the apparent rate of nucleosome disassembly
at the oleate responsive promoters was not dramatically different in htz1Δ cells. After
initial disassembly, the nucleosomes reassemble (~1 h after induction). Interestingly,
these new nucleosomes do not appear to contain Htz1p, but levels of transcription are
nonetheless higher in WT cells than in cells lacking Htz1p. These data suggest the initial
presence of Htz1p ensures a normal transcriptional response and provides an epigenetic
mark that persists after its loss, ensuring high levels of expression. Close examination of
the data from nucleosome protection assays suggest that, in the absence of Htz1p,
nucleosomes in the promoter regions of oleate responsive genes are relatively more
assembled, which may cause reduced expression levels at these later time points. The
reassembly of nucleosomes during the coincident high levels of gene expression, suggests
that transcriptional activity is not simply related to an overall openness of chromatin at
activated promoters and obstruction at repressed promoters. Rather, the precise dynamic
placement and specific constituents of individual nucleosomes at promoters
mechanistically regulates transcription by modulating access of transacting factors to
specific sites. Further, characterization of the dynamics of the epigenetic marks, protein
components of the nucleosomes and chromatin remodeling complexes at these promoters is required to delineate the mechanistic basis of the links between chromatin state and transcriptional activation.

The observed lower expression levels in cells lacking Htz1p may also be contributed by Isw2p. Isw2p is an energy-dependent chromatin remodeling factor and negative regulator of gene expression (8, 9). When assayed for genome binding by ChIP-chip Isw2p association with the POT1, POX1, FOX2 and CTA1 promoters was not detected (9). Similarly, we found no enrichment of Isw2p at these promoters in WT cells. However, Isw2p bound to each promoter in the absence of Htz1p, and this association persisted through the six hours of induction. Therefore, the increased association of Isw2p to these four oleate responsive promoters may account for the reduced expression levels in $htz1\Delta$ cells. It is also possible that in WT cells Isw2p provides a complementary mechanism for chromatin structural changes independent of Htz1p. Loss of Htz1p provides an opportunity for Isw2p binding that is not normally functional in Htz1p-containing regions of chromatin. Further studies are required to understand the global relationship between Isw2p and Htz1p.

In mammalian cells histone variant H2A.Z can serve as a novel epigenetic marker of breast cancer progression as it is associated with lymph node metastasis and decreased breast cancer survival (13). In the plant Arabidopsis thaliana, histone H2A.Z is required for immune resistance to the phytopathogenic bacteria Pseudomonas syringae pv. tomato (22). In zebrafish, histone variant 2a z (H2afza) is essential for larval development through the generation of a lethal locus with a truncation of conserved carboxy-terminal residues in the protein (31). Taken together these studies implicate histone H2A.Z in a number of diverse functions in different organisms. Because peroxisomes are highly dynamic and responsive eukaryotic organelles whose dysfunction are linked to a host of human conditions (4, 5), it is important to understand the roles of proteins like Htz1p, that control aspects of chromatin structure and transcriptional responses preceding the proliferation of peroxisomes and fatty acid metabolism in S. cerevisiae.
Acknowledgements

We thank Bradley R. Cairns, Haiying Zhang and Michael Grunstein for providing plasmids and strains; Jeff Ranish and members of the Aitchison laboratory for helpful comments and discussion during the course of this project. This work was supported by grants GM067228, GMO76547 and RR022220 from the U.S. National Institutes of Health.
References


Figure Legends

Figure 1. Robust expression of oleate-responsive genes expression is dependent on HTZ1. (A) Comparison of changes in mRNA levels of all yeast genes in WT (left column) and htz1∆ cells (middle column) after induction in oleate medium for 6 h. Shown are the relative expression levels (Log_{10}) of genes that were determined to be significantly (λ≥100) altered in cells on oleate (compared to WT cells on glucose). Relative expression levels are shown using the scale of the yellow-blue heat map (top). Genes are ordered top-bottom based on relative expression in WT cells on oleate. Approximately 1000 genes were significantly induced and 1000 genes were repressed and changed in expression at least 2-fold. Genes that were reduced in expression were significantly enriched for functions related to ribosomal biogenesis (hypergeometric distribution analysis of GO terms – p ~10^{-50}). Induced genes were enriched for oxidative phosphorylation, electron transport chain and aerobic respiration (p ~ 10^{-10}); components of the mitochondrial respiratory chain (p ~ 10^{-13}), fatty acid oxidation and peroxisome organization and biogenesis related (~ p ~ 10^{-6}), and the peroxisomal compartment (p ~ 10^{-12}). For comparison the relative expression of each gene in htz1∆ cells in oleate (middle column) and glucose (right column) are shown. (B) As in Panel A, but shown are the relative expression levels of 292 genes significantly (λ≥100) altered in WT cells on oleate and expressed at least two-fold less than their expression levels in WT cells. This list is enriched for genes linked to fatty acid and lipid oxidation (p~4.0x10^{-12}) and peroxisomes (p~9 x10^{-20}). (C) As in B, but shown are genes encoding peroxisomal proteins significantly (λ≥100) altered in WT cells on oleate and expressed at least 2-fold less in htz1∆ cells.

Figure 2. Deletion of HTZ1 leads to delayed peroxisome biogenesis. (A) Deletion of HTZ1 impairs cell growth on oleate-containing media. Strains were grown to mid-logarithm phase in liquid YPD medium, and equal amounts of cells were serially diluted ten-fold onto YPD and incubated at 30 °C for 3 days and onto oleate-containing YPBO and incubated at 30 °C for 5 days. (B) Fluorescent images of WT and htz1∆ cells shown are expressing the peroxisomal matrix protein Pot1p fused with GFP (Pot1p-GFP) at
different time points of oleate incubation were captured on a TCS SP2 Laser Scanning Spectral Confocal Microscope.

**Figure 3. Htz1p dynamically dissociates from oleate responsive promoters upon induction.** (A) **POT1**, **POX1**, **FOX2** and **CTA1** mRNA levels were determined by RT-PCR in WT and *htz1Δ* strains over a time course of oleate induction. The signal obtained from **ACT1** mRNA was used as a loading control for normalization. Error bars represent standard deviation from the mean of three independent experimental values. (B) Htz1p enrichment at four promoters was determined by qPCR during oleate induction. Relative enrichment values (Y axes) are the average of three independent ChIPs with qPCR determination performed twice per each biological replicate. Non-promoter IGRi **YMR325W** was used as an internal control to normalize signals of promoter enrichment. In response to oleate induction, Htz1p was lost from the **POT1**, **POX1**, **FOX2** and **CTA1** promoters.

**Figure 4. Swr1p, Chz1p and Gcn5p - dependent association of Htz1p to promoters.** (A) In vivo association of Htz1p with **POT1**, **POX1**, **FOX2** and **CTA1** promoters was measured by ChIP in the WT, *chz1Δ*, *gcn5Δ* and *swr1Δ* strains in 2% glucose medium. ChIP was performed in glucose-containing medium. Error bars represent standard deviation from the mean of three independent experimental values and two technical replicates of each. (B) Comparison of changes in mRNA levels of all yeast genes in WT, *chz1Δ*, *gcn5Δ* and *swr1Δ* strains after induction in oleate medium for 6 h. As in Figure 1 C, genes are encoding peroxisomal proteins significantly (λ ≥ 100) altered in WT cells on oleate and expressed at least 2-fold less in *htz1Δ* cells.

**Figure 5. Acetylation of Htz1p is required for efficient transcriptional induction.** (A) **POT1**, **POX1**, **FOX2** and **CTA1** mRNA levels were determined by RT-PCR in WT and *htz1Δ* and Htz1p K14A mutant strains over a time course of oleate induction. The signal obtained from **ACT1** mRNA was used as a loading control for normalization. Error bars represent standard deviation from the mean of three independent experimental values. (B) Enrichment of WT Htz1p and Htz1p K14A mutant at four promoters was determined by
qPCR during glucose and oleate induction for 6 hours. Relative enrichment values (Y axes) are the average of three independent ChIP experiments with two technical replicates of each. Non-promoter IGRi YMR325W was used as an internal control to normalize signals of promoter enrichment.

**Figure 6. Recruitment of TBP during oleate induction requires Htz1p.** (A) The association of TBP with POT1, POX1, FOX2 and CTA1 promoters was determined by chromatin immunoprecipitation (ChIP) using anti-Myc antibodies, followed by gene-specific PCR. The relative enrichment ratio is plotted at 4 time points (0, 1, 4, 6 h) of induction in oleate. ACT1 was used as an internal control to normalize signals of promoter enrichment. Error bars show the standard deviation from three independent experimental values with two technical replicates of each. (B) Deletion of Htz1p did not affect TBP expression during oleate induction. The WT strain and HTZ1 deletion strains expressing genomically integrated TBP were grown in 2% glucose overnight and then transferred to oleate-containing SCIM medium at the indicated time points. Samples containing equal protein were analyzed by Western blotting with anti-Myc antibody to visualize TBP expression. A polyclonal antibody directed against Gsp1p was used as loading control.

**Figure 7. Htz1p regulates the occupancy of specific nucleosomes on POT1, POX1, FOX2 and CTA1 promoters.** The NuSA assay was used to determine the nucleosome positioning and density at POT1, POX1, FOX2 and CTA1 promoters during oleate induction (time of induction is indicated at left) in WT and HTZ1 deletion strains. Each point represents the relative protection of each PCR amplicon, quantified by real-time PCR and normalized to a centromeric control. The position of each amplicon (referenced to the middle of each amplicon) within the promoter is shown on the x-axis. The approximate location of nucleosome is represented by grey circle with the nucleosome number referred to in the text shown on the circle.

**Figure 8. Isw2p can associate with oleate responsive promoters in the absence of Htz1p.** (A) The NuSA assay was used to determine the nucleosome positioning and
density at \textit{POT1}, \textit{POX1}, \textit{FOX2} and \textit{CTA1} promoters during repression (2\% glucose) in WT, \textit{htz1}\Delta and \textit{isw2}\Delta strains. Each point represents the relative protection of each PCR amplicon, quantified by real-time PCR and normalized to a centromeric control. (B) The association of Isw2p (as a C-terminal myc fusion) with \textit{POT1}, \textit{POX1}, \textit{FOX2} and \textit{CTA1} promoters was determined by chromatin immunoprecipitation (ChIP) using anti-Myc antibodies, followed by gene-specific PCR. The relative enrichment ratio is plotted at 4 time points (0, 1, 4, 6 h) of induction in oleate. \textit{ACT1} was used as an internal control to normalize signals of promoter enrichment. Error bars show the standard deviation from three independent experimental values with two technical replicates of each.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0</td>
<td>Open</td>
</tr>
<tr>
<td>BY4742</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0</td>
<td>Open</td>
</tr>
<tr>
<td>YWY004</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, het1Δ::kanMX4</td>
<td>Open</td>
</tr>
<tr>
<td>YWY007</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, POT1-GFP::natMX, pRS416</td>
<td>This study</td>
</tr>
<tr>
<td>YWY009</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, het1Δ::kanMX4, POT1-GFP::natMX</td>
<td>This study</td>
</tr>
<tr>
<td>YWY010</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, het1Δ::kanMX4, POT1-GFP::natMX, pCM305</td>
<td>This study</td>
</tr>
<tr>
<td>YWY011</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, het1Δ::kanMX4, POT1-GFP::natMX, pCM330</td>
<td>This study</td>
</tr>
<tr>
<td>YWY012</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, het1Δ::kanMX4, POT1-GFP::natMX, pCM314</td>
<td>This study</td>
</tr>
<tr>
<td>YWY013</td>
<td>MATa, leu2Δ0, ura3Δ0, HA-HTZ1</td>
<td>Zhang haiying, 2005</td>
</tr>
<tr>
<td>YWY0165</td>
<td>MATa, leu2Δ0, ura3Δ0, HA-HTZ, chc1Δ::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>YWY0166</td>
<td>MATa, leu2Δ0, ura3Δ0, HA-HTZ, gen5::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>YWY0177</td>
<td>MATa, leu2Δ0, ura3Δ0, HA-HTZ, swr1Δ::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>YWY206</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, SPT15-13MYC::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>YWY207</td>
<td>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, SPT15-13MYC::kanMX4, het1Δ::hphMX</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>pRS416</td>
<td>CEN6-ARS4 URA3</td>
<td>Open</td>
</tr>
<tr>
<td>pCM314</td>
<td>CEN6-ARS4 URA3 HA-leu1K144</td>
<td>Millar CB et al, 2006</td>
</tr>
<tr>
<td>pCM330</td>
<td>CEN6-ARS4 URA3 HA-leu1K14R</td>
<td>Millar CB et al, 2006</td>
</tr>
<tr>
<td>pCM305</td>
<td>CEN6-ARS4 URA3 HA-HTZ1</td>
<td>Millar CB et al, 2006</td>
</tr>
</tbody>
</table>
Fig 1
Fig 2

A

<table>
<thead>
<tr>
<th>WT</th>
<th>YPD</th>
<th>YPBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>pex3Δ</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>htz1Δ</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>WT</th>
<th><img src="image5" alt="Image" /></th>
<th><img src="image6" alt="Image" /></th>
</tr>
</thead>
<tbody>
<tr>
<td>htz1Δ</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

After shifting to oleate (h)

0 1 2 4 6 8 12

Pot1p-GFP

DIC
Fig 3

A

WT

leader mRNA

0 1 2 3 4 6

After shifting to oleate (h)

WT

leader mRNA

0 1 2 3 4 6

After shifting to oleate (h)

WT

leader mRNA

0 1 2 3 4 6

After shifting to oleate (h)

WT

leader mRNA

0 1 2 3 4 6

After shifting to oleate (h)

B

POT1

HTz1p enrichment

After shifting to oleate (h)

FOX2

HTz1p enrichment

After shifting to oleate (h)

POX1

HTz1p enrichment

After shifting to oleate (h)

CTA1

HTz1p enrichment

After shifting to oleate (h)
Fig 4

A

Hsp enrichment

POT1

POX1

FOX2

CTAI

B

MLM1

POT1

CTAI

HSP2

CAE2

FAA2

SPS9

PES18

REP9

ETI1

TIM8

LPX1

PES2

CT2

DCS5

PSA1

PES31

MDH5

PES1

PES15

PES21

PES28

PES23

ANT1
Fig 5

A

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5a}
\caption{Graphs showing the expression levels of different genes over time post-shift to oleate.}
\end{figure}

B

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5b}
\caption{Bar charts depicting the enrichment levels of certain genes over time post-shift to oleate.}
\end{figure}
Fig. 7

![Graph showing relative protection over time for different proteins.](image-url)

- **WT**
- **het1Δ**
Fig. 8