Published online 7 April 2010 *Nucleic Acids Research, 2010, Vol. 38, No. 14* ***4599–4606***
doi:10.1093/nar/gkq218

Genome-wide mapping of histone H4 serine-1 phosphorylation during sporulation in *Saccharomyces cerevisiae*

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Received December 22, 2009; Revised March 12, 2010; Accepted March 15, 2010

ABSTRACT

We previously showed that histone H4 serine-1 phosphorylation (H4S1ph) is evolutionarily conserved during gametogenesis, and contributes to post-meiotic nuclear compaction and to full completion of sporulation in the yeast *Saccharomyces cerevisiae*. Previous studies showed that H4S1ph and another modification of the same histone, H4 acetylation (H4ac), do not occur together and have opposing roles during DNA double-strand break (DSB) repair. In this study, we investigated the relationship between these marks during yeast sporulation. H4S1ph and H4ac co-exist globally during later stages of sporulation, in contrast to DSB repair. Genome-wide mapping during sporulation reveals accumulation of both marks over promoters of genes. Prevention of H4S1ph deposition delays the decline in transcription that normally occurs during spore maturation. Taken together, our results indicate that H4S1ph deposition reinforces reduced transcription that coincides with full spore compaction, without disrupting the local acetylation signature. These studies indicate distinctive features of a histone H4 modification marking system during sporulation compared with DSB repair.

INTRODUCTION

Chromatin function and folding are regulated by complex mechanisms, involving post-translational modifications of histones, histone variants and remodeling complexes (1). Histone modifications are involved in many chromatin events, including transcriptional regulation, double-strand break (DSB) repair and sub-nuclear organization. Modifications can directly alter the surface of nucleosomes to affect chromatin organization and higher order structure (2), or create new docking interfaces and recruit specific protein complexes (3).

We have previously shown that a specific histone modification, phosphorylation of serine-1 of histone H4 (H4S1ph), promotes nuclear compaction during sporulation in the budding yeast *Saccharomyces cerevisiae* (4). Sporulation is a key cellular differentiation pathway in the yeast life cycle (5). Meiosis generates four genetically distinct haploid nuclei that further differentiate into highly specialized spores. H4S1ph is deposited during the post-meiotic maturation of haploid nuclei, and is required to fully compact the spore nuclei.

In addition to phosphorylation at Ser-1 in the amino terminus of histone H4, adjacent residues (K5/8/12/16) are acetylated (Figure 1A), and thus the functional relationship between these modifications has been investigated. H4S1ph is detected during the DSB repair pathway in budding yeast, where H4S1ph follows a decrease of H4 acetylation (H4ac) (6,7). This suggests that H4ac and H4S1ph can be inversely correlated and indicates that the marks have opposing functions in chromatin regulation during DNA repair.

In this study, we investigated the genome-wide distribution and function of H4S1ph during sporulation, and compared its location to H4ac. Our results show that, unexpectedly, in contrast to the pattern during the DNA repair pathway, these marks are present at the same time

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

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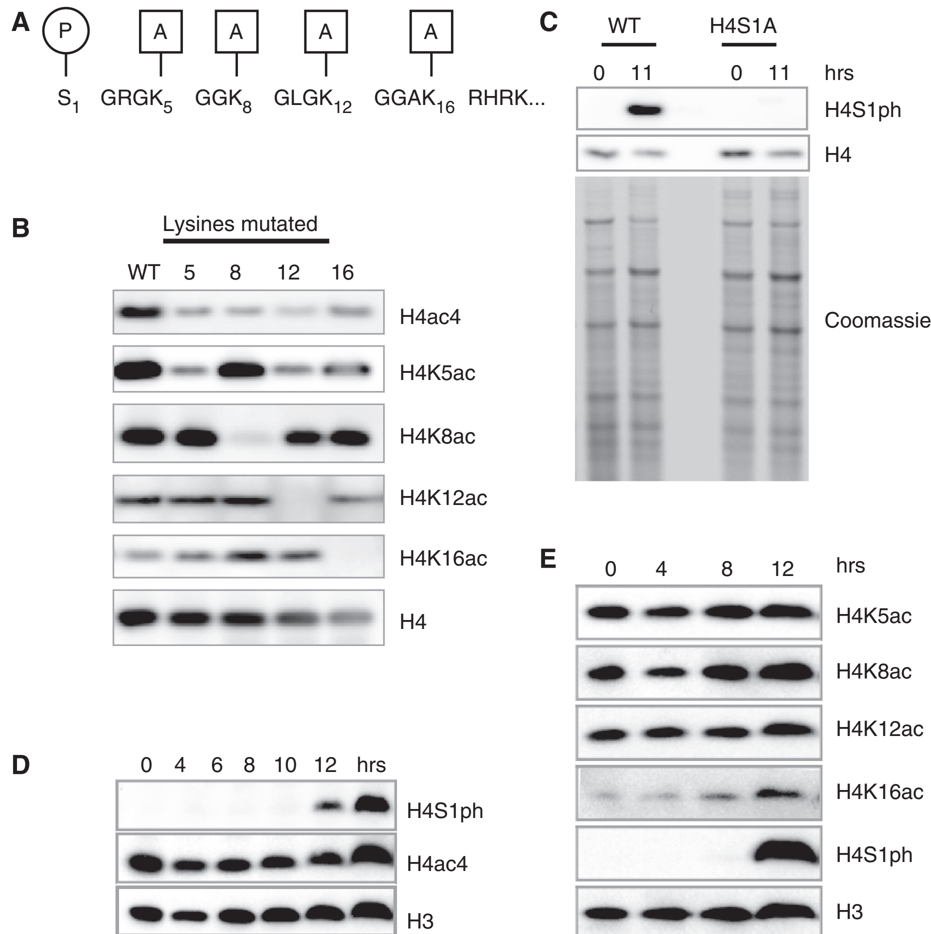

Figure 1. H4S1ph and H4ac occur at the same time during the sporulation pathway. (**A**) Schematic representation of the H4 tail, including known post-translational modifications (P, phosphorylation; A, acetylation). (**B**) Specificity of acetylated H4 antibodies. The specificity of acetylated H4 antibodies was assessed using substitution mutant strains, in which lysines are mutated to arginine, a non-acetylable mimic of lysine. Poor specificity of the anti-H4K5ac antibody confirms previous characterization of the same antibody (20). (**C**) H4S1ph antibody specificity. Extracts from WT and H4S1A strain were analyzed by western blot during sporulation. (**D**) and (**E**) H4ac and H4S1ph coincide temporally during sporulation. Histone modifications were analyzed by western blotting of whole cell extracts though the sporulation program.

during sporulation and map to similar gene locations. In addition, H4S1ph helps to reinforce low gene expression late in the sporulation process.

MATERIALS AND METHODS

Antibodies

H3, H4ac4, H4K5ac, H4K8ac, H4K12ac, H4K16ac antibodies were obtained, respectively, from Abcam (Ab1791) and Millipore-Upstate (08-866 lot 31992, 07-327 lot 30417, 07-328 lot 30399, 07-595 lot 28885, 07-329 lot 26818). H4 antibodies were obtained from active motif (39269 lot 11908001) for western blot detection, and from Millipore (05-859 lot JBC1361900) for ChIP. H4S1ph was a kind gift from David Allis at Rockefeller and described previously (8).

Strains

All strains are in the SK1 background. The genotype of the yeast WT diploid used for sporulation is *MATa/MATalpha leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2-SK1/lys2-SK1 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2*. A histone shuffling strain was created in the SK1 background by deletion of endogenous copies of the histone H3 and H4 genes, which are then provided on plasmid pDM1 (*HHF2-HHT2 CEN-ARS1 URA3*). Mutations were introduced using QuickChange kit (Stratagene 200518-5) into the pRM204 plasmid (*HHF2-HHT2 CEN-ARS1 TRP1*), which was then shuffled into a diploid strain (*MATa/MATalpha leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2-SK1/lys2-SK1 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2 hhf1-hht1::LEU2/hhf1-hht1::LEU2 hhf2-hht2::trp1::KanMX3/hhf2-hht2::trp1::KanMX3*). The presence of mutations was confirmed by sequencing the histone genes and by western blot.

Sporulation induction and spore analysis

For sporulation, diploid yeast in the SK1 background were grown in YPA to an OD of 0.5–0.8, and then transferred into sporulation media (K acetate 2%) at

OD 2 supplemented for auxotrophic amino acids. Samples were collected at indicated times, washed twice in water, and stored at −80°C. Whole cell extracts and western blots were performed as described previously (4). For chromatin immunoprecipitation, cells were washed twice in water, crosslinked 10 min in 1% formaldehyde under agitation, then quenched 5 min in 125 mM glycine under agitation. Cells were washed twice in cold water, and samples stored at −80°C. Sporulation efficiency was assayed on fresh spores as previously described (4).

Chromatin immunoprecipitation

Approximately 50 OD of crosslinked cells were collected at each time point. Extracts were made using conventional techniques. In brief, cells were beadbeat six times for a period of 1 min with intermediate incubation on ice in lysis buffer [Hepes 50 mM pH 7.5, NaCl 140 mM, EDTA 1 mM, Triton X-100 0.1%, PMSF 0.5 mM, aproteinin 2 mg/l, leupeptin 2 mg/l, pepstatin A 2 mg/l, TSA 100 mg/l, phosphatase inhibitor cocktail (Sigma P2850)]. Extracts were sonicated 20 times for 30 s with intermediate incubation of 30 s (Bioruptor, Diagenode). Cellular debris was removed by centrifugation at 20 000*g*, 15 min at 4°C. The protein concentration of the supernatant was quantified by Bradford assay.

The immunoprecipitation protocol for genome-wide and single loci analyses used published procedures with minor changes (9). Fifty micrograms of extract was used, with 3–5 µl of antibody. A quantity of 10–20 µl of Protein A Dynabeads (Invitrogen 100.02D) was used. Controls without antibody and with isogenic antibodies were routinely performed and did not show specific signals. Each data point acquired by qPCR was the average of three independent PCR reactions. Data presented are an average of at least two biological independent replicates. Error bars are standard error of the mean (SEM). Statistical significance was determined using the *T*-test. DNA sequencing and data analysis for genome-wide localization are described in Supplementary Methods section.

RNA analysis

RNA was purified using Qiagen RNAeasy purification kit according to the manufacturer's instructions. RT was performed using TaqMan Reverse Transcription (ABI). cDNA were quantified using standard procedures on a 7900HT Fast-Real-time PCR (ABI). cDNA levels were normalized to NUP85, whose mRNA appears to be constant in three independent genome-wide transcriptome analyses (10–12). Primers are presented in Supplementary Table S2. Four independent biological replicates were analyzed. Statistical significance was determined using the *T*-test.

RESULTS

H4S1ph and H4ac coincide temporally during yeast sporulation

Using specific antibodies for H4S1ph and H4ac and western analysis, we examined the global levels of these marks during the progression of the sporulation program. The specificity of each antibody was assayed using yeast strains in which serine or lysines were altered to alanine or arginines, respectively, which provide non-modifiable, conservative substitutions of each amino acid (Figure 1B and C). Consistent with our previous observations (4), H4S1ph increased at 10 h after induction of sporulation (Figure 1D). In contrast to H4S1ph, H4ac was present before sporulation begins and was maintained through the sporulation program (Figure 1D). Similar results were obtained using an antibody that recognizes all four amino terminal acetylation sites, or using antibodies that detect individual H4 acetylation states (K5ac, K8ac, K12ac and K16ac; Figure 1E). Thus, H4S1ph and H4ac co-exist globally during yeast sporulation. This contrasts with the DSB repair pathway, where H4S1ph appearance correlates with a concomitant global decrease in H4 acetylation (7).

Genome-wide analysis of H4S1ph and H4ac in spores

We investigated the genomic landscapes of H4 acetylation and phosphorylation, by genome-wide location analysis of the modifications. Cells were collected when H4S1ph is first detected, at 10 h after synchronous initiation of the sporulation program. Regions of the genome enriched in each histone mark were isolated by chromatin immunoprecipitation (ChIP), and sequenced by massive parallel DNA sequencing (ChIP-SEQ). Specificity of the H4K12ac and H4S1ph antibodies in ChIP were confirmed using substitution mutations at the sites, i.e. strains bearing H4K12R and H4S1A (Figure 2A and B).

We examined the position and abundance of nucleosomes using an antibody to unmodified histone H4 (Figure 2C). Importantly, nucleosome abundance was in agreement with published data (13). For example, regions of low nucleosome abundance, referred to as nucleosome-depleted regions (NDRs), are localized within the promoters of almost every gene (arrows on Figure 2C and Supplementary Figure S1).

We then analyzed the distribution of H4S1ph and H4K12ac. The local abundance of each mark was assessed in comparison to the H4 level along two stretches of genes (Figure 2D and Supplementary Figure S1B). The genes present in both regions show an exclusion of both H4S1ph and H4K12ac in the NDR regions (arrows on Figure 2D and Supplementary Figure S1B). This absence of signal in the NDR troughs indicates an exceedingly low level of noise for both modifications in the ChIP-SEQ. Moreover, compared with H4, both marks are clearly enriched around the transcription start site (TSS) of each gene, just downstream of the NDRs (asterisks in Figure 2D and Supplementary Figure S1B). The enrichment is confirmed by ratio analysis of each modification compared with H4 along the genes, in which there is a higher enrichment of each mark normalized to the level of overall H4 (asterisks in Figure 2E and Supplementary Figure S1C).

The average modification profiles were generated by calculating the signals surrounding the TSSs of all genes (Figure 2F). The NDR region is clearly seen (Figure 2F,

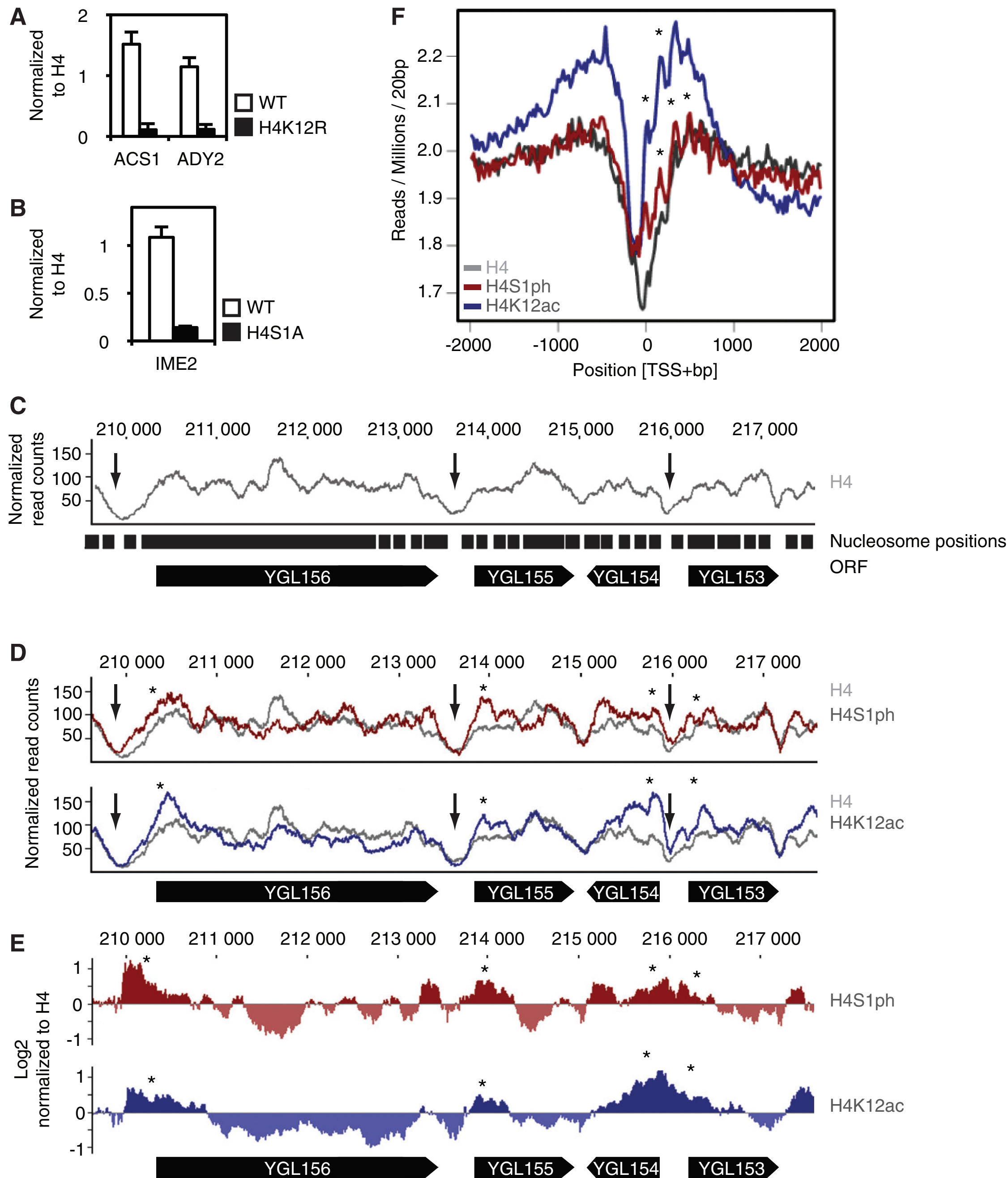

Figure 2. Genome-wide localization of H4S1ph and H4K12ac. (**A**) H4K12ac antibody specificity in ChIP assay. ChIP analysis of H4 K12ac was done on extracts prepared from WT and H4K12R cells. Two different loci were analyzed. H4K12ac signal was significantly decreased in the H4K12R strain, demonstrating the antibody specificity in ChIP. (**B**) H4S1ph antibody is specific in ChIP. ChIP analysis was done on extracts prepared from WT and H4S1A cells after induction of sporulation (10 h). H4S1ph signal was significantly decreased in the substitution mutant strain, demonstrating antibody specificity in ChIP. (**C**) Histone H4 distribution within a representative genomic region. ChIP-SEQ reads along a portion of chromosome VII are presented. Gene ORFs are represented by oriented black blocks, labeled with systematic gene names. An NDR localized at the promoter of each gene (black arrows) match nucleosomes positions previously identified (13). (**D**) H4S1ph and H4K12ac distribution within the same region as in panel A. The number of reads for each modification has been normalized to total number of reads for the H4 sample. H4 data are presented in grey (same as panel A). The 5′ regions of each gene appear enriched in H4S1ph and H4K12ac levels compared with H4 levels. (**E**) H4S1ph and H4K12ac enrichment, normalized to the local abundance in H4. Data are presented in Log2 scale. (**F**) Composite profile of an averaged TSS of all genes. Histone H4 content is represented in grey. H4S1ph and H4K12ac distributions are represented in red and blue, respectively.

grey line). As previously described, H4K12ac (Figure 2F, blue line) is highly enriched over the TSS, and spreads into the first portion of the transcribed region (14,15). The H4S1ph profile shows a more modest, but still clear enrichment downstream of the NDR region (Figure 2F, red line). The location of individual nucleosomes enriched in the modifications can be discerned in both H4S1ph and H4K12ac averages, over their regions of maximum

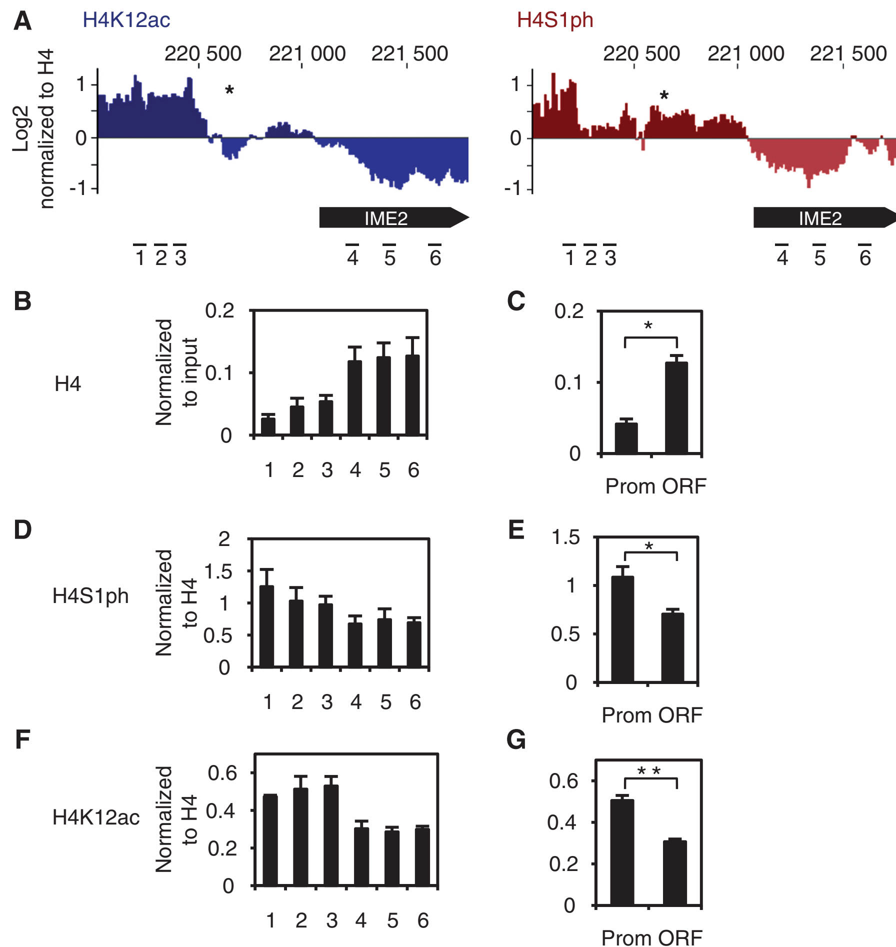

Figure 3. Analysis of *IME2* and promoter confirms the genome-wide survey. (**A**) *IME2* data acquired in the genome-wide analysis. Please refer to Figure 2E figure legend. TSS of the *IME2* gene and the primer pairs used for qPCR are indicated. (**B**), (**D**) and (**F**) ChIP-qPCR analysis of the histone H4 content, H4S1ph, H4K12ac on individual locations of *IME2,* respectively, 11 h after induction of sporulation. Data are normalized to total DNA input levels in (B) and to H4 levels in (D) and (F). (**C**), (**E**) and (**G**) represent data of panel B, D and F, respectively, averaged for primers located in the promoter or the ORF of *IME2*. Error bars are standard deviation of the mean (SEM). $*P < 0.05$ and $**P < 0.01$, respectively.

enrichment at the TSS (Figure 2F, asterisks over red and blue lines).

Taken together, the genome-wide analysis indicates an accumulation of H4S1ph over and slightly downstream of the TSS. Moreover, this analysis demonstrates that H4S1ph and H4ac occur in similar regions locally during sporulation.

Single locus analysis confirms genome-wide profiles

To confirm and extend these genome-wide observations, we examined a sporulation-specific gene, *IME2*, in greater detail before and during sporulation. The *IME2* gene is induced at the beginning of sporulation and remains high through 10 h, at which time it starts to decline (see Figure 4D). The ChIP-SEQ profile at 10 h of *IME2* shows H4K12ac in the promoter; H4S1ph is also enriched in the same locations, but also in the trough between the peaks of H4K12ac (see asterisks on Figure 3A).

We designed several pairs of primers (primer sets 1–6, Figure 3A) to analyze specific regions by ChIP-qPCR in the promoter and in the transcribed region of *IME2*. Unfortunately, no primer set successfully amplified the TATA region of *IME2*, which is probably due to the extremely high AT content of this region (Figure 3A, between the asterisk and the TSS). The locations of each primer set are indicated (Figure 3A), and each location was analyzed individually (Figure 3B, D and F), and average levels for each region—promoter versus transcribed region—are also presented (Figure 3C, E and G). ChIP-qPCR analysis of the histone H4 content of the promoter and *IME2* confirms that the transcribed region of *IME2* was significantly enriched in nucleosomes, compared with its promoter. This trend is clear for both individual locations within the promoter and the transcribed region (Figure 3B), and averaged levels within these two regions (Figure 3C, $P < 10^{-10}$).

We then analyzed H4S1ph and H4K12ac enrichment levels at the same locations in *IME2* using ChIP-qPCR, to examine whether the modifications are higher at the promoter compared with the transcribed region. The data show that both H4S1ph and H4K12ac are significantly enriched on the promoter compared with the

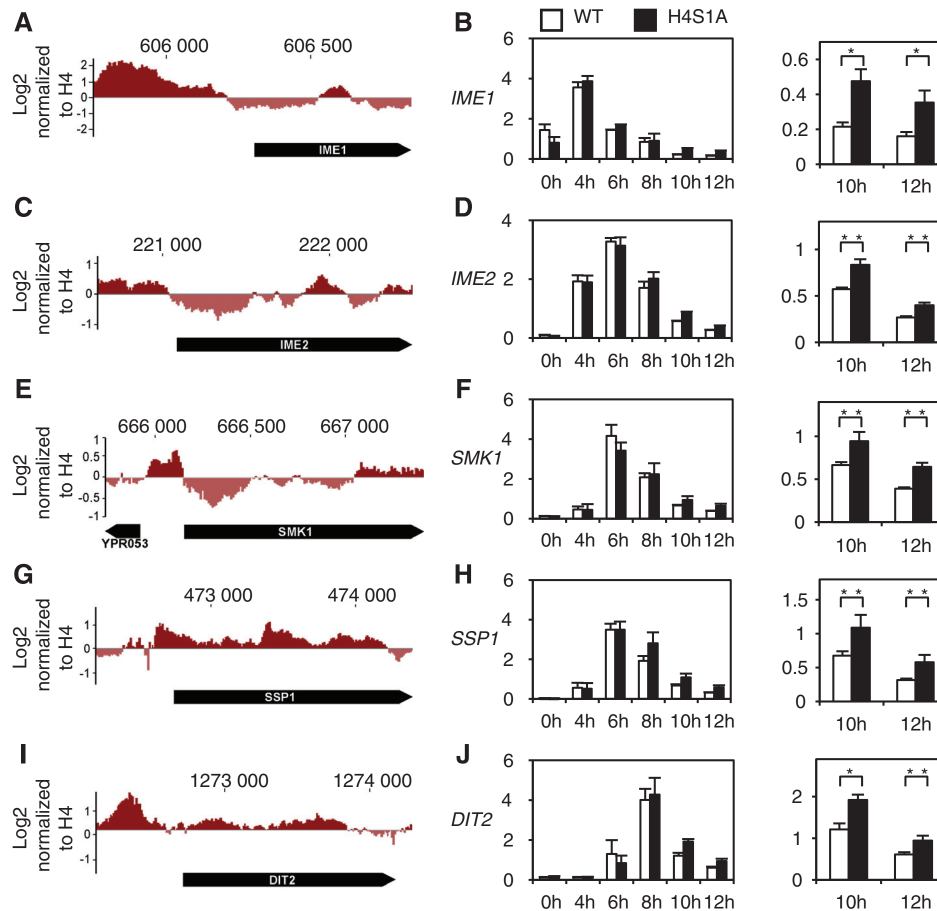

Figure 4. Loss of H4S1ph affects transcription repression. H4S1ph profiles (normalized to the H4 content, Log_2 scale) and expression profiles are shown, of early (**A–D**), middle (**E–H**) and late genes (**I** and **J**) during sporulation of WT and H4S1A strains. Panels A, C, E, G and I represent H4S1ph profiles for early, middle and late sporulation genes. Black boxes represent transcripts, and start at the TSS (21). Gene *YPR053* is located near to *SMK1*. Panels B, D, F, H and J represent expression profiles of indicated genes for the full time course or the 10 and 12 h time points. Error bars are standard deviation of the mean (SEM). $^*P < 0.05$ and $^{**}P < 0.01$, respectively.

transcribed region of *IME2* (Figure 3D and F). This was also the case for the averaged levels for the three primers sets in each region (Figure 3E and G, $P < 0.03$ and $<10^{-4}$, respectively). Taken together, the results confirm the genome-wide analysis on a local scale at the *IME2* gene, i.e. both H4 S1ph and H4K12ac are enriched on the TSSs compared with the downstream transcribed regions.

H4S1ph promotes transcription repression

Promoters of genes are enriched in histone modifications involved in the control of their transcription status (1). However, budding yeast is generally devoid of negative-acting histone modifications. Because we detected H4S1ph principally while transcription is globally declining during late stages of sporulation, we investigated using bioinformatics whether any particular class of genes is enriched in H4S1ph, and whether this enrichment might be related to reduction of gene expression. To examine this, we employed gene set enrichment analysis (GSEA) to look for the enrichment of collections of co-regulated genes in yeast, called gene modules (Supplementary Methods section and Supplementary Table S3). We found that, out of 778 modules representing 2951 genes, 20 modules are significantly enriched in H4S1ph (Supplementary Tables S4 and S5). Strikingly, more than half of these modules contain 'middle-sporulation genes', as categorized by the GSEA software. This correlation suggests that the role of H4S1ph could include regulation of transcription of certain sporulation-dedicated genes.

Based on this analysis, we tested whether H4S1ph regulates sporulation-specific genes. We noted, however, that some key regulators of sporulation are not represented in the modules used for the GSEA analysis, and thus we expanded our analysis to include a wide selection of well-characterized sporulation genes. Several classes of genes are expressed in succession during sporulation, and we chose representatives within each class: (i) early genes: *IME1* and *IME2*; (ii) middle genes: *SMK1* and *SSP1*; (iii) late genes: *DIT2* [for review, see ref. (16)]. The ChIP-SEQ profiles at 10 h show that H4S1ph is enriched on the promoters of these genes, and some of the genes display additional enrichment over their transcribed regions, in particular, two genes induced mid- or late-sporulation, *SSP1* and *DIT2* (Figure 4A, C, E, G and I).

We prepared strains in which H4S1 was substituted to alanine (H4 S1A), a non-phosphorylatable mimic of serine

(Figure 1C), to examine the effect of absence of phosphorylation at H4S1 on the transcription of these key genes expressed during sporulation. We found that H4S1A substitution leads to a significant delay in the re-repression of each sporulation-specific gene following their peak expression (Figure 4B, D, F, H and J; right panels). This effect is most pronounced at the time that H4S1ph is normally present, i.e. at 10 and 12 h; at these times the defect in re-repression of transcription is statistically significant (Figure 4B, D, F, H and J; right panels). This delay in re-repression is not due to a general delay in sporulation progression, as there is no delay in the meiotic progression in the H4S1A mutant strain in an SK1 background (data not shown). Furthermore, the time of onset of transcriptional activation of these genes was not altered by the H4S1A mutation (Figure 4). Finally, the delay in re-repression is not caused by lower global levels of the histone H4 mutated in H4S1A (Figure 1C), or by a decrease of H4 occupancy in the H4S1A mutant (Supplementary Figure S2).

Thus, our results show that during late sporulation, H4S1ph generally accumulates in the promoters of genes. In genes specifically expressed during sporulation, the H4S1A mutant results in a delay in re-repression following peak transcription. We note, as discussed below, that this delayed re-repression correlates with decreased compaction and lowered sporulation efficiency, as we previously reported (4).

DISCUSSION

We previously discovered H4 S1ph as a post-meiotic histone modification involved in genome compaction and required for optimal efficiency of sporulation (4). In the current study, to further understand the function and mechanisms underlying the physiological role of this modification, we analyzed its genome-wide localization and the effect of eliminating the modification on transcription during sporulation. Our novel findings are summarized here. First, the H4S1ph mark is mainly localized in the TSS region, and this occurs genome-wide as indicated by the composite TSS profile (Figure 2D). This peak coincides with the highest level of H4 acetylation. Indeed, there is an unexpected local co-localization of phosphorylated and acetylated histone H4, which previously were inversely correlated during DSB repair (7).

Thus, in contrast to the DSB repair pathway, H4S1ph deposition during sporulation is not associated with the erasure of H4 acetylation. Indeed, while H4S1ph prevents subsequent acetylation of H4 during DSB repair (7), H4 acetylation does not appear to prevent deposition of H4S1ph during sporulation. We note that our analysis does not rule out that the acetylation and phosphorylation occur on different neighboring nucleosomes or different H4 tails in single nucleosomes; however, in clear contrast to DSB repair, our data shows a similar location of the modifications.

A second finding is that the H4S1ph mark appears to be required for optimal and timely re-repression of sporulation-specific genes following their peak induction during sporulation. Negative-acting histone modifications are unusual in budding yeast, as only sumoylation has previously been correlated with gene repression (17). Moreover, histone phosphorylation has not previously been associated with transcription repression. It may be that the re-repression is functionally related to the role of H4S1ph in promoting chromatin compaction in the later stages of sporulation (4), rather than a classical transcriptional repression mechanism. Indeed a key correlation is established between our new findings of gene-linked H4S1ph and delay in re-repression, with our previous findings of an important physiological role of H4S1ph to promote nuclear compaction and optimal sporulation efficiency (4).

H4S1ph function during sporulation and DSB repair therefore appears to be different, and hence related to a specific role within each process. Indeed, a DSB is an unexpected event, affecting a random region of the genome and jeopardizing genome integrity. Repair of a DSB not only mends the genetic sequence, but also appears to restore a naïve, unmodified epigenetic state. During DSB repair, H4S1ph deposition helps to restore the genetic integrity of the genome, through promotion of non-homologous end joining (6), but also maintains a neutral epigenetic state by preventing any subsequent aberrant deposition of H4 acetylation marks (7).

In contrast, during sporulation, H4S1ph acts during a differentiation program. It is distributed throughout the entire genome, but shows highest accumulation in open regions of the genome, such as promoters and some transcribed regions. H4S1ph-mediated compaction seems to overcome the effect of existing modifications, such as H4ac.

Altogether, these data suggest that H4 acetylation is part of a promoter signature; it remains to be determined whether acetylation is maintained in the mature spores and used during germination. H4S1ph deposition appears to promote transcriptional re-repression and full compaction of spore nuclei, while still preserving the promoter acetylation signature. This dual modification may thus be somewhat analogous to 'bivalent domains', which combine active and repressive histone modifications at master regulator genes in mammalian embryonic stem cells (18). The master regulator genes are critical for specific differentiation pathways, and, depending on the eventual cell type, will either remain repressed or become active, and then retain only the relevant histone modifications.

Recent findings in mammalian sperm show a similar bivalent histone signature at these developmental master regulator genes (19). Hence, our findings suggest that spores may be analogous to sperm cells in their need to maintain genes in a status where genes are compacted and repressed (H4 S1ph), but maintain potential for renewed transcription (H4ac) upon germination.

Finally, given the evolutionary conservation of H4 acetylation and phosphorylation during gametogenesis in more complex eukaryotes (4), it remains to be determined how these modifications regulate spermatogenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors wish to thank former and present members of the Berger laboratory for valuable discussions.

FUNDING

Research grant GM55360 from the National Institutes of Health (to S.L.B.); Program 'Espoirs de la Recherche' of the 'Fondation pour la Recherche Médicale Française', and by the Philippe Foundation to J.G. SK laboratory is supported by Episperm and Empreinte ANR programs. Funding for open access charge: GM55360 from the National Institutes of Health (S.L.B.).

Conflict of interest statement. None declared.

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