Chromatin remodelers Isw1 and Chd1 maintain chromatin structure during transcription by preventing histone exchange

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Set2-mediated methylation of histone H3 Lys36 (H3K36) is a mark associated with the coding sequences of actively transcribed genes, but it has a negative role during transcription elongation. It prevents trans-histone exchange over coding regions and signals for histone deacetylation in the wake of RNA polymerase II (RNAPII) passage. We have found that in Saccharomyces cerevisiae the Isw1b chromatin-remodeling complex is specifically recruited to open reading frames (ORFs) by H3K36 methylation through the PWWP domain of its Ioc4 subunit in vivo and in vitro. Isw1b acts in conjunction with Chd1 to regulate chromatin structure by preventing trans-histone exchange from taking place over coding regions. In this way, Isw1b and Chd1 are important in maintaining chromatin integrity during transcription elongation by RNAPII.

Under physiological conditions chromatin represents a strong barrier to transcription by RNAPII that has to be overcome as well as restored following polymerase passage. Modulation of chromatin structure is achieved by the concerted actions of chromatin remodelers, histone-modifying enzymes and histone chaperones. One such enzyme, the lysine methyltransferase Set2, is associated with the elongating form of RNAPII, and methylates Lys36 on histone H3 over coding sequences1,2. H3K36 methylation is required for efficient deacetylation of histones by the Rpd3S histone deacetylase complex3–5. Rpd3S also associates with RNAPII6,7 and thereby maintains coding regions in a hypoacetylated state in the wake of transcription elongation. Loss of Set2 or Rpd3S activities leads to an accumulation of acetylated histones over coding regions and a disruption of chromatin organization, as evidenced by the accumulation of cryptic transcripts—the result of inappropriate transcription initiation from inside ORFs1,8. Recent studies from our laboratory have shown that Set2-mediated H3K36 methylation further maintains chromatin integrity by preventing histone exchange over coding regions, thereby limiting the incorporation of new, acetylated histones9. In the current study, we sought to identify mechanisms by which H3K36 methylation achieves and perpetuates the stable organization of transcribed chromatin.

Chromatin-remodeling factors use the energy generated by ATP hydrolysis to slide or evict nucleosomes, or to alter their composition, thus affecting chromatin organization. They have important roles in a number of cellular processes, including gene transcription, replication and recombination. ISWI (imitation switch) and CHD (chromodomain helicase DNA-binding) represent two families of remodeling enzymes that are conserved from yeast to humans, although there is substantial diversity with respect to the subunit composition of individual complexes (reviewed in ref. 10).

The Iswi remodeler was initially identified in Drosophila melanogaster11 and has two homologs in S. cerevisiae12, Isw1 and Isw2. Isw1 associates with Ioc3 (Isw one complex protein 3) or Ioc2 and Ioc4 to form two distinct remodeling complexes, Isw1a and Isw1b, respectively13. Although Isw1 can interact with nucleosomes through its SANT and SLIDE domains14,15, it is thought that the associated Ioc proteins are involved in targeting the remodeling complexes to different genomic locations. Indeed, previous experiments found that Ioc3 associated with the promoter of MET16, whereas Ioc2 and Ioc4 localized to the MET16 ORF16.

Chd1 is the sole representative of the CHD family in S. cerevisiae and contains a characteristic double chromodomian at its N terminus. In yeast, chromodomain mutations result in the dissociation of Chd1 from chromatin17; no large-scale effects are observed for the fly mutant homolog18. However, Chd1 interacts with several known elongation factors such as the PAF complex, Spt5 and FACT19–21, and it colocalizes with RNAPII19,22,23, thus linking Chd1 to transcription elongation.

Isw1 and Chd1 chromatin remodelers fulfill partially redundant functions. An isw1Δ isw2Δ chd1Δ strain shows synthetic phenotypes12 as well as widespread disruption of nucleosome positioning throughout the yeast genome24,25. In vitro both Iswi and Chd1 remodelers are particularly effective in repositioning nucleosomes and generating spaced arrays26,27, a feature of remodeling enzymes that is often associated with transcriptional repression rather than activation.

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Deletion of *ISW1* or *CHD1* in a yeast reporter strain resulted in increased cryptic transcription at the *FLO8* locus\(^{17,28}\), further underlining a repressive function for both remodeling factors.

In this study, we have determined that recruitment of Isw1 to ORFs is mediated by interaction of its Ioc4 subunit with H3K36-monomethylated nucleosomes both *in vivo* and *in vitro*. Furthermore, the chromatin remodelers Isw1 and Chd1 act synergistically in the Set2 pathway by antagonizing histone exchange and preventing increased incorporation of acetylated histones over coding regions. These results suggest a previously undescribed mechanism by which the chromatin-remodeling activities of Isw1 and Chd1 maintain chromatin integrity in the wake of transcription elongation by RNAPII.

**RESULTS**

**Isw1 and Chd1 associate with H3K36me3 nucleosomes *in vivo***

Trimethylated H3K36 (H3K36me3) is a histone mark that is deposited on the ORFs of actively transcribed genes and signals for desacetylation of nucleosomes by the Rpd3S complex\(^3–5\). We were interested in determining what other factors were recruited either directly or indirectly by H3K36me3. To this end, we prepared a chromatin fraction from wild-type yeast cells and obtained mononucleosomes using micrococcal nuclease (MNase) digestion. We immunoprecipitated yeast mononucleosomes using either H3K36me3 or a control IgG antibody, and we analyzed the eluates by multidimensional protein identification technology (MudPIT) mass spectrometry (MS) (Table 1). In addition to histones, substantial amounts of RNAPII were present in the eluates, as expected for nucleosomes representative of actively transcribed chromatin. Furthermore, we also identified all subunits of the Rpd3S complex as well as several histone chaperones and chromatin-remodeling factors. Among the most abundant were the Isw1 remodeling complexes and Chd1. In particular, we focused on the Isw1 remodeler, consisting of the catalytic Isw1 subunit complexed with Ioc2 and Ioc4, as this complex has two potential methyllysine interaction domains. Ioc2 contains a PHD-like domain, whereas Ioc4 features an N-terminal PWPP domain (Fig. 1a,b).

**Ioc4 interacts directly with H3K36me3 nucleosomes**

Next we wanted to determine whether Ioc2 and Ioc4 can interact directly with H3K36me3. TAP-purified Isw1b complex as well as purified recombinant Ioc2, Ioc4 and Ioc1 proteins used in histone peptide pull-down assays did not interact preferentially with histone H3 peptides methylated on the Lys36 residue (data not shown). However, nucleosomes might be required for binding. Thus, we used recombinant, histone H3 methyllysine analog (MLA) mononucleosomes. Histone H3 used for nucleosome reconstructions contained a K36C mutation that was chemically modified\(^29\) to mimic either unmethylated or trimethylated Lys36 (Fig. 2). Electromobility gel shift assays (EMSAs) showed that Ioc4 had a higher affinity for trimethylated than unmethylated mononucleosomes (Fig. 2a,c). This preference is dependent on the presence of the Ioc4 PWPP domain, as Ioc4 bearing an N-terminal deletion (Ioc4\(^\Delta_{PWPP}\)) showed equal binding to both unmethylated and trimethylated nucleosomes (Fig. 2b,c). The overall affinity of Ioc4\(^\Delta_{PWPP}\) for nucleosomes was also reduced, suggesting that the PWPP domain represents a major interface for nucleosome binding. Analogous experiments for Ioc2 showed no binding of purified, recombinant Ioc2 to either unmethylated or trimethylated mononucleosomes (Supplementary Fig. 1a). Recombinant Ioc3 also did not interact preferentially with peptides methylated on Lys36 (data not shown), whereas the Ioc3-containing Isw1a complex showed similar affinities for both unmethylated and trimethylated H3K36 MLA mononucleosomes (Supplementary Fig. 1b).

**Ioc4 localizes to ORFs in a Set2-dependent manner**

As Ioc4 interacts with H3K36me3-containing nucleosomes *in vitro*, we investigated whether this interaction also occurred *in vivo*. We used chromatin immunoprecipitation coupled with microarrays (ChIP-chip) to determine the genome-wide localization of Flag-tagged Ioc3 and Ioc4 *in vivo* for both wild-type and set2∆ yeast strains. Using average gene analysis (Supplementary Fig. 2a,b), we determined that Ioc4 localized primarily to the mid- and 3′-coding regions of genes (Fig. 3a), which are also generally associated with H3K36me3 (refs. 8,30). In contrast, Ioc3 localized mostly to the regions surrounding the transcription start and end sites (Fig. 3b). Deletion of SET2 resulted in an almost complete abrogation of Ioc4 occupancy over ORFs (Fig. 3a). We also observed reductions in the association of

### Table 1 Proteins associated with H3K36me3-containing mononucleosomes

| Protein | H3K36me3 | IgG control |
|---------|----------|-------------|
| Hta1/2 (H2A) | 34 | 45.5 | 3 | 17.4 |
| Htb1/2 (H2B) | 21 | 50.4 | 1 | 6.9 |
| Hht1/2 (H3) | 11 | 44.1 | 2 | 5.9 |
| Hhf1/2 (H4) | 727 | 63.1 | 139 | 51.5 |
| Hho1 (H1) | 10 | 14.3 | 2 | 5.4 |
| Rbp1 | 376 | 33.3 | 79 | 11.3 |
| Rbp2 | 242 | 38.0 | 45 | 11.2 |
| Rbp3 | 26 | 19.5 |
| Rbp4 | 7 | 28.1 | 1 | 3.6 |
| Rbp5 | 116 | 21.9 | 29 | 13.0 |
| Rbp7 | 12 | 26.9 | 2 | 7.6 |
| Rbp8 | 21 | 18.5 |
| Rbp10 | 21 | 37.1 |
| Rbp11 | 26 | 55.8 | 3 | 9.2 |
| Rbp12 | 17 | 40.0 | 3 | 28.6 |
| Eaf3 | 12 | 15.7 | 3 | 6.7 |
| Rco1 | 11 | 14.9 | 3 | 5.6 |
| Rpd3 | 23 | 24.7 | 4 | 6.5 |
| Sin3 | 82 | 20.1 | 2 | 1.2 |
| Ume1 | 14 | 21.5 |
| Isw1 | 185 | 40.8 | 4 | 3.6 |
| Ioc2 | 124 | 32.5 | 18 | 7.8 |
| Ioc3 | 138 | 36.0 | 19 | 8.9 |
| Ioc4 | 60 | 24.4 |
| Chd1 | 108 | 20.0 | 2 | 1.8 |
| Isw2 | 39 | 6.2 |
| Dpb4 | 6 | 20.4 |
| Dpb1 | 12 | 13.3 |
| Itc1 | 9 | 6.2 |
| Spt16 | 397 | 46.5 | 26 | 12.6 |
| Pob3 | 141 | 39.0 | 7 | 4.9 |
| Spt6 | 42 | 16.3 |
| Hir1 | 5 | 5.6 |
| Hir2 | 26 | 21.7 |
| Hir3 | 69 | 17.7 |
| Rtt106 | 31 | 25.3 |

Proteins associated with wild-type mononucleosomes were co-immunoprecipitated with antibodies against H3K36me3 or an IgG control and analyzed by MudPIT MS.

aSpectral count, total spectra matching peptides detected by MudPIT MS for the indicated protein.

bSequence coverage, percentage of protein sequence represented in peptides identified by MudPIT MS.
Flag-tagged loc2 and Isw1 over ORFs in a set2Δ background, as determined by ChIP-quantitative PCR (qPCR) and ChIP-chip, respectively (Supplementary Fig. 2c–f). Taken together, these data suggest that the H3K36me3 mark is involved in mediating Isw1b occupancy over coding regions. loc3 localization at intergenic regions was not affected by deletion of SET2, although loc3 occupancy over ORFs increased slightly in a set2Δ mutant background (Fig. 3b), perhaps as a consequence of loc4 delocalization. Given the reduction of loc4, loc2 and Isw1 occupancy over ORFs in the set2Δ mutant, we conclude that Isw1b occupancy depends on Set2 and H3K36 methylation.

Isw1 and Chd1 prevent intragenic transcription

Previous experiments have shown that Set2-dependent H3K36 methylation is vital for the catalytic activity of the Rpd3S histone deacetylase complex to maintain chromatin in a hypoacetylated state following transcription5. In the absence of H3K36 methylation, nucleosomes in ORFs become hyperacetylated, resulting in aberrant transcription initiation from cryptic promoters inside ORFs3.

Association of the Isw1b remodeling complex with H3K36-methylated nucleosomes (Table 1) suggested that it could be involved in maintaining ordered chromatin structure following transcription elongation. Thus, we wanted to assess the involvement of Isw1 in suppressing cryptic transcription, as well as its involvement in the Set2 pathway. Indeed, previous studies using a yeast reporter strain for cryptic transcription showed that deletion of ISW1 did result in a selective phenotype28. Deletion of ISW1 alone did cause the production of low-to-moderate amounts of cryptic transcripts at most genes tested when assessed by northern blotting (Fig. 4 and Supplementary Fig. 3). We observed the same phenotype for a strain containing the ISW1K227R catalytic mutant (Fig. 4c,d,g,h), suggesting that Isw1 remodeling activity is required for the prevention of cryptic initiation in wild-type cells.

Previous reports have indicated that Isw1, Isw2 and Chd1 can have overlapping functions12. Although we have not observed cryptic transcription in an isw2Δ strain (Supplementary Fig. 3, data not shown), deletion of CHD1 alone caused low levels of cryptic transcripts to accumulate in some instances28 (Supplementary Fig. 3a,c). However, the isw1Δ chd1Δ double deletion strain did show a substantially stronger cryptic transcript phenotype that was completely unaffected by the additional deletion of ISW2 (Fig. 4a,b,e,f), in agreement with previously published data17. These results suggest that Isw1 and Chd1, but not Isw2, affect chromatin structure during transcription. Deletion of SET2 in an isw1Δ chd1Δ background further exacerbates the cryptic transcript phenotype of this strain. However, the total levels of cryptic transcripts produced when SET2 is deleted in either the single-deletion (Supplementary Fig. 3e) or double-deletion backgrounds (Fig. 4) are similar to those obtained for set2Δ alone. In all instances, the ISW1K227R catalytic mutant phenocopies an isw1Δ mutant (Fig. 4c,d,g,h). These results implicate ISW1, CHD1 and SET2 as being involved in the same pathway.

Isw1 and Chd1 prevent widespread cryptic transcription

It was recently shown that deletion of ISW1 and CHD1 results in large-scale changes in chromatin structure24. With this in mind, we asked...
whether deletion of both remodelers resulted in the appearance of cryptic transcripts genome wide. For this purpose, we purified mRNA from both wild-type and isw1Δ chd1Δ yeast cells, directly labeled the mRNA using fluorescent compounds and carried out competitive hybridizations to strand-specific yeast genome tiling arrays. These experiments allowed us to obtain strand-specific gene expression data.

Whereas regularly transcribed genes show uniform signals all across ORFs, cryptic transcripts transcribed from the sense strand cause a signal increase toward the 3′ end of genes. The reverse is true for cryptic transcripts initiated from the antisense strand. Previously, our laboratory has used K-means clustering to analyze this type of data. Although this approach worked well for the antisense transcription data, regular transcriptional background proved problematic in the analysis of the sense data set. Instead, we directly compared the probe signals from the 3′ to the 5′ ends of ORFs and selected genes that showed at least a 1.5-fold signal increase at the 3′ end for the sense data set.

To validate our methodology, we reanalyzed data for sense cryptic transcripts in a set2Δ mutant. This approach proved to be more stringent than K-means cluster analysis and underestimates the number of cryptic transcript genes for set2Δ, as only 327 out of 621 genes were identified. However, these genes retain the same characteristics, being predominantly long (89% >1 kb) and infrequently transcribed (95% <5 mRNA per hour), and include known cryptic transcript genes for set2Δ, such as STE11 and PCA1. We used the same approach for the data analysis of gene expression in the isw1Δ chd1Δ mutant, which showed widespread cryptic initiation. We identified many genes with sense (646 genes) and/or antisense (962 genes) cryptic transcripts (Fig. 5a). Thus, even though the approach used to identify sense cryptic transcript genes probably led to an underestimate, a substantial number of genes were identified, indicating that both sense and antisense cryptic transcription is widespread in the isw1Δ chd1Δ mutant. Again, both STE11 and PCA1 were identified, in agreement with our northern analysis (Fig. 4).

Recent experiments from our laboratory have shown that the presence of H3K36-methylated nucleosomes is important for the retention of the original histones at the ORFs of transcribed genes by preventing

![Figure 3](Image)

**Figure 3** Deletion of SET2 abrogates localization of loc4 to coding regions. ChIP-chip experiments were performed using yeast genomic tiling arrays. The log$_2$ ratios of immunoprecipitate over input were subjected to average gene analysis (Supplementary Fig. 2a). Whole-genome average data were calculated and plotted as mean ± s.e.m. (gray) and represent three independent experiments. The transcription start site (TSS) and termination site (TES) are indicated. Flag-tagged loc4 (a) and loc3 (b) were immunoprecipitated from wild-type (WT) and set2Δ yeast strains.

![Figure 4](Image)

**Figure 4** ISW1 and CHD1 have overlapping functions during transcription within the Set2 pathway. (a–d) Total RNA for each strain was isolated and used for northern blot analysis. The probes used were directed against the 3′ ends of the STE11 and PCA1 genes. ACT1 and rRNA were used as loading controls. The full-length (●) and cryptic transcripts (♦) are indicated. (e–h) For each lane all cryptic transcript bands were quantified and normalized against the ACT1 loading control. Total levels of cryptic transcription were expressed relative to set2Δ. Data were plotted as mean ± s.e.m. for three independent experiments.
trans-histone exchange—that is, the replacement of existing histones with histones from the free cellular pool\(^{2}\). Histone exchange affects many more genes in a set2\(\Delta\) mutant when compared to the number of cryptic transcript genes (Supplementary Fig. 4a,b), presumably because many genes possess no cryptic promoters. Hence, we wanted to compare the genes that show cryptic initiation in either the set2\(\Delta\) or isw1\(\Delta\) chd1\(\Delta\) mutants to the group of genes with altered histone exchange over ORFs in a set2\(\Delta\) background. Figure 5b shows that ~60% of genes with cryptic transcripts in an isw1\(\Delta\) chd1\(\Delta\) mutant also show increased histone exchange over ORFs in a set2\(\Delta\) strain. Similarly, ~60% of set2\(\Delta\) cryptic transcript genes also show intragenic initiation in an isw1\(\Delta\) chd1\(\Delta\) mutant. However, an appreciable number of cryptic transcript genes in isw1\(\Delta\) chd1\(\Delta\) show no overlap with genes associated with either increased histone exchange or cryptic transcription in set2\(\Delta\) and are presumably regulated by Isw1 and Chd1 independently of Set2. In summary, these data show that deletion of ISW1 and CHD1 causes widespread intragenic transcription initiation in the yeast genome.

**Figure 5** Deletion of ISW1 and CHD1 causes widespread intragenic transcription. (a) Heatmap of cryptic transcript genes in an isw1\(\Delta\) chd1\(\Delta\) mutant. Probe intensities for the 5’ and 3’ ends of all genes were determined. Resulting 3’/5’ ratios with a cutoff value of log\(_2\) > 0.5 were used to define sense cryptic transcripts (\(n = 646\)). K-means clustering of gene expression profiles was used to identify antisense cryptic transcripts (\(n = 962\)). Only cryptic transcript genes are shown. (b) Venn diagram showing the overlaps between genes displaying increased histone exchange over ORFs in set2\(\Delta\) (mean log2 > 0; \(n = 3,728\)) and genes with cryptic transcription in set2\(\Delta\) (\(n = 865\)) and/or isw1\(\Delta\) chd1\(\Delta\) (\(n = 1,437\)). Cryptic transcript sets contain genes with sense and/or antisense cryptic transcripts, whereby 68 and 171 genes show intragenic initiation in both the sense and antisense direction for set2\(\Delta\) and isw1\(\Delta\) chd1\(\Delta\), respectively. For the purpose of direct comparison, we selected cryptic transcript genes for set2\(\Delta\) according to the same criteria applied to isw1\(\Delta\) chd1\(\Delta\) (a), rather than the previously published data\(^{8}\).

Isw1 and Chd1 prevent histone exchange over ORFs

Given the substantial overlap of genes showing cryptic initiation in an isw1\(\Delta\) chd1\(\Delta\) background with Set2-regulated genes, and as the Isw1b complex is a reader of the H3K36 methyl mark in particular, we asked whether these proteins were involved in trans-histone exchange (Fig. 6). We studied histone exchange using a yeast strain containing constitutively expressed, Myc-tagged H3 as well as Flag-tagged H3 under the control of the GAL1 promoter\(^{31}\). To study transcription-coupled exchange, we used α-factor to arrest yeast in G1, and then briefly induced expression of Flag-H3 by growth in galactose-containing medium. Using microarray and average gene analysis, transcription-coupled histone dynamics can be followed by comparing occupancy levels for Flag-H3 relative to Myc-H3. In agreement with previously published work, histone exchange in wild-type yeast is high at the promoters of genes and low over coding regions\(^{31,32}\) (Supplementary Fig. 5a,b). We focused our analysis primarily on genes known to show increased exchange over ORFs in a set2\(\Delta\) background (\(n = 3,728\))\(^{8}\) (Supplementary Fig. 4a), although the profiles for the whole-genome data are similar (Fig. 6a and Supplementary Fig. 5). Comparing data for both isw1\(\Delta\) and chd1\(\Delta\) relative to the wild type, both mutants showed increased levels of histone exchange from mid-ORF to the 3’ ends of genes (Fig. 6a), areas that are particularly associated with H3K36me3\(^{33}\). Furthermore, the effect of each gene deletion was clearly additive (Fig. 6a), in agreement with the results obtained for cryptic transcription by northern blotting (Fig. 4 and Supplementary Fig. 3a–d). We also assessed the effect of deletion of IOC4 on histone exchange and saw an increase in exchange over coding regions, similar to that observed for isw1\(\Delta\) (Fig. 6b). Slight differences in the exchange profiles between isw1\(\Delta\) and ioc4\(\Delta\) presumably result from the fact that both the Isw1 and Isw1b remodelers are affected in the isw1\(\Delta\) background. Genes that show increased exchange over ORFs in either an ioc4\(\Delta\), isw1\(\Delta\) or chd1\(\Delta\) background overlap to large extents with those known to be affected in a set2\(\Delta\) mutant (Fig. 6e,f). The proportion of genes affected in isw1\(\Delta\) and chd1\(\Delta\) was even greater when we assessed increases in histone exchange specifically for the 3’ halves of ORFs (Supplementary Fig. 4c). Also, genes showing cryptic transcription in an isw1\(\Delta\) chd1\(\Delta\) background largely overlapped with those genes that are affected by increased histone exchange over ORFs in both isw1\(\Delta\) chd1\(\Delta\) as well as set2\(\Delta\) (Fig. 6g).

To confirm that deletion of ISW1 and CHD1 increases histone exchange over ORFs, we also investigated incorporation of histone H3 acetylated on Lys56 (H3K56ac). Acetylation of H3K56ac is mediated specifically by the Rtt109 acetyltransferase (KAT11), which acts on soluble histones, but not on chromatin\(^{33}\). Distribution of H3K56ac correlates with both replication-dependent and replication-independent histone exchange\(^{32,34}\) and can therefore be used as a marker for genomic regions undergoing trans-histone exchange. Using ChIP-chip we determined the distribution of H3K56ac and histone H3 in G1-arrested strains in a BY4741 background to confirm that increases in histone exchange in the mutant strains were not due to overexpression of histone H3. We observed increased levels of H3K56ac from mid-ORF to the 3’ ends of genes (Fig. 6c) for isw1\(\Delta\) and chd1\(\Delta\), as also seen for the histone-exchange strains (Fig. 6a). Similarly, the effect of each gene deletion was additive (Fig. 6a,c). The increases in histone exchange for isw1\(\Delta\), chd1\(\Delta\) and isw1\(\Delta\) chd1\(\Delta\), whether represented as a Flag/Myc ratio in the histone-exchange strains or by H3K56ac/H3, are highly correlated (Fig. 6h). The ISW1K227R catalytic mutant strain also showed increased levels of H3K56ac over coding regions (Fig. 6d), similar to exchange in an ioc4\(\Delta\) background (Fig. 6b). This indicates that the catalytic activity of Isw1 is required to suppress histone exchange and maintain ordered chromatin structure in wild-type yeast, in agreement with results on cryptic transcription obtained by northern blotting (Fig. 4c,d,g,h).

Clustering all yeast genes according to transcription rates, we determined that exchange increased over the ORFs of lowly transcribed genes in an isw1\(\Delta\) background relative to the wild type, but it did not affect exchange in highly transcribed genes (Fig. 6i). These results conform to expectations, as genes known to rely on Set2 for accurate transcription are generally long and transcribed at low levels\(^{8}\). Notably, deletion of CHD1 increased exchange over both lowly and highly transcribed genes when compared to the wild type (Fig. 6j). Similar analysis of H3K56ac levels over ORFs for both
isw1Δ and chd1Δ show the same distributions (Fig. 6k,l), hinting at the complementary action of these two chromatin remodelers (see Discussion). From these experiments, we conclude that the Isw1b and Chd1 chromatin-remodeling factors both prevent co-transcriptional trans-histone exchange at largely overlapping, but somewhat distinct, groups of genes.

Deletion of ISW1 and CHD1 increases acetylated H4 over ORFs

The appearance of cryptic transcripts in a set2Δ background is associated with a lack of H3K36 methylation and hyperacetylation of ORF nucleosomes. Also, we have recently shown that increased trans-histone exchange in a set2Δ mutant results in greater levels of histone acetylation over ORFs. Therefore, we sought to determine whether deletion of ISW1 or CHD1 affected either of these histone marks. For the purpose of direct comparison, we focused our analysis on the same group of genes that showed increased histone exchange in set2Δ. However, the trends seen for each deletion are the same genome-wide (Supplementary Fig. 6).

Microarray analysis of H3K36me3 ChIPs clearly showed that the distribution and occupancy levels of H3K36me3 in an isw1Δ strain were highly similar to what was observed in the wild type (Fig. 7a). In contrast, deletion of CHD1 clearly affected the overall distribution but not the absolute levels of H3K36me3. Using histone H3 ChIP-chip data, we determined the overall distribution of nucleosomes for both the isw1Δ and chd1Δ mutants. Histone H3 occupancy was slightly reduced in an isw1Δ background relative to the wild type. However, deletion of CHD1 caused an overall redistribution of nucleosomes toward the 5′ end of genes (Fig. 7b), suggesting that Chd1 prevents loss of nucleosomes from the 3′ end of genes.

As deletion of ISW1 or CHD1 did not seem to affect absolute H3K36me3 levels in vivo, we asked whether deletion of either gene could cause an increase in co-transcriptional ORF acetylation, thought to result in sustained decompaction of chromatin. In wild-type cells histone H4 acetylation (AcH4) is high at promoters and low over the ends of genes. Thus, increased histone exchange in a wild-type, deletion of ISW1/Myr-H3) was determined for wild-type, over wild type. Average gene analysis for acetylated H3K56ac immunoprecipitated from wild-type, only genes known to show increased exchange over ORFs in a set2Δ mutant (n = 3,728) (Supplementary Fig. 4a) were used for average gene analysis and plotted as mean ± s.e.m. (gray) for three independent experiments. The TSS and TES are indicated. Histone exchange (Flag-H3/Myr-H3) was determined for wild-type, strains, and presented as difference profiles for mutant (mut) over wild type. Average gene analysis for acetylated H3K56ac immunoprecipitated from wild-type, strains, and the ISW1/K27R catalytic mutant (d) strains is shown. H3K56ac occupancy was normalized to histone H3 levels. Difference profiles are shown for mutant over wild type. Genes were clustered into two groups on the basis of their average histone-exchange signals for mutant relative to wild-type profiles over ORFs (mean log2 < 0, mean log2 > 0). Venn diagrams show overlaps between genes showing increased histone exchange over ORFs (mean log2 > 0) for the mutants indicated. (g) Venn diagram showing the overlap between genes that show cryptic transcription in an isw1Δ chd1Δ mutant (Fig. 5a,b) and genes that show increased histone exchange over ORFs in an isw1Δ chd1Δ and set2Δ background. (h) Pearson correlation coefficients and P values were calculated for histone-exchange (a) and H3K56ac/H3 (c) profiles for each mutant background using R. (i–k) For whole-genome analysis, genes (n = 4,894) were clustered into two groups (<10 mRNA per hour (4,250 genes) and ≥10 mRNA per hour (644 genes)) based on published transcription rates. Average gene analysis for histone-exchange (i,j) or H3K56ac/H3 (k,l) difference profiles clustered according to transcription rates are shown. Data were plotted for isw1Δ (i,k) or chd1Δ (j,l) relative to the wild type.

Figure 6 Deletion of ISW1 and CHD1 increases histone exchange over 3′ ends of ORFs. (a–d) ChIP-chip experiments were performed using yeast genomic tiling arrays. Only genes known to show increased exchange over ORFs in a set2Δ mutant (n = 3,728) (Supplementary Fig. 4a) were used for average gene analysis and plotted as mean ± s.e.m. (gray) for three independent experiments. The TSS and TES are indicated. Histone exchange (Flag-H3/Myr-H3) was determined for wild-type, and presented as difference profiles for mutant (mut) over wild type. Average gene analysis for acetylated H3K56ac immunoprecipitated from wild-type, only genes known to show increased exchange over ORFs in a set2Δ mutant (n = 3,728) (Supplementary Fig. 4a) were used for average gene analysis and plotted as mean ± s.e.m. (gray) for three independent experiments. The TSS and TES are indicated. Histone exchange (Flag-H3/Myr-H3) was determined for wild-type, for whole-genome analysis, genes (n = 4,894) were clustered into two groups (<10 mRNA per hour (4,250 genes) and ≥10 mRNA per hour (644 genes)) based on published transcription rates. Average gene analysis for histone-exchange (i,j) or H3K56ac/H3 (k,l) difference profiles clustered according to transcription rates are shown. Data were plotted for isw1Δ (i,k) or chd1Δ (j,l) relative to the wild type.
indicating a secondary role for Chd1 at promoters. Notably, deletion of either ISW1 or CHD1 resulted in a rise of ORF histone H4 acetylation, with small additive effects in the isw1Δ chd1Δ mutant (Fig. 7c).

We also assessed the effects of simultaneous deletion of ISW1 or CHD1 in a set2Δ background on histone H4 acetylation levels. As expected for proteins that act in the same pathway, AcH4 levels and distribution of either isw1Δ set2Δ or chd1Δ set2Δ mimic those of set2Δ alone (Fig. 7d), which also agrees with our results on cryptic transcription for those mutants (Supplementary Fig. 3e). Taken together, our results show that, in wild-type yeast, Isw1 and Chd1 function to suppress trans-histone exchange, thereby preventing the incorporation of soluble, highly acetylated histones over ORFs.

**DISCUSSION**

We have shown here that the Isw1b chromatin remodeler is recruited to coding sequences by association of its Ioc4 subunit with H3K36-trimethylated nucleosomes, as interaction of Ioc4 with chromatin was reduced both in vivo and in vitro in the absence of H3K36 methylation (Figs. 2 and 3). By contrast, Ioc3, representative of the Isw1a complex, was found preferentially at intergenic regions. Notably, its association with coding sequences increased in a set2Δ background, perhaps as a consequence of Ioc4 decomplexation. The increased Ioc3 occupancy in a set2Δ mutant, taken together with the overall genome-wide distributions of Ioc3 and Ioc4 in the wild type (Fig. 3), point toward the existence of a dynamic equilibrium between the Ioc3a and Ioc3b complexes in the cell. This idea is further supported by the observation that estimates on protein abundance imply that Ioc3 is the least abundant of all ISWI subunits.

In an earlier study, Isw1 was reported to preferentially pull down histone H3 Lys4-trimethylated (H3K4me3) nucleosomes. On the basis of our ChIP-chip data for Ioc3 (Fig. 3b), we speculate that Isw1a may preferentially bind to H3K4me3-containing nucleosomes, which are generally associated with promoters. In our hands, TAP purifications of Isw1 result in near-equal amounts of Ioc3 and Ioc2–Ioc4 being pulled down. Furthermore, in vitro gel-shift experiments on mononucleosome binding by Isw1a and Isw1b have shown that Isw1a has a much higher affinity for mononucleosomes, owing to its high affinity for DNA when compared to Isw1b. Taken together, these observations indicate that in the absence of cross-links, any nucleosomes isolated from Isw1 pull-down experiments are more likely to be associated with Isw1a rather than Isw1b. Previous ChIP-qPCR experiments at the inducible MET16 locus suggested that Isw1a is recruited to the promoter only when the gene is inactive. However, we observed Ioc3 occupancy at many genes known to be actively transcribed, implying that Isw1a may have a more widespread role than previously anticipated.

Chd1 is known to associate with elongating RNAPII through interaction with the PAF complex and/or other RNAPII-associated proteins such as Spt5 and FACT19–21. Previous experiments using ChIP-qPCR could not establish differential association of Chd1 with chromatin when comparing wild-type cells to a set2Δ background. Neither did Chd1 preferentially bind to H3K36-methylated nucleosomes in vitro. Although it is possible that Chd1 preferentially remodels H3K36-methylated nucleosomes, it is likely that its association with RNAPII mediates localization of its activity at coding sequences. This is in agreement with the observation that both histone exchange and H3K56ac in a chd1Δ but not an isw1Δ background are increased over genes with higher transcription rates (Fig. 6i–l).

Recent ChIP-seq experiments on nucleosome positioning in strains bearing single deletions of ISW1 or CHD1, as well as the combination of isw1Δ chd1Δ or isw1Δ isw2Δ chd1Δ, show that nucleosome positioning is greatly perturbed across the genome in the double and triple deletion strains. The coding regions were severely affected in an isw1Δ chd1Δ background, in agreement with our own results showing that deletion
of ISW1 and CHD1 has additive effects with respect to both cryptic transcription (Figs. 4 and 5) and histone exchange (Fig. 6a,c) and leads to widespread intragenic transcription initiation. Examining each deletion individually, single deletion of CHD1 had more severe effects overall compared to deletion of ISW1 alone.24,38, also in agreement with our own data (Fig. 6a,c). These results presumably reflect the fact that Chd1 can compensate for the loss of Isw1 better than vice versa. Chd1 association with RnAPII may well account for this effect; conversely, even though highly transcribed genes contain high levels of H3K36me3, their lower nucleosome density overall may translate as a reduced ‘interaction surface’ for Isw1b recruitment.

Previous studies have shown that histone exchange of H3–H4 tetramers generally takes place only over promoters, and it is limited to highly transcribed genes over ORFs.31,32,39 In contrast, the passage of multiple RnAPII elongation complexes, reminiscent of the transcription of highly transcribed genes, causes complete dissociation of histone octamers from the DNA, which are subsequently reassembled behind the polymerase. We hypothesize that Isw1b and Chd1 may exert their overlapping but distinct functions in this context. Studies on D. melanogaster Chd1 and the Iswi-containing ACF complex have shown that both enzymes can catalyze the transfer of histones from the histone chaperone Nap1 onto DNA and that they generate regularly spaced nucleosomal arrays in vitro.26 Further experiments in D. melanogaster established that Chd1 together with the Hira chaperone is important for the replication-independent deposition of histone variant H3.3 in male fly pronuclei.41 The yeast Chd1 and Isw1 chromatin remodelers are similarly known to efficiently space nucleosomes.13,27,44 This raises the possibility that yeast Isw1 and Chd1 may differ functionally in preventing histone exchange and promoting chromatin integrity at weakly and strongly transcribed genes, respectively: Isw1 in the form of the Isw1b complex by remodeling nucleosomes that were retained on the DNA in spite of transcription and, Chd1 by reassembling nucleosomes in cis in the wake of RnAPII (Fig. 7e). In agreement with this hypothesis, we see that, in the wild-type, Isw1 catalytic activity is required to suppress both cryptic transcription (Fig. 4c,d,g,h) and histone exchange (Fig. 6d) and thus maintains chromatin integrity. Such different functional roles for Isw1 and Chd1 could also account for the reduced capacity of Isw1 or Isw1b to compensate for the loss of Chd1 activity, assuming that Isw1b is less able to recapture histones displaced by transcription. A number of histone chaperones have been implicated in RnAPII transcription elongation, and it will be interesting to determine which may function in concert with these remodelers in vivo.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: ChIP-chip and gene expression data sets have been deposited with accession number GSE32071.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.S. designed and carried out experiments, analyzed the data and wrote the manuscript. M.S., M.M.G., S.V. and H.L. carried out bioinformatics analyses. Y.Z., L.F. and M.P.W. performed MS and analyzed the results. J.L.W. supervised experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Yeast strains and plasmids.** All yeast strains used in this study are listed in Supplementary Table 1.

**Antibodies.** All antibodies and the dilutions used in this study are listed in Supplementary Table 2.

**Co-immunoprecipitation of H3K36me3 mononucleosomes and MudPIT mass spectrometry.** Chromatin was isolated from wild-type yeast and digested to mononucleosome level with MNase (Worthington) (Supplementary Note). Anti-H3K36me3 antibody was cross-linked to Dynabeads (Invitrogen) and used to co-immunoprecipitate proteins associated with H3K36me3-containing mononucleosomes. Rabbit IgG was used as a negative control. Binding reactions were set up with 100 µl of mononucleosomes in binding buffer (40 mM HEPES (pH 7.5), 200 mM NaCl, 0.1% (w/v) Tween-20, 1 mM PMSE, 2 µg ml⁻¹ leupeptin and 1 µg ml⁻¹ pepstatin A) and incubated overnight at 4 °C. Samples were washed three times with binding buffer and eluted in three steps with elution buffer 1 (E1; 70 mM citrate (pH 4.6), 1 M NaCl and 0.1% (v/v) Tween-20), elution buffer 2 (E2; 10 mM Tris (pH 8.0) and 0.2% (w/v) SDS) and elution buffer 3 (E3; 10 mM Tris (pH 8.0) and 1% (w/v) SDS). Specificity of co-immunoprecipitations was confirmed by western blotting. Eluates E1–E3 were analyzed independently by MudPIT MS as described, and the data were subsequently combined for analysis.

**Electromobility shift assays.** Cy5-labeled 216-bp DNA fragments containing the 601 positioning sequence were reconstituted with recombinant Xenopus laevis core histones by serial dilution as described. Mononucleosomes were isolated and EMSAs performed as described. Gels were scanned using a Typhoon Phosphorimaging system (GE Healthcare) and quantified using ImageQuant TL software (GE Healthcare). For binding reactions, 5 fmol of reconstituted RNA is isolation.

**Northern blots.** Northern blots and hybridization were done as described. We used 20 µg of total RNA to assess cryptic transcript phenotypes. Blots were exposed onto phosphorimaging screens, scanned using a Typhoon Phosphorimaging system and quantified using ImageQuant TL software (GE Healthcare). Cryptic transcript levels were determined for each sample, normalized to ACT1 for equal loading and expressed as a percentage relative to total set2Δ cryptic transcript levels. The mean of triplicate experiments was plotted. Error bars represent the s.e.m.

**Chromatin immunoprecipitation assays.** For ChIPs of Flag-tagged Ioc4 or Ioc3, yeast strains were grown in 200 µl of YPD medium at 30 °C until they reached an optical density at 600 nm (OD600) of 0.8. mRNA was subsequently purified from TL software (GE Healthcare). For binding reactions, 5 fmol of reconstituted reconstituted reached an optical density at 600 nm (OD600) of 0.8. Total RNA was isolated using M. Cells were cross-linked, collected and processed for ChIP as described. For histone-modification ChIPs, we used deletion strains with a BY4741 backbone to ensure that histone-modification patterns were not the result of histone overexpression in the exchange strain. Yeast strains were grown in YPD medium at 30 °C and arrested in G1 by addition of α-factor at a final concentration of 2 µM. Cells were cross-linked, collected and processed for ChIP as described. G1 arrest of cells was confirmed by FACs analysis of samples collected before and after treatment with α-factor. ChIP lysates were used to immunoprecipitate AcH4, H3K56ac and H3.

**Microarray analysis.** ChIP-chip assays for the genome-wide distribution of Flag-tagged Ioc3 and Ioc4 were performed using 4 × 44k yeast genome DNA arrays (Agilent, cat. no. 014810) with an average probe spacing of ~280 bp. All other ChIP-chip experiments were performed using 8 × 60k yeast genome DNA arrays (Agilent, cat. no. 031697) with an average probe spacing of ~200 bp. We used 20–50 ng of input and immunoprecipitation samples for double T7 linear amplification (Epiconcept) and labeling as described. Inputs were labeled with Cy3 dye and immunoprecipitations with Cy5 dye (GE Healthcare). We combined 4 µg of each input and immunoprecipitation for hybridization.

For transcription profiling, mRNA was labeled directly with Cy dyes using the Kreatech ULS system. Labeled RNA (2 μg) from wild-type and sw1Δ chd1Δ strains were competitively hybridized to 4 × 44k arrays (Agilent cat. nos. 037746, 037680) designed to detect transcripts specifically from either the sense or antisense strands. Three biological repeats were done for all microarray-based experiments.

**Data analysis.** Normalized ChIP-chip data were analyzed using a modified gene average analysis. ORFs were subdivided into 6 (4 × 44k array data) or 14 (8 × 60k array data) equal-sized bins each. Intergenic regions (500 bp or 480 bp upstream and downstream of genes) were allocated into two or three bins each, for 4 × 44k and 8 × 60k array data, respectively (Supplementary Fig. 2a,b). Microarray enrichment ratios (log2(IP/input)) for each probe were assigned to the closest bin. For whole-genome average gene plots, all probes within a bin were averaged and plotted as mean ± s.e.m. Genes not regulated by RNAPII, including tRNA and snRNA genes as well as most dubious ORFs (~450 genes), were removed from the analysis. Histone exchange was calculated as the ratio of Flag–H3 enrichment relative to M. Cells were cross-linked, collected and processed for ChIP as described. G1 arrest of cells was confirmed by fluorescence-activated cell sorting (FACS) analysis of samples collected before and after treatment with α-factor. ChIP lysates were used to immunoprecipitate Flag–H3, Myc–H3 and H3K36me3 and processed as described.

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