Molecular Cell, Vol. 12, 1565–1576, December, 2003, Copyright ©2003 by Cell Press

A Snf2 Family ATPase Complex Required for Recruitment of the Histone H2A Variant Htz1

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Summary

Deletions of three yeast genes, *SET2*, *CDC73*, and *DST1*, involved in transcriptional elongation and/or chromatin metabolism were used in conjunction with genetic array technology to screen ~4700 yeast deletions and identify double deletion mutants that produce synthetic growth defects. Of the five deletions interacting genetically with all three starting mutations, one encoded the histone H2A variant Htz1 and three encoded components of a novel 13 protein complex, SWR-C, containing the Snf2 family ATPase, Swr1. The SWR-C also copurified with Htz1 and Bdf1, a TFIID-interacting protein that recognizes acetylated histone tails. Deletions of the genes encoding Htz1 and seven nonessential SWR-C components caused a similar spectrum of synthetic growth defects when combined with deletions of 384 genes involved in transcription, suggesting that Htz1 and SWR-C belong to the same pathway. We show that recruitment of Htz1 to chromatin requires the SWR-C. Moreover, like Htz1 and Bdf1, the SWR-C promotes gene expression near silent heterochromatin.

Introduction

The eukaryotic genome is packaged into nucleosomes, which consist of two tightly superhelical turns of DNA (~146 bp) around a histone octamer that contains two copies each of the four most abundant histones: H2A, H2B, H3, and H4. Nucleosomes are connected by 20–80 bp of linker DNA and compacted into hierarchically folded higher-order assemblies in a process aided by the linker histone H1, numerous nonhistone proteins, and divalent metal ions (Hansen, 2002; Luger, 2003).

Nucleosomal histones are subjected to many types of modifications that can facilitate or inhibit processes such as transcription, DNA replication, DNA repair, and chromosome segregation. These posttranslational chemical modifications include acetylation, phosphorylation, ADP-ribosylation, ubiquitination, and methylation (Fischle et al., 2003). Variation in nucleosome structure also results from the incorporation into chromatin of variant histone proteins that differ in amino acid sequence from their more abundant counterparts. Variant histones allow for specialization of nucleosome structure for specific purposes (Wolffe and Pruss, 1996). For example, a specific histone 3 variant, CENP-A (Cse4 in *S. cerevisiae*), is incorporated exclusively at centromeres, creating a specialized chromatin structure needed for efficient kinetochore function (Henikoff et al., 2000; Stoler et al., 1995). In *Drosophila* chromatin, a different H3 variant, H3.3, replaces the canonical histone 3 in highly transcribed regions through a replication-independent mechanism (Ahmad and Henikoff, 2002). A variant of H2A (H2A.X) also plays locus-specific roles, since it is recruited to sites of DNA damage (Paull et al., 2000), and macroH2A is localized to the inactive X chromosome in mammals (Ladurner, 2003).

H2A.Z, or Htz1 in *S. cerevisiae*, is another H2A variant that is conserved from yeast to humans (Jackson et al., 1996). This particular histone variant is found to be associated with transcriptionally active chromatin in Tetrahymena and has therefore been thought to be involved in a chromatin structure that favors gene transcription (Stargell et al., 1993). Adam et al. (2001) further implicated *S. cerevisiae* Htz1 in transcriptional activation by showing it is required in vivo for RNA polymerase II (RNAPII) recruitment under certain conditions. Htz1 is also important for preventing the spread of silent heterochromatin into active regions near telomeres and silent mating loci and is partially redundant with nucleosome remodeling complexes such as SWI/SNF (Meneghini et al., 2003; Santisteban et al., 2000). We now describe what appears to be a new chromatin remodeling complex, the SWR-C, which was identified in a genetic screen for proteins involved in chromatin modification and transcriptional elongation by RNAPII. The SWR-C is required for the recruitment of Htz1 into chromatin.

Results and Discussion

Synthetic Genetic Array Analyses with *SET2*, *DST1*, and *CDC73*

In an effort to discover novel genes involved in transcriptional elongation or chromatin modification, we used the automated synthetic genetic array (SGA) technique (Tong et al., 2001) to cross deletions of each of three

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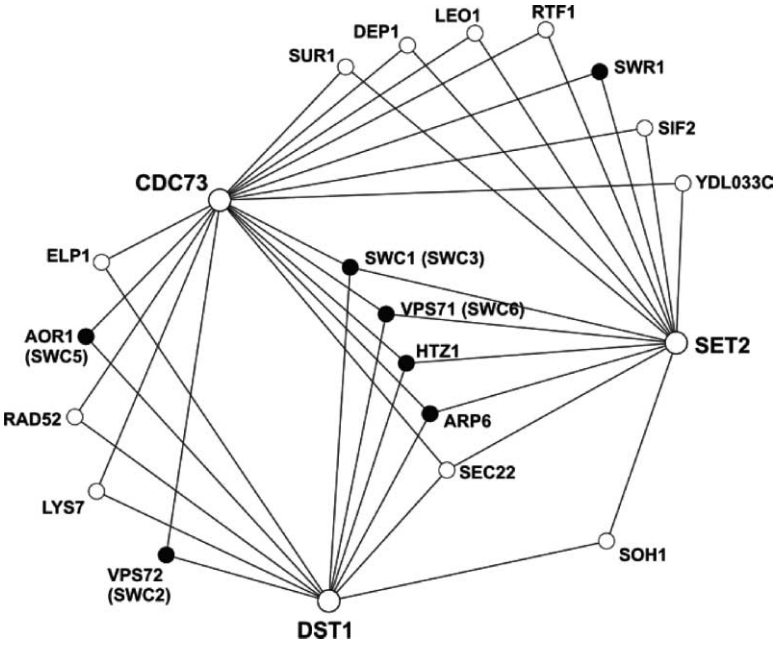


Figure 1. Genes Whose Deletions Cause Synthetic Growth Defects When Combined with the Deletion Mutants *set2*Δ, *dst1*Δ, or *cdc73*Δ

SGA technology (Tong et al., 2001) was used to combine each of these three deletions with ~4700 deletions in the yeast deletion set. All the double mutant combinations that caused synthetic growth defects are listed in Supplemental Data. Shown here are genes whose deletion caused synthetic growth defects with at least two of the three query deletions. Of these 19 genes, one is *HTZ1* and six encode nonessential subunits of the SWR-C (indicated by filled circles). All of the synthetic genetic interactions shown were confirmed by tetrad dissection (data not shown).

genes, *SET2*, *CDC73*, and *DST1*, separately to an array of ~4700 viable gene deletion mutants. Double mutant progeny exhibiting inviability or slow growth are thought to identify functional relationships between genes.

Set2, Dst1, and Cdc73 have all been implicated in chromatin modification and/or transcriptional elongation by RNAPII. Set2 physically interacts with RNAPII and is a histone 3 lysine 36-specific methyltransferase. At least one activity of the enzyme is linked to the process of transcriptional elongation (reviewed by Hampsey and Reinberg, 2003). Cdc73 is a subunit of the five-component PAF complex, which interacts physically with RNAPII and the elongation factor FACT (Spt16/Pob3) (Krogan et al., 2002; Mueller and Jaehning, 2002; Squazzo et al., 2002). The PAF complex is recruited to the coding regions of actively transcribed genes (Krogan et al., 2002; Pokholok et al., 2002), and is required for the recruitment and activities of both Set2 and the Set1-containing complex, COMPASS, which methylates histone 3 on lysine 4 (Hampsey and Reinberg, 2003). Dst1, or TFIIS, physically associates with RNAPII, enabling backtracked RNAPII to transcribe through pause and arrest sites, and enhances transcriptional fidelity by cleaving the transcript to generate a new 3′ end (Fish and Kane, 2002).

To carry out SGA analysis, deletions of *SET2*, *CDC73*, and *DST1*, marked by Nat^R, were first introduced individually into a haploid starting strain of mating type *MAT*α, and then each of the three Nat^R deletion strains was crossed to an array of ~4700 Kan^R gene deletion mutants of the opposite mating type (*MAT***a**) constructed by the yeast deletion consortium (Winzeler et al., 1999). Sporulation of the resulting diploid cells and selection for Kan^R Nat^R haploids then resulted in an ordered array of double mutant haploid strains whose growth rate was monitored by visual inspection. Putative genetic interactions for the *SET2*, *CDC73*, and *DST1* screens were then confirmed by either tetrad dissection or random sporulation (data not shown), resulting in 63, 111, and 16 confirmed genetic interactions, respectively, as listed in Supplemental Data at http://www.molecule.org/cgi/content/full/12/6/1565/DC1. Shown in Figure 1 are the genetic interactions that arose from at least two of the three initial screens and were confirmed by tetrad dissection. Only five interacting genes were common to all three screens: *HTZ1*, encoding a variant histone 2A; the actin-related gene *ARP6* (Frankel and Mooseker, 1996); *SEC22*, encoding a v-SNARE involved in membrane fusion during ER-to-Golgi transport (Newman et al., 1990); and two functionally uncharacterized genes, *SWC1* and *VPS71*. An additional 13 genes were identified in two of the three screens. It should be noted, however, that SGA screens of the entire ~4700 strain deletion set characteristically miss 20 to 40% of the interacting genes (A.T. et al., unpublished data).

Purification of an Swr1-Containing Complex

VPS71 interacted genetically with our three query genes encoding RNAPII elongation or chromatin modifying factors and is located in the nucleus (Huh et al., 2003; Kumar et al., 2002). After placing a tandem affinity purification (TAP) tag (Rigaut et al., 1999) containing a calmodulin binding peptide and *Staphylococcus aureus* protein A, separated by a TEV protease cleavage site, at the C terminus of Vps71, the tagged protein was purified sequentially on IgG and calmodulin columns and analyzed by SDS-PAGE followed by staining with silver (Krogan et al., 2002). Protein bands absent from a control preparation and corresponding to the tagged protein and any associated proteins were then identified by MALDI-TOF and tandem mass spectrometry (Figure 2A, lane 6) (Krogan et al., 2002). Tagged Vps71 copurified with seven other nonessential proteins, Swr1, Arp6, Yaf9, Vps72, Swc1, Ylr385c, and Ybr231c (or Aor1, for Actin overexpression resistant), and with five essential proteins, Arp4, Act1, God1, Rvb1, and Rvb2. We call this 13 pro-

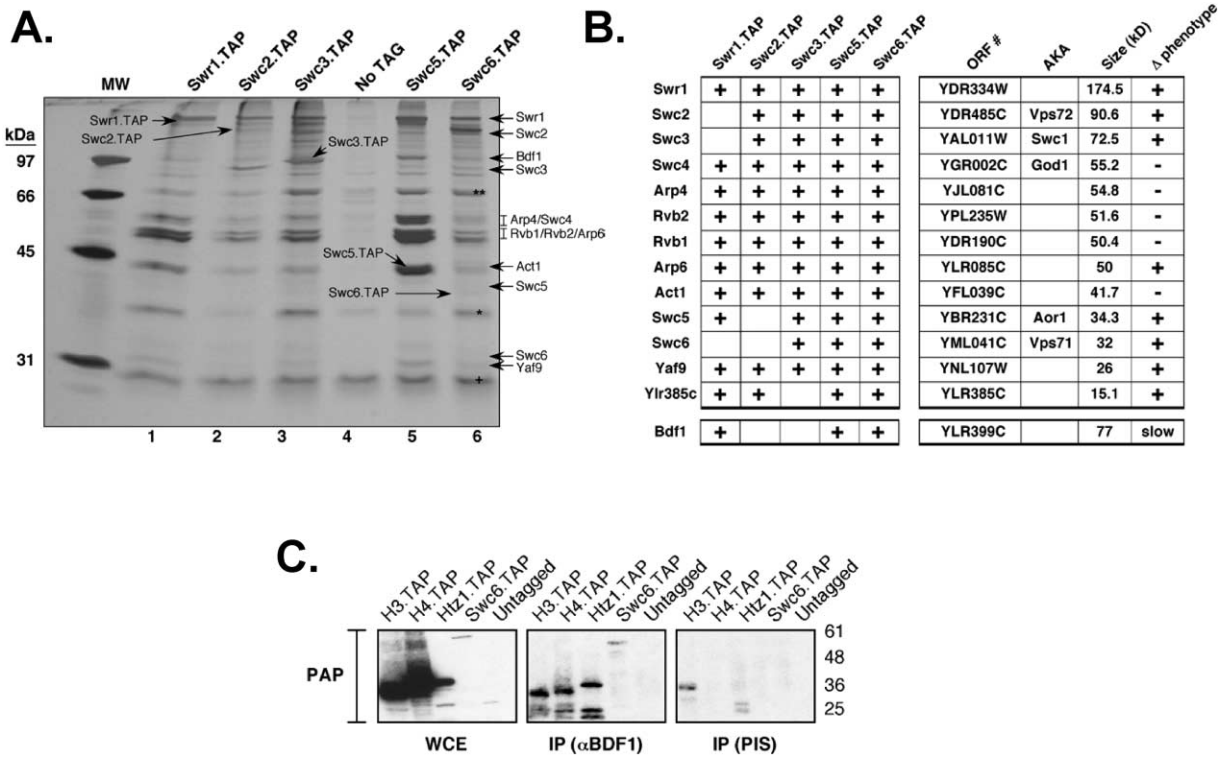


Figure 2. Isolation of the SWR Protein Complex

(A) Tandem affinity purifications of the SWR-C were carried out on strains containing either no tagged protein or TAP-tagged versions of Swr1, Swc2, Swc3, Swc5, and Swc6. The protein complexes were purified in the presence of 100 mM NaCl and were then analyzed by SDS-PAGE and silver-staining. The subunits of the SWR-C were identified by trypsin digestion and MALDI-TOF mass spectrometry or else by tandem mass spectrometry after subjecting an aliquot of the eluate from the final column directly to trypsin. Contaminating bands are as follows: **, the heat shock proteins, Ssb1, Ssa1, and Sse1; *, glyceraldehyde-3-phosphate dehydrogenase 3 (Tdh3); and +, TEV protease.

(B) Summary of purified proteins identified by mass spectrometry. Proteins that were present in at least three of the five purifications are represented. Several other proteins that were detected only once (Cka1, Isw1, Ski2, Ski3, and Ski8) are not shown. Ylr385c (Swc7) ran off the bottom of the gel and was detected only by LC-MS/MS. Protein size (kDa) was predicted by amino acid composition (SGD) and not determined experimentally.

(C) The SWR-C and Htz1 are associated with Bdf1. Bdf1 coimmunoprecipitations from the indicated TAP-tagged strains were performed as described in Experimental Procedures. Bdf1 copurifies with Htz1 and the SWR-C (represented by Swc6.TAP), as well as with the nonvariant histones H3 and H4 as previously described (Matangkasombut and Buratowski, 2003). PAP, peroxidase anti-peroxidase; PIS, preimmune serum.

tein complex the SWR-C and have assigned the names Swc2, Swc3, Swc4, Swc5, Swc6, and Swc7 (for SWR complex polypeptides in descending order of molecular weight) to Vps72 (Ydr485c), Swc1 (Yal011w), God1 (Ygr002c), Aor1 (Ybr231c), Vps71 (Yml041c), and Ylr385c, respectively (Figure 2B). Swr1 contains a DEAH box and is a putative ATPase of the Snf2-DNA helicase family (Shiratori et al., 1999), while Act1 and the actin-related protein Arp4 are known to be components of the NuA4 histone acetylation complex (Galarneau et al., 2000). The Ino80 chromatin remodeling complex also contains Act1 and Arp4, as well as the DNA helicases Rvb1 and Rvb2 (Shen et al., 2000). Yaf9, also a member of the NuA4 complex (Le Masson et al., 2003), is the yeast homolog of human AF9 and contains a YEATS domain that is implicated in chromatin modification and transcriptional regulation. Other members of this family include yeast and human Sas5 proteins, human Gas41, which is implicated in oncogenesis, and Taf14, which is a component of several transcription factors, including the transcriptional initiation factor TFIIF and the chromatin remodeling complex SWI/SNF (Le Masson et al., 2003). Interestingly, AF9 is often fused to the SET domain protein MLL in acute myeloid leukemia, and an AF9-MLL fusion results in acute myeloid leukemia in transgenic mice (Dobson et al., 1999).

To further define the composition of the SWR-C, we also purified and characterized complexes containing TAP-tagged Swr1, Swc3, Swc2, and Swc5 (Figure 2A, lanes 1, 2, 3, and 5). Our results confirmed that the SWR-C consists of all 13 proteins, since all were identified in at least three of the five purifications (Figure 2B). Remarkably, in the three original genetic screens with *set2*Δ, *cdc73*Δ, and *dst1*Δ, six of the eight nonessential components of the SWR-C were identified: Swc3, Swc6, and Arp6 were each identified by all three query genes, whereas Swc2, Swc5, and Swr1 were each identified by two of the three query genes (Figure 1). We have since found that the *yaf9*Δ strain in our copy of the yeast deletion set does not grow well, probably explaining why we did not identify it as genetically interacting with the three starting mutations. We also did not identify the small, 15 kDa protein, Ylr385c (Swc7), in our genetic screen, which may indicate it plays a minor role in

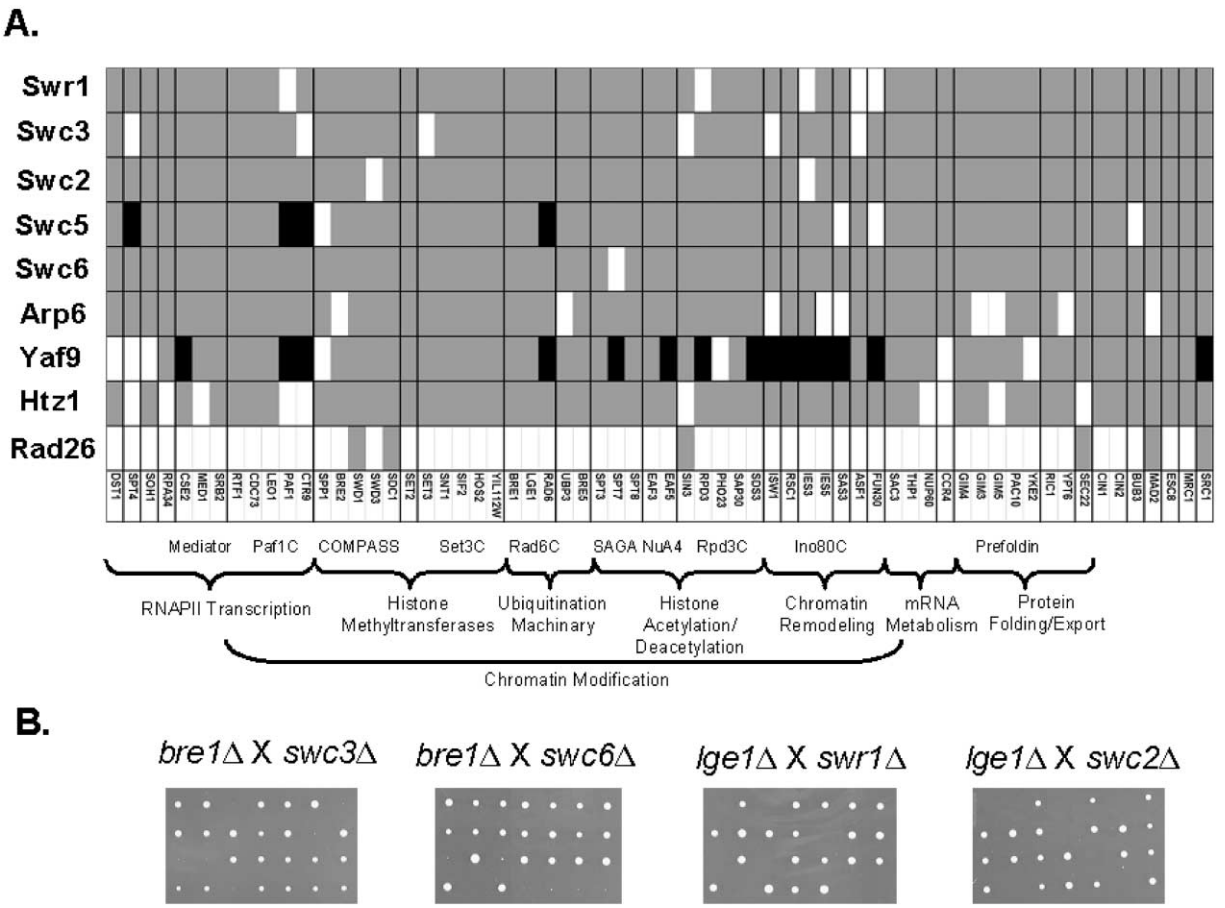


Figure 3. Htz1 and the SWR-C Have Similar Genetic Interactions

(A) Nine Nat^R strains harboring individual gene deletions of *HTZ1*, *RAD26*, and seven nonessential components of the SWR-C were generated and crossed with a transcription-targeted 384 deletion strain miniarray to create sets of haploid double mutants. Growth rates were assessed by automated image analysis of colony size. Gray blocks indicate that a genetic interaction was observed while white blocks indicate that no synthetic growth defect was observed with this technique. Black blocks represent double deletion combinations that were not tested.

(B) Representative confirmations by tetrad dissection of synthetic growth defects detected by the SGA technique using components of the Rad6 histone ubiquitin complex, Bre1 and Lge1. 40 of the 455 synthetic genetic interactions shown in (A) were tested by tetrad dissection, and all were confirmed.

SWR-C function. Seven of the eight nonessential SWR-C components have also been identified, along with many other genes, in a large screen designed to uncover defects in vacuolar protein sorting (VPS) (Bonangelino et al., 2002). In a recent genome-wide localization study in *S. cerevisiae* (Huh et al., 2003), all 11 components of the SWR-C that were analyzed localized to the nucleus, implying that its effect on vacuolar protein sorting is indirect.

Bdf1, a loosely associated component of the general transcriptional initiation factor, TFIID (Matangkasombut et al., 2000), also copurified with Swr1, Swc5, and Swc6 (Figure 2). Bdf1 has two bromodomains that interact with the acetylated tails of histones 3 and 4, and Bdf1 stimulates gene expression near silent heterochromatin (e.g., near telomeres) (Ladurner et al., 2003; Matangkasombut and Buratowski, 2003; Pamblanco et al., 2001). Therefore, Bdf1 may recruit the SWR-C to sites where H3 and H4 are acetylated and possibly to specific promoters. We did not, however, identify any TFIID subunits accompanying Bdf1 when we purified the SWR-C. One possibility is that the Bdf1-TFIID interaction is sufficiently weak that TFIID was lost during the purification. Another possibility is that Bdf1 cannot interact with TFIID and the SWR-C at the same time. In that case, Bdf1 may simply recruit the SWR-C to nonpromoter sites where histones H3 and H4 are acetylated.

In order to confirm the association of Bdf1 with the SWR-C, we immunoprecipitated from a Swc6-TAP strain with a polyclonal antibody against Bdf1 and Western blotted with IgG that binds the protein A component of the TAP tag. We detected a physical association between Bdf1 and Swc6, not observed when we used preimmune serum instead of anti-Bdf1 (Figure 2C). Interestingly, anti-Bdf1 also coprecipitated the histone H2A variant, Htz1, as well as histones H3 and H4 (Figure 2C). Western blotting of extracts containing TAP-tagged histones indicated that there is far less Htz1 than the nonvariant histones H3 and H4 (WCE in Figure 2C), about 50–100 times less according to Ghaemmaghami et al. (2003). Nevertheless, Bdf1 appears to be strongly associated with nucleosomes containing Htz1, since similar

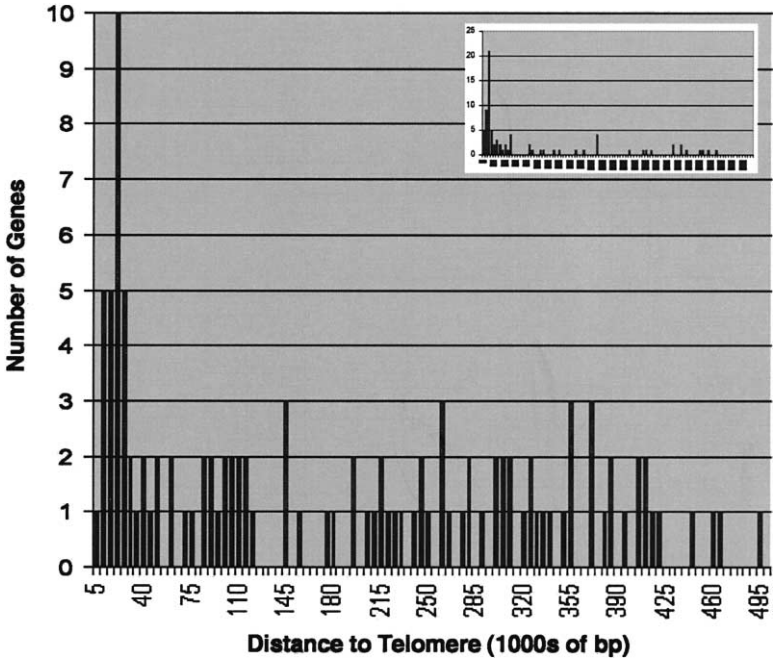


Figure 4. The SWR-C Preferentially Affects the Expression of Genes in Subtelomeric Regions

The chromosomal distribution of genes that are downregulated at least 1.7-fold when *SWR1* is deleted is shown. The histogram represents the Swr1-activated genes plotted as a function of their distances from a chromosomal end. The inset plots the distance from the telomeres of genes identified by Meneghini et al. (2003) as being downregulated at least 1.7-fold when *HTZ1* is deleted. The scale of the *x* axis on the inset is the same as in the main panel.

amounts of Htz1, H3, and H4 were coimmunoprecipitated with Bdf1.

Htz1 and the other histones would have run off the bottom of the 10% gel shown in Figure 2A. Therefore, to further confirm a physical interaction between the SWR-C and Htz1, we trypsin digested and used LC-MS/MS on an ion-trap system to analyze an aliquot of the SWR-C obtained by the purification of TAP-tagged Swc3. Htz1 was detected with 99% confidence, confirming the association of Htz1 with the SWR-C. This interaction appears to be highly specific, because we have identified Htz1 by mass spectrometry in only five of the approximately 3500 purifications of yeast TAP-tagged proteins carried out in our laboratory thus far (N.J.K., G.C., A.E., and J.G., unpublished data). Two of these other proteins, the importin Kap114 and the uncharacterized ORF, Ybr267w, are associated with the nucleosome assembly protein Nap1 (Gavin et al., 2002; N.J.K. et al., unpublished data). In addition, two other core histones, Hht1 (histone H3) and Htb2 (histone H2B), and the previously uncharacterized ORF, Ykl023w, also copurified with Htz1.

Genetic Analysis of the SWR-C

Genes encoding proteins of similar function in the same protein complex should, in principle, have similar sets of genetic interactions. To test this concept and provide independent evidence that the nonessential proteins of the SWR-C are indeed likely to be components of the same protein complex, we turned again to the SGA technique (Tong et al., 2001). For this purpose, a miniarray containing 384 deletion strains was constructed in which each deletion represents a protein known or suspected to function in some aspect of transcription and/or chromatin modification or remodeling (N.J.K. and J.G., unpublished data). Seven Nat^R strains harboring individual gene deletions of seven nonessential components of the SWR-C were then generated and crossed with the 384 deletion strain miniarray to create sets of haploid double mutants. Growth rates were assessed by automated image analysis of colony size. Of the 455 putative genetic interactions identified in the automated screens shown in Figure 3A, 40 were tested by tetrad dissection and all 40 were confirmed, suggesting a low false positive rate in the automated analysis of our miniarray. Several representative tetrad dissections are shown in Figure 3B.

Components of the SWR-C were found to interact genetically not only with the elongation factors Dst1 and Cdc73, but also with all the remaining subunits of the PAF complex (Rtf1, Ctr9, Leo1, and Paf1) (Krogan et al., 2002; Mueller and Jaehning, 2002; Squazzo et al., 2002) and with Spt4, a nonessential component of the RNAPII elongation factor Spt4/Spt5 (Hartzog et al., 1998; Wada et al., 1998) (Figure 3A). A genetic interaction was also uncovered with Soh1 (Fan and Klein, 1994), which was originally identified on the basis of a genetic interaction with the elongation and RNA processing complex, TREX (Strasser et al., 2002). The seven nonessential subunits of the SWR-C also interacted genetically with three of the twelve nonessential subunits of the transcriptional initiation complex, Mediator (Cse2, Med1, and Srb2) (Myers and Kornberg, 2000) (Figure 3A).

In addition, interactions were uncovered not only with Set2, but also with other factors required for histone modification: five subunits of the Set1-containing methyltransferase, COMPASS (Hampsey and Reinberg, 2003); five subunits of the Set3 complex, including the putative methyltransferase, Set3, whose substrate is unknown, and two histone deacetylases, Hos2 and Hst1 (Pijnappel et al., 2001); all three known subunits of the Rad6 histone H2B ubiquitination complex (Rad6, Bre1, and Lge1) (Bach and Ostendorff, 2003); and both subunits of the deubiquitinating enzyme Ubp3/Bre5 (Cohen et al., 2003). Also included are components of the histone acetyl-

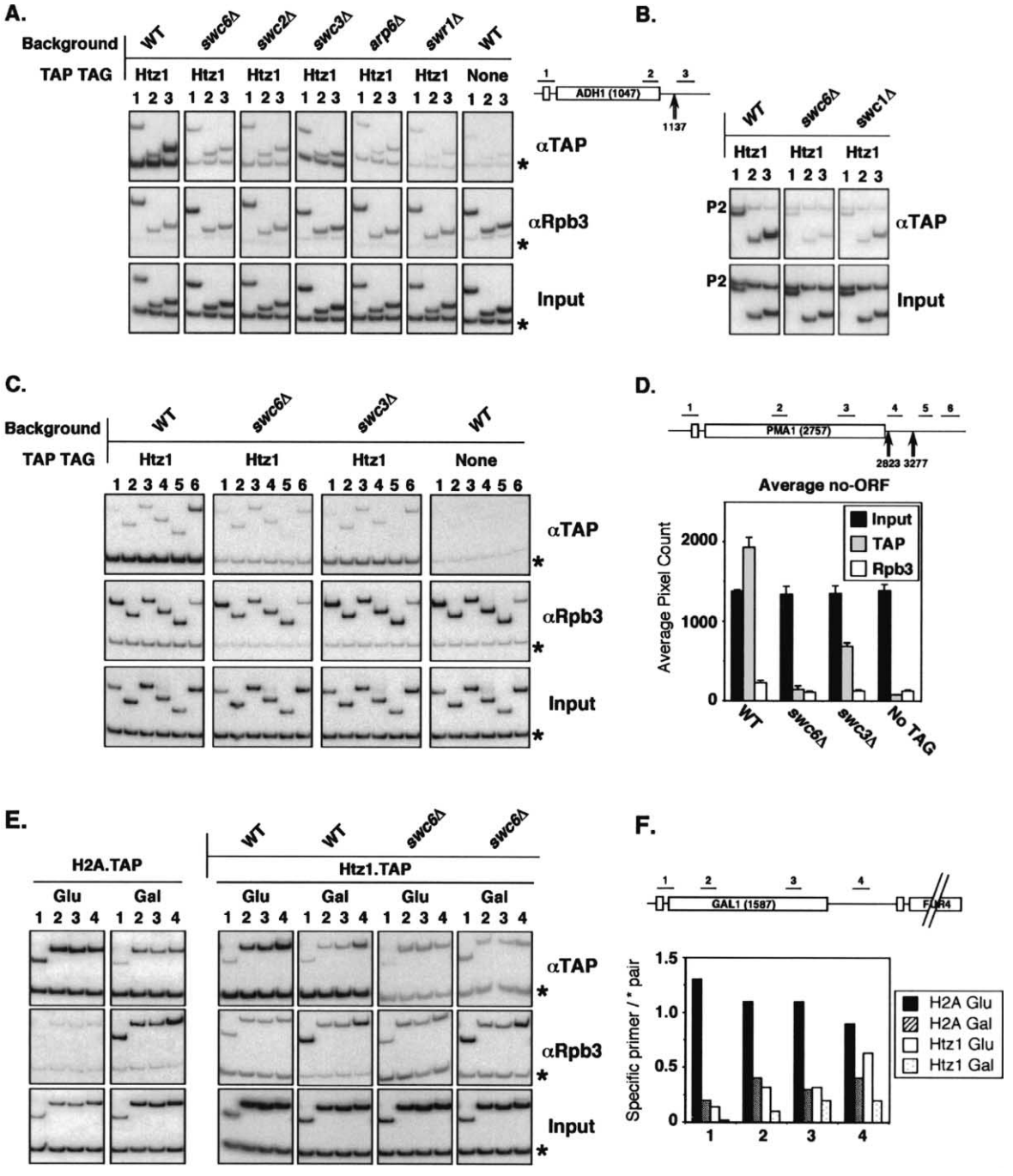


Figure 5. The SWR-C Is Required for Recruitment of Htz1 to Transcribed and Nontranscribed Regions

(A) ChIP analysis at the *ADH1* gene. A schematic of the *ADH1* locus is shown with the location of the ChIP primer pairs 1–3 (above) and the major polyadenylation/cleavage site at +1137 (below) (M. Kim et al., personal communication). * indicates a noncoding region of Chromosome V (9716-9823) (see Figure 6). Chromatin samples from each strain were analyzed after the indicated precipitation. Lowest row is input, used to normalize the PCR amplification efficiency of each primer pair. Equivalent crosslinking of Rpb3 in each case is used as a control for sample integrity. Note that deletion of members of the SWR-C leads to either a complete (*swc6*Δ, *swc2*Δ, *arp6*Δ, *swr1*Δ) or significant (*swc3*Δ) loss of Htz1 occupancy at the * region and throughout *ADH1*.

(B) Htz1 occupancy at *ADH1* was analyzed as in (A), except instead of comparing to the * primer pair, we used *PMA1* Primer 2 (P2) from (D). Note that Htz1 occupancy is higher throughout *ADH1* than at *PMA1*. This difference is intriguing since, as far as we can tell, neither gene is regulated by Htz1.

(C) ChIP analysis at the *PMA1* gene. As at *ADH1*, loss of the SWR-C components Swc6 and Swc3 significantly reduces Htz1 recruitment throughout *PMA1*.

(D) Quantitation of the *PMA1* results. A schematic of the *PMA1* locus is shown with the location of the ChIP primer pairs 1–6 (above) and the major polyadenylation/cleavage sites at +2823 and +3277 (below) (M. Kim et al., personal communication). Note that elongating RNAPII (represented by αRpb3) is displaced between primers 5 and 6. To demonstrate the integrity of the chromatin samples, the average intensity

transferase complexes SAGA (Spt3, Spt7, Spt8) (Sterner and Berger, 2000) and NuA4 (Eaf3 and Eaf5) (Galarneau et al., 2000) and components of the histone deacetylase complex, Sin3/Rpd3 (Lechner et al., 2000), as well as the Isw1 and Rsc1 components of chromatin remodeling complexes (Cairns et al., 1996; Tsukiyama et al., 1999). Since the Snf2-like helicase family, of which Swr1 is a member, includes the known chromatin remodelers Chd1 and Ino80 (Shiratori et al., 1999), these genetic interactions suggested that the SWR-C functions in some aspect of chromatin remodeling and is functionally redundant to some extent with other chromatin remodeling and modifying factors. The genetic interactions between the SWR-C and various transcription factors suggest that the SWR-C is involved in transcription or aspects of chromatin modification that are linked to transcription.

Genetic interactions were also detected with a number of other proteins with varied function. These included Ccr4, the catalytic component of the main cytoplasmic mRNA deadenylase (Denis and Chen, 2003); the mRNA export complex, Sac3/Thp1/Nup60; two genes required for proper chromosome segregation, Cin1 and Cin2 (Fleming et al., 2000); two genes implicated in the spindle-assembly checkpoint, Mad2 and Bub3 (Gardner and Burke, 2000); and Mrc1, a gene involved in the DNA replication checkpoint (Osborn and Elledge, 2003). The fact that members of SWR-C genetically interact with a wide variety of genes again implies that its proposed role in chromatin integrity impinges on a large number of biological processes, either directly or indirectly.

Involvement of the SWR-C in the Functioning of Htz1

We have individually crossed Nat^R versions of each of the 384 deletions in our transcriptional elongation/chromatin modification miniarray against the entire Kan^R miniarray, thereby creating a 384 $\times$ 384 matrix of double mutant strains (N.J.K. and J.G., unpublished data). Growth rates were evaluated by automated image analysis and the results of all the screens were analyzed by two-dimensional hierarchical clustering. This clustering process groups genes according to the degrees of similarity of their genetic interactions. One important expectation is that genes with the same or very similar functions would have similar sets of genetic interactions and would fall into the same cluster.

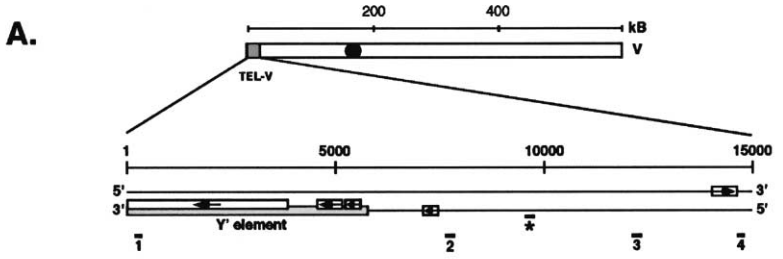
As expected, all nonessential components of the SWR-C, with the exception of *SWC7,* have virtually identical sets of genetic interactions and clustered together (Figure 3A), as did the gene encoding Htz1 (clustering data not shown). *HTZ1* was also one of the five genes identified by all three query genes in our original genetic screens (Figure 1). The characterized genes in the 384 strain miniarray whose deletions genetically interact with *htz1*Δ are also shown in Figure 3A, and they represent most of the genes, and essentially all of the protein complexes, that genetically interact with genes encoding the SWR-C. By way of contrast, the gene encoding Rad26, which is involved in coupling transcription to DNA repair (Woudstra et al., 2002), has very few of the same genetic interactions (Figure 3A). This result was consistent with our observation that the SWR-C is associated with Htz1 and implicated the SWR-C in the functioning of Htz1 and perhaps also in transcriptional elongation. An involvement of Htz1 in transcriptional elongation would be consistent with the finding that deleting *HTZ1* causes sensitivity to both 6-azauracil and mycophenolic acid, both of which deplete nucleotide pools in the cell and indicate a possible involvement in transcriptional elongation (Desmoucelles et al., 2002).

To further test the possible involvement of the SWR-C in Htz1 functioning, we turned to microarray analysis of gene expression. Meneghini et al. (2003) showed that deleting *HTZ1* reduces the expression of 84 genes by more than 1.7-fold and that many Htz1-activated genes cluster near telomeres (see insert in Figure 4). Interestingly, groups of Htz1-activated genes form 18 small, local clusters (ranging from 3–17 genes), termed HZADs (for Htz1-activated domains). For comparison purposes, we carried out a microarray experiment with a strain harboring a deletion of *SWR1* and found that 112 genes had mRNA levels reduced more than 1.7-fold (raw microarray data on our website, http://www.utoronto.ca/greenblattlab/swrcmicroarraydata). Of these 112 genes, only 32 (or 28%) are contained within the 18 HZADs (see website), suggesting that the SWR-C may also have one or more roles that is independent of Htz1. Furthermore, there was a significant enrichment of Swr1-activated genes near telomeres (Figure 4), which is not due to an increase in overall gene density near telomeres (Meneghini et al., 2003). Htz1 contributes to telomeric silencing (Dhillon and Kamakaka, 2000), protects genes located near silent telomeric regions and flanking the HMR silent mating cassette from Sir2-dependent silencing, and inhibits heterochromatin formation (Dhillon and Kamakaka, 2000; Meneghini et al., 2003). Because Htz1 and the SWR-C are required for activating overlapping sets of genes and have similar genetic interactions, it is probable that the SWR-C participates in demarcating heterochromatin boundaries. Mutations in *BDF1* also

(and standard deviation) of the no-ORF band for each sample is represented. This demonstrates that the various chromatin preparations are equivalent in concentration (see the input samples) and that the immunoprecipitation background for each sample is similar (see the αRpb3 samples).

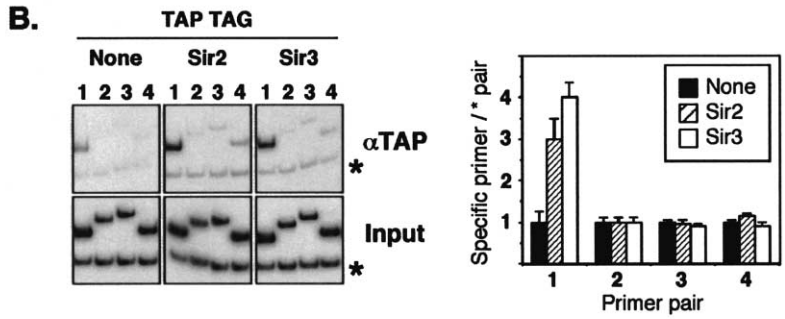
(E) ChIP analysis at the *GAL1* gene. Chromatin samples from the indicated strains were analyzed after each indicated precipitation. Strains were grown on YP medium with glucose or galactose as indicated. Growth on the former represses *GAL* genes, while the latter induces them. There is a significant reduction in histone occupancy (both H2A and Htz1) on induction of *GAL1*. Loss of the SWR complex (represented by *swc6*Δ) leads to loss of Htz1 recruitment, but does not affect Rpb3 crosslinking.

(F) A schematic of the *GAL1* locus is shown with the location of the ChIP primer pairs 1–4 indicated above. Quantitation data are expressed as the intensity of each band relative to the * control. The occupancy of the nonvariant histones (represented by H2A) and Htz1 at *GAL1* are strongly reduced on inducing (Gal) conditions. Similar profiles are seen for histones H2B and H3 (not shown). We presume that the exclusion of histones from *GAL1* on induction is due to reduced nucleosome occupancy on transcription, as they are also excluded from the promoters of *GAL2*, *GAL3*, *GAL7*, and *GAL10* on galactose addition (data not shown).



Gene Name	ORF #	mRNA /hr	Location
Y' element	-	N/A	1 - 6278
-	YEL077C	11.4	1 - 3834
-	YEL076C	1.2	4464 - 5114
-	YEL075C	0.7	5345 - 5713
-	YEL073C	ND	7230 - 7553
RMD6	YEL072W	0.2	13720 - 14415

Primer pair	Location
1	373 - 578
2	7699 - 7930
*	9716 - 9823
3	12161 - 12415
4	14075 - 14278



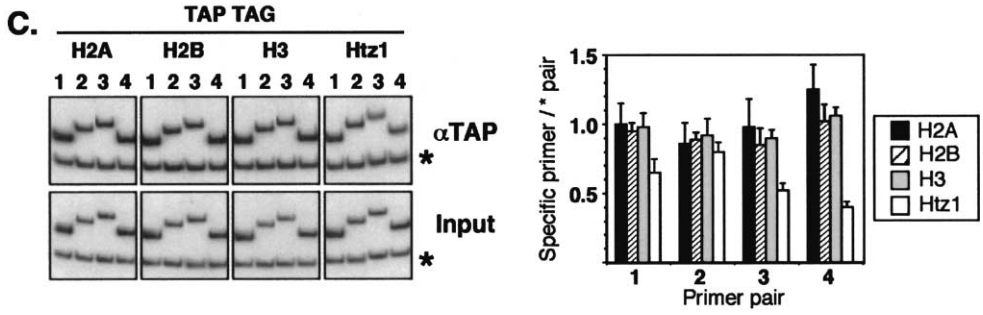


Figure 6. Htz1 Association at the Chromosome V Telomere Proximal Region

(A) A schematic of the Chr V telomere (TEL-V) is shown with the location of coding sequences, structural elements, and ChIP primer pairs depicted. * indicates a portion of this region we frequently use as a no-ORF control when analyzing transcriptionally active regions (Komarnitsky et al., 2000; Krogan et al., 2002). Transcriptional frequency of each gene (mRNA/hr) is from Holstege et al. (1998) (http://web.wi.mit.edu/young/expression/halflife.html).

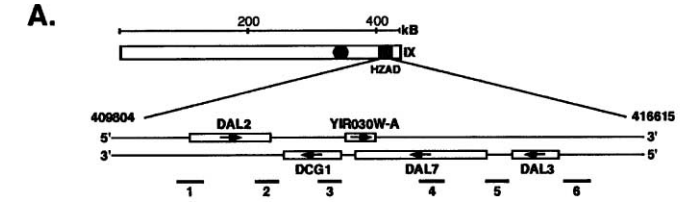
(B) ChIP analysis at this region. Chromatin samples from the indicated strains were analyzed after precipitation via the TAP tag. Bottom row is input, used to normalize the PCR amplification efficiency of each primer pair relative to the * pair (9716-9823) as described in Experimental Procedures. Sir2 and Sir3 crosslink solely to the extreme telomere, as previously reported. Quantitation of the αTAP ChIP panels is shown on the right. Data are presented as the ratio of each primer pair relative to the * pair.

(C) While occupancy of the nonvariant histones H2A, H2B, and H3 remains nearly constant, Htz1 crosslinking varies significantly, peaking at the * region. This observation supports previous work suggesting that Htz1 may act at boundary elements to prevent the spread of silent heterochromatin (Meneghini et al., 2003).

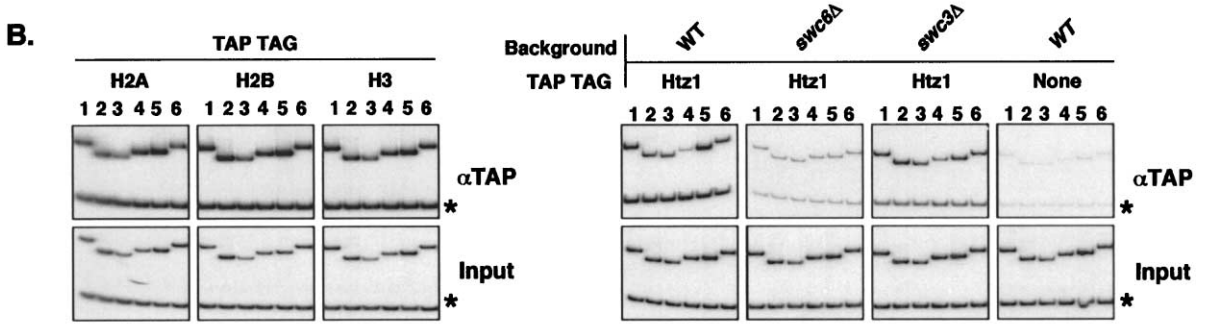
result in downregulation of genes located near telomeric heterochromatin (Ladurner et al., 2003), consistent with our observation that the SWR-C copurifies with Bdf1 (Figure 2).

Recruitment of Htz1 Relies on the SWR-C

To analyze the recruitment of Htz1 into chromatin, we used strains harboring TAP-tagged versions of Htz1 and carried out chromatin immunoprecipitation (ChIP) after



Gene	ORF #	mRNA/hr	Fold change Vs WT		
			*htz1*Δ	*swr1*Δ	*swc6*Δ
DAL2	YIR029W	4.7	-1.59	-1.65	-2.18
DCG1	YIR030C	0.1	-2.93	-1.94	-2.14
-	YIR030W-A	ND	ND	ND	ND
DAL7	YIR031C	0.3	-1.13	+1.02	-1.05
DAL3	YIR032C	0.2	-1.44	-3.26	-1.33



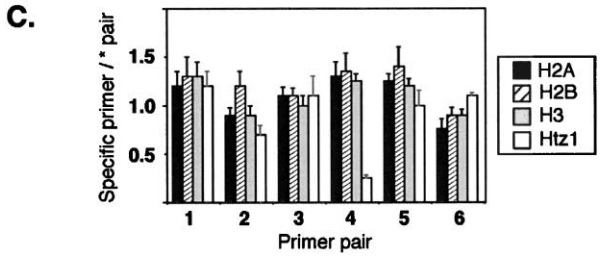


Figure 7. Htz1 Recruitment to an Htz1-Activated Domain

(A) An HZAD (Meneghini et al., 2003) was chosen for more detailed study. The schematic indicates the location of this region on chromosome IX. The region contains five open reading frames (see SGD; http://www.yeastgenome.org) and six ChIP primer pairs were designed as indicated. The size of each ORF and the locations of the ChIP primer pairs are drawn to scale. Microarray analysis of this HZAD is shown on the right. Transcriptional frequency of each gene (mRNA/hr) is from Holstege et al. (1998) (http://web.wi.mit.edu/young/expression/halflife.html). The observed relative expression levels compared to wild-type for each ORF in this HZAD in *htz1*Δ (Meneghini et al., 2003), *swr1*Δ (this work), and *swc6*Δ (this work) backgrounds are indicated. The results indicate that *DAL7* gene expression is not significantly perturbed by removal of Htz1 or the SWR-C in contrast to the ORFs that surround it.

(B) ChIP analysis on the indicated strains was performed as described. The occupancy of Htz1 and the nonvariant histones H2A, H2B, and H3 was examined. As in Figure 5, the loss of the SWR-C (represented by *swc6*Δ and *swc3*Δ) abrogates or reduces Htz1 recruitment at the HZAD.

(C) The relative occupancy of each histone throughout the HZAD is quantitated. While the crosslinking of the nonvariant histones (H2A, H2B, and H3) remains constant, Htz1 occupancy is significantly reduced at primer pair 4 within *DAL7*.

proteins were crosslinked in vivo to DNA using formaldehyde. Unlike strains with a deletion of *HTZ1*, such strains are not sensitive to 6-azauracil or mycophenolic acid (data not shown), suggesting that Htz1-TAP retains its normal function. Yeast cells were lysed and, following isolation and shearing of chromatin, the presence of Htz1 near specific DNA sequences was monitored by immunoprecipitating (IP) with IgG that binds to the protein A component of the TAP tag. After reversal of the crosslinks, the coprecipitated DNA was analyzed by PCR amplification with specific primer pairs. These were directed against the promoter regions, coding regions, and 3′ untranslated regions of *ADH1*, encoding alcohol dehydrogenase, and *PMA1*, encoding plasma membrane ATPase, as well as the inducible gene *GAL1*, encoding galactokinase (Figure 5). In each PCR reaction, primers directed against a nontranscribed region of chromosome five (Chr V, see Figure 6) were also present and are indicated by an asterisk. As a control for the integrity of each of the chromatin preparations, the crosslinking of RNAPII to various regions was also analyzed by using an antibody directed against Rpb3.

As previously observed by Santisteban et al. (2000), we found that Htz1 crosslinked more efficiently to the *GAL1* gene when it was repressed by glucose than when its expression was induced by growth in the presence of galactose (Figures 5E and 5F). Interestingly, the nonvariant histone H2A also crosslinked more efficiently when *GAL1* was repressed (Figures 5E and 5F). Although Htz1 was previously found to bind to the promoter region of the *GAL1* gene (Santisteban et al., 2000), we found that Htz1 occupancy is much higher beyond the polyadenylation/cleavage site relative to the promoter region of each gene we investigated, *ADH1*, *PMA1*, and *GAL1*

(Figure 5). In addition, Htz1 was strongly recruited to the control nontranscribed region of Chr V. In each case, precipitation from a strain containing no tag served as a negative control.

Importantly, deletion of *SWC6* almost completely abolished the recruitment of Htz1 to all these regions. A similar abrogation of Htz1 binding was observed for other components of the SWR-C, including *swc2*Δ, *arp6*Δ, the ATPase component *swr1*Δ and, to a lesser extent, *swc3*Δ (Figures 5A and 5B). The effects of the *swc6*Δ and *swc3*Δ mutations on the recruitment of Htz1 to the nontranscribed region on Chr V are substantial and were quantified in Figure 5D. These results cannot be explained by an effect of the SWR-C on the synthesis of Htz1, which Western blotting showed to be present at similar levels in wild-type and all SWR-C knockout strains (data not shown).

Because the nontranscribed region we used on Chr V as our "no-ORF" control in Figure 5 is located ≈10 kB from the telomere end, we designed four more primer sets that further encompass this area (Figure 6A). Using TAP-tagged versions of the silencing proteins Sir2 and Sir3, we demonstrated by ChIP that these proteins bind only very close to the telomere (Figure 6B). Interestingly, Htz1 binds not only to the subtelomeric region, but also near the telomere where Sir proteins are present (Figure 6C). The nonvariant histones H2A, H2B, and H3 are also found throughout this region (Figure 6C). It has been previously suggested that Htz1 acts as a barrier for the spread of hetereochromatin near telomeric regions (Meneghini et al., 2003). Because Htz1 is found throughout the region near the telomere of Chr V, including the area where histone deacetylation by Sir2 silences gene expression, some other protein must also be necessary to demarcate the barrier between hetereochromatin and euchromatin in this region.

The presence of Htz1 was also monitored along several genes in one of the HZADs where transcription is stimulated by Htz1 and Swr1 (Figure 7A). The recruitment of Htz1 to the *DAL2*, *DCG1*, and *DAL3* genes in the HZAD was also strongly affected by *swc6*Δ and only modestly affected by *swc3*Δ (Figure 7B). The genes in this region are not expressed strongly enough for ChIP to detect recruitment of RNAPII (data not shown). Although the amount of Htz1 bound to these genes does not correlate with their expression levels (compare Figures 7A and 7B), it is interesting that Htz1 is not strongly recruited to the *DAL7* gene (Figure 7B), the only gene in this region whose expression was not significantly affected by Htz1 or the SWR-C (Figure 7A). On the other hand, Htz1 was recruited to the *ADH1* gene and just downstream of the *PMA1* gene (Figures 5A and 5C), and the expression of these genes is not affected by the SWR-C (data not shown) or Htz1 (Meneghini et al., 2003). Therefore, Htz1 is recruited to both nontranscribed and transcribed regions and only sometimes affects transcription and so it is difficult for the moment to predict whether Htz1 and the SWR-C will affect gene expression in a transcribed region to which Htz1 is recruited. Wherever Htz1 is recruited, however, its recruitment depends on the SWR-C.

We conclude that the recruitment of the histone 2A variant Htz1 depends in vivo on a novel 13 subunit complex, the SWR-C, containing the Snf2-family ATPase Swr1. Involvement of the SWR-C and Htz1 in the same pathway was predicted by the similarity of the genetic interactions of *htz1*Δ and deletions of genes encoding nonessential subunits of the SWR-C, as well as by a physical association between the SWR-C and Htz1. We also suggest that the SWR-C could be recruited to chromatin by virtue of its physical interaction with Bdf1, which binds to the acetylated tails of histones H3 and H4 (Ladurner et al., 2003; Matangkasombut et al., 2000; Matangkasombut and Buratowski, 2003). This makes sense since similar sets of genes at heterochromatin/euchromatin boundaries are regulated by Htz1 (Meneghini et al., 2003), Bdf1 (Ladurner et al., 2003) and the SWR-C (this work). Htz1 and Bdf1 prevent the spread of Sir2-dependent silencing (Ladurner et al., 2003; Meneghini et al., 2003), and we predict that is also the case for the SWR-C.

Similar observations on the role of the SWR complex in the insertion of Htz1 into yeast chromatin have been made by the laboratories of Carl Wu, Jasper Rine, and Hiten Madhani. Their laboratories and ours have agreed on the nomenclature for the subunits of the SWR complex that is found in this paper.

Experimental Procedures

Purification of the SWR Complex

TAP-tagged components of the SWR complex were purified on IgG and calmodulin columns from extracts of yeast cells (3 liters) grown in YPD medium to an OD_{600} of 1.0–1.5 and analyzed by SDS-PAGE, MALDI-TOF mass spectrometry, and tandem mass spectrometry essentially as previously described (Krogan et al., 2002).

Chromatin Immunoprecipitations

ChIP assays using the TAP-tagged strains were performed essentially as described (Krogan et al., 2002). In most experiments yeast strains were grown in YPD (yeast extract-peptone-dextrose) medium at 30°C to an OD_{595} of 0.4 to 0.6 before processing. The exception was analysis of the *GAL* genes (Figures 5E and 5F), where cells were grown on YP-Raffinose prior to the addition of glucose or galactose (to 2%) as indicated. The antibody against Rpb3 was purchased from Neoclone. IgG agarose for the precipitation of TAP tagged proteins was from Sigma. After resolution by PAGE, PCR products were quantitated using a Fujix BAS 2040 PhosphoImager and allied Fuji *ImageGauge* software. The input sample was used to calculate the normalization value (NV) between each specific primer pair and the control "No-ORF" primer pair (*). This ratio compensates for any variation in PCR efficiency and label content by converting the signal from different primer pairs into normalized units of the control primer pair. This generates the corrected value (CV) for each specific primer pair in each immunoprecipitation. Finally each CV is divided by the No-ORF (*) signal from each immunoprecipitation to give a relative value (x-fold compared to the * internal control) that allows trend comparison across samples to be performed. All primer sequences are listed in Supplemental Data at http://www.molecule.org/cgi/content/full/12/6/1565/DC1.

RNA Isolation and Microarray Analysis

The isogenic wild-type, *swr1*Δ, and *swc6*Δ strains were grown in parallel in SC medium at 30°C and RNA was prepared and extracted for microarray analysis as previously described (Hughes et al., 2000).

Synthetic Genetic Array Analysis

SGA analysis was carried out as previously described (Tong et al., 2001). Automated analysis of the results was carried out by procedures that will be described elsewhere (H.D. et al., unpublished data). For random spore analysis, spores were inoculated into 3 ml of liquid haploid selection medium [synthetic dextrose (SD) medium lacking histidine and arginine but containing canavanine: SD - His/

Arg + canavanine] and incubated at 30°C for 2 days. The germinated *MAT***a** spore progeny were diluted in sterile H_2O and plated out on medium that selects for the query gene mutation [(SD/MSG) – His/Arg + canavanine/clonNAT], the deletion array mutation [(SD/MSG) – His/Arg + canavanine/G418], or both the query gene and deletion array mutations [(SD/MSG) – His/Arg + canavanine/clonNAT/G418], then incubated at 30°C for ~2 days. Colony growth under the three conditions was compared and the double mutants were scored.

Bdf1 Coimmunoprecipitations

4 l of each strain (Figure 2C) was grown to mid log phase ($OD_{600} \approx$ 1.0), centrifuged, and washed twice with 500 ml ice-cold H_2O. All manipulations from this point on were performed on ice at 4°C and all buffers contained protease inhibitors. The yeast pellet (≈5g/l) was resuspended in 2 ml Buffer A (200 mM Tris [pH 7.9], 390 mM NH_4SO_4, 10 mM $MgSO_4$, 1 mM EDTA, 20% Glycerol, and 2 mM DTT) per gram wet weight and subjected to glass bead disruption. After centrifuging at 5000 rpm for 10 min, the supernatant was collected and 1/7 Volume of 4 M potassium acetate (pH 7.6) was added dropwise. The resulting mix was rotated at 4°C for 30 min and successively centrifuged at 10,000 rpm for 20 min and then in a Beckman 45Ti rotor at 30,000 rpm for 60 min. The supernatant was dialyzed overnight against three 2 l changes of buffer B (50 mM HEPES [pH 8], 50 mM KOAc, 10 mM MgOAc, 1 mM EDTA, and 10% Glycerol), centrifuged in a Beckman 45Ti rotor at 30,000 rpm for 60 min, and frozen at –80°C in aliquots at 10 mg/ml. Thawed samples (3 mg/assay) were precleared for 2 hr with 10 μl protein A Sepharose and the supernatant transferred to a new tube. Samples were then incubated for 4 hr with rotation with 10 μl protein A Sepharose plus 1 μl polyclonal rabbit α-Bdf1 or preimmune serum (PIS). The complexes were collected by centrifugation, washed with buffer B, and eluted with 100 mM Glycine (pH 3). Eluates were neutralized with 1 M Tris (pH 8), resolved on 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Bdf1 or Peroxidase Anti-Peroxidase (Sigma) followed by enhanced chemiluminescence (ECL).

Acknowledgments

We thank C. J. Ingles for critically reading the manuscript, C. Wu for sharing unpublished data, and M. Grunstein for stimulating discussion. N.J.K. was supported by a PGS-B Scholarship Award from the Natural Sciences and Engineering Research Council of Canada (NSERC) and a Doctoral Fellowship from the Canadian Institutes of Health Research (CIHR). This research was supported by grants to J.F.G. from the Canadian Institutes of Health Research, the Ontario Genomics Institute, and the National Cancer Institute of Canada with funds from the Canadian Cancer Society, and to S.B. by grant GM46498 from the U.S. NIH. S.B. is a Scholar of the Leukemia and Lymphoma Society.

Received: September 16, 2003
Revised: December 2, 2003
Accepted: December 2, 2003
Published: December 18, 2003

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