Characterization of the Isw1a and Isw1b ATP-dependent chromatin remodeling complexes from the budding yeast, *Saccharomyces cerevisiae*

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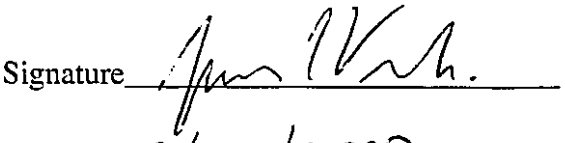
Signature 

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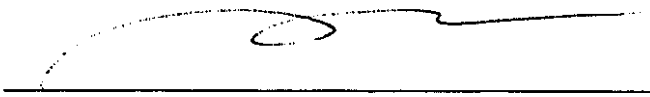

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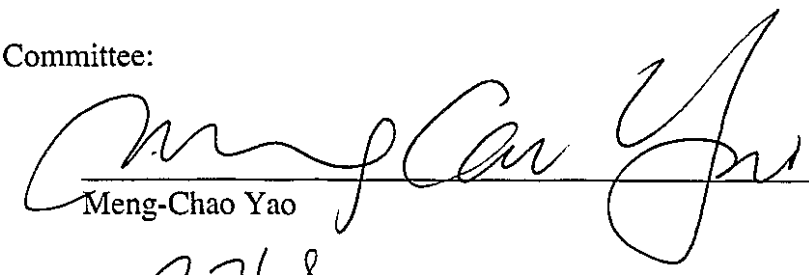

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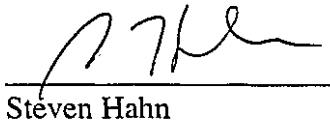

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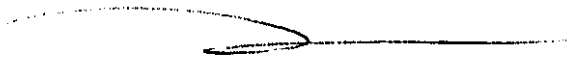

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Date: 7/28/2003

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Abstract

Characterization of the Isw1a and Isw1b ATP-dependent chromatin remodeling complexes from the budding yeast, *Saccharomyces cerevisiae*

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Members of the ISWI class of ATP-dependent chromatin remodeling complexes have been found in nearly every eukaryote studied, suggesting important functions that have been conserved throughout evolution. The budding yeast, *S. cerevisiae*, has two homologs of the conserved ISWI ATPase, Isw1p and Isw2p. Although these complexes share similar biochemical activities to remodel chromatin, their *in vivo* roles are unclear. Chromatographic separations and immunoprecipitations of Isw1p and its associated proteins revealed that Isw1p forms two distinct complexes *in vivo*. Each complex interacts with DNA and nucleosomes *in vitro*, and can reposition nucleosomes in an ATP-dependent manner. *In vivo*, these complexes have overlapping, yet distinct roles in transcriptional regulation, and each shows different genetic interactions with other genes. To identify factors that operate in parallel to these Isw1-containing complexes, deletions of the histone H3 and H4 N-terminal tails were made. *ISW1* genetically interacts with both histone mutants at elevated temperatures suggesting that a histone tail-dependent pathway operates in parallel to one or both Isw1 complexes. Mutational analysis of histone H4 revealed that *ISW1* interacts with the four acetylatable lysines on the H4 N-terminal tail, closely matching the substrate preference of the NuA4 histone acetyltransferase complex. Mutants of NuA4 components have *ISW1*-dependent phenotypes similar to the histone mutants at elevated temperatures and on media containing the drug rapamycin. The specificity of these interactions strongly suggests a role for TOR (*t*arget *o*f *r*apamycin)-signaling in a parallel pathway to one or both Isw1 complexes. The identification of the nature of this interaction will yield clues as to the functions of Isw1 complexes *in vivo* that may be extended to higher eukaryotes.

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Dedication

To Tillie

Chapter I: Introduction

Chromatin Structure

As eukaryotes have evolved larger and more complex genomes, they have required the co-evolution of enzymatic and structural mechanisms to physically organize large amounts of genetic material. For example, nearly every human cell packages approximately two meters of DNA into a nucleus with a diameter five to six orders of magnitude smaller. This extraordinary level of compaction must, however, be flexible and organized for such processes as replication, repair, mitotic and meiotic chromosome segregation, and transcription to occur throughout the genome. The nucleosome, the primary unit of this chromatin organization, was described over 30 years ago (79), and is composed of approximately 146 base pairs of DNA wrapped in two superhelical turns around an octamer of histone H2A, H2B, H3, and H4 proteins. A non-nucleosomal segment of linker DNA remains between each pair of nucleosomes, to a portion of which a single lysine-rich linker histone H1 protein (or H5 protein in some non-mammalian erythrocytes) may bind in higher eukaryotes. The combined nucleosomal and spacer DNA results in an average spacing of from ~165 base pairs of DNA per histone octamer in yeast, which lack an H1-type linker histone to ~200 base pairs in humans (and higher in some invertebrates) (124). This nucleosomal DNA may then fold into larger 30 nm chromatin fibers, which subsequently fold into more compacted structures themselves, again, and again. The extremely organized nature of this compaction is evident in mitotic chromosomes. Each displays a unique banding pattern that is faithfully reproduced from division to division, even in different individuals of the same species. However, these higher order structures of chromatin and their regulation are still largely undefined (for reviews see references (77, 131).

Histone Modifying Enzymes

Two groups of enzymatic activities have evolved in eukaryotes to remodel nucleosomes to regulate a variety of cellular processes. One such group, the histone modifying enzymes, covalently modifies histone proteins. Although these modifications have been found in the core of the histone proteins, the majority of known modifications are found on the basic N-terminal tails. The positively charged residues on these tails may interact with their associated negatively charged DNA within a nucleosome, thereby making the DNA inaccessible to many other factors. Allfrey originally proposed that modification of these basic residues by acetylation might help to neutralize their charge, creating more accessible DNA (7). Indeed, acetylation of lysine residues on these tails is generally associated with higher levels of transcription. In addition to acetylation, many other modifications have been identified on these tails, such as lysine or arginine methylation, serine phosphorylation, lysine ubiquitination, and ADP-ribosylation; however, the correlation between charge neutralization and transcriptional activation has not been found in all cases. For example, methylation of histone H3 lysine 4 is generally associated with transcriptional activation, while methylation of nearby lysine 9 is associated with transcriptionally silent regions, although each modification results in the same charge neutralization (15, 86, 99, 110). These discrepancies led Strahl and Allis to develop the "histone code hypothesis" (115). They propose that the specific pattern of covalent modification on a given histone may be recognized by other proteins to result in recruitment of enzymatic activities, thereby marking the associated DNA to a specific fate such as transcriptional activation. As predicted by this hypothesis, the ability of some proteins to interact with histone tails appears to be modification-sensitive. For example, the bromodomain motif that is found in a number of transcriptionally important proteins binds to histone tails, often in an acetylation-dependent manner (38, 67, 94). In addition, the heterochromatin-associated protein, HP1, utilizes its chromodomain to specifically bind histone H3 tails that are methylated at lysine 9 (12, 80).

ATP-dependent Chromatin Remodeling enzymes

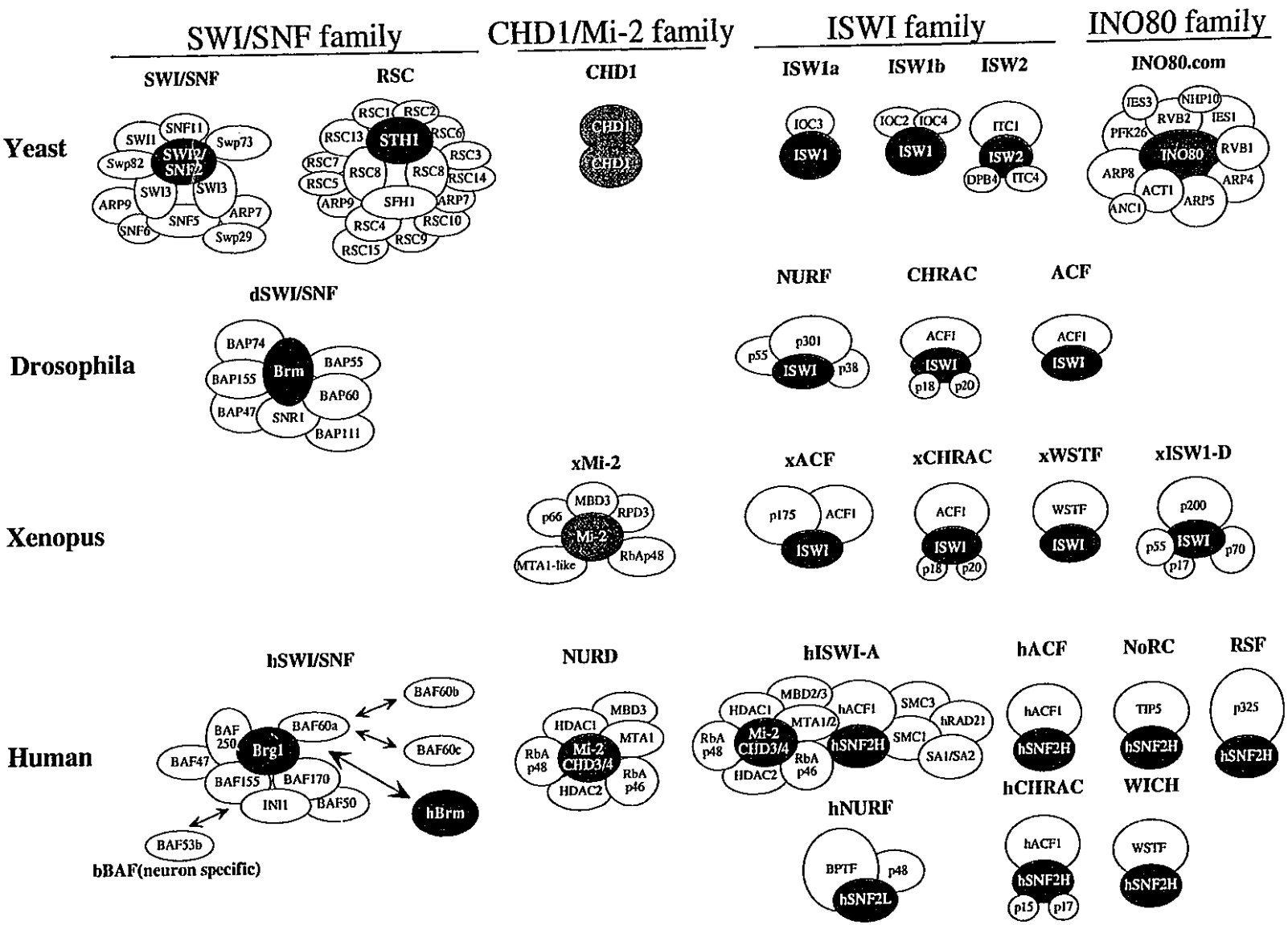
In contrast to the histone modifying enzymes, the ATP-dependent chromatin remodeling factors utilize the energy of ATP hydrolysis to reposition or alter the structure of nucleosomes to affect DNA accessibility. The first, and best characterized, of this group is the [Mating-type] *Swi*tch/*S*ucrose *N*on-*F*ermentor (SWI/SNF) complex from *S. cerevisiae*, a large (~1.1 MDa) complex consisting of at least 10 subunits including the ATPase, Swi2/Snf2p. Homologs of the SWI/SNF complex have been found in nearly all eukaryotes studied, with several members often present in the same organism (44, 45, 52, 102, 111). Three other classes of ATP-dependent chromatin remodeling complexes have also been identified and are classified by the sequence of their Swi2/Snf2p-like ATPase subunit: the *I*mitation-*Swi*tch (ISWI) class, the *C*hromodomain *H*elicase *D*NA-binding (CHD)/Mi-2 class, and the most recently identified INO80 class. Like the SWI/SNF class, members of the ISWI, CHD/Mi-2, and INO80 classes have also been found in a wide variety of eukaryotes, from yeast to human, suggesting that each class has important cellular functions that may have been evolutionarily conserved (see Figure 1; for review see reference (48).

Drosophila ISWI

The founding member of the ISWI class, *Drosophila Iswi*, was originally identified by sequence homology to *brahma*, a *SWI2/SNF2* homolog (45). Purification of this ISWI protein as a recombinant monomer revealed that it is indeed an ATP-dependent chromatin remodeling enzyme. It shows the ability to reposition nucleosomes in the presence of a transcription factor, and a nucleosome spacing activity (31). In addition, its ATPase activity is stimulated by nucleosomes, but not free DNA, suggesting that ISWI recognizes a specific structural feature present only in nucleosomal DNA (31). Consistent with this specificity, stimulation of ISWI ATPase activity is dependent upon the histone H4 tail, and that loss of the H4 tail prevents ISWI-mediated remodeling *in*

Figure 1: ATP-dependent chromatin remodeling complexes

Illustrated are the known ATP-dependent chromatin remodeling complexes identified as of June 2003 in the budding yeast, *Saccharomyces cerevisiae*; the fruit fly, *Drosophila melanogaster*; the African clawed frog, *Xenopus laevis*; and the human, *Homo sapiens*. The SWI/SNF, CHD/Mi-2, ISWI, and INO80 classes each have unique ATPases that are conserved in each class and are colored similarly in each complex shown.


SWI/SNF family
CHD1/Mi-2 family
ISWI family
INO80 family
SWI/SNF
RSC
CHD1
ISW1a
ISW1b
ISW2
INO80.com
Yeast
SWI1
SNF11
Swp73
SWI2/SNF2
Swp82
SWI3
ARP9
ARP7
SNF5
SNF6
Swp29
RSC1
RSC2
RSC13
STH1
RSC6
RSC3
RSC7
RSC8
RSC14
RSC5
SFH1
RSC4
RSC10
RSC15
RSC9
CHD1
IOC3
ISW1
IOC2
IOC4
ITC1
ISW2
DPB4
ITC4
IES3
NHP10
RVB2
IES1
PFK26
INO80
RVB1
ARP8
ARP4
ACT1
ARP5
ANC1
dSWI/SNF
NURF
CHRAC
ACF
Drosophila
BAP74
Brm
BAP55
BAP155
BAP60
BAP47
SNR1
BAP111
p55
p301
ISWI
p38
ACF1
ISWI
p18
p20
ACF1
ISWI
xMi-2
xACF
xCHRAC
xWSTF
xISW1-D
Xenopus
p66
MBD3
RPD3
Mi-2
MTA1-like
RbAp48
p175
ACF1
ISWI
ACF1
ISWI
p18
p20
WSTF
ISWI
p200
p55
ISWI
p70
p17
hSWI/SNF
NURD
hISWI-A
hACF
NoRC
RSF
Human
BAF60b
BAF60a
BAF250
Brg1
BAF60c
BAF47
BAF155
BAF170
INI1
BAF50
hBrm
BAF53b
bBAF(neuron specific)
HDAC1
MBD3
MTA1
RbA p48
Mi-2 CHD3/4
RbA p46
HDAC2
MBD2/3
HDAC1
hACF1
SMC3
MTA1/2
hRAD21
RbA p48
Mi-2 CHD3/4
hSNF2H
SMC1
SA1/SA2
RbA p46
HDAC2
hACF1
hSNF2H
TIP5
hSNF2H
p325
hSNF2H
hCHRAC
WICH
hNURF
BPTF
p48
hSNF2L
hACF1
hSNF2H
p15
p17
WSTF
hSNF2H

vitro (26, 60). Interestingly, exchange of this H4 tail to another histone protein (H3 or H2A) renders nucleosomes incapable of stimulating ISWI activity, suggesting that ISWI recognizes this tail only in its correct nucleosomal context (27). Deletion and mutation analysis of this tail showed that an RHR motif at residues 17-19 is absolutely required for ISWI ATPase, sliding, and spacing activities; and acetylation of the nearby lysines 12 or 16 can diminish this ATPase activity, suggesting that ISWI recognizes a defined protein-DNA "epitope" (27, 29, 60). It has been proposed that these residues form an α-helical structure with DNA as the tail exits the nucleosome (11, 42, 68); however, nucleosome crystal structures show these residues interacting with an adjacent nucleosome, contacting either its H2A-H2B dimer surface (*Xenopus* nucleosome (89)) or its DNA (Yeast nucleosome (130)). It is unclear if this is an artifact of crystal packing, however, as the position of only one of the two H4 tails in each structure can be resolved. It is possible that ISWI interacts with the histone tail through a C-terminal SANT domain (*S*WI3, *A*DA2, *N*-CoR, and *T*FIIIB B") that is found in many ISWI homologs. This domain interacts functionally with histone tails in other SANT-containing proteins (20), and the SANT domain of N-CoR interacts directly with histone tails in a modification-insensitive manner (119).

The *Drosophila* ISWI ATPase is shared among three complexes, the *Nu*cleosome *R*emodeling *F*actor (NURF), the *Chr*omatin *A*ccessibility *C*omplex (CHRAC), and the *A*TP-dependent *C*hromatin-assembly and Remodeling *F*actor (ACF), each of which utilizes the enzymatic activity of ISWI; however, these complexes can also exhibit ISWI-dependent activities that are different than ISWI monomer. In addition, the specific activities of these complexes are significantly higher than monomer alone, highlighting the importance of the ISWI-associated proteins in each complex (31, 61, 66, 82, 132).

Drosophila NURF complex

The first of these complexes to be identified was the NURF complex, consisting of ISWI, NURF301, NURF55 (identical to dCAF1 p55, a WD-repeat protein homologous

to RbAp48), and NURF38 (an inorganic pyrophosphatase) (50, 92, 120, 123, 132). NURF was purified by chromatographic separation of its activity to disrupt chromatin structure at the *hsp70* promoter in the presence of the GAGA transcription factor, suggesting a role for NURF in transcriptional activation (122). In support of this, NURF was shown to facilitate transcription in the presence of a Gal4-HSF fusion (95), and the binding of Gal4 can affect NURF-mediated nucleosome positioning (72). Although the precise mechanism by which these nucleosomes are repositioned remains unclear, NURF and other ISWI-containing complexes appear to slide nucleosomes in *cis*, as histones do not appear to be lost during remodeling, nor are they transferred to competing DNA in *trans* (61, 72, 82).

ISWI is expressed in all cells of early *Drosophila* embryos and is an extremely abundant protein, estimated at 100,000 copies/cell (120). As *Iswi* is also conserved throughout eukaryotes, it might be expected that ISWI proteins would have critical *in vivo* functions. Indeed, there are number of phenotypes associated with loss of ISWI function in *Drosophila*, which can be phenocopied by *nurf301* mutants, suggesting that the NURF-dependent ISWI activity is requireed (10, 37). Loss of the NURF complex is incompatible with life as homozygous mutants die at late larval or early pupal stages of development, likely coinciding with depletion of maternal pools of NURF. The expression of two homeotic genes, *engrailed* (*en*) and *Ultrabithorax* (*Ubx*), are both significantly reduced in *iswi* and *nurf301* mutants suggesting that the NURF complex is required in transcriptional activation of some homeotic genes (10, 37). In fact, *nurf301* mutants enhance *bithorax* (*bx*) mutant phenotypes (10). In contrast to these apparent roles of NURF in transcriptional activation, polytene staining reveals mutually exclusive localization of RNA polymerase II and ISWI proteins, suggesting a role for ISWI at transcriptionally inactive loci (37). NURF may also play a role in hematopoietic development as *iswi* and *nurf301* mutants display a high incidence of neoplastic transformation of larval blood cells (10).

The NURF complex may also have more global effects on chromatin structure. A role in dosage compensation and/or higher order chromosome structure at the *Drosophila*

male X chromosome is suggested by data showing that both *isw1* and *nurf301* mutants display short, broad X chromosomes in males only (10, 37). *Drosophila* males have only a single X chromosome, while females have two. To compensate for the resulting gene dosage difference, X-linked genes in males are transcribed at twice the levels found in females. This relative overexpression in males is highly regulated and requires the dosage compensation complex, containing *M*ale-specific *l*ethal-1 (MSL1), MSL2, MSL3, *M*ale*l*ess (MLE), and the histone acetyltransferase *M*ales-absent *o*n the *f*irst (MOF). The acetylation of histone H4 lysine 16 by MOF is required for this compensation (103). Interestingly, expression of MSL2 in female *iswi* mutants phenocopies the male X defect. In addition, overexpression of MOF in males or females enhances a small eye phenotype observed by overexpressing a dominant negative ISWI protein in the eye-antennal imaginal disc (29). This suggests that the dosage compensation complex and/or lysine 16 acetylation may antagonize ISWI function. This is consistent with the previously described work showing that acetylation of lysine 12 or lysine 16 of the histone H4 tail prevents stimulation of ISWI ATPase activity (27, 29).

Drosophila ACF and CHRAC complexes

Although no *in vivo* functions have yet been ascribed to them, the ACF and CHRAC complexes have a number of biochemical activities that suggest they may have important roles as well. Both the ACF and CHRAC complexes are composed of ISWI and ACF1; in addition, the CHRAC complex contains two small histone-fold proteins, CHRAC-14 and -16 (30, 40, 64, 66, 125). In contrast to the NURF complex, both ACF and CHRAC complexes show the ability to create regularly spaced nucleosomes *in vitro* (64, 125). In addition, the ATPase activity of the CHRAC complex can be stimulated by naked as well as nucleosomal DNA, unlike monomer ISWI or NURF complex which are not efficiently stimulated by naked DNA alone (31, 122, 125). These differences illustrate the importance of the ISWI-associated proteins in modifying the activity of the ISWI ATPase. The role of these other subunits is clearly demonstrated by a nucleosome

sliding assay in which a remodeling complex is incubated with mononucleosomes that can occupy several positions along a piece of DNA. In such an assay, ISWI monomer alone slides a mononucleosome to the DNA ends, while intact NURF, CHRAC, or ACF complex slide it toward the middle (40, 61, 82, 132).

ACF1 is a protein with several sequence motifs commonly found in transcription factors and chromatin-associated proteins: two *P*lant *H*omeo*d*omain (PHD) fingers, a bromodomain, a WAC (*W*STF, *A*CF1, *c*bp146) motif, and a WACZ (*W*STF, *A*CF1, *c*bp146, ZK783.4) motif (66). The largest subunit of the NURF complex, NURF301, has a remarkably similar arrangement of these same domains, and it also contains an N-terminal HMGA domain, which is necessary for biochemical activity of NURF (132). Proteins bearing similar motifs to those of ACF1 are associated with ISWI homologs in human and *Xenopus* cells, as well as one of the *S. cerevisiae* ISWI homologs, Isw2p (17, 21, 51, 57, 84, 104, 116). The high degree of evolutionary conservation of ISWI complex components suggests important roles for these subunits in ISWI-mediated activity.

Mammalian ISWI

In addition to these ACF-like complexes, however, several unique ISWI-containing complexes have been found in other eukaryotes as well. These discreet complexes often share or compete for a common catalytic ISWI ATPase. For example, mammals have two ISWI homologs, *SNF2H*, and *SNF2L* (4, 45). The hSNF2H homolog forms at least 6 complexes. It forms an ACF like complex (17, 84), and a CHRAC like complex (104). The human hAcf1 protein (identical to BAZ1A and WCRF180) is a homolog of the *W*illiams *S*yndrome *T*ranscription *F*actor (WSTF), a protein encoded by a gene deleted in the developmental disorder Williams-Beuren Syndrome, a contiguous gene deletion syndrome. WSTF also associates with hSNF2H to form the *W*STF-*I*SWI *Ch*romatin (WICH) chromatin remodeling complex (21). A screen for proteins interacting with an RNA polymerase I transcription termination factor identified *T*TF-I

*I*nteracting *P*rotein-5 (TIP5), yet another homolog of ACF1. Purification of TIP5 showed that it is found in a novel complex, termed the *N*ucle*o*lar *R*emodeling *C*omplex (NoRC), with hSNF2H and possibly two additional uncharacterized proteins (116). These four complexes all can mobilize nucleosomes *in vitro*, similar to their homologs in *Drosophila*, suggesting some conserved functions. In addition to these ACF-like complexes, hSNF2H also associates with a 325 kDa protein to form the two-subunit *R*emodeling and *S*pacing *F*actor (RSF), which facilitates transcriptional initiation from chromatin templates *in vitro* in an activator-dependent manner (85). Hakimi, *et al.* purified a much larger hSNF2H complex of ~1500-2000 kDa (currently unnamed, but labeled as "hISWI-A" in Figure 1) and found that it contained hAcf1 in addition to the *Nu*cleosome *R*emodeling and *D*eacetylase (NuRD) complex, and components of the cohesin complex. Chromatin immunoprecipitation showed that this cohesin-containing complex associates with unmethylated Alu repeats and the ATPase activity of hSNF2H is required for this association (59). Interestingly, two other groups have found that ISWI-containing complexes from humans and *Xenopus laevis* associate with mitotic chromosomes, although it is unclear if cohesin components are associated with ISWI in these studies (21, 90).

Another mammalian ISWI homolog, hSNF2L, has only recently begun to be characterized. Purification of hSNF2L reveals that it forms a 3-subunit complex with BPTF (a NURF301 homolog) and a 48 kDa protein, suggesting that it may be a human NURF homolog (58). Staining of SNF2L in mice is limited to the cerebellum and hippocampus, and SNF2L is required for neurite growth (58, 83). Both BPTF and SNF2L associate with the promoters of *Engrailed*-1 & -2 near the binding site of β-catenin, and preliminary evidence suggests that SNF2L coactivates β-catenin transcription, indicating a possible role of this complex in the Wnt-signaling transcriptional cascade (58). Overexpression of a dominant negative version of xISWI in *X. laevis* embryos often results in early embryonic lethality. Of the few that survive to later embryonic stages, however, a variety of defects are observed in neural tube structure

and/or proper neural tube closure, again suggesting that ISWI homologs may have critical roles in neuronal development in vertebrates (39).

Yeast Isw2 complex

The budding yeast, *Saccharomyces cerevisiae*, encodes two homologs of the ISWI ATPase, Isw1p and Isw2p. Purification of each revealed that Isw1p associates with three other proteins, while Isw2p associates with the WAC domain-containing protein, Itc1p. Both Isw1 and Isw2-containing complexes show nucleosome-stimulated ATPase activity, as well as the ability to create an evenly spaced array of nucleosomes (121).

The mechanisms of action of these ISWI complexes, as well as those in other eukaryotes, are still largely unclear. The Isw2 complex interacts with either naked or nucleosomal DNA *in vitro* in an ATP-independent manner, suggesting that its nucleosome-stimulated catalytic activity is utilized at a step subsequent to substrate binding (51). By utilizing a photochemical cross-linking technique, Kassabov, *et al.* showed that Isw2-mediated remodeling results in a canonical nucleosome in which the histone is repositioned to a new sequence, yet the DNA path in the nucleosome is identical to the unremodeled nucleosome. This suggests that Isw2-remodeled chromatin is not inherently different from unremodeled chromatin, *i.e.* the nucleosomes are structurally intact. Consistent with these findings in yeast, other work has confirmed that DNA accessibility is not altered after remodeling by SNF2H (46). In addition, Isw2 requires at least 23 base pairs of DNA flanking the mononucleosome for remodeling to occur, suggesting that it may require non-nucleosomal linker DNA for binding and/or catalytic function (73).

In vivo, the Isw2 complex acts in a parallel pathway with the Rpd3-Sin3 histone deacetylase complex to repress transcription. A number of early meiosis specific genes have an *u*pstream *r*epressive *s*equence element (URS1), to which the Ume6p transcription factor binds. In haploids cells, the expression of these genes is repressed, in part, by Ume6-mediated recruitment of the Rpd3-Sin3 complex. Deletion of the genes encoding

Rpd3p or Sin3p results is associated with increased acetylation and a partial loss of repression of these genes in haploid cells (70, 71). Mutation of *ISW2* results in changes in nucleosome positions at these genes, which is associated with a partial loss of repression; however, an *isw2rpd3* mutant exhibits a nearly complete loss of repression, suggesting that these two complexes act in parallel to repress these Ume6-dependent genes (53). DNA microarray analysis revealed that in addition to acting in parallel in the repression of early meiosis specific genes, the Isw2 and Rpd3-Sin3 complexes also act in parallel at a number of loci throughout the genome, many of which do not contain the URS1 binding site for Ume6p (47). Consistent with a role in transcriptional repression, Isw2 complex has the ability to displace the Gal4-VP16 activator during remodeling *in vitro* (73).

Yeast Isw1 complexes

Purification of Isw1p from yeast revealed a four-subunit complex that, like Isw2 complex, exhibits a nucleosome-stimulated ATPase activity, as well as a robust nucleosome spacing activity. The Isw1-containing complex also showed the ability to disrupt chromatin at a promoter in the presence of the GAGA transcription factor, suggesting a role in transcriptional regulation (121). Consistent with this, both *isw1* and *isw2* mutants show changes in gene expression as assayed by DNA microarray analysis (63). Isw1p has been shown to physically interact with the Cbf1p transcription factor, and both are required for transcriptional repression at the *PHO8* promoter (96). *ISW1* has also been shown to be required for silencing of a *TRP1* reporter gene ectopically located at the *HMR* locus (35). Isw1-dependent remodeling of chromatin at gene promoters has been observed at a number of genes, some of which show mild derepression in *isw1* mutants (75). However, other evidence suggests that Isw1 complex may play a role in transcriptional activation. Chromatin immunoprecipitation shows that Isw1p colocalizes with trimethylated lysine 4 of histone H3 at *MET16*. Further analysis of *MET16* has

shown that Isw1-dependent activation requires the ATPase activity of Isw1p, as well as Set1p-dependent methylation of H3 lysine 4 (110).

There is also emerging evidence that the Isw1 complex may be involved in transcriptional elongation and/or termination. *CHD1* of the CHD/Mi-2 class of ATP-dependent chromatin remodeling complexes is required for proper transcriptional termination at the *GAL10* locus. The defect observed in a *chd1* mutant is enhanced in an *isw1isw2chd1* mutant, suggesting a redundant role for at least one of these ISWI complexes (5). Unpublished work by the same group has shown that *isw1* single mutants show a similar defect in the same assay, and Isw1p localization is detected at the 3' end of the open reading frame when the gene is active. Mutation of *isw1* suppresses the sensitivity of *spt4* and *dst1* mutants to 6-azauracil, suggesting a role in transcriptional elongation. Indeed, phosphorylation of serine 2 and 5 on the CTD of RNA Polymerase II, which are normally associated with transcriptional initiation and elongation, respectively, are not detectable in *isw1* mutants (98).

Thesis description

The thesis project described herein investigates the Isw1-containing complexes in *S. cerevisiae*. Much of the work in Chapters II and III has been previously published (126). Chapter II describes purification of the Isw1-containing complexes. Isw1p co-purifies with three other proteins of 110, 105, and 74 kDa sizes (121). These associated proteins were identified, and found to associate with Isw1p to form two distinct complexes *in vivo*, termed Isw1a and Isw1b. Chromatographic separation and co-immunoprecipitation were used to show that these two complexes are separable and share only the Isw1 enzymatic subunit in common. Chapter III describes the initial characterization of these two complexes, *in vitro* and *in vivo*. These two Isw1 complexes both exhibit equivalent nucleosome-stimulated ATPase activity, suggesting that each complex is purified in its active form. However, the two complexes differ in their abilities to interact with DNA and nucleosomes and have different specific activities to

alter chromatin structure *in vitro*. DNA microarray analysis and phenotypic characterization revealed that the two Isw1 complexes have overlapping, but distinct functions *in vivo*. Chapter IV describes approaches to identify the *in vivo* functions of the Isw1-containing complexes. Expression analysis was used to investigate genetic interactions of the *ISW1* gene and genes encoding components of chromatin modifying enzymes. Mutations of the histone H3 and H4 N-terminal tails were also found to interact with mutations of the Isw1 components. Targeted mutational analysis of the histone H4 tail showed that *ISW1* interacts with all four lysines of this tail that are substrates for acetylation. The possibility that this interaction occurs due to loss of a NuA4 histone acetyltransferase-dependent pathway that operates in parallel to Isw1 activity is explored.

Chapter II: Identification of Isw1a and Isw1b complexes

Introduction

Isw1p was previously shown to co-purify with three proteins, p110, p105, and p74. All four proteins comigrate in a Superose 6 size exclusion column at around 240 kDa, and coelute at the same salt concentration during ion exchange chromatography, suggesting that they form a single four-subunit complex (Toshio Tsukiyama, unpublished observations, and reference (121). To identify these three proteins, each subunit was subjected to mass spectrometric analysis. The sequences of the 110 kDa, 105 kDa, and 74 kDa proteins were encoded by previously uncharacterized open reading frames, identified as *YLR095c*, *YFR013w*, and *YMR044w*, respectively (Yeast Protein Database (32, 33). However, it was unclear what function each subunit contributes to a larger Isw1-containing complex.

This chapter describes the characterization of the Isw1-associated proteins and the Isw1 complexes that each forms. I describe the sequence analysis of these three proteins in order to gain clues as to what their roles may be *in vivo*. In addition, several lines of evidence suggested that there are two separable Isw1-containing complexes *in vivo*, either in addition to or in place of the single 4-subunit complex originally described. First, the observed size of the Isw1 complex by size exclusion chromatography (~240 kDa) is significantly smaller than predicted by the masses of its four component proteins (~370 kDa). Although the shape of a protein complex may alter its mobility during chromatography, resulting in an erroneous estimation of its mass, it is also possible that Isw1p forms two smaller complexes that are not separable in the conditions tested. Second, during elution from ionic exchange chromatography, the stoichiometry of the four proteins changes slightly in the off-peak fractions, which is consistent with overlapping elution profiles of two independent complexes. Third, loss of any individual subunit had little effect on the nucleosome-stimulated ATPase activity of purified Isw1 complexes, while activity of the Isw1 monomer is reduced by more than ten-fold

(described in Chapter III). One possibility to explain this finding is that two of the subunits are redundant for their ability to stimulate that ATPase activity of Isw1p in the presence of nucleosomes. However, it is also possible that there are two enzymatically active complexes, and deletion of any single Isw1-associated protein will disrupt only one of these, leaving another active complex intact. This chapter describes the verification of the latter possibility: identification of two Isw1 complexes by immunoprecipitation of individual Isw1 subunits as well as partial chromatographic separation of the complexes from whole cell extract. The two complexes have also been purified separately to near homogeneity, and their components identified.

Results

The Isw1-interacting proteins, Ioc2p, Ioc3p, and Ioc4p are previously uncharacterized

The three Isw1-associated proteins were previously identified by mass spectrometric analysis as the uncharacterized open reading frames, *YLR095c*, *YFR013w*, and *YMR044w* (Toshio Tsukiyama, unpublished results). We have designated them as I*swi* O*ne* C*omplex* (*IOC*) 2, 3, and 4, respectively (Figure 2A) (Yeast Protein Database (32, 33)). Ioc2p has a putative PHD finger domain between amino acids 557-659, a domain found in many chromatin-associated proteins. PHD fingers typically have seven universally conserved cysteine residues and one histidine residue (C_4HC_3), which are thought to coordinate two zinc ions (23). While Ioc2p has a high degree of conservation around the central four conserved residues, the homology to other PHD fingers is lost in the N-terminal region, and separated by 70 residues (12 to 46 residues is the normal range) from the C-terminal region (see alignment in Figure 2B). It is unclear if this represents a recent degeneration of an ancestral PHD finger structure, or if this region of Ioc2p is an atypical PHD-like domain. A truncated PHD finger is also found in human Peregrin (hBR140), though this protein also contains a separate intact PHD finger (Figure 2B, hBR140-a & b). Ioc2p also has homology to human *A*ndrogen *R*eceptor (hAR) near

its C-terminal end, though this region has a poly-glutamine tract that accounts for much of this homology. The function of this region of hAR is unclear, but mutations within it are associated with prostate cancer (55).

Ioc3p has significant homology (27%) to a protein involved in silencing, Esc8p ((35) and Yeast Protein Database (32, 33), yet has no identifiable conserved motifs. Ioc4p is a glutamic acid-rich protein with a PWWP domain between amino acids 4-76. The PWWP domain is named for a motif containing two moderately conserved proline and tryptophan residues followed by two highly conserved tryptophan and proline residues, although the larger domain encompasses many other conserved residues flanking this motif. The PWWP domain is found in a number of proteins involved in transcriptional and chromatin regulation, such as the putative *de novo DNA methyltransferase 3* (Dnmt3); the nuclear co-repressor, Adenovirus E1A Binding Protein (BS69); and the $TAF_{II}250$ homolog, Peregrin (BR140) (Figure 2B). The PWWP domain of murine Dnmt3b can bind DNA *in vitro*, and mutations of this domain within Dnmt3a prevent its heterochromatin association (105). In addition to PWWP homology, Ioc4p also has overall homology to PWWP-containing *S. cerevisiae* and *S. pombe* proteins, Ylr455p and SPBC29A3.13 (24% identity for both), respectively (Yeast Protein Database (32, 33)). In addition to Isw1p associated with Ioc proteins, a significant amount of Isw1p was purified as an apparent monomer (Figure 2A, lane 2) from both *wild-type* and *ioc* mutants using FLAG-affinity and Bio-Rex cation exchange chromatography (see Chapter V: Materials & Methods).

Isw1p forms two distinct complexes *in vivo*

In order to reveal interactions among Isw1 complex subunits, Isw1p was immunoprecipitated from strains bearing deletions of the *IOC2*, *IOC3*, or *IOC4* genes (Figure 2A, lanes 3, 4, and 5). When Isw1p was immunoprecipitated from each deletion strain, the corresponding protein was absent from the complex, confirming the identities of the subunits. However, in an *ioc4* strain we also found that Ioc2p failed to co-

Figure 2: Identification of the proteins associated with Isw1p

A) Isw1-2xFLAGp (Isw1-2FLp) was purified from strain (YTT449, lane 1—see Table I), or congenic strains bearing *ioc2* (YTT512, lane 3), *ioc3* (YTT610, lane 4), or *ioc4* (YTT548, lane 5) mutations. Isw1-2FLp monomer was also purified from all of these strains (Isw1-2FLp from *ioc4* deletion strain YTT548 shown, lane 2).

B) Protein motifs for Ioc2p, Ioc3p, and Ioc4p were determined by database searching. Residue positions for each motif are shown. Ioc2p has a PHD finger-like motif and limited homology to human Androgen Receptor in its C-terminal region. Alignment to other PHD finger-containing proteins is shown (The core PHD cysteine and histidine residues are shown in black with white type, while other conserved residues are shown in grey), as is alignment to the human Androgen Receptor homology region. Ioc3p has no recognized sequence and/or structural motifs. Ioc4p is a glutamate-rich protein with a PWWP domain near its N-terminus. Alignment to other PWWP domains is shown. Residues conserved in >80% of aligned sequences are shown in black with white type if identical, or grey if similar (NCBI Conserved Domain database (8) and Blocks database (62)). Accession numbers and complete names for the aligned proteins are shown in Table II.

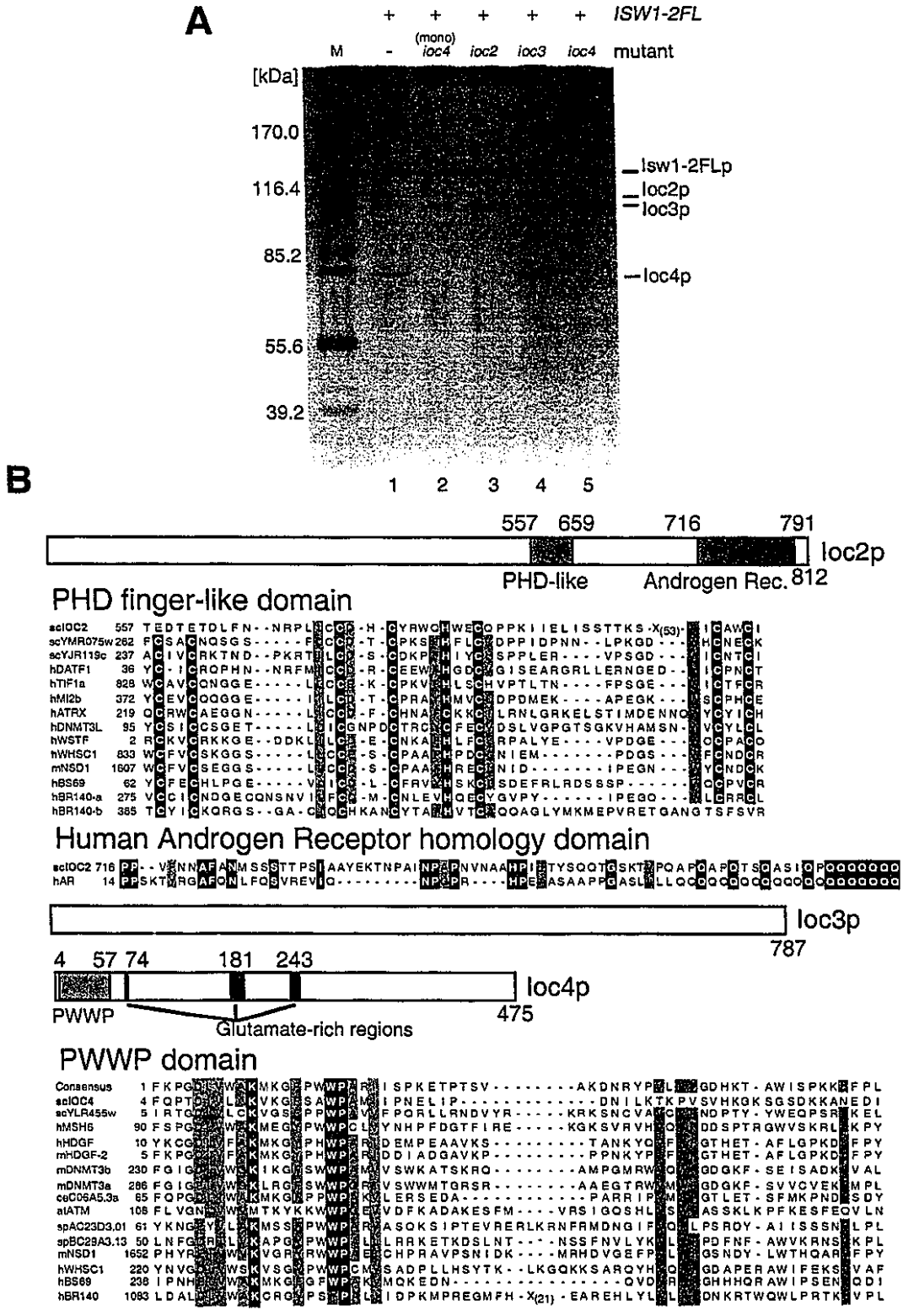

A
+ + + + + ISW1-2FL
M - (mono) ioc4 ioc2 ioc3 ioc4 mutant
[kDa]
170.0
116.4
85.2
55.6
39.2
Isw1-2FLp
Ioc2p
Ioc3p
Ioc4p
1 2 3 4 5
B
557 659 716 791
Ioc2p
PHD-like
Androgen Rec.
812
PHD finger-like domain
Human Androgen Receptor homology domain
Ioc3p
787
4 57 74 181 243
Ioc4p
PWWP
Glutamate-rich regions
475
PWWP domain

Table I: *S. cerevisiae* strains used in this dissertation

Strain[a]	Genotype
W303-1A	MATa; *ade2-1*; *can1-100*; *his3-11,15*; *leu2-3,112*; *trp1-1*; *ura3-1* (W303 background)
W1588-4C	MATa; same as W303-1A, but *RAD5*
YTT187	MATα; *isw1::ADE2*
YTT199	MATa; *isw1::ADE2*; *isw2::LEU2*
YTT219	MATa; *isw2::HisG*
YTT227	MATa; *isw1::ADE2*; *isw2::LEU2*; *chd1::TRP1*
YTT396	MATα; *pep4::HIS3*
YTT441	MATa; *isw1::kanMX*
YTT442	same as YTT441
YTT443	MATa; *isw1::kanMX*; *isw2::HISG*
YTT449	MATα; *ISW1-3'2xFLAG* (*ISW1-2FL*); *pep4::HIS3*
YTT512	MATa; *ISW1-2FL*; *ioc2::kanMX*; *pep4::HIS3*
YTT522	MATa; *ioc2::kanMX*; *isw2::LEU2*; *chd1::TRP1*
YTT548	MATa; *ISW1-2FL*; *ioc4::kanMX*; *pep4::HIS3*
YTT610	MATα; *ISW1-2FL*; *ioc3::HIS3*; *pep4::HIS3*
YTT642	MATa; *ISW1-2FL*; *IOC2-3MYC*; *IOC3-3HA*
YTT693	MATa; *hda1::kanMX*
YTT694	MATa; *rpd3::kanMX*
YTT695	MATa; *hos1::kanMX*
YTT696	MATa; *hos2::kanMX*
YTT697	MATa; *hos3::kanMX*
YTT698	MATa; *isw1::ADE2*; *isw2::LEU2*; *hda1::kanMX*
YTT699	MATa; *isw1::ADE2*; *isw2::LEU2*; *rpd3::kanMX*
YTT700	MATa; *isw1::ADE2*; *isw2::LEU2*; *hos1::kanMX*
YTT701	MATa; *isw1::ADE2*; *isw2::LEU2*; *hos2::kanMX*
YTT702	MATa; *isw1::ADE2*; *isw2::LEU2*; *hos3::kanMX*
YTT705	MATα; *isw1::ADE2*; *rpd3::kanMX*
YTT717	MATa; *isw2::HisG*; *rpd3::kanMX*
YTT728	MATα; *isw1::ADE2*; *isw2::HisG*; *rpd3::kanMX*
YTT823	MATa; *ioc2::kanMX*
YTT825	MATa; *ioc3::natMX*
YTT829	MATa; *ioc2::kanMX*; *ioc3::natMX*
YTT853	MATa; *ioc3::natMX*; *isw2::LEU2*; *chd1::TRP1*
YTT855	MATa; *ioc4::hphMX*; *isw2::LEU2*; *chd1::TRP1*
YTT861	MATa; *ioc2::kanMX*; *ioc3::natMX*; *isw2::LEU2*; *chd1::TRP1*
YTT1167	MATα; *IOC2-TAP::TRP1*; *pep4::HIS3*
YTT1168	MATα; *IOC3-TAP::TRP1*; *pep4::HIS3*
YTT1970	MATα; *hht1hhf1::hphMX*; *hht2hhf2::natMX*; pRM200U (*CEN4/ARS1/URA3/HHT2-HHF2*)
YTT1971	MATα; *isw1::kanMX*; *hht1hhf1::hphMX*; *hht2hhf2::natMX*; pRM200U (*CEN4/ARS1/URA3/HHT2-HHF2*)
YTT2329	MATa; *eaf1::natMX*
YTT2331	MATa; *isw1::kanMX*; *eaf1::natMX*
YTT2267	MATa; *isw1-K227R-3'-3XFLAG-kanMX* (*isw1-K227R-kanMX*)
YTT2333	MATa; *isw1-K227R-kanMX*; *eaf1::natMX*
YTT2256	MATa; *yng2::natMX*
YTT2258	MATa; *isw1::kanMX*; *yng2::natMX*
YTT2323	MATa; *esa1::natMX*; pLP795 (*CEN/ARS/URA3/ESA1*)
YTT2325	MATa; *isw1::kanMX*; *esa1::natMX*; pLP795 (*CEN/ARS/URA3/ESA1*)
YTT2343	MATα; *isw1-K227R-kanMX*; *hht1hhf1::hphMX*; *hht2hhf2::natMX*; pRM200U (*CEN4/ARS1/URA3/HHT2-HHF2*)
YTT2360	MATa; *isw1-K227R-kanMX*; *esa1::natMX*; pLP795 (*CEN/ARS/URA3/ESA1*)
UCC6583[b]	MATα; *ade2::HISG*; *his3-200*; *leu2Δ0*; *lys2Δ0*; *met15Δ0*; *trp1-63*; *ura3Δ0*; *hht1hhf1::LEU2*; *hht2hhf2::MET15*; *ADE2-VR* (S288C background); pRM200U (*CEN4/ARS1/URA3/HHT2-HHF2*)
YTT1475	MATα; *isw2::kanMX*; otherwise congenic to UCC6583
YTT1479	MATα; *isw1::kanMX*; *isw2::hphMX*; otherwise congenic to UCC6583
YTT1543	MATα; *isw1::kanMX*; otherwise congenic to UCC6583
140[c]	MATa; *met15Δ0* (S288C background)
139[c]	MATa; *leu2Δ0*; *met15Δ0*; *eaf3*; otherwise congenic to 140
YTT2359	MATa; *leu2Δ0*; *met15Δ0*; *isw1::kanMX*; otherwise congenic to 140
83[c]	MATa; *met15Δ0*; *eaf3*; *isw1*; otherwise congenic to 140
133[c]	MATa; *met15Δ0*; *eaf3*; *isw2*; otherwise congenic to 140
540[d]	MATa; *his3Δ0*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *rad52::kanMX* (S288C background)

[a] All YTT strains are derived from W1588-4C, except YTT1543, YTT1475, YTT1479, and YTT2359
[b] a gift of Dan Gottschling
[c] a gift of Jacques Côté
[d] from the Research Genetics MATa haploid deletion set

Table II: Sequences used for alignment

Protein[a]	Accession #	Full protein name
atATM	gi:7529272	Ataxia-Telangiectasia Mutated protein
ceC06A5.3a	gi:14573766	
hAR	gi:113830	Androgen Receptor
hARTX	gi:6274548	α-Thalassemia/mental Retardation X-linked Syndrome
hBR140	gi:4757865	Peregrin/BRPF1
hBS69	gi:10719919	Adenovirus 5 E1A-binding protein (BS69 protein)
hDATF1	gi:2224607	Death Associated Transcription Factor
hDNMT3L	gi:7019367	DNA MethylTransferase 3-Like protein
hHDGF	gi:1708157	Hepatoma-Derived Growth Factor
hMl2b	gi:5921744	CHD4 Chromodomain Helicase DNA-binding protein 4
hMSH6	gi:4504190	MutS Homolog 6
hTIF1a	gi:12746552	Transcription Intermediary Factor 1a
hWHSC1	gi:4378019	Wolf-Hirschhorn Syndrome Critical region protein
hWSTF	gi:4049922	Williams Syndrome Transcription Factor
mDNMT3a	gi:6681209	DNA MethylTransferase 3A
mDNMT3b	gi:6753662	DNA MethylTransferase 3B
mHDGF-2	gi:6680201	Hepatoma Derived Growth Factor, related protein 2
mNSD1	gi:6679138	Nuclear receptor-binding SET-Domain protein 1
scYJR119c	gi:1352920	YJ89
scYLR455w	gi:6323488	
scYMR075w	gi:2497139	YMW5
spAC23D3.01	gi:2130333	
spBC29A3.13	gi:3006149	

[a] Abbreviations: at, *Arabidopsis thaliana*; ce, *Caenorhabditis elegans*; h, *Homo sapiens*; m, *Mus musculus*; sp, *Schizosaccharomyces pombe*; sc, *Saccharomyces cerevisiae*

immunoprecipitate with Isw1p. Conversely, Ioc4p did not co-immunoprecipitate with Isw1p in an *ioc2* strain. This suggests that Ioc2p and Ioc4p are mutually dependent for their stable association with Isw1p.

We hypothesized that the complexes purified from *ioc3* strains (composed of Isw1p, Ioc2p and Ioc4p) and those purified from either *ioc2* or *ioc4* strains (composed of Isw1p and Ioc3p) might represent two distinct and separable complexes, which normally coexist in *wild-type* strains. We constructed a strain (YTT642) that bears unique epitope tags on Isw1p, Ioc2p, and Ioc3p, to directly test this hypothesis. Whole cell extract was made from this strain and subjected to size exclusion chromatography. The presence of Isw1p, Ioc2p, and Ioc3p was detected by western blotting and the relative levels of each protein across the collected fractions were determined. As shown in Figure 3A, the elution profiles of Isw1p, Ioc2p, and Ioc3p overlap significantly. Ioc3p elutes in a single peak in which Isw1p is also detected (fraction 5); however, Ioc2p reproducibly forms two distinct peaks (fraction 3 and 7) that are out of phase with the Ioc3p peak. It is probable that one such population represents its associated Isw1 complex, while the other may represent Ioc2p in either monomeric form, or in another as yet unidentified complex. Isw1p was detected in all of the fractions in which Ioc2p and Ioc3p are detected; however, the peak of Isw1p (fraction 8) is smaller in molecular mass than any of the Ioc2p and Ioc3p peaks (fractions 3 and 7, fraction 5, respectively). This suggests the presence of an Isw1p population that is not associated with Ioc2p or Ioc3p; therefore, this likely represents a monomer form of Isw1p.

The distinct elution profiles of Ioc2p and Ioc3p are consistent with their presence in two separable complexes, however the extensive overlap of each profile does not rule out a single 4-subunit Isw1 complex. In order to more rigorously separate these complexes, we utilized the distinct epitope tags in this extract to immunoprecipitate each protein individually. As shown in Figure 3B (lanes 3-5), immunoprecipitation of Isw1p co-precipitated Ioc2p and Ioc3p, as observed previously (Figure 2A and reference (121). Although Isw1p was nearly immunodepleted from the whole cell extract, a significant portion of Ioc2p and Ioc3p remained in the supernatant. This suggests that some of these

proteins may exist as either monomers or in another complex *in vivo*; however, it is also possible that their associations with Isw1p became unstable during immunoprecipitation. Anti-Myc antibodies were used to immunoprecipitate Ioc2p. As expected, Isw1p was co-precipitated; however, no detectable Ioc3p was precipitated (Figure 3B, lanes 6-8). Conversely, immunoprecipitation of Ioc3p with anti-HA antibodies pulled down Isw1p, but not Ioc2p (Figure 3B, lanes 9-11). This is consistent with our hypothesis that Isw1p forms two distinct complexes *in vivo*.

In order to independently verify these results, as well as to identify what other proteins, if any, Ioc2p and Ioc3p associate *in vivo*, we used the *T*andem *A*ffinity *P*urification (TAP) method to purify each protein separately. TAP-tagged strains were created for each protein, as described (107). TAP purification from whole cell extract of mid-log phase cells resulted in highly purified complexes containing the tagged protein. Purification from either mid-log phase or saturated cultures yielded identical complexes (data not shown). Ioc2p-TAP co-purified two other proteins in approximately stoichiometric amounts (Figure 3C). These proteins were subsequently identified by mass spectrometry as Isw1p and Ioc4p (data not shown). Notably, there was no band corresponding to Ioc3p. Ioc3p-TAP co-purified one other protein at near stoichiometric amounts, which was subsequently identified as Isw1p by mass spectrometry (data not shown). Ioc2p and Ioc4p were notably absent from this purified complex, as were any other proteins with which Ioc3p might associate. A control purification from an untagged strain (YTT396) had no detectable proteins by silver staining (data not shown).

We saw no evidence by the approaches above that Isw1p forms a single complex with all three Ioc proteins. As a result, we conclude that there are two stable complexes containing Isw1p that coexist within yeast. We have designated the Isw1p-Ioc3p and Isw1p-Ioc2p-Ioc4p complexes as Isw1a and Isw1b, respectively. This nomenclature will be used throughout the remainder of this dissertation.

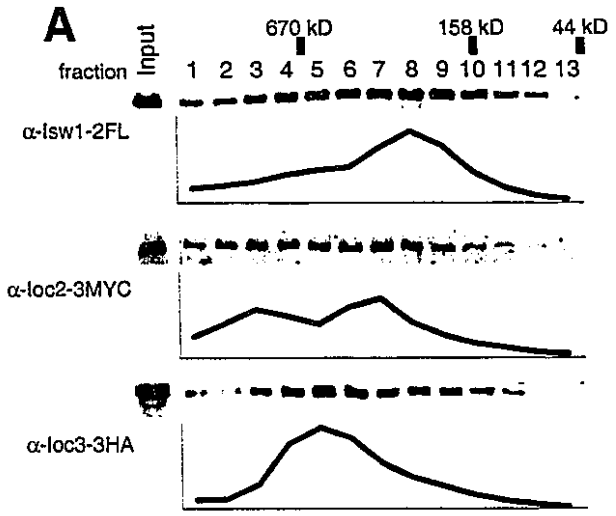
TAP purification of the Isw1b complex followed by mass spectrometry also revealed an association of all four core histones. Using dilutions of known quantities of Isw1b and recombinant histone octamer, a stoichiometric ratio of approximately 1:9

Figure 3: Isw1p is found in two distinct complexes in vivo

A) Whole cell extract from strain YTT642 was separated by size exclusion chromatography. Isw1p, Ioc2p, and Ioc3p were detected by western blotting using α-FLAG, α-MYC, or α-HA antibodies, respectively. The relative amounts of protein in each fraction were quantified and the results were averaged across three identically loaded western gels for each antibody to generate the elution profiles shown. The positions of size standards run in identical conditions are indicated. Note that the protein levels shown are in arbitrary units and are relative to the other fractions for each antibody/protein pair only; therefore, direct comparison of the relative levels of different proteins is not possible.

B) Immunoprecipitation of YTT642 whole cell extract was done using α-FLAG (lanes 3-5), α-MYC (lanes 6-8), or α-HA (lanes 9-11) resin, or resin alone as a control (lanes 12-14). The presence of Isw1p, Ioc2p, or Ioc3p in each experiment was determined by separate western blots of identically loaded 8% SDS-PAGE gels. Starting material was loaded at either 1x (undiluted) or at 1/4x dilutions (lanes 1 & 2) and undiluted unbound (U) samples from each IP were loaded for comparison (lanes 3, 6, 9, & 12). The immunoprecipitate was eluted in SDS-PAGE sample buffer, and appropriate volumes were loaded to correspond to either 4x or 1x concentrations of the starting material (lanes 4-5, 7-8, 10-11, 13-14), as indicated.

C) Isw1-2FLp was purified from strain YTT449, as before, and loaded in lane 1 of an 8% SDS-PAGE gel. Ioc2-TAPp and Ioc3-TAPp were purified using the Tandem Affinity Purification (TAP) method from strains YTT1167 and YTT1168, respectively. The most concentrated eluates from each purification were loaded into lanes 2 and 3, respectively. The proteins were visualized by silver-stain. Note that the 2x FLAG epitope on Isw1p that adds ~2 kDa to this protein is in lane 1 only. In addition, the Calmodulin Binding peptide that remains after TAP purification adds ~5.1 kDa to Ioc2p in lane 2 or to Ioc3p in lane 3.


A
Input
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158 kD
44 kD
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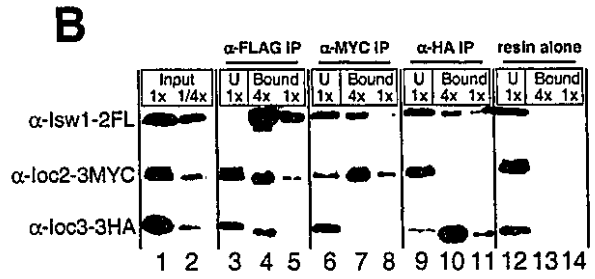
α-Ioc2-3MYC
α-Ioc3-3HA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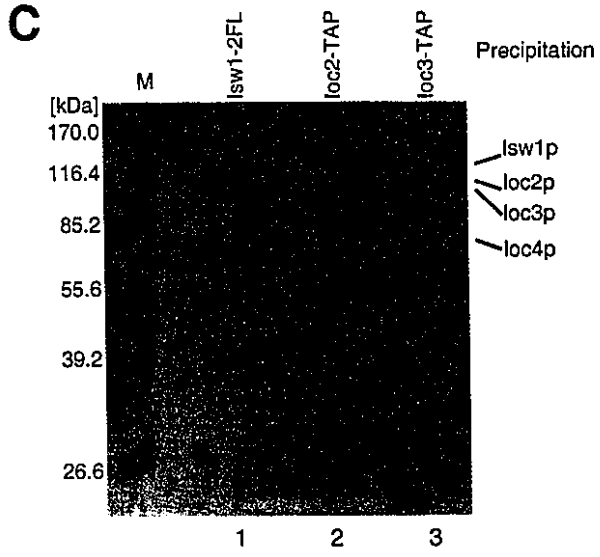
α-FLAG IP
α-MYC IP
α-HA IP
resin alone
Input 1x 1/4x
U 1x
Bound 4x 1x
α-Isw1-2FL
α-Ioc2-3MYC
α-Ioc3-3HA
1 2 3 4 5 6 7 8 9 10 11 12 13 14


C
M
Isw1-2FL
Ioc2-TAP
Ioc3-TAP
Precipitation
[kDa]
170.0
116.4
85.2
55.6
39.2
26.6
Isw1p
Ioc2p
Ioc3p
Ioc4p
1 2 3

(Isw1b:octamer, data not shown) was estimated by silver-stained SDS-PAGE gel. Purification of Ioc3p-TAP or purification of Isw1p in an *ioc2* mutant both failed to copurify histones, suggesting that this interaction is specific to the Isw1b complex. However, purification of Isw1p in an *ioc4* mutant does result in copurification of histone proteins, suggesting that Ioc4p is not required in the Isw1b complex for this interaction. At this time, it remains unclear if this interaction is direct, or if it is mediated indirectly through the coincidental association of each complex with DNA. DNA can be detected in the final TAP eluates of Ioc2p, suggesting that it could be mediating such an interaction (data not shown). Ethidium bromide (EtBr) has been shown to disrupt protein-DNA interactions of sequence specific DNA-binding proteins, thereby providing a means to distinguish these two possibilities (81). Addition of EtBr to 50 μg/ml prior to immunoprecipitation did not disrupt the interaction, suggesting that it is not mediated through DNA (data not shown). As neither Isw1b nor histone octamer has sequence-specific DNA-binding activity, however, it is not clear how to interpret this negative result. To test for a DNA requirement directly, DNAse and MNase were added during TAP purification of Ioc2p following TEV cleavage, but prior to the final Calmodulin affinity precipitation. Octamer was still detectable by silver-stained SDS-PAGE, but the ratio of histone proteins to Isw1b proteins was significantly reduced (data not shown). The abundance of all proteins was markedly reduced following nuclease treatment, however, suggesting that a protease contaminant is present in one of the preparations. There are three possibilities regarding this potential Isw1b interaction with histone octamer. First, Isw1b can interact directly with several histone octamers, but these histones were particularly sensitive to proteases present during nuclease treatment. Second, Isw1b can interact directly with only a single histone octamer, but several more are copurified by their association with the same chromatin fragment. Following nuclease treatment, these "contaminating" histones are released, leaving only a single Isw1b-bound histone. Third, Isw1b binds DNA in close proximity to a histone, thereby preventing nuclease accessibility and subsequent release of this histone. Antibodies to acetylated histone H4 lysine 5 strongly bind to Isw1b-associated histone H4 by western,

but not to recombinant histones, suggesting that H4 K5 acetylation is enriched in this population. It is intriguing to speculate that the Isw1b associated histones may bear particular modifications that are required for this interaction. Technical limitations primarily due to low substrate concentrations have prevented the characterization of other possible histone tail modifications in this population by western or mass spectrometry.

Discussion

I have shown that yeast Isw1p associates with the previously uncharacterized proteins Ioc2p, Ioc3p, and Ioc4p to form two distinct Isw1 complexes *in vivo*. By utilizing epitope tags on individual Ioc proteins, each complex can be purified to near homogeneity without any evidence of its sibling, showing that these are not subsets of a larger complex. Recently, Ito, *et al.*, found that Ioc2p interacts with Ioc3p in a high-throughput two-hybrid screen (65). As we have seen no evidence of this interaction in our work, this may have resulted from endogenous Isw1p acting as a "bridge" between the overexpressed fusion proteins of Ioc2p and Ioc3p in the screen. If this were the case, it would suggest that Isw1p associates with each protein via unique interaction domains. It is possible, however, that unstable interactions between these complexes may occur, or that direct association of Ioc2p and Ioc3p may exist under growth conditions different from what we have tested.

None of the Ioc proteins have similarity to each other, nor do they have overall homology to any other proteins for which a function has been described. However, Ioc2p has a putative PHD finger motif, and Ioc4p has a PWWP motif; these domains are found in a number of proteins involved in chromatin or transcriptional regulation and have been proposed to be involved in protein-protein interaction. Several proteins in higher eukaryotes such as mNSD1, hBR140, hWHSC1, and hBS69 contain both of these domains, suggesting that these domains might be cooperatively required for some cellular process(es). It is possible that in yeast, this requirement is fulfilled by the proximity of each separate protein within the Isw1b complex. Ioc3p does not have any known

conserved motifs, yet it is required for the strong DNA and nucleosome interactions of the Isw1a complex. This suggests that Ioc3p may contain as yet unidentified DNA and/or chromatin association domains.

Given that a significant portion of Isw1p is purified as a monomer in *wild-type* as well as *ioc* mutant strains, it is possible that Isw1p monomer may represent a third *in vivo* form of the protein. Although the purification of this monomer could result from disruption of an intact Isw1 complex during immunoprecipitation, it is likely that Isw1p monomer exists *in vivo* for several reasons. When whole cell extract is applied at 0.2 *M* KCl to a Bio-Rex 70 cation exchange column prior to α-FLAG immunoprecipitation, Isw1p monomer is found in the flow-through fraction, while intact Isw1 complexes are retained in the column matrix (data not shown). In addition, the size exclusion chromatography data (Figure 2A) suggests that Isw1p monomer may be the most abundant form of Isw1p within the cell. While other groups have shown that *Drosophila* ISWI monomer exhibits enzymatic activity *in vitro*, this monomer has much lower specific activities than the complete complexes (31, 40, 61, 132).

An Isw1 complex-independent form was also suggested for Ioc2p. Size exclusion chromatography reveals that Ioc2p elutes in two apparently distinct populations of similar abundance, consistent with either Ioc2 monomer or a separate Ioc2-containing complex. TAP purification of Ioc2p failed to yield any non-Isw1b complex proteins near stoichiometric levels, so we conclude that either Ioc2p also is present as a monomer, or in another complex that is unstable during TAP purification.

The presence of two Isw1p-containing yeast chromatin remodeling complexes may parallel the situation found in higher eukaryotes. *Drosophila*, *Xenopus*, and humans all express several forms of ISWI complexes, which share a common ATPase subunit, yet have distinct activities *in vitro*, and may exhibit unique functions *in vivo*.

Chapter III: Biochemical and *in vivo* characterization of the Isw1a and Isw1b complexes

Introduction

The Isw1p protein from the budding yeast, *S. cerevisiae*, forms two separable complexes *in vivo*, designated Isw1a and Isw1b, and it may also exist as a monomer as well. As many higher eukaryotes also express distinct ISWI-containing complexes which must share or compete for a common ISWI ATPase subunit, a greater understanding of these two Isw1 complexes *in vitro* and *in vivo* may yield clues as to how ISWI complexes function in more complex organisms.

This chapter describes the biochemical characterization of these two complexes, as well as the effect of deletion of individual components of these complexes *in vivo*. A mixture of the Isw1a and Isw1b complexes (purified by immunoprecipitation of their common Isw1p subunit) shows a number of biochemical activities. For example, this mixture has an intrinsic ATPase activity that is stimulated by nucleosomes, but not histones or naked DNA in isolation. In addition, this mixture shows a strong nucleosome spacing activity that can space nucleosomes along DNA every 175 base pairs (121). Separately purified Isw1a or Isw1b complexes were tested for these same biochemical activities to determine if one or both complexes are responsible for conferring these activities to the mixture. In addition, in collaboration with Vamsi Gangaraju and Blaine Bartholomew at the Southern Illinois University, the individual complexes were tested for a nucleosome sliding activity that has been observed for Isw2 complex as well as ISWI complexes in higher eukaryotes (61, 73, 82). As will be described, this data prompted an analysis of the abilities of each complex to bind to their substrates *in vitro* as well.

While exploring the biochemical activities of these individual complexes is crucial to understanding their functions, it is unclear how these activities may be utilized within the cell. To gain further insight into their roles *in vivo*, individual *IOC* genes were

deleted to selectively inactivate each complex. The *ISW1* gene is known to interact genetically with *isw2chd1* mutants, so individual *ioc* mutants were made in this strain to look for contributions of either complex to this interaction (5, 121). In addition, the known role of Isw1 and other ISWI complexes in transcriptional regulation, prompted an analysis of the expression changes that occur in *isw1* mutants as compared to *ioc* mutants.

Results

Both Isw1a and Isw1b complexes show nucleosome-dependent ATPase activity

As it had previously been shown that a mixture of the two Isw1 complexes purified from a *wild-type* strain exhibited an ATPase activity that was stimulated by nucleosomes, but not by histones or DNA alone, the activities of the two Isw1p-containing complexes were compared (121). Approximately equimolar amounts of Isw1p monomer, Isw1a, or Isw1b complex (as determined by normalizing the Isw1p subunit in silver-stained SDS-PAGE gels) were assayed for ATPase activity in the presence or absence of nucleosomes. Both Isw1 complexes had similar nucleosome-stimulated ATPase activities, while the ATPase activity of Isw1 monomer was not stimulated by nucleosomes (Figure 4A). Using varying amounts of Isw1 complexes, these ATPase activities were confirmed to be within linear ranges (data not shown). This suggests that Ioc3p as well as Ioc2p and/or Ioc4p play essential roles in stimulating the ATPase activity of Isw1 complexes in a nucleosome-dependent manner. As complexes purified from either *ioc2* or *ioc4* strains had identical subunit compositions (Isw1p and Ioc3p only—see Figure 2A), it was expected that these complexes would have similar activities (Figure 4A, lanes 6-7 and 10-11). The slight differences observed were likely due to variability in quantitation of the complexes and/or experimental procedures.

The Isw1a complex exhibits greater specific activity in nucleosome spacing

To further compare these two complexes, different forms of Isw1 were tested for the ability to facilitate the regular spacing of nucleosomes. We used an *in vitro* system to assemble recombinant yeast histones onto naked DNA in the presence of yeast histone chaperone, Nap1p (51). In the absence of any exogenous spacing activity, Nap1p will randomly assemble nucleosomes along a DNA template. This array can then be digested with *m*icrococcal *nucle*ase (MNase), which preferentially cuts between nucleosomes. Digestion of such a randomly spaced array results in a population of differently sized fragments of DNA that appear as a smear when the purified DNA is run on an agarose gel (see Figure 4B, lanes 3-4). However, the addition of a mixture of the two Isw1 complexes and ATP produces a "nucleosome ladder" in which distinct bands are observed approximately every 175 bp (121). This is due to an ATP-dependent activity of Isw1 complex(es) that positions a majority of the assembled nucleosomes in a regularly spaced manner.

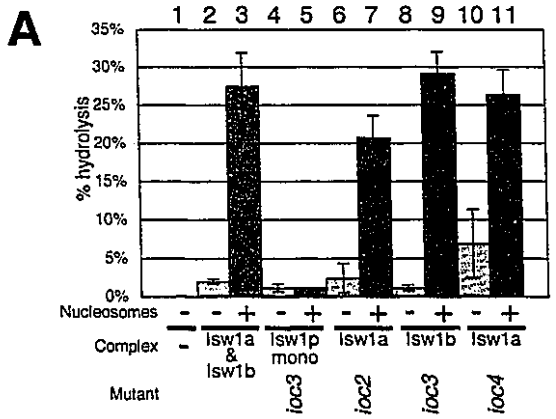
To determine whether this spacing activity depends upon one or both Isw1 complexes, each Isw1 complex was tested in such a nucleosome spacing assay. As previously shown, a clear nucleosome ladder was observed when the mixture of Isw1 complexes was used (Figure 4B, lanes 5-6). However, consistent with its ATPase activity, Isw1p monomer did not affect the MNase digestion pattern (compare Figure 4B, lanes 3-8). The Isw1a complex, purified from either *ioc2* or *ioc4* strains, exhibited nucleosome spacing activity (Figure 4B, lanes 9-10, and 13-14). The Isw1b complex also exhibits this activity, however, at equimolar amounts, the activity of the Isw1b complex (lanes 11-12) was less than that of the Isw1a complex. The qualitative difference between the two complexes was reproducible in several independent assays and is most noticeable after short MNase digestions (arrowheads, compare lanes 9 and 13 to lane 11) in which the "ladder" is visible much higher in the gel. This suggests that the Isw1a complex has greater specific activity and precision in spacing multiple nucleosomes as these higher bands represent long arrays of regularly spaced nucleosomes.

Figure 4: Isw1a and Isw1b complexes exhibit different specific activities in biochemical assays.

A) In vitro nucleosome-stimulated ATPase assays for each complex. Reconstituted nucleosomes (+, lanes 3,5,7,9,11) or buffer alone (-, lanes 1,2,4,6,8,10) were incubated with [γ-^{32}P]ATP and either no complex (lane 1), or 30 fmoles of Isw1 complexes in the form of: co-purified Isw1a and Isw1b complexes (lanes 2-3), Isw1p monomer (lanes 4-5), Isw1a complex from an *ioc2* strain (lanes 6-7) or *ioc4* strain (lanes 10-11), or Isw1b complex from an *ioc3* strain (lanes 8-9). The %ATP hydrolysis was determined by the [^{32}P]Pi/[γ-^{32}P]ATP ratio. The results shown are the average of three typical assays +/- 1 S.D.

B) Nucleosome spacing assays for each complex. Recombinant Nap1p (lanes 1-14) was allowed to assemble yeast histones (lanes 3-14) onto λ DNA in the presence of either no complex (lanes 1-4), or 30 fmoles of Isw1 complexes in the form of: co-purified Isw1a and Isw1b complex (lanes 5-6), Isw1p monomer (lanes 7-8), Isw1a complex from an *ioc2* strain (lanes 9-10) or *ioc4* strain (lanes 13-14), or Isw1b complex from an *ioc3* strain (lanes 11-12). After assembly, DNA was digested with Micrococcal nuclease (MNase) for 3 minutes or 15 minutes (odd and even lanes, respectively), purified, and run on 1.3% agarose and visualized with Ethidium Bromide.

C) Nucleosome sliding assays for Isw1a and Isw1b complexes. Recombinant octamer was assembled onto 5S rDNA (214 bp). The efficiency of sliding was checked with 48 fmoles labeled nucleosomes and either Isw1a (lanes 2 –5) or Isw1b (lanes 6-9) in the ratios shown and 400 μM ATP. Nucleosome positions prior to mobilization are labeled N1-N4. The asterisk (*) denotes a band that is specific for the Isw1a complex. It is not observed with DNA alone.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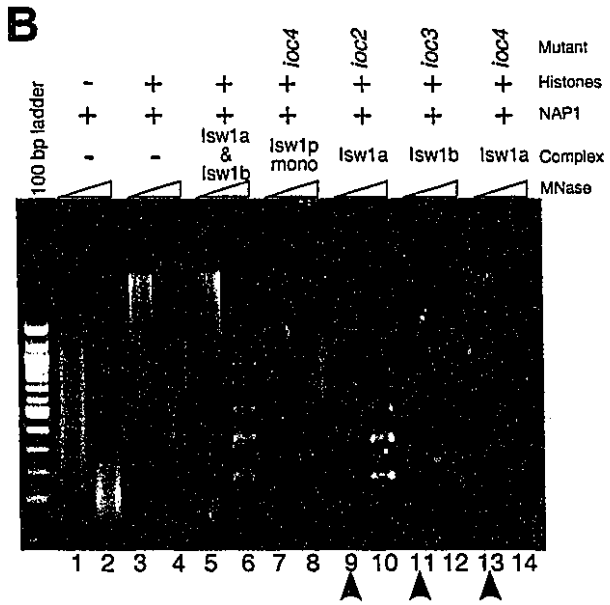
B
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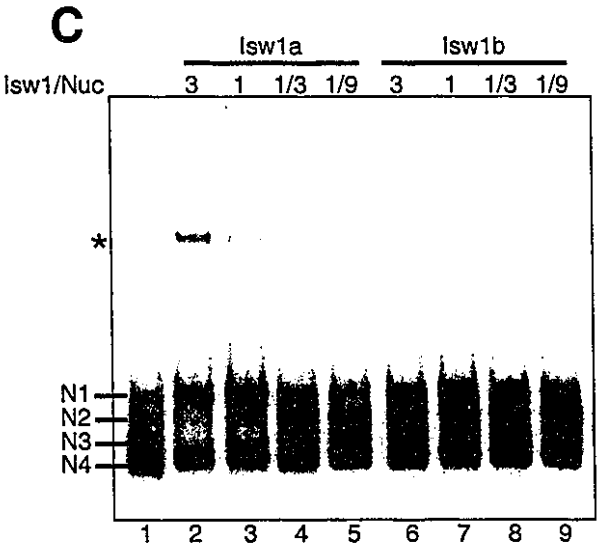
1 2 3 4 5 6 7 8 9 10 11 12 13 14


C
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1 2 3 4 5 6 7 8 9

The Isw1a complex exhibits greater specific activity in nucleosome sliding

Remodeling of nucleosomal arrays, as in the spacing assay, could occur by a sliding mechanism in which nucleosomes track along the DNA template. Several ATP-dependent chromatin remodeling complexes exhibit such sliding activity, often differing from each other by the direction in which nucleosomes are moved (*i.e.* whether they slide nucleosomes to the ends of a DNA fragment, or to the middle). The directionality of this activity can be modified by the ATPase-associated proteins. For example, *Drosophila* ISWI monomer slides histones to the DNA ends, while ISWI-containing NURF, ACF, and CHRAC complexes slide histones to a more central position (40, 61, 82, 132). To investigate which, if any, of the Isw1 complexes could mobilize nucleosomes, we collaborated with Blaine Bartholomew's group at Southern Illinois University, as they had developed a nucleosome sliding assay for Isw2 complex. They incubated our purified Isw1a or b complexes with yeast nucleosomes assembled by salt dialysis with recombinant histones and a 214 base-pair 5S rDNA fragment. In the absence of any Isw1 complex, nucleosomes adopted four separable positions that were designated as N1-N4 (Figure 4C, lane 1). Previous work has shown that DNA fragments with nucleosomes positioned at either end migrate more quickly (*e.g.* N4) than do those with centrally positioned nucleosomes (*e.g.* N1) (61, 82). Following incubation with increasing amounts of Isw1a complex, the N4 position remained unchanged, while nucleosomes positioned at N2 and N3 were reduced and the N1 population was increased (compare Figure 4C, lanes 1-5). This shows that the Isw1a complex exhibits a nucleosome mobilization activity, in agreement with the observed nucleosome spacing activity (Figure 4B). Interestingly, using equivalent amounts of Isw1b complex, relatively little change in positions was observed (Figure 4C, lanes 6-9), though at higher Isw1b:nucleosome ratios there may be a slight increase in the N1 population (compare lane 6 to lanes 1 and 9). We estimate that the Isw1b complex has a specific activity at least 9-fold lower than the Isw1a complex in this assay. These results support those obtained in the nucleosome spacing assay, which may be mechanistically similar. We

also noted the presence of a lower mobility band that possibly represents the Isw1a complex bound to the nucleosomal substrate.

Isw1a complex binds to DNA and nucleosomes in an ATP-independent manner

In order to determine the mechanisms underlying the differences in specific activities that we observed for the two Isw1 complexes, as well as to investigate the possible binding of Isw1a complex to its nucleosomal substrates observed in the sliding assay, we tested how these complexes interact with DNA and nucleosomes. To this end, we employed two assays, an immobilized template assay (51) and an *e*lectrophoretic *m*obility *s*hift *a*ssay (EMSA).

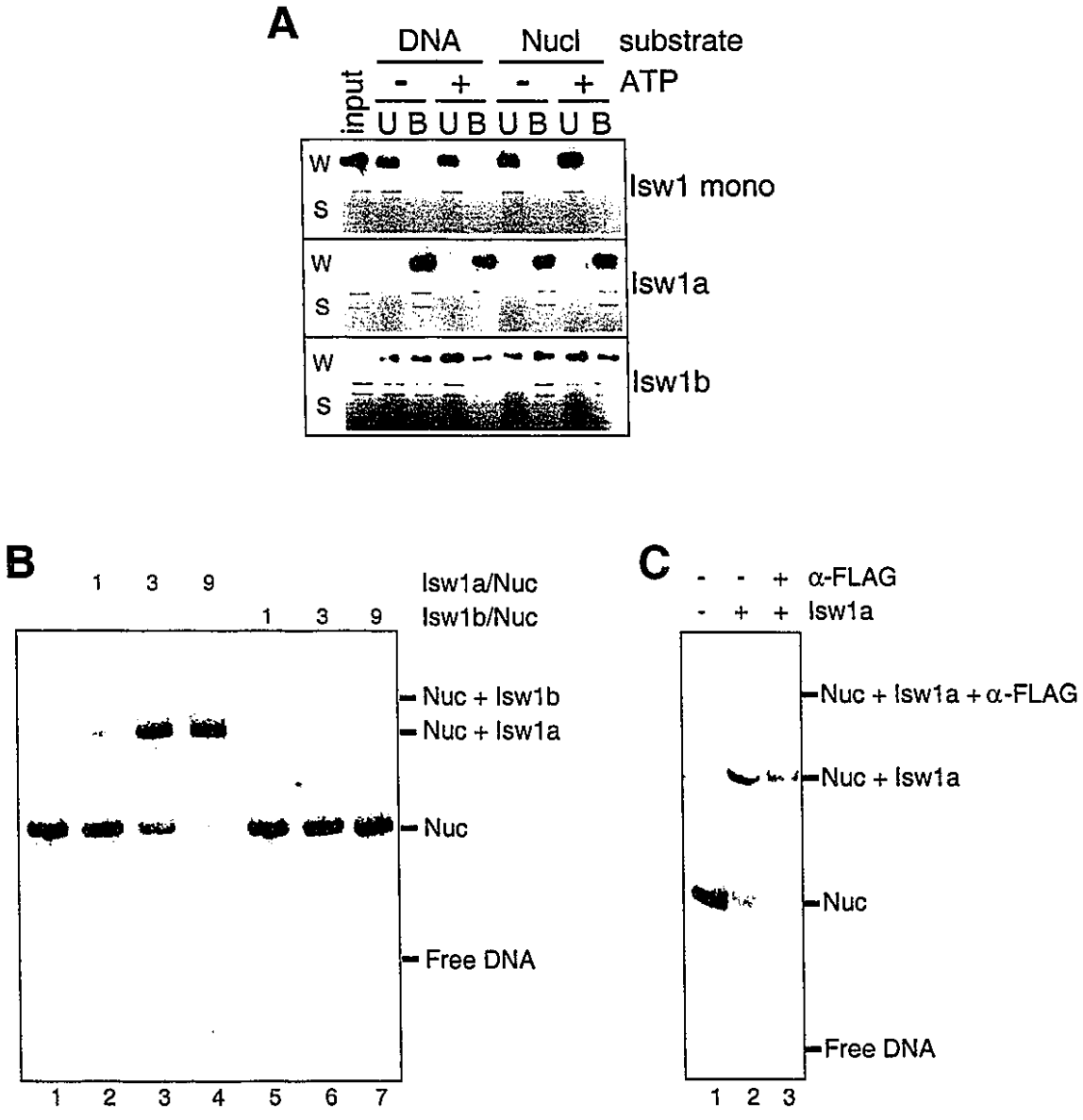
In the first assay, nucleosome arrays were assembled using recombinant histones and DNA immobilized onto magnetic beads. After mixing immobilized naked DNA or nucleosomal templates with Isw1 complexes, unbound and bound Isw1 species were detected by α-FLAG western blotting or by silver-stained gels (Figure 5A). Consistent with our ATPase and nucleosome spacing assays, Isw1 monomer failed to interact with DNA or nucleosome arrays. However, Isw1a complex interacted strongly with nucleosomal, as well as naked DNA templates. Isw1b complex interacted with both templates as well, though this interaction was not as robust as that of Isw1a. The Ioc proteins associated with Isw1p are visible by silver staining in all fractions in which Isw1p is detected by western, confirming that binding requires the intact complexes. All of these interactions were ATP-independent, suggesting that the enzymatic activity of Isw1 complexes is utilized at a step following binding, as observed previously for Isw2 complex (51). None of the Isw1 species interacted with beads alone (data not shown). It is possible that Isw1b does not interact with these templates as strongly as Isw1a due to intrinsic differences in their affinities. However, it is also possible that a portion of Isw1b in these preparations is enzymatically active, yet not competent for binding to these substrates. Tandem incubations were done in which the bound and unbound fractions of Isw1b were incubated for an additional 30 minutes with fresh buffer or

Figure 5: Isw1a and Isw1b bind DNA and nucleosomes in an ATP-independent manner

A) Immobilized template assays for DNA and nucleosome interactions with Isw1 complexes. Magnetic beads were bound with either naked DNA or nucleosomal DNA. Isw1 monomer, Isw1a or Isw1b were allowed to interact with beads in the presence or absence of ATP. Following incubation, Unbound (U) and Bound (B) fractions were detected by silver stained SDS-PAGE gels (S, bottom) or by western using α-FLAG antibodies to detect Isw1p (W, top). 7.6 fmoles (1 ng Isw1p equivalent) or 46 fmoles (6 ng Isw1p equivalent) of each Isw1 complex was used for either western or silver staining detection, respectively.

B) Electrophoretic mobility shift assay for Isw1a and Isw1b binding to mononucleosomes. 41 fmoles mononucleosomes were assembled from recombinant histone octamer and a labeled 255 bp DNA fragment and incubated in the indicated ratios with either Isw1a (lanes 2-4), Isw1b (lanes 5-7) or no complex (lane 1) without ATP. Reactions were analyzed on a 4% native polyacrylamide gel.

C) Antibody supershift assay. 41 fmoles mononucleosomes were incubated alone (lane 1) or with 369 fmoles Isw1a complex (lanes 2-3). To one pre-bound sample, 46 pmoles α-FLAG antibody was added for an additional 15 minutes (lane 3). Reactions were analyzed on a 4% native polyacrylamide gel.


A
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Isw1b/Nuc
Nuc + Isw1b
Nuc + Isw1a
Nuc
Free DNA
1 2 3 4 5 6 7
C
- - + α-FLAG
- + + Isw1a
Nuc + Isw1a + α-FLAG
Nuc + Isw1a
Nuc
Free DNA
1 2 3

immobilized template beads, respectively. The originally bound fraction remained mostly bound after the second incubation as well; the originally unbound fraction could also bind to the substrate, however, at lower levels than observed in the original incubation (data not shown). These results are consistent with the hypothesis that a portion of the Isw1b fractions used may not be competent for binding. However, a control experiment in which the first incubation was extended for a full hour also showed a smaller percentage of the Isw1b interacting with the substrate. Therefore, it is also possible that upon incubation, a portion of the Isw1b in the assay becomes incompetent for binding. It is unclear if this is due to destabilization of the complex during the course of the experiment or if the Isw1b adopts a new conformation in which it cannot bind the substrate as avidly. Interestingly, there was a minor, but reproducible decrease in binding of the Isw1b complex in the presence of ATP. This could be due to the ability of ATP to chelate Mg^{++} ions, thereby slightly lowering their concentration in the buffer and altering the Isw1b binding affinity. Alternatively, this could be a biologically relevant phenomenon; *i.e.* it is possible that the Isw1b complex can bind, but is inhibited from releasing its substrate until an ATP-dependent enzymatic event allows it to let go. These two possibilities have not yet been explored.

To confirm the finding that Isw1a and Isw1b complexes bind to nucleosomes, we also utilized an EMSA assay. This assay was done in collaboration with Blaine Bartholomew's group, and utilized mononucleosomes assembled from a 255 bp DNA fragment and recombinant histone octamer. These were incubated with increasing amounts of our Isw1a or Isw1b complexes in the absence of ATP and separated on a 4% native polyacrylamide gel. The presence of slower migrating bands was associated with increasing amounts of either Isw1a or Isw1b, suggesting that each complex was binding to mononucleosomes (Figure 5B, lanes 2-4 and 5-7). This is consistent with our previous results, as is the observation that the Isw1a complex appeared to bind more avidly than the Isw1b complex. As this reaction contained no ATP, it is unlikely that these slower migrating bands were due to Isw1-mediated remodeling of the labeled nucleosomes, but rather represented Isw1a or Isw1b bound to nucleosomes. In addition, these bands could

be competed away with excess DNA, consistent with our hypothesis that they represent Isw1a or Isw1b bound to nucleosomes (data not shown). To confirm the identity of the putative nucleosome-Isw1a band, α-FLAG antibody was added to this reaction mixture, resulting in the supershift of some of the putative nucleosome-Isw1a species (Figure 5C). The band caused by Isw1b was too weak to characterize by antibody supershift assay.

The behavior of each Isw1 complex in template interaction assays correlates well with its activities in both our nucleosome spacing and sliding assays. This suggests that the differences we observe in specific activity for the complexes can be attributed to their abilities to interact with their substrates *in vitro*, rather than inherent differences in their enzymatic activities. These results also highlight the requirement of the Ioc proteins in the interaction of Isw1p with templates. Although either Ioc2p and Ioc4p or Ioc3p alone are sufficient to target Isw1p, there is an apparent lack of amino acid homology among them, suggesting that each may utilize unique substrate interaction domains.

The Isw1a and b complexes show different genetic interactions with other chromatin remodeling factors

The differences in the biochemical properties of Isw1a and Isw1b complexes prompted us to compare their functions *in vivo*. It was previously found that an *isw1isw2chd1* triple mutant fails to form single colonies at elevated temperatures, while any double mutant combinations are affected to significantly lesser degrees (121). To determine whether defects in one or both Isw1 complexes are required for this phenotype, strains YTT522, YTT853, YTT855, and YTT861 were created, which have deletions in specific *IOC* genes, as well as *ISW2* and *CHD1* (Table I). Deletion of either *IOC2* or *IOC4* specifically disrupts the Isw1b complex, while deletion of *IOC3* disrupts the Isw1a complex (Figure 2A, lanes 3-5). Substitution of these *ioc* mutations for the *ISW1* deletion, therefore, allows us to separately examine the contributions of each Isw1 complex to this phenotype.

In a serial dilution assay, all strains grew as well as *wild-type* at 30° C. As expected, the *isw1isw2chd1* strain exhibited a severe growth defect when grown at 37° C (Figure 6A, row 4); it was unable to form single colonies, and only grew at the most saturated dilution (10^0). When a deletion of *IOC2* was substituted for the *ISW1* deletion, the cells still showed a strong growth defect, though it was not as severe, growing at the 10^{-2} dilution (Figure 6A, row 5). Deletion of *IOC3* rather than *ISW1* resulted in no detectable growth defects, suggesting that Isw1b complex is sufficient for cells to grow at elevated temperature (Figure 6A, row 6). However, deletion of *IOC3* in an *ioc2isw2chd1* mutant made the temperature-sensitive phenotype more severe (compare rows 5 and 8), showing that Isw1a complex has a minor involvement in growth at elevated temperature. We would expect that deletion of *IOC4* in this assay would phenocopy an *IOC2* deletion. However, *IOC4* deletion in an *isw2chd1* mutant caused only slight growth retardation at 37° C (Figure 6A, row 7), suggesting that either Ioc4p function has only a minor contribution to this particular Isw1b function, or that Ioc2p has functions *in vivo* that are independent of its role in the Isw1b complex. We also noticed that *isw1isw2chd1* cells exhibited a significantly more severe phenotype than that of *ioc2ioc3isw2chd1* cells (or *ioc2ioc3ioc4isw2chd1* cells, which showed no additional growth defects—data not shown) (compare Figure 6A, rows 4 and 8). This suggests that a portion of the *ISW1* requirement at elevated temperatures is due to functions of Isw1p monomer, or another, as yet unidentified Ioc-independent complex. It is interesting to note that this temperature-sensitivity is completely abrogated by plating on media containing 1 *M* sorbitol (data not shown). This type of temperature-dependent osmotic-sensitivity has also been observed in *rsc3*, *rsc30*, mutants (9), and suggests that this phenotype may result from effects on the integrity of the cell wall.

Mutation of Isw1a and Isw1b complex subunits cause distinct expression profiles

The Isw2 complex is required for transcriptional repression at a number of loci, including early meiotic genes. *ISW1* has also been shown to affect transcriptional

regulation (35, 63, 75, 96, 110), although it is not clear if each Isw1 complex has unique roles *in vivo*. To study the relative contributions of each complex, we prepared RNA from *ioc2*, *ioc3*, *ioc2ioc3*, and *isw1* strains. These samples were used for Northern blotting and DNA microarray analysis using a PCR-based array of all putative yeast open reading frames. As previously observed for mutants of the Isw2 complex (47, 53, 63), mutations of components of the Isw1 complexes resulted in modest derepression of many genes, while they resulted in deactivation of relatively few (Figure 6B and http://parma.fhcrc.org/JVary). If the Isw1a and Isw1b complexes have parallel functions in transcriptional repression, one would expect that deletion of either single mutant would result in little change, while deletion of both complexes would result in a greater number of genes being derepressed. This did appear to be the case for many genes, as the number of genes derepressed at least 1.5 fold was higher in both *ioc2ioc3* and *isw1* mutants, than in either *ioc2* or *ioc3* mutants (Figure 6B). This trend was confirmed by Northern analysis, and three such genes, *YEL070w*, *YMR090w*, and *SPR28*, are shown (Figure 6C).

We also found evidence that each complex may function independently at other loci. Cluster analysis identified a large group of 667 genes that were derepressed in *isw1* and *ioc* mutants; this cluster includes a significant number of genes involved in carbohydrate transport ($p < 0.00002$). While the *ioc2ioc3* double mutant strain exhibited a transcriptional profile similar to an *isw1* single mutant, *ioc2* and *ioc3* single mutants had distinct transcriptional profiles that, when additively combined, produce a profile similar to an *ioc2ioc3* strain. This suggests that at many loci within this cluster, Isw1a and Isw1b complexes may have independent functions. In addition, the transcriptional profiles of the *isw1* and *ioc* mutants were dramatically different than those of an *isw2* mutant or mutants of the Rpd3-Sin3 complex (47) within this cluster as well as the rest of the genome, (Figure 6D and data not shown).

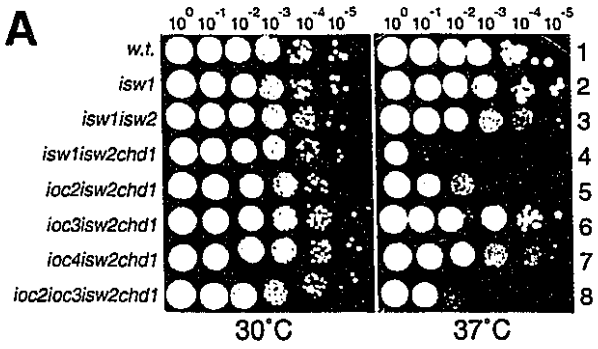
Figure 6: Isw1a and Isw1b complexes have partially overlapping, yet distinct functions *in vivo*.

A) Temperature sensitivity assays. Ten-fold serial dilutions of overnight cultures were spotted onto YEPD plates and allowed to grow at 30° C or 37° C for 2 or 3 days, respectively. The strains used were YTT166 (row 1) YTT441 (row 2), YTT443 (row 3), YTT227 (row 4), YTT522 (row 5), YTT853 (row 6), YTT855 (row 7), and YTT861 (row 8).

B) DNA microarray expression analyses. RNA was prepared and labeled from YTT823 (*ioc2*), YTT825 (*ioc3*), YTT829 (*ioc2ioc3*) or YTT441 or YTT442 (*isw1*) strains and hybridized to a microarray of yeast open reading frames. Shown are the numbers of yeast genes that were changed either 1.5 or 2.0 fold in each mutant background. Genes whose expression was increased in a mutant were defined as "derepressed", while those that were decreased in a mutant were defined as "deactivated". The numbers marked by an (*) asterisk represent two genes directly adjacent to *ISW1*, which may show reduced expression due to the *ISW1* deletion rather than to loss of Isw1p.

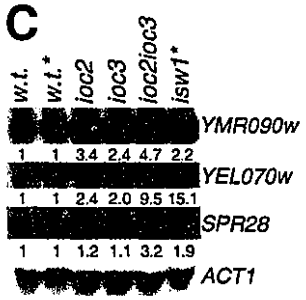
C) Northern hybridizations. RNA from these mutant strains was probed for the corresponding genes shown and quantified by Phosphorimager. The ratios relative to *wild-type* (normalized to *ACT1*) are shown below each image. The two strains marked by an (*) asterisk were prepared and normalized separately from the other samples.

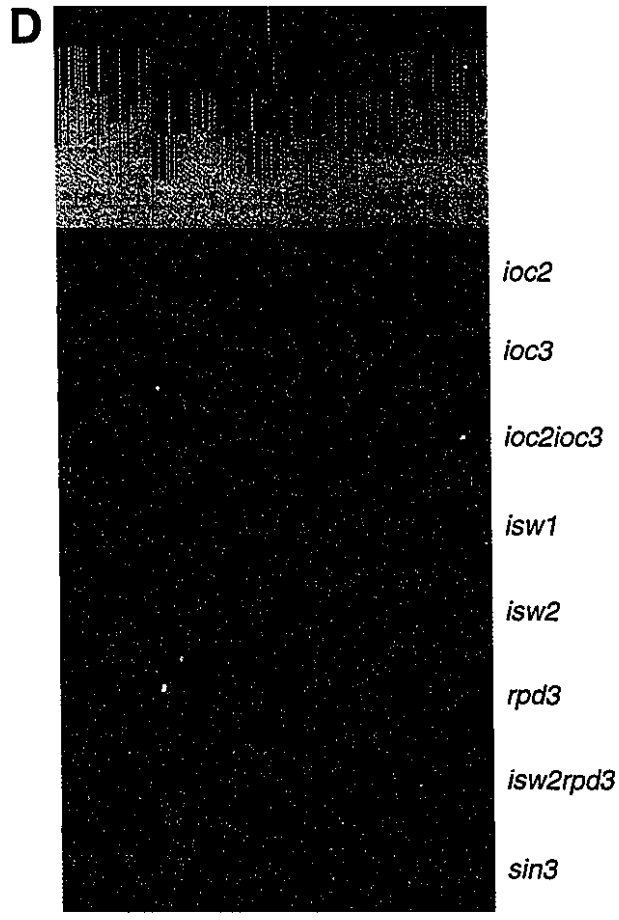
D) Expression clustering analysis. Microarray data from *ioc2*, *ioc3*, *ioc2ioc3*, and *isw1* mutants from this study were clustered with data from previously published *isw2*, *rpd3*, *isw2rpd3*, and *sin3* strains. Shown is a cluster of 667 genes that were derepressed in the *isw1* strains. The green tree at top represents smaller clusters of gene subgroups. For each deletion strain, the colored vertical lines correspond to the expression levels of individual genes. Red is indicative of genes which were derepressed in the mutant, while blue is indicative of genes which were deactivated in the mutant. Purple indicates little to no change.



B

	deactivated		derepressed	
strain	>2x	>1.5x	>1.5x	>2x
ioc2	0	4	22	1
ioc3	1	13	24	3
ioc2ioc3	7	28	48	4
isw1	1+*1	22+*2	139	36





Discussion

I have shown that the two separable complexes associated with the *S. cerevisiae* Isw1p protein have distinct and overlapping functions *in vitro* and *in vivo*. In order to characterize each separate complex, several established assays were used to study their biochemical activities. Both Isw1a and Isw1b showed equivalent nucleosome-stimulated ATPase activity, suggesting that each can utilize the catalytic activity of Isw1p. An equimolar amount of Isw1p monomer is relatively inactive in this assay, illustrating that the Ioc proteins are required for full specific activity of Isw1p. However, at equimolar levels the Isw1a complex exhibited stronger nucleosome spacing and sliding activities than the Isw1b complex. These *in vitro* differences are consistent with differences in interaction of each complex to immobilized DNA and nucleosome arrays. Indeed the spacing assay shows a dose-dependent effect for the Isw1b complex (data not shown), suggesting that the differences in specific activity observed may be a direct result of their differing abilities to interact with substrates in these systems. As the recombinant histones utilized in this study do not bear modifications, it is also possible that the Isw1b complex requires covalently modified histones for substrate interaction and/or full activity. Consistent with this, histone H4 K5 appears to be acetylated in the fraction of histones that interacts with the Isw1b complex, as described in Chapter II. If this is the case, the nucleosome-stimulated ATPase activity observed in these assays does not require such a modification, as the ATPase activity was equivalent between Isw1a and Isw1b. It is similarly possible that full activity and/or substrate binding of the Isw1p monomer also requires histone modifications that were not present in our assays. However, unlike Isw1b complex, Isw1p monomer did not exhibit any nucleosome stimulated ATPase activity showing that it has different requirements for its activity.

Isw1a and Isw1b have different roles *in vivo* as well. By exploiting a synthetic temperature-sensitive phenotype to study the contributions of each complex, it was found that deletion of components of the Isw1a and Isw1b complexes resulted in different phenotypes. While the Isw1a complex appeared to play a very minor role for growth at

elevated temperature, deletion of components of the Isw1b complex resulted in strains that grew more slowly at 37° C. Such an *in vivo* difference has been observed for other ATP-dependent chromatin remodeling complexes that utilize the same ATPase subunit. For example, the Sth1p ATPase of the yeast RSC chromatin remodeling complexes also forms complexes with distinct functions *in vivo*. At least three forms of the RSC complex have been purified, two of which are distinguished only by their associations with either Rsc1p or Rsc2p (9, 22). Mutants of *RSC1* and *RSC2* differed not only in their sensitivities to elevated temperature and hydroxyurea, but further showed different genetic interactions with components of the SAGA histone acetyltransferase complex, suggesting unique roles *in vivo* for each complex (22). In *Drosophila*, mutants of *Iswi* exhibit a variety of developmental phenotypes described in the introduction to this thesis. Mutants of *nurf301* exhibit the same phenotypes, suggesting that the NURF complex is responsible for these functions *in vivo*, while the ISWI-containing ACF and CHRAC complexes are not. As no phenotype has been ascribed to either the ACF or CHRAC complexes, it is formally possible that all three ISWI complexes share these functions, but each is limiting in its concentration. Therefore, disruption of any one of these complexes (as in a *nurf301* mutant) might phenocopy an *iswi* mutant in which all three are disrupted.

In both *in vivo* assays, there was evidence that Isw1p may have functions within the cell that are independent of the Ioc subunits. The *isw1isw2chd1* mutant was significantly more temperature-sensitive than any similar combination with *ioc* mutants. In addition, the expression data consistently showed that deletion of *ISW1* resulted in transcription changes in a greater number of genes than those observed in an *ioc2ioc3* mutant. Given that a significant portion of Isw1p is purified as a monomer in *wild-type* as well as *ioc* mutant strains, it is possible that these differences are due to functions of Isw1p monomer.

An Isw1 complex-independent role was also suggested for Ioc2p. Both chromatographic profiles and co-immunoprecipitation data suggest that an Ioc2p monomer or other Ioc2-containing complex may exist. Deletion of either *IOC2* or *IOC4*

results in disruption of the Isw1b complex, so one would expect these phenotypes to be identical if their activities were limited to the Isw1b complex. However, the *ioc2isw2chd1* mutant was more temperature-sensitive than the *ioc4isw2chd1* mutant, and deletion of *IOC4* in the *ioc2isw2chd1* mutant did not result in additional growth defects (data not shown). However, deletion of *IOC4* does not disrupt the physical interaction of Isw1b complex with histone octamer, as described in Chapter II. It is possible that Isw1p and Ioc2p may interact in the absence of Ioc4p *in vivo*, but cannot be copurified in the conditions described in this dissertation.

Analyses of transcription profiles in *isw1* and *ioc* mutants revealed that the two Isw1 complexes function in parallel pathways to affect transcription of a number of genes, while they appear to act separately at many genes. It is unclear if these disparities represent differences in targeting of the two complexes *in vivo*. It is also possible that they represent indirect changes that result from other unique functions of each complex.

The data presented in this chapter also demonstrate that lower specific activities in biochemical assays do not necessarily correlate with lesser *in vivo* activity, as the Isw1b complex has more significant roles in growth at elevated temperatures and transcription *in vivo* than the more biochemically active Isw1a complex.

Chapter IV: Genetic interaction of *ISW1* with histone N-terminal tails and the NuA4 complex

Introduction

The effects on gene expression that result from deletion of Isw1 complex components are subtle, rarely exhibiting more than two-fold changes as compared to *wild-type* cells. There are at least three models to explain these results. First, it is possible that Isw1 complexes function in parallel with (an) unidentified factors in transcriptional regulation. If this were the case, these factors could mask the effects of mutations in the Isw1 components. Indeed, parallel functions of the Isw2 and Rpd3-Sin3 complexes make the phenotypes of *isw2* mutants subtle, due to this reason (47, 53, 63). *S. cerevisiae* has at least ten known or putative histone deacetylases that could serve such a function for *ISW1*. It is also possible that the Isw1 complexes regulate nuclear processes other than transcription. Kent, *et al.*, recently reported that the deletion of *ISW1* results in changes in chromatin structure at several genes *in vivo*. However, these changes are not associated with changes in transcription levels ((75) and http://parma.fhcrc.org/JVary), suggesting that there may be many Isw1-dependent chromatin changes that do not affect transcription *in vivo*. A third possibility is that our assay for transcription is unable to detect *ISW1*-dependent effects on transcription. As mentioned in the introduction to this dissertation, other groups have proposed that Isw1-containing complexes may play a role in transcriptional elongation and/or termination. Defective elongation or termination may result in RNA transcripts that have not been polyadenylated. DNA microarray analyses introduce a bias in detection by utilizing either polyadenylated RNA as the starting material, and/or using polydeoxythymidine as a primer for dye-incorporation. This method of analysis of transcriptional changes, therefore, might fail to detect changes in non-polyadenylated transcripts. Indeed, preliminary results indicate that the fraction of total RNA that is polyadenylated is lower

in *isw1* mutants than *wild-type* (97). Northern hybridizations utilizing total RNA should not be affected by this bias, and our Northern analyses do not support this possibility.

This chapter describes two approaches used to reveal factors that act in parallel with the yeast Isw1-containing complexes, either in transcription, or other as yet unidentified processes. First, to assess if Isw1 complexes act in parallel to a histone deacetylase complex in transcriptional regulation, as has been observed for the Isw2 complex, targeted deletions were made of known or putative histone deacetylases to look for synthetic effects with *isw1* mutants on transcription. Second, in order to identify unknown factors that may act in parallel to the Isw1 complexes, we assumed that such factors would likely be involved in chromatin regulation and might, therefore, require specific histone modifications for their activities. To this end, deletions of the histone H3 or histone H4 tails were made in *wild-type* and *isw1* mutant backgrounds. Surprisingly, *isw1* mutants showed synthetic growth defects with either histone mutant. The histone H4 phenotype was very strong, prompting a further dissection of the residues required. Both deletion and substitution analysis showed that *ISW1* interacts with four lysine residues of the histone H4 tail. The lysine residues required match very closely the pattern of acetylation mediated by the essential NuA4 histone acetyltransferase complex *in vivo*, prompting an analysis of mutants of the NuA4 complex in an *isw1* strain.

Results

Transcriptional changes in *isw1* mutants are not synthetic with *rpd3*, *hst1*, *hda1*, or *tup1* mutants

To find genetic interactions between *ISW1* and *h*istone *dea*cetylase *c*omplexes (HDAC's), deletions were made of the known or putative HDAC's, *RPD3, HDA1, HOS1, HOS2, HOS3,* and *HST1*. In an *isw1isw2* double mutant, the addition of *hda1*, *hos1*, or *hos3* mutations had no effect on growth at any temperature tested. However, the addition of a *hos2* mutation resulted in a slight temperature-sensitive growth defect, and

the addition of an *rpd3* mutation caused a severe temperature-sensitive growth defect at 37° C (Figure 7A). As the Isw2 complex and the Rpd3-Sin3 complexes act in parallel pathways to repress transcription, this latter result was not surprising. To determine which *ISW* gene was responsible for these interactions, these strains were transformed with *CEN* plasmids expressing either one or both *ISW1* and *ISW2* genes. Expression of both *ISW* genes suppressed the growth defects of either *isw1isw2hos2* or *isw1isw2rpd3* mutants, while vector alone had no effect. Expression of either *ISW1* or *ISW2* alone were sufficient to suppress the growth defect of the *isw1isw2hos2* mutant, suggesting that they may be redundant for whatever process is defective in this strain. Surprisingly, however, neither *ISW1* nor *ISW2* alone could suppress the growth defect in the *isw1isw2rpd3* mutant, suggesting that both genes interact with *RPD3* separately (Figure 7B). In order to independently verify these results, without relying upon potentially misregulated ectopic expression of plasmid-borne genes, separate strains were created bearing *rpd3*, *isw1*, and *isw2* mutations alone or in combination. The *isw1rpd3* and *isw2rpd3* mutants again exhibited temperature-sensitive growth defects, although the defect in an *isw1rpd3* mutant was much more severe (Figure 7C). This suggests that the Rpd3-Sin3 complex may have important roles in parallel to the Isw1 complex. As several of the genes that are mildly derepressed in an *isw1* mutant are mid-meiosis specific genes, deletions were made of *HST1* (an HDAC) and its DNA-binding factor partner *SUM1*. Both of these also showed mild temperature-sensitive growth defects with *isw1* mutants that were not observed in any of the single mutants (data not shown).

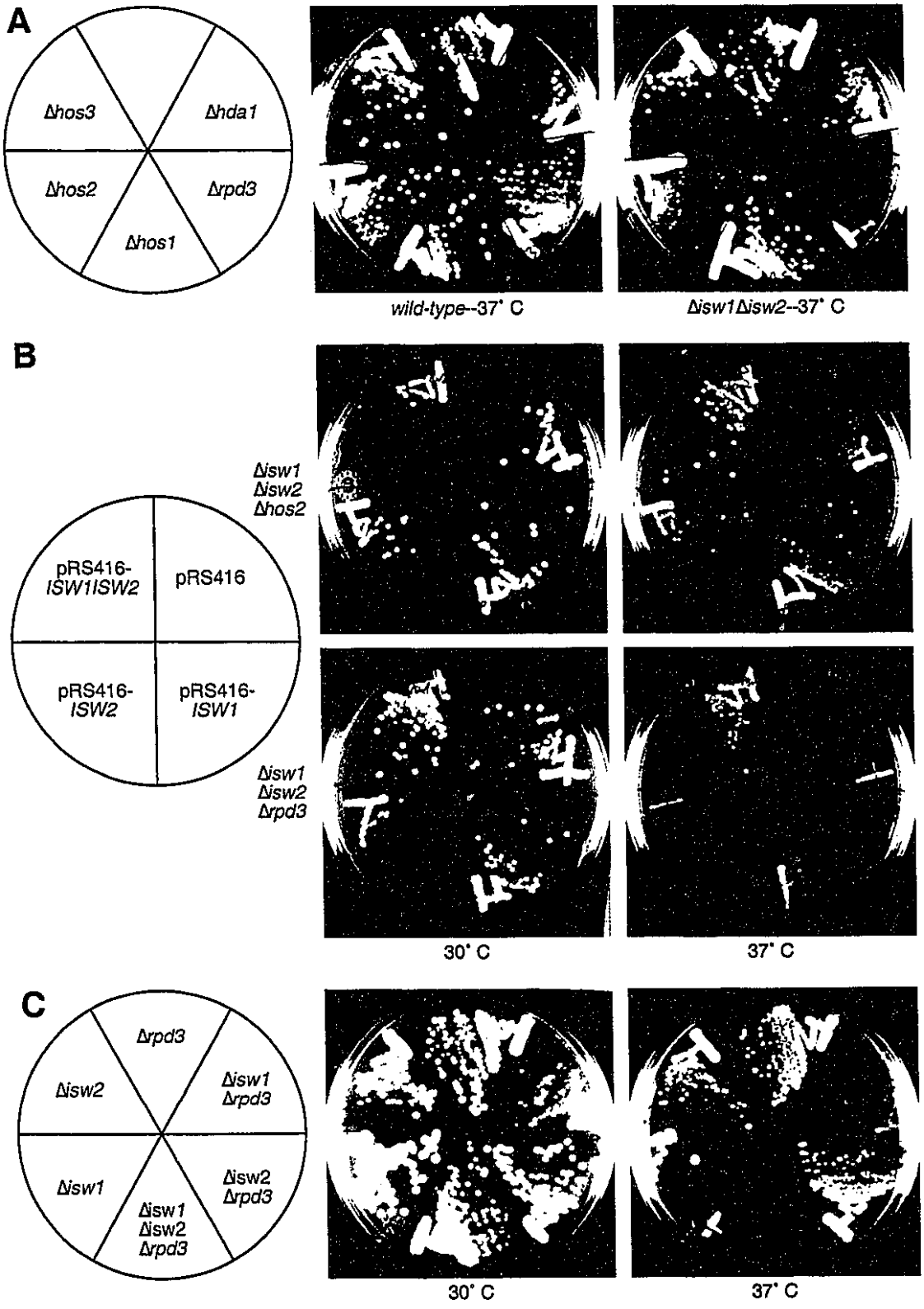
In order to determine if the genetic interactions between *ISW1* and *RPD3* or *HST1* were caused by effects on transcriptional regulation, changes in gene expression in these mutants were assayed using DNA microarray and northern hybridization analyses. In addition, mutants of *HDA1* were tested due to an apparent ability of *isw1* to suppress *isw2hda1* growth defects (data not shown). Mutants of the general repressor *TUP1* were also tested as Tup1 interacts functionally with both Hda1 and the Isw2 complex (Marnie Gelbart, unpublished results). The use of DNA microarray and northern hybridizations were very effective in illustrating the collaboration between the Isw2 and Rpd3-Sin3

Figure 7: The histone deacetylases *RPD3* and *HOS2* genetically interact with *ISW1* at elevated temperatures

A) Genes encoding the known or putative histone deacetylases Hda1, Rpd3, Hos1, Hos2, or Hos3 were deleted in *wild-type* (YTT166, YTT693, YTT694, YTT695, YTT696, YTT697) or *isw1isw2* (YTT199, YTT698, YTT699, YTT700, YTT701, YTT702) backgrounds. Strains were plated on YEPD media and grown at 30° or 37° C.

B) The mutants *isw1isw2hos2* (YTT701—upper pair of images) or *isw1isw2rpd3* (YTT699—lower pair of images) were transformed with pRS416 (*CEN6/ARSH4/URA3)* vectors expressing *ISW1*, *ISW2*, *ISW1/ISW2* or vector alone, plated onto YC-URA media, and grown at 30° or 37° C.

C) Strains bearing deletions of the *ISW1*, *ISW2*, and/or *RPD3* genomic loci in the combinations indicated (YTT187, YTT219, YTT694, YTT705, YTT717, YTT728) were plated onto YEPD media and grown at 30° or 37° C.


A
Δhos3
Δhda1
Δhos2
Δrpd3
Δhos1
wild-type--37° C
Δisw1Δisw2--37° C
B
Δisw1
Δisw2
Δhos2
pRS416-
ISW1ISW2
pRS416
pRS416-
ISW2
pRS416-
ISW1
Δisw1
Δisw2
Δrpd3
30° C
37° C
C
Δrpd3
Δisw2
Δisw1
Δrpd3
Δisw1
Δisw2
Δrpd3
Δisw1
Δisw2
Δrpd3
30° C
37° C

complexes, and therefore might be expected to detect a similar collaboration between Isw1 and another complex (47, 53). RNA was prepared from *rpd3*, *hda1*, *hst1*, *sum1*, and *tup1* single mutants and the corresponding *isw1* double mutants and hybridized to a DNA microarray. Although the double mutant strains often exhibited transcriptional changes that were distinct from either corresponding single mutant, in no instance was a synergistic effect on transcriptional regulation apparent (data not shown). In addition, no group of genes that was misregulated in the double mutants could be assigned to known biological pathways, nor did they share cis-element sequences that might be required for coordinate regulation. Northern hybridization was used to confirm this by probing for genes that showed significant expression differences in these mutants (data not shown). Although it is possible that a small group of genes exists that requires both Isw1 and one of these other complexes for proper transcriptional regulation, the widespread collaboration observed for Isw2 and Rpd3-Sin3 complexes was not present in any of these mutants. However, we cannot rule out the possibility that such collaboration exists under different environmental conditions or genetic backgrounds than have been tested.

ISW1 interacts with histone H3 and H4 tail deletions

There are several other known or putative HDAC's in yeast that have not yet been tested for synthetic effects with *ISW1*. In addition, there may be other non-HDAC factors that act in parallel to Isw1 complexes to mask its role in transcriptional regulation *in vivo*. Alternatively, any of these factors may act in parallel to Isw1 complexes in processes other than transcription. This is supported by the fact that mutations of *RPD3*, *HOS2*, *HST1*, and *SUM1* all show synthetic growth defects with *isw1*, yet none have clear synthetic effects on transcription. In an attempt to identify any factors that operate in parallel to an Isw1 complex, we made an assumption that such a factor was likely to be involved in chromatin metabolism to some extent, and might, therefore, be dependent upon some portion of the histone H3 or H4 N-terminal tails for its activity.

To this end, deletions were made of histone H3 and H4 from residues 2-30 and 4-19, respectively, and these mutant histones were introduced into *isw1* mutants by plasmid shuffling. Briefly, both genomic loci of histone H3 and histone H4 were deleted in otherwise *wild-type* or *isw* mutant backgrounds, ensuring viability was dependent upon a *URA3*-marked plasmid expressing *wild-type* versions of each histone. A similar *TRP1*-marked plasmid expressing desired alleles of histone H3 and H4 was introduced, and 5-*f*luoro*o*rotic *a*cid (5-FOA) was used to counter-select against cells that retained the *URA3*-marked plasmid. This effectively resulted in cells whose sole source of histones was from the *TRP1*-marked plasmid, thereby allowing the assessment of a variety of specific histone mutations.

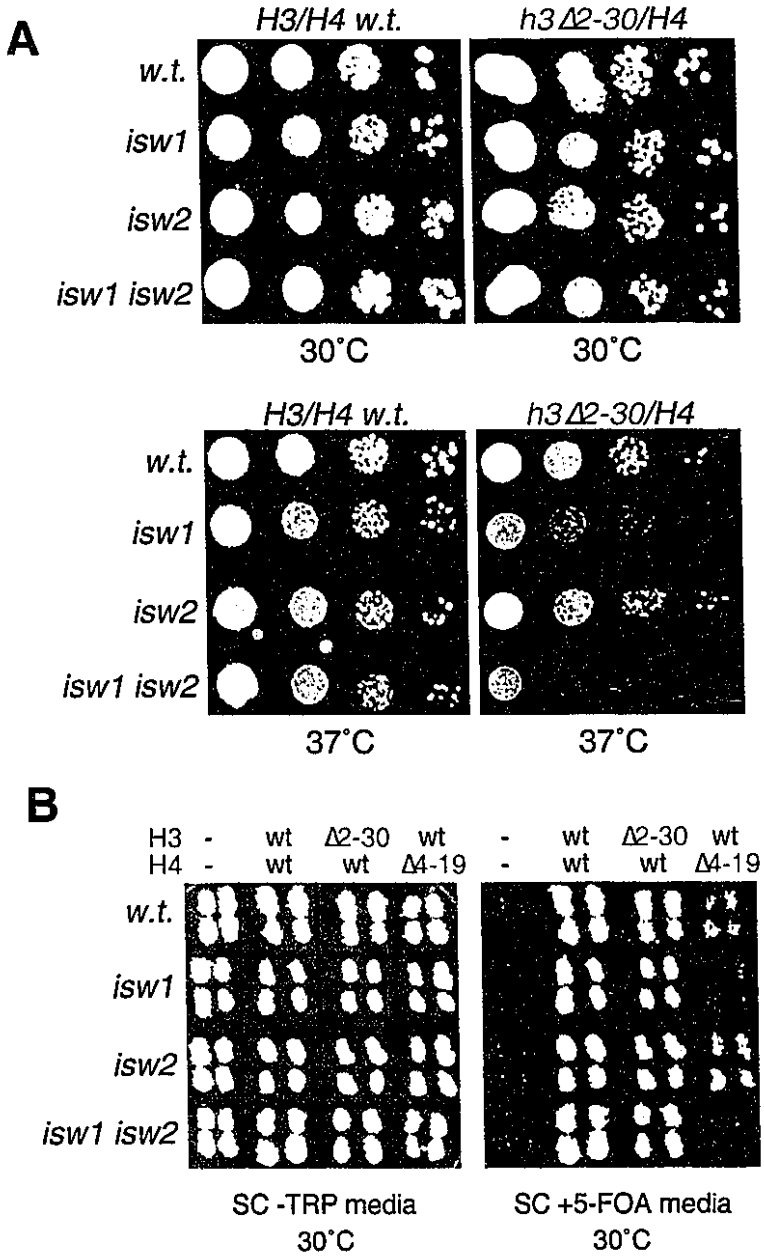
To assess the requirement of the histone H3 tail, a *TRP1*-marked plasmid expressing *wild-type* histone H4, and tailless histone H3 (Δ2-30) was transformed into S288C histone deficient strains in otherwise *wild-type*, *isw1*, *isw2*, or *isw1isw2* backgrounds. Following 5-FOA counterselection, 10-fold serial dilutions were spotted onto solid media and grown at 30° C or 37° C (Figure 8A). At elevated temperature, there is a clear growth defect in *isw1* mutants, yet not in *isw2* mutants. The defect is somewhat more severe in an *isw1isw2* mutant, however, suggesting that *ISW2* may have a minor role in this phenotype when *ISW1* is absent. To determine the portion of the H3 tail that was responsible for this interaction, alleles with smaller deletions in this region as well as alleles with lysine to arginine substitutions were created and tested using the same plasmid shuffling experiment. However, due to the mild nature of this phenotype even with the largest (Δ2-30) deletion, it was technically difficult to reliably determine which of the more specific H3 tail mutants interacted with *isw1* mutants (data not shown).

To assess the requirement of the histone H4 tail, a similar plasmid was created containing *wild-type* histone H3, and tailless histone H4 (Δ4-19). Following transformation of these *TRP1*-marked plasmids, and 5-FOA counterselection, the resulting colonies grew extremely slowly, even in the *wild-type* background, making spot tests an impractical method of rapidly assessing phenotypes. Consequently, for each

Figure 8: *ISW1* interacts genetically with tailless histones H3 and H4

A) H3 tailless mutants. Strains whose sole source of *wild-type* histones H3 and H4 was on the *URA3*-marked plasmid pRM200U (YTT1284, YTT1543, YTT1475, and YTT1479) were transformed with plasmids expressing *wild-type* histone H4 and either *wild-type* or tailless histone H3 (pMP3 and pFVL85.8). Following 5-FOA counter-selection against pRM200U, 10-fold serial dilutions of OD_{660}=0.1 cultures were spotted onto YEPD media and grown at 30° or 37° C.

B) H4 tailless mutants. Identical strains as in (A) were transformed with an empty vector or one expressing either *wild-type* or tailless histone H3 and either *wild-type* or tailless histone H4 (pRS414, pMP3, pFVL85.8, and pRS414-HHT2-hhf2del4-19). Four individual transformants from each were patched onto YC-TRP media, subsequently replica plated onto YC +5-FOA media to counter-select against pRM200U, and grown at 30° C.


A
H3/H4 w.t.
h3Δ2-30/H4
w.t.
isw1
isw2
isw1 isw2
30°C
30°C
H3/H4 w.t.
h3Δ2-30/H4
w.t.
isw1
isw2
isw1 isw2
37°C
37°C
B
H3 - wt Δ2-30 wt - wt Δ2-30 wt
H4 - wt wt Δ4-19 - wt wt Δ4-19
w.t.
isw1
isw2
isw1 isw2
SC -TRP media
30°C
SC +5-FOA media
30°C

transformation, four separate TRP+ transformants were patched onto synthetic media lacking tryptophan. These patches were then replica plated onto synthetic media containing 5-FOA for counterselection against the *URA3*-marked plasmid expressing *wild-type* histones. A mutation in the plasmid-borne *URA3* gene can confer resistance to 5-FOA, thereby permitting a cell to retain *wild-type* histones at this last selection step. Although such mutations were rare, the analysis of four independent transformants allows the rapid identification of such novel mutants. A clear *isw1*-dependent growth defect was observed for strains transformed with this tailless histone H4 allele at all temperatures tested (Figure 8B and data not shown). Notably, as for histone H3 tail mutants, this defect was specific for *isw1* as growth in an *isw2* background was similar to *wild-type*. The strong *isw1*-dependence prompted us to make similar histone deficient strains in a W303 strain both to look for differences due to strain backgrounds as well as to rule out the presence of a cryptic mutation in our S288C *isw1* mutants. The results in W303 were similar to those in S288C, thus independently confirming the *isw1* interaction (data not shown and Figures 9A and B).

ISW1 interacts with lysines 5,8,12, and to a lesser extent 16 of histone H4

The very strong genetic interaction between *isw1* and histone H4 Δ4-19 mutants, is consistent with our hypothesis that another factor that operates in parallel with an Isw1 complex may utilize the histone H3 or H4 tails. The portion of the histone H4 tail deleted in this allele encompasses four acetylatable lysines as well as a basic patch thought to be important for silencing as well as ISWI function in *Drosophila*, and may also have other functions that have not yet been described (27, 60, 69). We sought to determine the minimal region of this H4 tail that was responsible for the *isw1* genetic interaction in order to reduce effects on the cell that were independent of the *ISW1*-interacting region. In addition, the identification of such a minimal region may provide clues as to what factor(s) might be acting in a parallel pathway with an Isw1 complex.

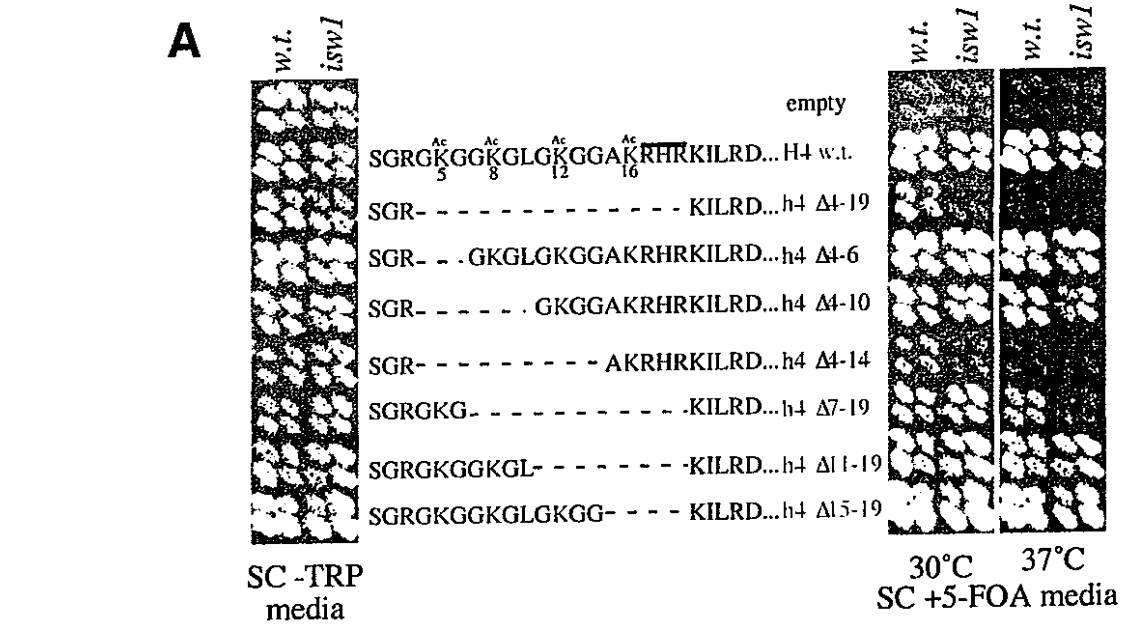
To this end, a set of similar *TRP1*-marked plasmids was constructed containing six smaller nested deletions of residues 4-19. Each deletion encompassed overlapping regions containing one, two, or three of the acetylatable lysines. Transformation into W303 *wild-type* or *isw1* strains lacking genomic histone loci and patching onto –TRP media was carried out as described earlier. Following replica plating onto media containing 5-FOA, patches were grown at either 30° or 37° C (Figure 9A). An *isw1*-dependent growth defect was observed for deletions of residues 4-14 or 7-19, and to a lesser extent residues 4-10. Interestingly, the growth defect was stronger for the largest deletions, yet the 4-14 deletion was more severe than the slightly larger 7-19 deletion. There are several possible explanations for these findings. First, *isw1* may interact primarily with a portion of the tail between residues 7-10 that is lacking in all three of these mutants. Second, *isw1* may interact with some feature present only in histone H4 tails of normal length, independent of the specific sequence on the tail, such that the larger the region deleted, the stronger the interaction. As the interaction was not strongest with the largest deletion (Δ7-19), this is unlikely; however, it is possible that following deletion, the sequence remaining in the Δ7-19 mutant is slightly more preferred as a substrate than that remaining in the Δ4-14 mutant. Third, *isw1* may interact with the four acetylatable lysines in the histone H4 tail, such that the interaction was stronger when fewer lysines were present. If correct, this would predict that lysine 16 is least able to rescue this *ISW1*-dependent phenotype as the Δ4-14 mutant was more severe than the Δ7-19 mutant.

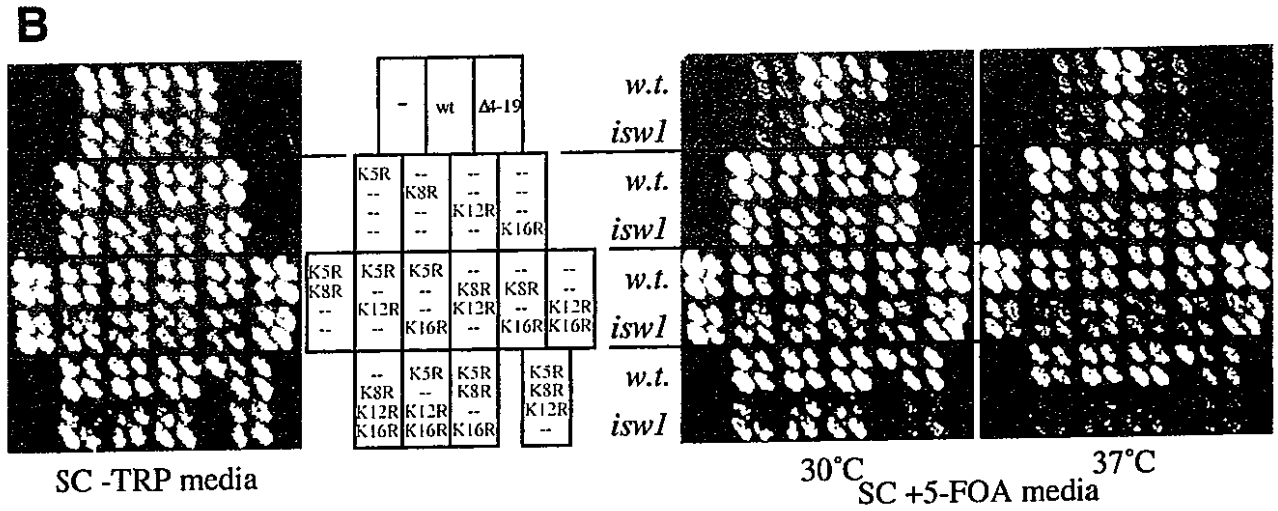
To test these three possibilities, a set of fourteen similar plasmids was acquired from our collaborator, Mark Parthun at the Ohio State University, in which lysine to arginine substitutions were made at each of these four lysines either as single substitutions, or in every double or triple combination. These plasmids were tested in our plasmid shuffling system as before and visualized on media containing 5-FOA at 30° or 37° C (Figure 9B). Very little, if any, genetic interaction with *isw1* was observed for any of the single lysine substitutions even at the highest temperatures, suggesting that it is unlikely that a single lysine could be mediating this phenotype. A slight growth defect

Figure 9: *ISW1* interacts genetically with histone H4 lysines 5, 8, 12, and to a lesser extent 16

A) H4 nested tail deletion mutants. Strains whose sole source of *wild-type* histones H3 and H4 was on the *URA3*-marked plasmid pRM200U (YTT1970 and YTT1971) were transformed with an empty vector or one expressing *wild-type* H3 and either *wild-type* or nested tail deletion mutants of histone H4 (pRS414, pMP3, pRS414-HHT2-hhf2Δ4-19, pJV101 (Δ4-6), pJV102 (Δ4-10), pJV103 (Δ4-14), pJV104 (Δ7-19), pJV105 (11-19), or pJV106 (Δ15-19)). Four individual transformants from each were patched onto YC-TRP media, subsequently replica plated onto YC +5-FOA media to counter-select against pRM200U, and incubated at 30° or 37° C. The sequence of *wild-type* histone H4 residues 1-24 is shown and the corresponding region deleted in each mutant is illustrated below. The four acetylateable lysines are shown in blue. The black bar above residues 17-19 corresponds to the residues required for ISWI function in *Drosophila* (see Chapter I)

B) H4 lysine to arginine tail mutants. Identical strains as in (A) were transformed with an empty vector or one expressing *wild-type* H3 and either *wild-type*, Δ4-19, or lysine to arginine mutants of histone H4 (pRS414, pMP3, pRS414-HHT2-hhf2Δ4-19, pMP13 (K5R), pMP23 (K8R), pMP14 (K12R), pMP24 (K16R), pMP15 (K5, 8R), pMP110 (K5, 12R), pMP121 (K5, 16R), pMP111 (K8, 12R), pMP122 (K8, 16R), pMP123 (K12, 16R), pMP124 (K8, 12, 16R), pMP125 (K5, 12, 16R), pMP63 (K5, 8, 16R) or pMP62 (K5, 8, 12R)). Transformants were patched onto YC-TRP media, and replica plated onto YC +5-FOA media as in (A). The chart illustrates the location of each mutant on the plates. Lysine to arginine substitutions are indicated in blue (e.g. K5R) at each location.


A
w.t.
isw1
empty
SGRGKGGKGLGKGGAKRHRKILRD... H4 w.t.
SGR- - - - - - - - - - - - - KILRD... h4 Δ4-19
SGR- - -GKGLGKGGAKRHRKILRD... h4 Δ4-6
SGR- - - - - -GKGGAKRHRKILRD... h4 Δ4-10
SGR- - - - - - - - -AKRHRKILRD... h4 Δ4-14
SGRGKG- - - - - - - - - - -KILRD... h4 Δ7-19
SGRGKGGKGL- - - - - - - -KILRD... h4 Δ11-19
SGRGKGGKGLGKGG- - - - KILRD... h4 Δ15-19
w.t.
isw1
w.t.
isw1
SC -TRP media
30°C
37°C
SC +5-FOA media


B
-
wt
Δ4-19
K5R
K8R
K12R
K16R
w.t.
isw1
w.t.
isw1
w.t.
isw1
SC -TRP media
30°C
37°C
SC +5-FOA media

was observed for all of the double substitution mutants in an *isw1* mutant. Notably, there was no double combination that could reproduce the phenotype of the Δ4-19 mutant suggesting that no pair of lysine residues were mediating this phenotype in isolation. All of the triple lysine substitutions interacted strongly with *isw1*, such that at 37° C, all four combinations were inviable in an *isw1* strain. This pattern of genetic interaction is consistent with only the last of the three possibilities described above, namely that *ISW1* interacts with all of the four acetylatable lysines on the histone H4 tail. Interestingly, the phenotype was most severe when lysines 5, 8, and 12 were mutated, leaving only lysine 16. This is exactly as predicted based upon observations of the deletion mutants shown in Figure 9A, *i.e.* lysine 16 is the least able to rescue the growth phenotype in an *isw1* mutant.

In order to confirm these results in a quantitative manner, serial dilutions of the histone deletion mutants and a subset of double and triple lysine to arginine substitution mutants were spotted onto YEPD media and grown at room temperature, 30°, or 37° C (Figures 10A and 11A). Consistent with the patch tests, both of the largest deletion mutants (Δ4-14 and Δ7-19) were synthetically lethal with *isw1* at 37° C, as were the two triple lysine to arginine substitution mutants tested (K5, 8, 16R and K5, 12, 16R). In addition, the severity of the growth defect was proportional to the number of lysines deleted or substituted in both assays.

ISW1 interacts with the NuA4 HAT complex

The histone acetyltransferase complex, NuA4, is a likely candidate factor that could mediate the genetic interaction with *ISW1*. NuA4 is an 11-subunit complex, composed of the essential proteins Esa1p, Tra1p, Arp4p, Act1p, Epl1p, and the non-essential proteins Yng2p, Eaf1, 2, 3, 5, & 6p (6, 32-34, 41, 43, 49, 87). Recombinant Esa1p can acetylate histones H2A lysines 4 and 7, H3 lysines 4 and 14, and H4 lysines 5, 8, 12, and 16 *in vitro*, but the NuA4 complex shows an *in vivo* preference for histone H4 lysines 5, 8, and 12 and histone H2A lysine 7, thereby matching very closely the

preferred histone residues required for the *ISW1* genetic interaction (6, 28, 117, 128). In addition, glutamine substitution of histone H4 lysines 5, 8, 12, and 16 results in hypersensitivity to DNA damaging agents; this sensitivity is thought to be mediated by the NuA4 complex as *esa1* mutants mimic this phenotype. The hypersensitivity of these histone mutants can be alleviated by acetylation of a single lysine residue at positions 5, 8, or 12, but not at position 16 (16).

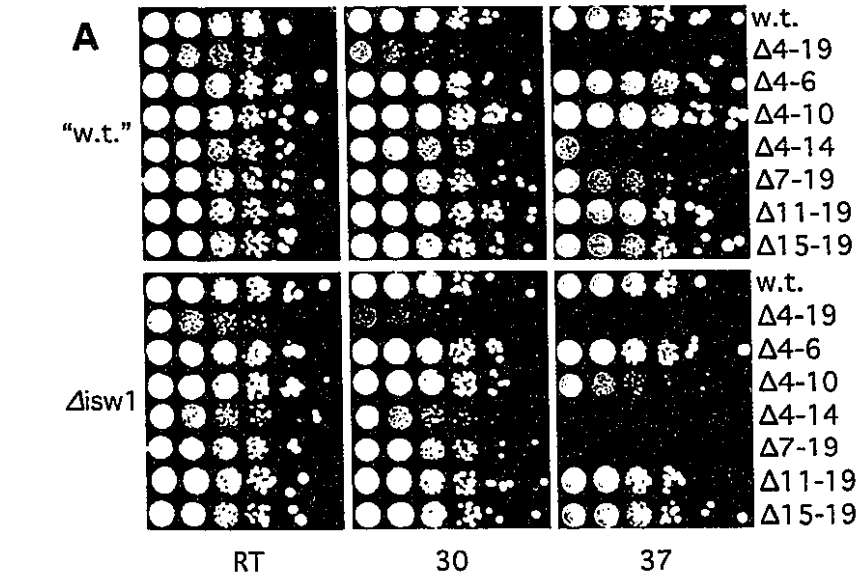
Several *in vivo* functions have been ascribed to the NuA4 complex. First, a role in DNA repair during S-phase is suggested by studies showing that in addition to *esa1* mutants, *yng2* mutants are also hypersensitive to DNA damaging agents, especially *m*ethyl*m*ethane*s*ulfonate (MMS), *h*ydroxy*u*rea (HU) and camptot*h*ecin (CT) (16, 24). Second, the NuA4 complex has roles in transcriptional activation at the *PHO5*, *HIS4*, *TRP4*, and *LYS2* genes and is required for p53-mediated or Gal4-VP16-mediated activation (43, 49, 100, 101, 127). In addition, Esa1p is required for transcription of a number of ribosomal protein genes, to which it may be specifically recruited by the DNA-binding proteins Rap1p or Abf1p (106). This recruitment is downstream of the nutrient sensing, *T*arget *o*f *R*apamycin (TOR) signaling pathway. When nutrients are plentiful, TOR signaling results in activated transcription of ribosomal protein genes. However, in conditions of nitrogen or other nutrient limitation, TOR signaling is decreased, resulting in a reduction of ribosomal protein synthesis and a concomitant induction of genes required for the cellular response to nitrogen and carbon starvation (108). Finally, the NuA4 complex has been implicated in "global" acetylation of the histone H4 tail throughout the genome. This non-targeted acetylation is independent of transcriptional activation and has been proposed as a mechanism to "reset" acetylation marks after perturbations due to other cellular processes (*e.g.* transcriptional regulation, recombination, repair, or replication) (106, 128).

To determine if the NuA4 complex operates in a parallel pathway to an Isw1 complex, deletion of the non-essential *EAF1* and *YNG2* genes were made in W303 *wild-type* and *isw1* strains. In addition, a set of *eaf3* and *isw* mutants in S288C was kindly provided by Jacques Côté at the Laval University Cancer Research Center. Mutants of

Figure 10: *ISW1* interacts genetically with histone H4 deletion mutants on rapamycin

A) H4 nested tail deletion mutants interact with *isw1* mutants at elevated temperatures. Strains whose sole source of *wild-type* histones H3 and H4 was on the *URA3*-marked plasmid pRM200U (YTT1970 and YTT1971) were transformed with a vector expressing *wild-type* H3 and either *wild-type* or nested tail deletion mutants of histone H4 (pMP3, pRS414-HHT2-hhf2Δ4-19, pJV101 (Δ4-6), pJV102 (Δ4-10), pJV103 (Δ4-14), pJV104 (Δ7-19), pJV105 (11-19), or pJV106 (Δ15-19)). Following 5-FOA counter-selection against pRM200U, a large patch of cells derived from a single colony was resuspended in sterile water and serial dilutions were spotted onto YEPD media and grown at room temperature, 30°, or 37° C.

B) H4 nested tail deletion mutants interact with *isw1* mutants on rapamycin. Identical dilutions as in (A) were spotted onto YEPD media containing 0.6 % DMSO, 30 μg/ml CT (0.6 % DMSO), 100 m*M* HU, 0.015% MMS, or 25 n*M* rapamycin and grown at room temperature.


A
"w.t."
Δisw1
w.t.
Δ4-19
Δ4-6
Δ4-10
Δ4-14
Δ7-19
Δ11-19
Δ15-19
RT
30
37

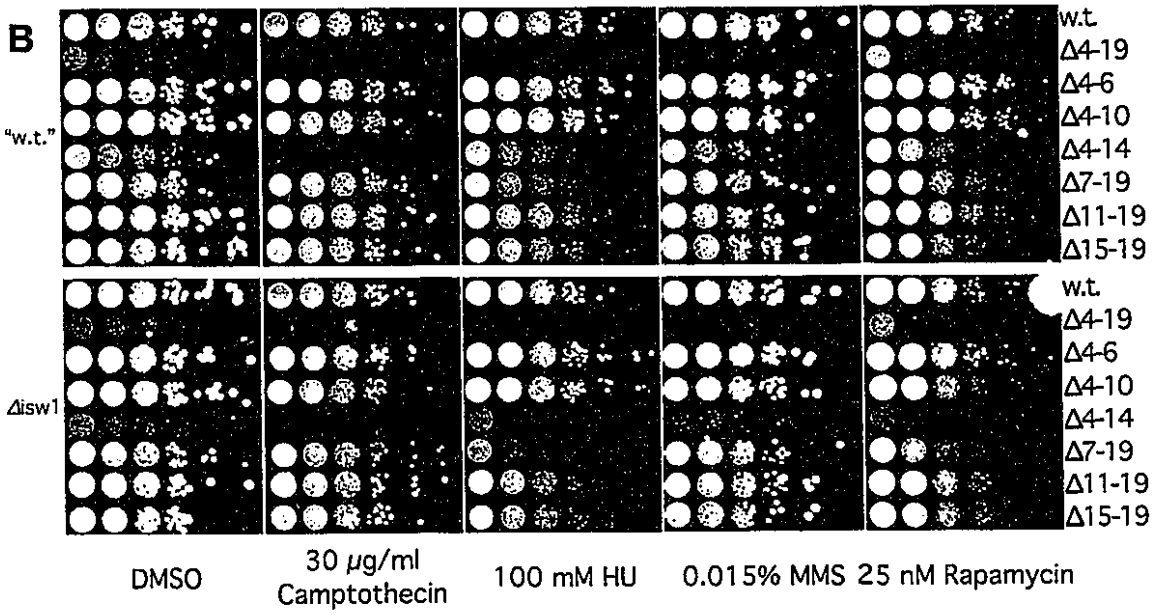
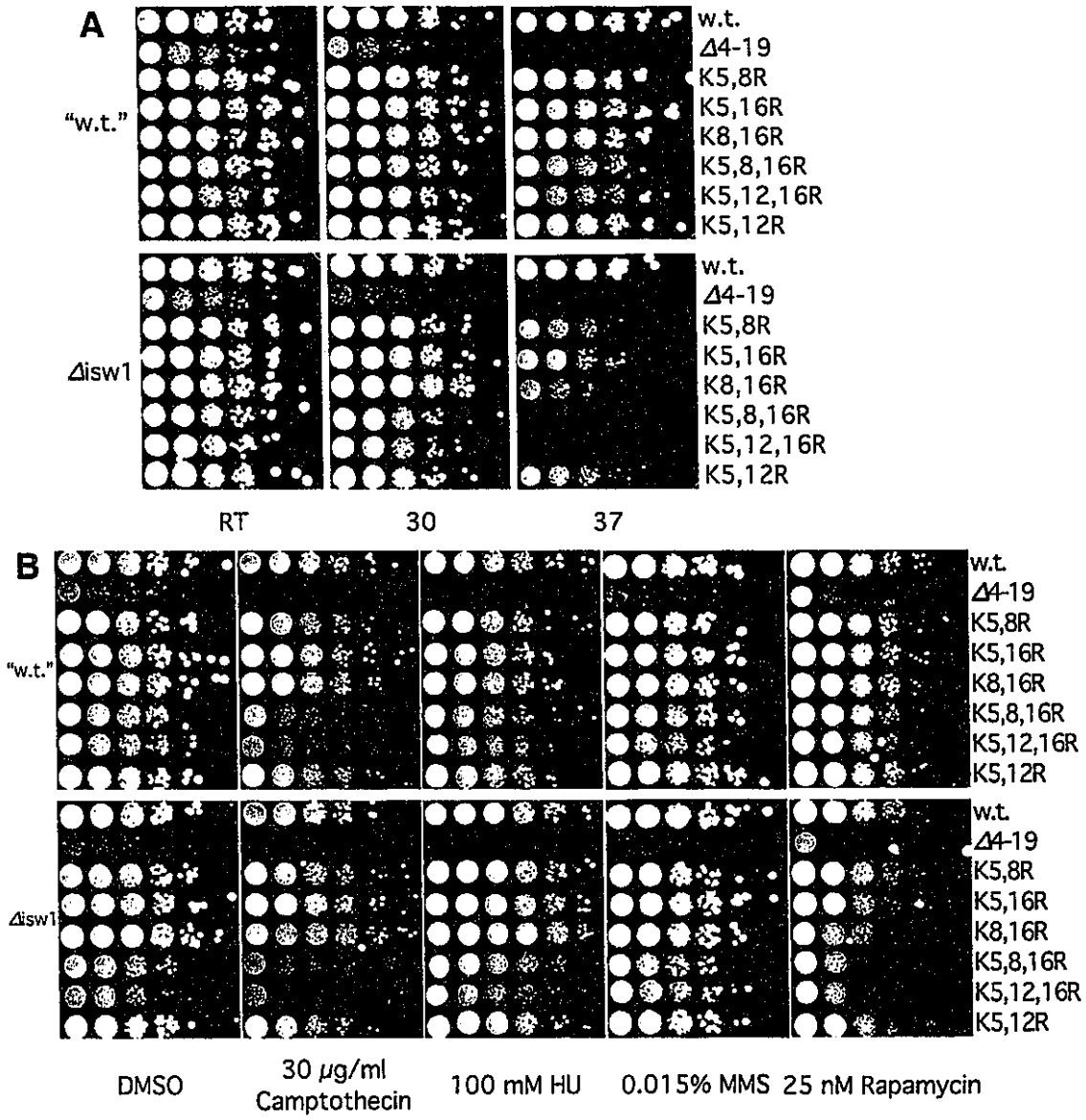

B
"w.t."
Δisw1
w.t.
Δ4-19
Δ4-6
Δ4-10
Δ4-14
Δ7-19
Δ11-19
Δ15-19
DMSO
30 μg/ml Camptothecin
100 mM HU
0.015% MMS
25 nM Rapamycin

Figure 11: *ISW1* interacts genetically with histone H4 lysine mutants on rapamycin

A) H4 lysine to arginine tail mutants interact with *isw1* mutants at elevated temperatures. Strains whose sole source of *wild-type* histones H3 and H4 was on the *URA3*-marked plasmid pRM200U (YTT1970 and YTT1971) were transformed with a vector expressing *wild-type* H3 and either *wild-type* or lysine to arginine mutants of histone H4 (pMP3, pRS414-HHT2-hhf2Δ4-19, pMP15 (K5, 8R), pMP121 (K5, 16R), pMP122 (K8, 16R), pMP63 (K5, 8, 16R), pMP125 (K5, 12, 16R), and pMP110 (K5, 12R)). Following 5-FOA counter-selection against pRM200U, a large patch of cells derived from a single colony was resuspended in sterile water and serial dilutions were spotted onto YEPD media and grown at room temperature, 30°, or 37° C.

B) H4 lysine to arginine tail mutants interact with *isw1* mutants on rapamycin. Identical dilutions as in (A) were spotted onto YEPD media containing 0.6 % DMSO, 30 μg/ml CT (0.6 % DMSO), 100 m*M* HU, 0.015 % MMS, or 25 n*M* rapamycin and grown at room temperature.


A
"w.t."
Δisw1
w.t.
Δ4-19
K5,8R
K5,16R
K8,16R
K5,8,16R
K5,12,16R
K5,12R
RT
30
37
B
"w.t."
Δisw1
DMSO
30 μg/ml Camptothecin
100 mM HU
0.015% MMS
25 nM Rapamycin

YNG2 reduce global histone H4 acetylation by at least 5-fold and show defects in DNA repair and gene-specific transcriptional activation (24, 25, 87, 100). In contrast, *eaf1* and *eaf3* mutants do not show defects in global HAT activity, yet do show defects in gene-specific transcription, and *eaf1* mutants are sensitive to DNA damaging agents (14, 34, 43). These mutants, therefore, provide a possible means to separate the global HAT activity from other NuA4 functions. To study the essential *ESA1* gene, plasmids expressing 6xHA-tagged *ESA1*, *esa1-G315E*, or *esa1-L254P* were kindly provided by Jacques Côté. The *L254P* allele disrupts a conserved leucine residue in the hydrophobic core required for HAT activity, and cannot support growth at 37° C, while the *G315E* allele grows normally, but has defects in HAT activity and transcriptional activation of *PHO5* (but not the other NuA4 target genes *HIS4* or *TRP4*) (1, 28, 43). Using the same parent plasmid, an *L357H* allele was created; this mutation has been reported to be inviable at 37° C, but does not exhibit sensitivity to the DNA damaging agents MMS or CT at permissive temperatures (16). These *esa1* alleles provide a means by which to further separate the functions of the NuA4 complex and were introduced into *wild-type*, *isw1*, and *isw1-K227R* (catalytically inactive) strains to determine if they show genetic interactions with *ISW1*.

Serial dilutions of *eaf1*, *eaf3*, and *yng2* mutants in *wild-type* or *isw* strains were spotted onto YEPD media and incubated at room temperature, 30°, or 37° C (Figure 12A). Mutants of *eaf1*, *eaf3*, and *yng2* all showed synthetic growth defects with *isw1* mutations at elevated temperatures (although the defect in an *isw1eaf3* mutant was mild), as was also observed for histone H4 tail mutants, supporting our hypothesis that NuA4 could be mediating this interaction.

In addition, all of the *esa1* point mutants showed a mild synthetic growth phenotype (Figure 13A). As previously published, *esa1-G315E* grows normally, while *esa1-L254P* and *esa1-L357H* are both inviable at 37° C (16, 28, 114). At lower temperatures, where *esa1-L254P* or *esa1-L357H* mutants can support viability, a synthetic growth defect is observed for all three mutants, supporting our hypothesis.

The synthetic growth defects observed with *isw1* mutants and the NuA4 components prompted an investigation to determine which, if any, of the cellular functions of the NuA4 complex might act in parallel with an Isw1 complex. To this end, identical dilutions of each strain were spotted onto media containing rapamycin (to inhibit the TOR pathway), MMS, HU, or CT (to assess sensitivity to DNA damaging agents. CT is prepared in DMSO, so a control plate with an equivalent concentration of DMSO was also tested.

Neither the histone deletion mutants nor the lysine to arginine substitution mutants showed growth defects on media containing MMS, HU or CT that were significantly more severe with an *isw1* mutant as compared to *wild-type*, nor were they sensitive to 500 Gy of γ-irradiation (Figures 10B and 11B and data not shown). However, both sets of mutants did show growth defects on media containing rapamycin in a manner that was proportional to the number of lysines that were deleted or substituted (Figures 10B and 11B). This suggests that a parallel pathway that requires these lysine residues may utilize the TOR signaling pathway, as does the NuA4 complex.

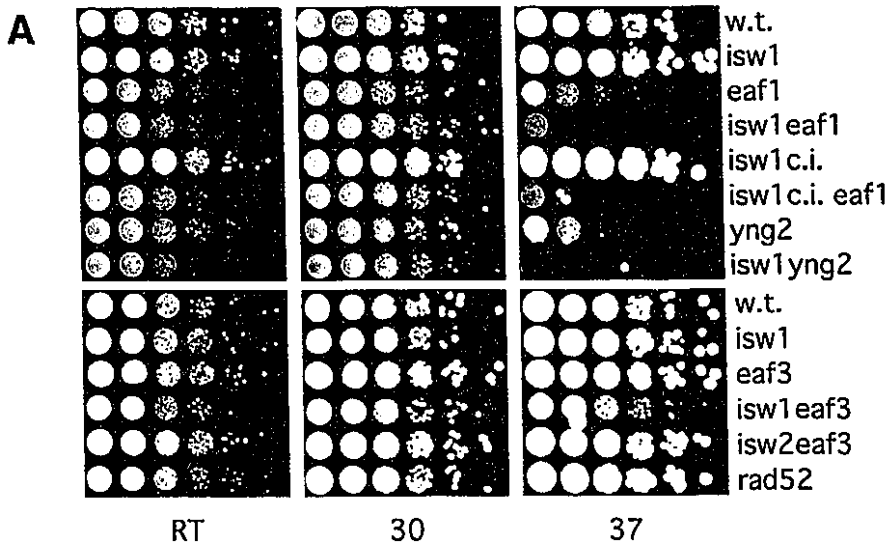
The *isw1eaf3* mutants showed a strong sensitivity to MMS (and a slight sensitivity to HU and CT, but not γ-irradiation) that was much more severe than either single mutant or an *isw2eaf3* mutant, illustrating specificity to *isw1* (Figure 12B and data not shown). As both *eaf1* and *yng2* single mutants were inviable at this concentration of MMS (0.03%), they were tested at a permissive concentration (0.015%), in which both also showed synthetic growth defects with *isw1*. Neither *isw1eaf1* nor *isw1yng2* mutants showed synthetic growth defects on media containing HU or CT or to γ-irradiation (Figure 12B and data not shown). None of the histone H4 tail mutants showed a synthetic growth defect to any of the DNA damaging agents (MMS, HU or CT), however (Figures 10B and 11B). This suggests that if *ISW1* interacts with the NuA4 complex in a DNA damage response pathway, it may be independent of the pathway that requires the four acetylatable lysines.

Both *isw1eaf1* and *isw1yng2* strains grew more slowly on media containing rapamycin than any of the single mutants, suggesting a function of NuA4-dependent

Figure 12: *ISW1* interacts genetically with *eaf1*, *eaf3*, and *yng2* mutants on MMS & rapamycin

A) *eaf1*, *eaf3*, and *yng2* mutants interact with *isw1* mutants at elevated temperatures. A large patch of cells derived from a single colony of *isw1*, *isw1c.i.* (*K227R* catalytically inactive mutant), *eaf1*, or *yng2* mutants in single and double combinations was resuspended in sterile water and serial dilutions were spotted onto YEPD media and grown at room temperature, 30°, or 37° C

B) *eaf1*, *eaf3*, and *yng2* mutants interact with *isw1* mutants on MMS and/or rapamycin. Identical dilutions as in (A) were spotted onto YEPD media containing 0.6 % DMSO, 30 μg/ml CT (0.6 % DMSO), 100 m*M* HU, 0.03 % MMS (or 0.015 % MMS for eaf1 and yng2 mutants), or 6.25 n*M* rapamycin and grown at room temperature.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rad52
RT
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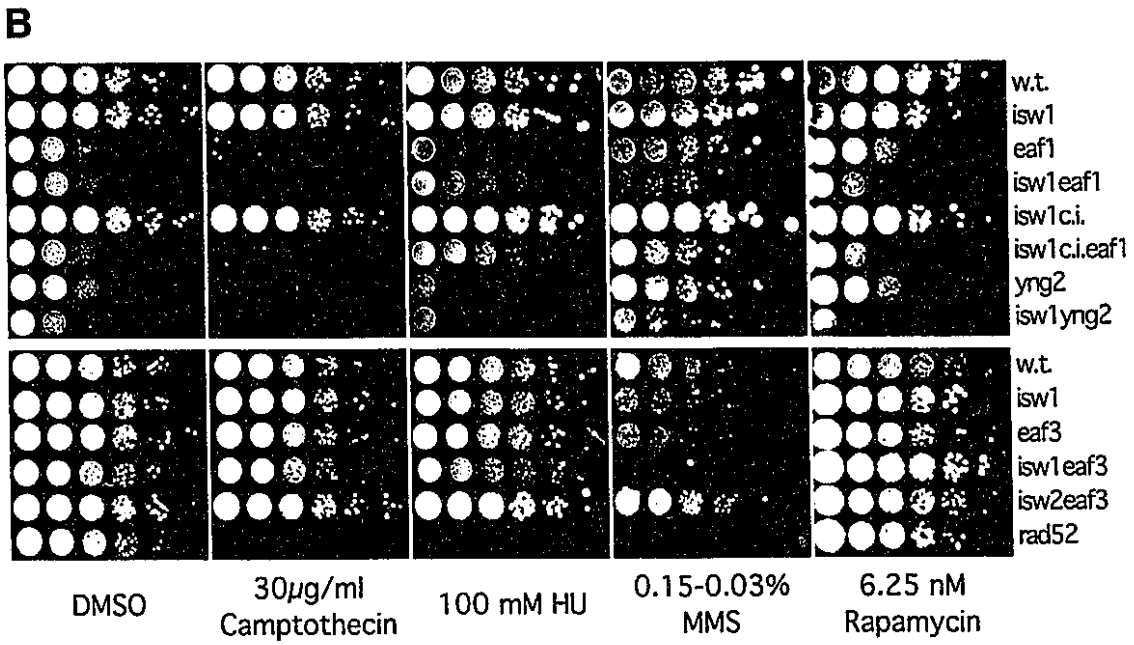
B
w.t.
isw1
eaf1
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isw1c.i.eaf1
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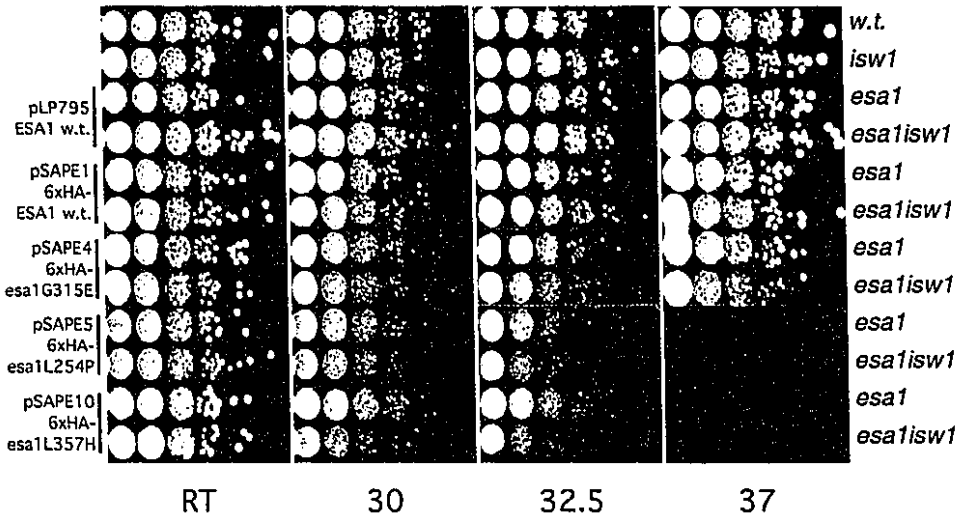
w.t.
isw1
eaf3
isw1eaf3
isw2eaf3
rad52
DMSO
30μg/ml Camptothecin
100 mM HU
0.15-0.03% MMS
6.25 nM Rapamycin

Figure 13: *ISW1* interacts genetically with *esa1* mutants on MMS & rapamycin

A) *esa1* mutants do not interact with *isw1* mutants at elevated temperatures. *wild-type* or *isw1* strains or *esa1* or *isw1esa1* mutant strains containing pLP795 (*CEN/ARS/URA3/ESA1*), pSAPE1 (*CEN/ARS/LEU2/6xHA-ESA1*), pSAPE4 (*CEN/ARS/LEU2/6xHA-esa1-G315E*), pSAPE5 (*CEN/ARS/LEU2/6xHA-esa1-L254P*), or pSAPE10 (*CEN/ARS/LEU2/6xHA-esa1-L357H*). Following 5-FOA counter-selection against pLP795 for the pSAPE-containing strains, a large patch of cells derived from a single colony was resuspended in sterile water and serial dilutions were spotted onto YPD media and grown at room temperature, 30°, 32.5° or 37° C

B) *esa1* mutants interact with *isw1* mutants on CT, HU, MMS and/or rapamycin. Identical dilutions as in (A) were spotted onto YPD media containing 0.6 % DMSO, 30 μg/ml CT (0.6 % DMSO), 100 m*M* HU, 0.03 % MMS, or 25 n*M* rapamycin and grown at room temperature.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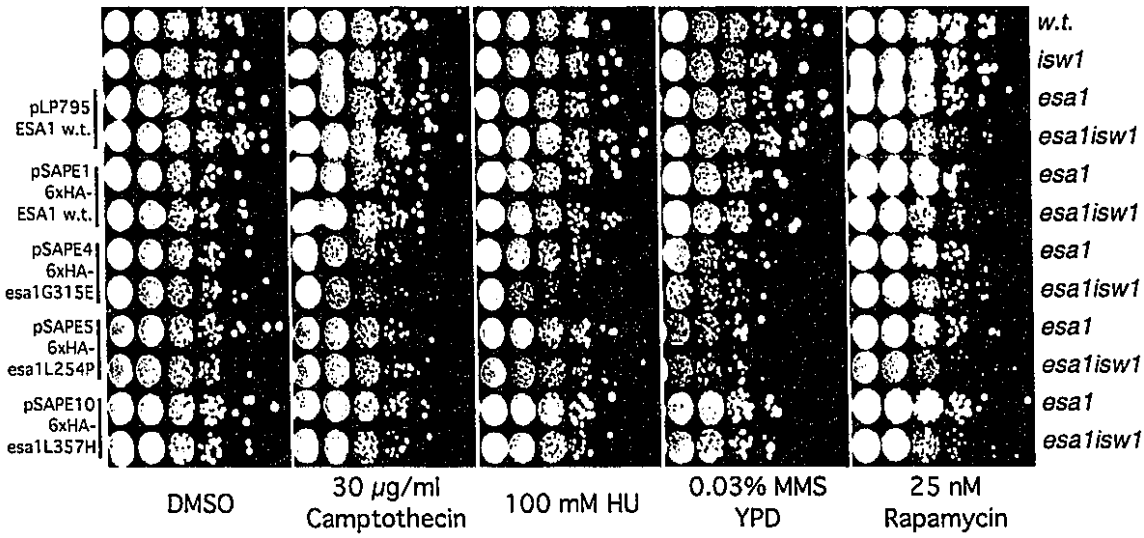
pLP795 ESA1 w.t.
pSAPE1 6xHA- ESA1 w.t.
pSAPE4 6xHA- esa1G315E
pSAPE5 6xHA- esa1L254P
pSAPE10 6xHA- esa1L357H
RT
30
32.5
37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esa1isw1
pLP795 ESA1 w.t.
pSAPE1 6xHA- ESA1 w.t.
pSAPE4 6xHA- esa1G315E
pSAPE5 6xHA- esa1L254P
pSAPE10 6xHA- esa1L357H
DMSO
30 μg/ml Camptothecin
100 mM HU
0.03% MMS YPD
25 nM Rapamycin

TOR signaling in parallel to an Isw1 complex. Unexpectedly, while *isw1eaf3* mutants showed synthetic growth defects at elevated temperatures and upon exposure to DNA damaging agents, both *isw1* and *isw1eaf3* mutants grew better on media containing rapamycin than either *wild-type* or *eaf3* single mutant. This may represent a difference in strain backgrounds as *isw1* single mutants in W303 showed sensitivity to rapamycin. This is currently being investigated by Kim Wachter, in the Tsukiyama lab, by creating *eaf3* mutants in a W303 background.

Strains bearing plasmid-borne *esa1* point mutants were also tested under these conditions. Unfortunately, variability in the results of separate experiments makes it difficult to draw strong conclusions from these data. This variability may be due to the ability of these strains to easily acquire mutations that suppress the *esa1* mutant (34). The *isw1esa1-G315E* mutants were variably sensitive to HU (Figure 13B) and MMS (data not shown), while the temperature-sensitive *esa1-L254P* or *esa1-L357H* mutants showed little, if any, synthetic effects on either drug. None of the mutants showed any defects on CT or to γ -irradiation, however (Figure 13B).

All of the *isw1esa1* strains bearing plasmid borne *ESA1* or *esa1* mutants were very sensitive to rapamycin, and those expressing *esa1-L254P* or *esa1-L357H* were the most sensitive. This may be due to a slight rapamycin sensitivity of *isw1* mutants that is exacerbated by slightly misregulated *ESA1* expression from the plasmids. Because sensitivity to rapamycin was observed for not only the histone mutants, but also the *esa1* and non-essential NuA4 mutants, this strongly suggests that NuA4 may be operating in a parallel to an Isw1 complex in a pathway that requires TOR signaling. Again, the histone mutants showed no synthetic sensitivities with *isw1* to DNA damaging agents, so any interaction between *ISW1* and *ESA1* must occur by a different pathway than the one that requires histone H4 lysines.

Discussion

The transcriptional changes that occur in response to deletion of Isw1 complex components are relatively mild, as has previously been observed for mutants of the Isw2 complex (47, 53). Only upon simultaneous disruption of the Isw2 complex and the HDAC complex Sin3-Rpd3 are the transcriptional effects of the Isw2 complex unmasked. In an attempt to identify any similar pathway(s) that may act in parallel to one or both Isw1 complexes, targeted deletions were made of several histone deacetylases. Although synthetic growth defects with *isw1* were observed for *rpd3*, *hos2*, and *hst1* mutants at elevated temperatures, the transcriptional changes that occurred in these mutants were not indicative of such a parallel pathway to Isw1 in transcriptional regulation. It is possible that such synthetic transcriptional changes might occur under different growth conditions than those tested (*e.g.* elevated temperatures or after the diauxic shift). However, it is possible that these mutants may interact with *isw1* for reasons independent of transcription. In addition, there may be other complexes not yet mutated that might interact with Isw1 complexes to regulate transcription or other processes not yet assayed.

In an attempt to find such a factor(s), deletions of the histone H3 and H4 tails were made. Mutants of *ISW1* genetically interacted very strongly with the histone H4 tail, specifically the acetylateable lysines 5, 8, 12, and 16—lysine 16 being the least able to rescue the synthetic phenotype. This not only matches the *in vivo* substrate preference for the NuA4 histone acetyltransferase complex, but it also mimics the pattern of lysines required for resistance to DNA damaging agents—a process thought to require *ESA1* (16).

To test the requirement of the NuA4 complex in a parallel pathway that requires histone H4 lysines, mutants of the enzymatically active Esa1p subunit as well as the Eaf1p, Eaf3p, and Yng2p non-essential subunits of the NuA4 complex were made in *isw1* strains. Both the *esa1* mutants and the non-essential NuA4 components interacted with *ISW1* at elevated temperatures, suggesting that the NuA4 complex is indeed operating in parallel to an Isw1 complex. In an attempt to identify which of the several *in vivo*

functions that have been ascribed to the NuA4 complex operates in parallel to an Isw1 complex, both the histone H4 tail mutants as well as the NuA4 complex mutants were tested on media containing DNA damaging agents to assess defects in repair or containing rapamycin to impede TOR signaling. While sensitivities to the DNA damaging agents MMS, HU, and CT differed in double mutants of *isw1* and NuA4 subunits, no *isw1*-specific sensitivities were observed for the histone H4 tail mutants suggesting that any such interaction is likely to be independent of the histone H4-dependent pathway being pursued in these studies (see Figure 14). To the contrary, however, the histone H4, *eaf1*, *yng2*, and *esa1* mutants tested were sensitive to rapamycin in an *isw1*-dependent manner, strongly suggesting that TOR signaling is required for any such parallel pathway. Interestingly, many genes that are repressed by TOR signaling, such as those involved in carbohydrate utilization and amino acid transport, are misregulated in *isw1* and *ioc* mutant strains (data not shown and Figure 6D). Unfortunately, none of the *esa1* point mutants or non-essential NuA4 mutants has been studied for its roles in TOR signaling and the downstream activation of ribosomal protein genes; therefore, none can be used as a potential separation of function mutant to test this hypothesis. It may be particularly useful to determine if the Eaf3p subunit has a unique role in TOR-dependent NuA4 transcriptional activation as addition of an *isw1* mutant suppresses *eaf3* sensitivity to rapamycin, however, this suppression may be due to a dominant *isw1* rapamycin resistance in S288C, rather than an *eaf3*-specific phenotype. There are precedents in the literature for chromatin regulation in the response to rapamycin and TOR signaling. Localization of components of the RSC chromatin remodeling complex are dramatically altered throughout the genome upon rapamycin treatment (36). In addition, disruption of TOR signaling by rapamycin is associated with Sir3p hyperphosphorylation and loss of subtelomeric silencing. *SIR3* deletion confers partial rapamycin resistance as well (3).

Recently, the Esa1p, Epl1p, and Yng2p components of NuA4 have been purified in a novel trimeric complex termed "*Pic*colo *NuA4*" (picNuA4). In addition, specific disruption of the larger NuA4 complex is not lethal, suggesting that the essential nature

of the NuA4 complex may be due to the smaller picNuA4 complex (19). As *ISW1* genetically interacts with components specific to the larger NuA4 complex, as well as those shared with the picNuA4 complex, it is possible that it interacts with one or both complexes. Deletion of the C-terminal half of the Epl1 protein appears to result in specific loss of the larger NuA4 complex (19). This mutant would be a useful tool to determine which complex interacts with an Isw1 complex. Although the larger NuA4 complex appears to be required for gene-specific transcriptional activation, it is not yet clear which of the other known "NuA4" functions are dependent upon the larger or smaller complexes. Such studies could greatly assist in determining the *in vivo* process that acts in parallel to an Isw1 complex.

Another yeast gene, *BDF1*, shows genetic interactions with both histone H4 lysine residues as well as *ESA1* (94). Bdf1p physically interacts with TFIID and may correspond to a "missing" portion of $TAF_{II}250$ in yeast (93). A $TAF_{II}250$ homolog, BR140, is one of a small collection of proteins that possess both a PHD finger as well as a PWWP domain, which are also found in Ioc2p and Ioc4p, respectively. It will be interesting to determine if Isw1p and Bdf1p act in the same pathway.

In *Drosophila*, ISWI and the *ESA1* homolog, MOF, genetically interact in the regulation of male X-chromosome structure (see Chapter I). However, the nature of the interaction is quite different than that observed in yeast. Overexpression of MOF can enhance the phenotype of dominant negative *iswi* mutants, suggesting that they may have competing activities in *Drosophila* (29). In addition, while Esa1p prefers histone H4 lysines 5, 8, and 12 as *in vivo* substrates, MOF specifically acetylates lysine 16 (the target of the Sas2p HAT in yeast). It is unclear if yeast Isw1 or Isw2 complexes require the basic RHR motif of histone H4 for their activity as is the case for *Drosophila* ISWI. Microarray expression analysis of histone H4 R17A/R19A mutants of this motif show similar changes as *isw1* or *isw2* mutants, suggesting that such a requirement may be conserved (Tom Fazzio, unpublished observations). However, histone H4 deletion mutants lacking this motif (*e.g. hhf2Δ4-19* or *hhf2Δ7-19* alleles) still show a synthetic growth defect upon deletion of *ISW1*, suggesting that this motif is not required for Isw1

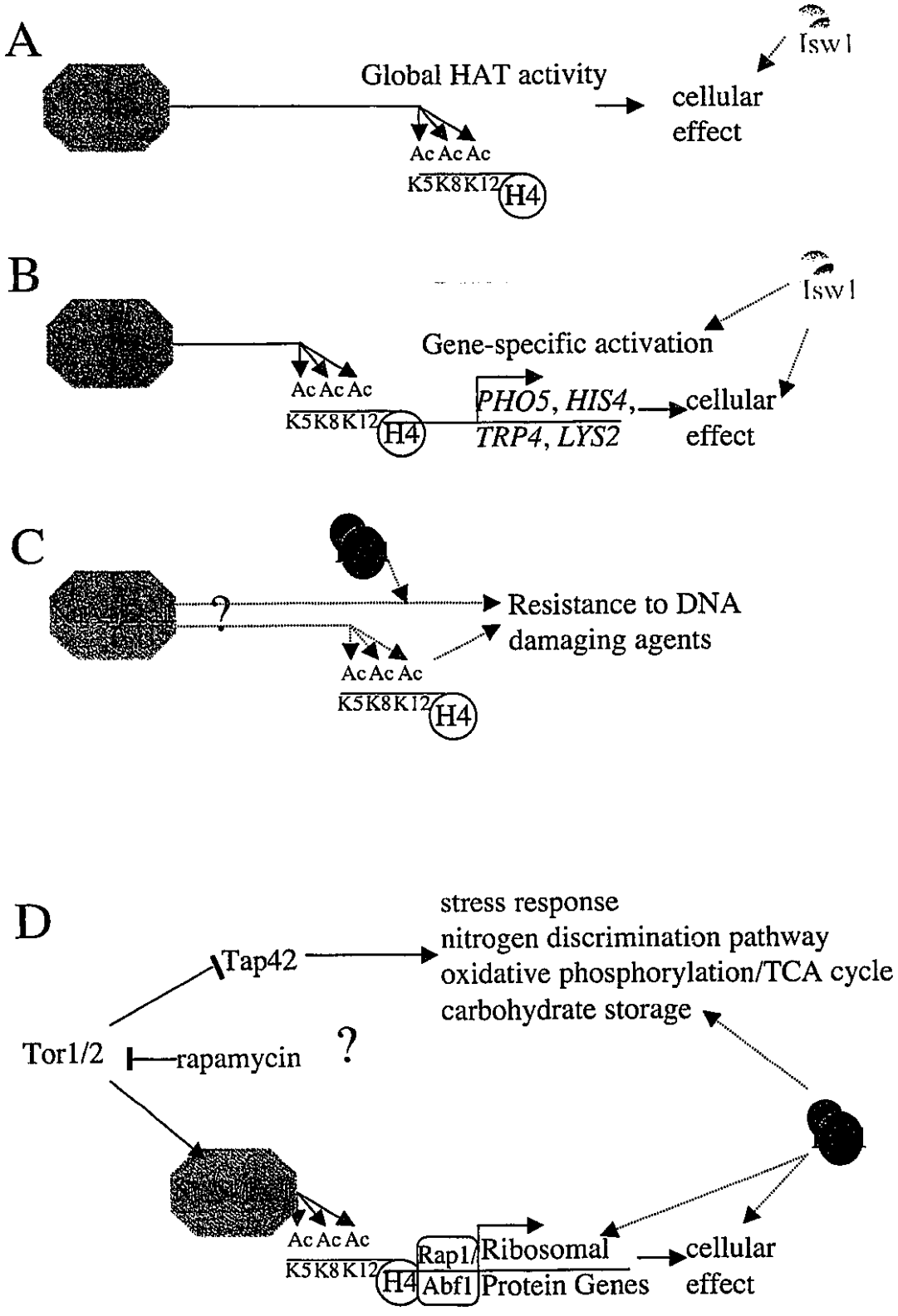
Figure 14: Model for NuA4 and Isw1 complex parallel activities

The NuA4 complex has four known functions *in vivo*: A) Global transcription-independent HAT activity; B) Gene-specific transcriptional activation; C) Resistance to DNA damaging agents; and D) TOR-pathway dependent activation of ribosomal protein gene transcription.

A) & B) The possibility of Isw1 complex activity in a pathway parallel to NuA4-mediated global histone H4 acetylation, or gene-specific transcriptional activation is not suggested by the data in this dissertation, yet it has not been excluded.

C) Genetic interactions between *ISW1* and gene encoding NuA4 components on media containing DNA-damaging agents suggests that an Isw1 complex may operate in parallel to NuA4 complex-dependent resistance to these agents. NuA4-dependent resistance to these agents is associated with acetylation of histone H4 lysines 5, 8, and 12; however, strains bearing mutations of these histone residues do not show *isw1*-dependent growth defects to DNA-damaging agents. This suggests that an Isw1 complex may operate in parallel to NuA4 in a histone H4-independent DNA damage resistance pathway.

D) TOR signaling both activates transcription of several ribosomal protein genes as well as inhibits the nuclear import of transcription factors involved in the cellular response to nutrient quality or limitation. As both pathways are inhibited by the drug rapamycin, the genetic interactions of *ISW1* and genes encoding NuA4 components on rapamycin suggests that an Isw1 complex may operate in parallel to the NuA4 complex in a TOR-dependent pathway. An Isw1 complex may act in parallel to ribosomal protein gene activation, which is known to require the NuA4 complex. Alternatively, it may act in parallel to the inhibition of the nutrient limitation response, although a role of the NuA4 complex in this inhibition has not yet been discovered.


A
Isw1
Global HAT activity
cellular
effect
Ac Ac Ac
K5K8K12
H4
B
Isw1
Gene-specific activation
Ac Ac Ac
K5K8K12
H4
PHO5, HIS4,
TRP4, LYS2
cellular
effect
C
?
Resistance to DNA
damaging agents
Ac Ac Ac
K5K8K12
H4
D
stress response
nitrogen discrimination pathway
oxidative phosphorylation/TCA cycle
carbohydrate storage
Tap42
Tor1/2
rapamycin
?
Ac Ac Ac
K5K8K12
H4
Rap1/
Abf1
Ribosomal
Protein Genes
cellular
effect

to function in this pathway. A human homolog of Esa1p, the HIV *T*at-*i*nteracting *P*rotein-*60* (TIP60), may be a more functionally similar homolog. TIP60 has a similar substrate preference to Esa1p, preferring to acetylate lysines 5, 8, 12, and 16 of histone H4 and lysine 5 of histone H2A (76). It would be interesting to determine if *TIP60* genetically interacts with the human ISWI homologs, *hSNF2H* or *hSNF2L* as has now been observed in yeast and flies.

While the involvement of a NuA4 complex in a pathway that operates in parallel to the Isw1 complex has not yet been proven, it remains the most likely candidate to be mediating the genetic interaction observed between *ISW1* and the histone H4 tail. Unpublished work by Kim Wachter, a graduate student in the Tsukiyama lab, shows that mutation of *IOC2*, but not *IOC3*, phenocopy mutation of *ISW1* in a histone H4 mutant showing that the interaction is due to the Isw1b complex specifically. Further dissection of the involvement of the NuA4 complex and/or picNuA4 complex in such a pathway may help to solidify our understanding of the nature of this interaction. In addition, the apparent specificity of *isw1* mutants to interact with mutants of NuA4 components and histone H4 mutants on rapamycin points to an involvement of the TOR signaling pathway in this interaction. Determining the cause of the synthetic sickness on rapamycin will be useful in identifying what may be a previously unknown function of the Isw1b complex *in vivo*, and may provide clues as to ISWI functions in higher eukaryotes, as well.

Chapter V: Materials & Methods

Media and Strains

Yeast strains were grown and manipulated according to standard procedures (2). All W303 strains used in this study were derived from a parent in which a weak *rad5* mutation was repaired (118, 134); these and all other strains are listed in Table I.

Construction of a 3' tandem FLAG epitope at the ISW1 genomic locus was accomplished by subcloning the ISW1-FL2 gene from pRS416-ISW1FL2 (121) into a pRS406 vector containing roughly 1 kb of ISW1 3' non-coding region, followed by integration by homologous recombination using the *URA3* pop-in/5-FOA pop-out method (18). Integrations of the 3' triple MYC and 3' triple HA epitope tags were done using a PCR-based strategy that utilized pMPY-3xMYC and pMPY-3xHA, respectively (112).

Disruption of genes with the dominant drug resistance cassettes kanMX, natMX, or hphMX was accomplished using a PCR-based strategy based upon the plasmids pUG6, pAG25, or pAG32, respectively (54, 56).

The ISW1 genomic locus was fused to the *T*andem *A*ffinity *P*urification (TAP) tag by a PCR-based strategy utilizing the plasmid pBS1479 as a template, generously provided by Bertrand Séraphin (107).

Plasmids

Plasmids used for histone plasmid shuffling experiments were the following: pRS414 (*CEN6/ARSH4/TRP1* (113)); pRM200U (*CEN4/ARS1/URA3*/expressing *HHT2* and *HHF2* from their native promoters (91, 129)); pMP3 (*CEN6/ARSH4/TRP1*/expressing *HHT2* and *HHF2* from their native promoters (74)); pFVL85-8 (pMP3-based with deletion of *HHT2* residues 2-30—a kind gift of Fred van Leeuwen and Dan Gottschling); pRS414-HHT2-hhf2Δ4-19 (the *Not*I/*Xho*I fragment of pMP3 was mutagenized using the Quick-Change Site-Directed Mutagenesis kit

(Stratagene) to create a deletion of *HHF2* residues 4-19, and was subcloned into pRS414—a kind gift of Thomas Fazzio); pJV101 (the *SacI*/*KpnI* fragment of pMP3 was mutagenized to create deletions of *HHF2* residues 4-6, and was subcloned into pRS414 after sequencing to confirm that no PCR errors had been generated), and similarly created pJV102 (Δ4-10), pJV103 (Δ4-14), pJV104 (Δ7-19), pJV105 (Δ11-19), pJV106 (Δ15-19); pMP13 (pMP3 was mutagenized to create a lysine to arginine substitution at *HHF2* residue 5 and the coding sequences were checked by DNA sequencing—this and all subsequent pMP plasmids were a kind gift of Mark Parthun), and similarly created pMP23 (K8R), pMP14 (K12R), pMP24 (K16R), pMP15 (K5, 8R), pMP110 (K5, 12R), pMP121 (K5, 16R), pMP111 (K8, 12R), pMP122 (K8, 16R), pMP123 (K12, 16R), pMP124 (K8, 12, 16R), pMP125 (K5, 12, 16R), pMP63 (K5, 8, 16R), pMP62 (K5, 8, 12R). All histone residue numbers refer to residues remaining after post-translational processing that removes the N-terminal methionine of both histone H3 and H4.

Other plasmids used were the following: pLP795 (*CEN/ARS/URA3/ESA1*), pSAPE1 (*CEN/ARS/LEU2/6xHA-ESA1*), pSAPE4 (*CEN/ARS/LEU2/6xHA-esa1-G315E*), and pSAPE5 (*CEN/ARS/LEU2/6xHA-esa1-L254P*)—all kind gifts of Jacques Côté; pSAPE10 (*CEN/ARS/LEU2/6xHA-esa1-L357H*) was derived from pSAPE1 utilizing the Quick-Change Site-Directed Mutagenesis kit (Stratagene) to introduce a leucine to histidine substitution at residue 357 of *ESA1* (16); pRS416 *(CEN6/ARSH4/URA3* (113)); pRS416-ISW2 and pRS416-ISW1-ISW2 (121); and pRS416-ISW1-AM (same as pRS416-ISW1 (121), except that an additional 430 base pairs of 3' non-coding sequence was added to ensure proper termination of the transcript.).

Purification of Isw1 complexes for *in vitro* assays

All whole cell yeast extracts described in this dissertation were created by growing cultures to saturation, unless otherwise noted. Cells were pelleted by centrifugation and washed twice in approximately 5 pellet volumes of H_2O with 2 m*M* DTT, 1 m*M* sodium metabisulfite ($Na_2S_2O_5$), and 0.4 m*M* *p*henyl*m*ethyl*s*ulfonyl*f*luoride

(PMSF). Cells were then washed once in 5 pellet volumes of Buffer H-0.3 (0.3 *M* KCl, 25 m*M* Hepes-KOH pH 7.6, 0.1 m*M* EDTA, 0.5 m*M* EGTA, 2 m*M* $MgCl_2$, 20 % Glycerol, 0.02 % NP-40, 1 m*M* DTT, 0.5 m*M* $Na_2S_2O_5$, 1 m*M* PMSF, 2 μM pepstatin A, 0.6 μM leupeptine, 2 μg/ml chymostatin, and 2 m*M* benzamidine—the number following Buffer H refers to the molar concentration of KCl). For most preparations, cells were then either frozen as "noodles" in liquid N_2, or immediately resuspended in an equal volume of Buffer H-0.3, and broken using 45 seconds of cycling through a microfluidizer (Microfluidics Corp.). For TAP precipitations, extracts were frozen after resuspension in Buffer H 0.3 and broken for 90 seconds at high speed in a coffee grinder (Krups, model 408) with dry ice pellets to prevent thawing. The resulting product from either method was centrifuged at 27,000 RPM for 90 minutes in a Beckman SW40Ti rotor to pellet unbroken cells and aggregates. The supernatant was frozen and stored at –80° C.

All complexes purified for *in vitro* assays were derived from saturated cultures. Purification of Isw1p monomer was done concurrently to Isw1 complex purification from either strain YTT449, or from congenic strains (YTT512, YTT548, YTT610) bearing deletions in single *IOC* genes. All such strains yielded identical Isw1p monomer in addition to Isw1a and/or Isw1b complexes. For each volume of extract corresponding to 1 liter of culture, approximately 0.2 ml of α-FLAG M2 antibody conjugated to agarose beads was equilibrated in Buffer H-0.3. This was incubated with extract for 3 hours at 4° C. The beads were gently pelleted and washed twice in 15 ml Buffer H-0.3 with 5 minute incubations at each wash step, followed by 6 x 1 ml washes in Buffer H-0.3 and 3 x 1 ml washes in Buffer H-0.1. One bed volume of FLAG peptide (2 mg/ml DYKDDDDK) in Buffer H-0.1 was added and incubated for 30 minutes at 4° C to elute the precipitated complex. Following four such elutions, the eluates were pooled and applied to a column containing 0.2 ml of BioRex 70 cation exchange resin (BioRad) that had been preequilibrated in Buffer H-0.2 in a Pharmacia AKTA FPLC system. Isw1p monomer does not bind to the resin under this condition, whereas both complexes are retained. The column was washed with Buffer H-0.2, and fractions collected, until the UV absorbance returned to baseline. A linear gradient of Buffer H from 0.2-0.6 *M* KCl,

was then applied and fractions were collected. Fractions containing high concentrations of the purified complex(es) were used for biochemical assays.

Size Exclusion Chromatography

Whole cell extract of YTT642 grown to saturation was prepared in Buffer H-0.3. 1 ml of this extract was applied to a Superose 6 10/30 size exclusion column (Amersham Pharmacia Biotech, Piscataway, NJ) pre-equilibrated in Buffer H-0.3 and 0.5 ml fractions were collected. Following complete elution, gel filtration size standards (Bio-Rad Laboratories, Hercules, CA) were run in identical conditions.

Fractions were loaded onto 9 identical 8% SDS-PAGE gels and transferred to nitrocellulose membranes. Detection of Isw1p, Ioc2p, or Ioc3p was done in triplicate using α-FLAG M2, α-MYC 9E10, or α-HA 16B12 monoclonal antibodies, respectively, and quantified using an Odyssey Infrared Imaging System and fluorescently labeled α-mouse antibody (Li-Cor Biosciences, Lincoln, NE). The integrated intensities of the bands in each gel were normalized to their mean intensity. To account for quantitative differences due to loading variability, these values were averaged with the corresponding lanes for each triplicate blot to determine the elution profiles for each protein, although the elution profiles for any individual blot were similar to those of the averaged values.

Immunoprecipitations

FLAG, Myc, and HA immunoprecipitations were done using extract from YTT642 grown to saturation. 20 μl of Protein G Sepharose (Amersham Pharmacia Biotech) was incubated with either 20 μl 16B12 α- HA monoclonal antibody, 9E10 α-Myc monoclonal antibody or buffer alone in 200 μl 1x PBS 0.05 % NP-40 and incubated for 1 hour at 25° C. The precoupled resin was then washed 3 x 1 ml Buffer H-0.3. Each precoupled resin preparation or 40 μl equilibrated α-FLAG M2 agarose resin or Protein G resin alone was added to 200 μl YTT642 extract and incubated at 4° C for 4 hours.

Each immunoprecipitate was washed once in Buffer H-0.3 and twice in Buffer H-0.1 (to reduce precipitation of SDS and DTT during subsequent gel loading) before being eluted in SDS loading buffer at 95° C for 5 minutes. Samples of starting material, supernatants and immunoprecipitates of each IP were loaded on 8% SDS-PAGE gels, transferred to nitrocellulose, probed with either α-Myc 9E10, α-HA polyclonal, or α-FLAG M2 and appropriate HRP-conjugated secondary antibodies, and visualized with SuperSignal West Femto chemiluminescent substrate (Pierce).

TAP precipitations were done using whole cell extract from 12 liter cultures grown to OD_{660}=0.7. For each extract, 0.8 ml of Pharmacia IgG Sepharose 6 Fast Flow suspension and 0.8 ml of Stratagene Calmodulin Affinity Resin were used. Purification was done as described (107) with the following changes: 1) The IgG precipitation was done in extract prepared in Buffer H-0.3 with NP-40 added to 0.1 %, without dialysis; 2) Both precipitations were done by batch method in 50 ml or 15 ml tubes rather than in a column, and one 5-minute wash step was added following both incubations before transferring to a column for subsequent washing; 3) KCl concentration of buffer used for washing the IgG precipitation was raised to 0.3 *M*; 4) NP-40 was omitted from the Calmodulin Binding and Elution buffers; and 5) Additional final elution fractions were collected to increase yields.

Mass Spectrometry

Samples from TAP purification were precipitated with *tri*chloro*a*cetic acid (TCA) and resuspended in 8 *M* Urea, 100 m*M* NH_2HCO_3 at 37° C for 5 minutes. DTT was added to 1 m*M* and incubated at 37° C for 20 minutes, and reactive thiol groups were covalently modified with 10 m*M* iodoacetimide for 20 minutes at 25° C. $CaCl_2$ was added to 1 m*M* and Lys-C endoproteinase was added at ~1:20 (w/w) dilution at 37° C for 4 hours. The digested sample was diluted to 2 *M* Urea in 100 m*M* NH_2HCO_3 and $CaCl_2$ was added to 5 m*M*. Trypsin was added at ~1:20 (w/w) dilution, and incubated overnight at 37° C. Sample was concentrated to 8 *M* Urea in a vacuum chamber and *tri*fluoro*a*cetic

acid (TFA) and *aceto*nitrile (ACN) were added to 2 % and 1 %, respectively. The digested sample was washed using a C18 resin Zip-Tip (Millipore) and dried. Peptide detection was done by liquid chromatographic electrospray ionization tandem mass spectrometry and analysis was done using the SEQUEST software package.

ATPase assays

Nucleosomes were assembled from 2 μg purified *Drosophila* histones and 1.5 μg λ DNA in the presence of 75 μg BSA and trace amounts of ^{32}P-labeled dHSP70 promoter DNA to confirm assembly. The reaction was assembled in 150 μl Hi Buffer (2 *M* NaCl, 10 m*M* Tris pH 7.6, 1 m*M* EDTA, 0.05 % NP-40 5 m*M* 2-mercaptoethanol) and gradually dialyzed to 50 m*M* NaCl in otherwise similar buffer over 15 hours.

Each 5 μl ATPase reaction used 25 ng DNA equivalents of these prepared nucleosomes, or equivalent control assemblies of histones, DNA, or buffer alone. The reactions contained 5 m*M* $MgCl_2$, 0.1 m*M* cold ATP, and 1-2 mCi/ml [γ-^{32}P] ATP and Isw1 complex normalized to 0.8 μg/ml of Isw1p by silver stained SDS-PAGE gels. Reactions were incubated at 30° C for 30 minutes. To separate the hydrolyzed [^{32}P] Pi from intact [γ-^{32}P] ATP, 0.5 μl of each reaction was spotted onto a 20 cm x 10 cm cellulose PEI-F sheet (J. T. Baker Inc.), dried, and then separated by thin layer chromatography in 0.8 *M* LiCl, 0.8 *M* Acetic acid. ATPase activity was determined by measuring the free [^{32}P] Pi/[γ-^{32}P] ATP ratio as determined by scintillation counting or phosphorimager.

Nucleosomal spacing assays

A reaction containing 220 ng recombinant yeast octamer (51), 750 ng Nap1p, and Isw1 complex (4 ng Isw1p equivalent) was assembled in ExB 5/50 buffer (10 m*M* Hepes-KOH pH 7.6, 50 m*M* KCl, 5 m*M* $MgCl_2$, 0.5 m*M* EGTA, 10 % glycerol, 0.1 mg/ml BSA), and incubated on ice 30 minutes. 250 ng λ DNA, ATP to 3 m*M* and an ATP

regeneration system (McNAP) (13) were then added to a final reaction volume of 30 μl, followed by incubation at 30° C for 4 hours. $CaCl_2$ was added to 2 m*M* and 5-15 units MNase were added. The reaction was allowed to proceed at 25° C for 3 or 15 minutes; 14.5 μl was removed at each time point and added to 5 μl of stop solution (4 mg/ml glycogen, 2 % Sarkosyl, 80 m*M* EDTA). Following Proteinase K digestion, DNA was precipitated and run on a 1.3 % agarose gel and stained with Ethidium Bromide.

Nucleosomal sliding assays

Sliding assays were done by our collaborators in the Bartholomew lab utilizing our purified Isw1 complexes. The DNA used for reconstitutions was a 214 bp *Eco*RI-*Dde*I fragment of DNA derived from the *Xenopus borealis* somatic 5S rRNA gene.

Mononucleosomes were assembled at 37° C by salt dilution with 10 μg (92 pmoles) of recombinant octamers, 100 fmoles of labeled 5S rDNA, 10 μg Salmon sperm DNA and 1.8 *M* NaCl in a starting volume of 10 μl. Salmon sperm DNA was prepared as in Sambrook and Manniatis (109), the DNA being sheared to a 100-700 base pair length by sonication. Stepwise dilution of NaCl was carried out in three stages: 1 *M*, 714 m*M*, and 270 m*M*, by the addition of 6.8 μl, 8.4 μl and 42 μl of Buffer D (25 m*M* Tris-HCl, pH 8.0; 1 m*M* BME), respectively, at ten-minute intervals. Nucleosome assemblies were analyzed on a 4 % native polyacrylamide gel (acrylamide:bisacrylamide—38.9:1.1) in 0.5X TBE at 4° C.

In a standard 12.5 μl reaction, Isw1a and Isw1b were incubated in the molar ratios shown with 12 ng of nucleosome assemblies and 400 μ*M* ATP, at 30° C for 30 minutes under the following reaction conditions: 30 m*M* HEPES-KOH, pH 7.6, 5 m*M* $MgCl_2$, 37 m*M* KCl, 8.8 m*M* NaCl, 0.1 m*M* EGTA, 0.02 m*M* EDTA, 10 % glycerol, and 0.1 μg/μl BSA. Of the 12.5 μl reaction, 4 μl was analyzed by electrophoretic mobility shift assay on a 5 % native polyacrylamide gel (acrylamide:bisacrylamide - 60:1) in 0.2X TBE with buffer recirculation at 4° C.

Electrophoretic mobility shift assays

EMSA assays were done by our collaborators in the Bartholomew lab utilizing our purified Isw1 complexes. Mononucleosomes were assembled onto a 255 bp "601" DNA fragment with a high affinity nucleosome positioning sequence (88). Binding assays were done under conditions similar to the sliding assays (see Nucleosomal sliding assays) but with no ATP. 41 fmoles of nucleosomes were incubated with 41 fmoles, 123 fmoles, or 369 fmoles of either Isw1a or Isw1b complex. These were analyzed by electrophoretic mobility shift assay on a 4 % native polyacrylamide gel in 2X TE (10 m*M* Tris pH 8.0, 1 m*M* EDTA) with buffer recirculation at 4° C. For antibody supershift, 369 fmoles of Isw1a complex was first pre-bound to 41 fmoles of mononucleosomes, as above. In a second incubation reaction, the pre-bound complex was incubated with 46 pmoles α-FLAG antibody for an additional 15 minutes. These were analyzed as above.

Immobilized template interaction assays

Streptavidin coated magnetic particles (Dynal Biotech, Lake Success, NY 11042) were bound to linearized pBluescript SK- DNA linearized with *Cla*I/*Eco*RI, which was endfilled with dCTP, dGTP, dTTP, and biotinylated dATP. Recombinant histones were loaded with rNAP1p, followed by a wash in buffer containing 600 m*M* NaCl to remove Nap1p and non-nucleosomal histones, as previously described (51). 12.5 ng (or 75 ng for silver detection) DNA equivalent of beads with nucleosomal or naked DNA were incubated with 1 ng (7.6 fmoles) Isw1 equivalent (or 6 ng for silver detection) of Isw1 monomer, Isw1a, or Isw1b complex with or without 1.7 m*M* ATP in 10 m*M* Tris pH 7.6, 50 m*M* NaCl, 5 m*M* MgCl2, 1 m*M* EDTA, 0.05 % NP-40, 0.1 mg/ml BSA for 30 minutes at 30° C in an Eppendorf Thermomixer at 1200 RPM. Bound fractions were washed for 1 minute in buffer at 30° C before boiling in sample buffer. Proteins were visualized by silver stained SDS-PAGE gel or Isw1p was detected by western using α-FLAG antibody.

Expression analyses

RNA was harvested from cultures grown to OD_{660}=0.7, and Northerns were run as described, except that 30 μg RNA was loaded into each lane (53). Blots were hybridized with [α-^{32}P]-dCTP labeled probes for the genes indicated and quantified by Phosphorimager.

DNA microarray analyses were done using 30 μg total RNA from each strain, as described (47). A number of different previously described methods were used to normalize our expression data. Briefly, a Bayesian background correction method was applied to reduce the variance of spots of low intensity (47, 78). This corrected data from each microarray slide was then normalized to account for bias due to spot intensity (Intensity-dependent normalization using a lowess smoother to account for nonlinearity) and each cDNA's position on the array grid (Within-print-tip-group normalization). In addition, conversely labeled slide pairs were normalized to each other to account for dye-specific differences in labeling efficiency and/or dye stability (Paired-slides normalization); and separate slides were normalized to each other to reduce absolute expression differences that introduce bias when making slide to slide comparisons (Multiple slide normalizations) (133).

The expression changes for each gene were then determined by calculating the median value from three (*isw1* mutants) or four (*ioc2*, *ioc3*, and *ioc2ioc3* mutants) independent microarray hybridizations. Clustering analysis was done utilizing GeneSpring 4.0.4 (Silicon Genetics, Redwood City, CA 94063) and data that had undergone only Bayesian background correction to allow direct comparison with previously published microarray data for *isw2*, *rpd3*, *isw2rpd3*, and *sin3* strains (47, 78). Clustering was weighted more heavily for the Isw1 complex mutants to prevent bias due to stronger phenotypes of Sin3/Rpd3 mutations.

Serial dilution spot tests

Histone H3 tailless mutant spot tests were done by James Sampietro, a rotating graduate student in the Tsukiyama lab. Strains expressing *wild-type* or *hht2Δ2-30* histone H3 alleles were grown overnight in liquid culture diluted to OD_{660}=0.1. 10-fold serial dilutions were manually spotted onto YEPD plates and grown at room temperature, 30° C or 37° C.

Strains expressing *hhf2* histone H4 alleles often grew very slowly, thereby increasing the risk of suppressor mutations overtaking a liquid culture. Consequently, single colonies were resuspended in 100 μl YEPD and immediately plated as a large patch. Following several days incubation at room temperature, the patch was resuspended in sterile H_2O to OD_{660}=0.5 (suppressor mutants were clearly visible by this method as rapidly growing colonies within the larger patch, and could easily be avoided). Using a Beckman Biomek 2000 robot, 10-fold serial dilutions were made of each strain and 5 μl aliquots of each dilution were spotted onto appropriate plates and incubated at room temperature unless otherwise indicated.

End Notes:

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1993	B.S., Biology, University of Illinois at Urbana-Champaign
1995-1997	Completed 2 years of medical school at the University of Washington, Seattle
1998-present	Medical Scientist Training Program (M.D./Ph.D. combined degree program)
2003	Ph.D., Molecular and Cellular Biology, University of Washington, Seattle

RESEARCH TRAINING

1992-1993	Undergraduate research project in the lab of Dr. M. Joan Dawson, Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, Illinois.
1993-1995	Research Technician in the lab of Dr. Phillip Tarr, Division of Gastroenterology, Children's Hospital and Medical Center, Seattle, Washington.

1996-1997 Independent Study in Medical Science in the lab of Dr. Kenneth Mackie, Department of Anesthesiology, University of Washington, Seattle, Washington.

1997-1998 Guest Researcher in the lab of Dr. Karl Pfeifer, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland.

1999-2003 Graduate work in the lab of Dr. Toshio Tsukiyama, Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington.

PUBLICATIONS

Tarr, P.I., S.S. Bilge, J. Vary, N.M. Tang, M.R. Baylor, K. Potter, and T.E. Besser. *Escherichia coli* O157:H7 Adherence and Colonization Mechanisms. Proceedings of the UJNR Joint Panel on Toxic Microorganisms. VIII International Symposium of Toxic Microorganisms: "Molecular Approaches to Food Safety Issues Involving Toxic Microorganisms", Alaken, Inc., Fort Collins, Colorado, 1995.

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Vary J.C., V.K. Gangaraju, J. Qin, C.C. Landel, C. Kooperberg, B. Bartholomew, and T. Tsukiyama. Yeast Isw1p forms two separable complexes in vivo. Molecular and Cellular Biology. 2003 Jan; 23(1): 80-91.

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ABSTRACTS

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AWARDS

1997-1998	Howard Hughes Medical Institute/National Institutes of Health Research Scholar Award.
2000-2003	Developmental Biology Training Grant, Department of Zoology, University of Washington
2000-2003	Achievement Rewards for College Scientists, ARCS Foundation Inc.

PATENTS

Tarr, P.I., S.S. Bilge, J.C. Vary. Nucleic Acid Probes for Detecting *E. coli* O157:H7. Patent #5654417, August, 1997.

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