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H2B ubiquitylation and the histone chaperone Asf1 cooperatively mediate the formation and maintenance of heterochromatin silencing

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ABSTRACT

Heterochromatin is a heritable form of gene repression, with critical roles in development and cell identity. Understanding how chromatin factors results in such repression is a fundamental question. Chromatin is assembled and disassembled during transcription, replication and repair by anti-silencing function 1 (Asf1), a highly conserved histone chaperone. Transcription and DNA replication are also affected by histone modifications that modify nucleosome dynamics, such as H2B ubiquitylation (H2Bub). We report here that H2Bub and Asf1 cooperatively promote transcriptional silencing at yeast telomeres and mating loci. Through real time monitoring of *HML* (Hidden MAT Left) locus silencing, we found that transcriptional repression was slowly initiated and never fully established in mutants lacking both Asf1 and H2Bub. These findings are consistent with impaired *HML* silencer-binding and spreading of repressor proteins, Sir2 and Sir3. In addition, mutants lacking H2Bub and Asf1 show defects in both nucleosome assembly and higher-order heterochromatin organization at the *HML* locus. Our findings reveal a novel role for H2Bub and Asf1 in epigenetic silencing at mating loci. Thus, the interplay between H2Hbub and Asf1 may fine-tune nucleosome dynamics and SIR protein recruitment, and represent an ongoing requirement for proper formation and maintenance of heterochromatin.

INTRODUCTION

Eukaryotic genomes are folded into hierarchically ordered configurations that comprise distinct domains within the nucleus. The most visible nuclear domains are composed of heterochromatin and are found clustered near the nuclear periphery. These condensed chromatin domains restrict gene expression (1–3) and play critical roles in development and cell identity (4). For example, X-inactivation in mammals entirely converts one of the two X chromosomes into gene-silenced heterochromatin (5). In a much simpler organism, the budding yeast *Saccharomyces cerevisiae*, a similar gene silencing mechanism is adopted to define sexual identity. Yeast possess three mating loci on chromosome III [*HML* (Hidden MAT Left), *MAT* (*a* or α), and *HMR* (Hidden MAT Right)]. *MAT* determines the mating type of the cell (*a* or α), while the other two, collectively termed homothallism (*HM*) loci, are packaged as heterochromatin and silenced (6). The establishment and maintenance of silenced chromatin in these two regions requires the physical spreading of chromatin-associated proteins (7) and histone modifications (8). Disruption of transcriptional silencing at *HML* or *HMR* loci in haploid cells leads to the expression of both *a*- and α-related genes, which inhibits haploid cells from responding to sex pheromones and prevents mating (9,10). Similar repression occurs within areas close to yeast telomeres (6). Investigations into the regulation of homothallic *HM* loci and/or telomeres have revealed some basic features of heterochromatin assembly (11). The presence of *cis*-acting sequences (silencers) and specific DNA binding factors are required to nucleate/initiate the recruitment of general repressors, and the *trans*-acting factors Sir2, Sir3 and Sir4 (silent information regulator; SIR) (9,12). Heterochromatin subsequently spreads from the initiation sites, followed by a change in the higher-order organization of silenced chromatin, which then clusters the heterochromatin near the nuclear envelope (7). *HML* and *HMR* interact despite a large separation on the chromosome (7,13), indicating the presence of heterochromatic sub-nuclear domains. The specific DNA binding factors, bound to silencers, are

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able to coordinate with a distal silencer and thereby promote the formation of SIR-mediated chromatin silencing (14–16).

Appropriate nucleosome assembly plays a role in gene silencing and spatial organization of silenced chromatin. Therefore, histone chaperones, which regulate nucleosome assembly, are important in regulating chromatin assembly and dynamics. For example, the chromatin assembly factor 1 (CAF-1) complex contributes to replication-dependent nucleosome assembly (17,18), whereas a second chaperone, the histone regulatory (HIR) complex, functions outside of DNA replication (19). A third chaperone, anti-silencing factor 1 (Asf1), which is required for histone H3K56 acetylation and replication-dependent and -independent nucleosome assembly (20–23), boosts the activities of both complexes (24,25). Interestingly, cells deficient in any one of these histone chaperones exhibit defects in telomere silencing, but no defects, or only slight changes, in *HM* silencing (17,26–29). For example, deletion of *ASF1* disrupts the *HML–HMR* long-range interaction but not gene silencing (7).

Post-translational modifications of histone proteins are also important for the establishment of gene silencing. Deacetylation of H4K16 (and potentially H3K56) occurs immediately after nucleation to enable SIR binding across the locus (30–34). H3K4 and H3K79 are demethylated at a later stage of chromatin silencing (35). The two active chromatin marks, methylated H3K4 and H3K79, are thought to prevent promiscuous binding of SIR proteins throughout the genome (36,37). Thus, yeast strains lacking the corresponding histone methyltransferases, Set1 and Dot1, exhibit unregulated SIR binding in regions near telomeres. (38,39). Dot1 methylates H3K79 upon recruitment by acetylated H4K16, further inhibiting the loading of the SIR complex. Thus, H3K79 methylation and H4K16 acetylation co-regulate SIR-mediated silencing and boundary formation between active and silent chromatin at telomeres (40). H2B ubiquitylation (H2Bub), a histone modification which promotes H3K4 and H3K79 methylation, enhances activity of RNA Polymerase II during transcription elongation, by mediating nucleosome reassembly in both yeast and human (41–43). In addition, H2Bub promotes replication-associated nucleosome formation in budding yeast (44,45). However, these processes are independent of H3K4 and H3K79 methylation. Interestingly, H2Bub is required for telomere silencing, but has not been associated with heterochromatin assembly at the *HM* loci (46,47).

Here, we investigated the functional consequences of a genetic interaction between H2Bub and Asf1. We found that depletion of both H2Bub and Asf1 led to an overall loss of the ability of the cell to respond to sex pheromone, caused by a severe disruption of *HML* silencing. Our results suggest that histone H2Bub cooperates with Asf1 histone chaperone to mediate the assembly of nucleosomes and the recruitment/maintenance of SIR proteins which in turn promotes higher-order heterochromatin organization at silenced mating-type (MAT) locus. Thus, our results show a previously unknown function of Asf1 and H2Bub for the formation and maintenance of facultative heterochromatin.

MATERIALS AND METHODS

Yeast strains and primers

Yeast strains used in this study were mainly derived from Y131 and JRY9107, which have been described previously (48). Yeast cells were collected at log-phase for analysis. All strains and primers are listed in Supplementary Tables S1 and 2, respectively. Gene replacement and tagging were performed using standard techniques.

Gene replacement

For gene disruptions, the indicated gene was deleted by high efficiency transformation, using a polymerase chain reaction (PCR) product in which the target was replaced with the *KanMX* gene (Yeast deletion project) or amplified using plasmid with selectable markers as templates.

Flow cytometry analysis

For DNA content analysis, $\sim 1 \times 10^7$ cells were collected at each time point and resuspended in 1 ml 70% ethanol (ice-cold), before being stored at −80°C for at least one night (samples were stored up to a maximum of 3 days). The cells were then washed twice with 1 ml 50 mM Tris–HCl (pH 8.0) followed by ribonuclease (RNAase) A digestion (1 mg/ml of RNAase A in 50 mM Tris–Cl, pH 8.0) and proteinase K digestion (16 units/ml in 30 mM Tris–Cl, pH 8.0). Finally, cells were stained with SYBR GREEN I buffer (in 50 mM Tris–Cl, pH 8.0) at 4°C overnight. The cell size and DNA contents of 50 000 cells were examined on a FACSCanto II (BD).

For green fluorescent protein (GFP) expression assays, cells were grown to 0.1 OD_{600} in SC medium over two sequential nights of growth at 30°C; the culture was diluted down each day. Cells were harvested by centrifugation, fixed in a 4% paraformaldehyde/3.4% sucrose solution for 15 min at room temperature (RT) and then washed and stored in a 1.2-M sorbitol, 0.1-M KPO4 solution (pH 7.5). Cells were stored at 4°C for a maximum of 24 h. GFP expression data were collected for each sample using FACSCanto II (BD). A total of 50 000 cells were measured per run and gated to identify those that were within a specific size and granularity (∼40 000 cells/experiment).

For analysis using nicotinamide, cells were grown in SC medium containing 5 mM nicotinamide over two consecutive nights at 30°C; the culture was diluted down each night. For time-course analyzes of silencing establishment, cells were grown in 5 mM nicotinamide in SC medium over two nights at 30°C, collected by centrifugation, washed and then resuspended in SC medium. Samples were collected during washing (0 min) and at 0.5, 1, 1.5, 2, 4, 6 and 24 h post-washing.

RT-PCR analysis

Transcripts for reverse transcriptase-polymerase chain reaction (RT-PCR) were extracted by the acid–phenol method with DNase digestion and then analyzed by real-time PCR after reverse transcription.

Pheromone Halo assay

This assay was performed as described (49). Briefly, small amounts of overnight-cultured cells were mixed well with 0.5% sterile agar at 55°C and then poured onto a pre-warmed plate containing the appropriate solid media. One paper disk containing 5 μl of different concentrations of α-factor was placed on the plate and then incubated at 30°C for 16–24 h.

Chromatin immunoprecipitation

Yeast strains were grown to an OD_{600} of 0.4–0.8 and then fixed with 1% formaldehyde at RT for 15 min. Fixation was stopped by the addition of glycine to a final concentration of 125 mM for 5 min and the cells were then collected and washed twice with ice-cold TBS (100 mM Tris, pH 7.5, 0.9% NaCl). Cell pellets were stored at −80°C or resuspended immediately in 1 ml of FA lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium deoxycholate, 0.1% sodium dodecylsulphate (SDS)) supplemented with fresh protease inhibitor cocktail (Sigma) and 0.4 mM dithiothreitol (DTT) and lysed by vortexing with glass beads for 45 min (15-min on, 1-min off and three times) at 4°C. Chromatin pellets were obtained by centrifugation at 4°C and then washed with FA lysis buffer three times. Chromatin pellets resuspended in FA lysis buffer were then sonicated using a Thermo Fisher Diagenode™ Bioruptor® Pico Ultrasonicator. The average size of the resulting DNA fragments was between 200 and 500 bp. Following centrifugation at 13 500 rpm for 30 min at 4°C, the soluble chromatin was collected for detection of protein concentration.

For immunoprecipitation, 250 μg chromatin per reaction was incubated overnight at 4°C, together with 20 μl of protein G dynabeads (Invitrogen) that had been pre-bound with anti-H3 or anti-Myc (Sir2 or Sir3–9Myc). Immunoprecipitates were collected by a step-wise washing protocol, consisting of 1.5 ml FA-lysis buffer, 1.5 ml WASH I (FA lysis buffer with 0.5 M NaCl), 1.5 ml WASH II (10 mM Tris–Cl, pH 7.5, 1 mM EDTA, 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate) and 1.5 ml TE (pH 8.0) for 5 min each at RT. The immuno-complexes were eluted by adding 0.25 ml elution buffer (50 mM Tris–Cl, pH 7.5, 10 mM EDTA, 1% SDS), followed by incubation first at 65°C for 20 min and then at RT for 10 min with vortexing. DNA was purified using Qiaquick PCR purification spin-columns (Qiagen) and used as template for quantitative-PCR. All the primers used are listed in Supplementary Table S2.

Transcript DNA microarrays

DNA microarray analysis was performed with Phalanx Yeast OneArray® chip (Phalanx Biotech). For each mutant, three independent experiments were performed for the statistical analysis. Yeast genome probe content for the array was selected from Operon Yeast Genome Array-Ready Oligo Set (yeast AROS) v1.1 and Yeast Brown Lab Oligo Extension (YBOX) v1.0. These two sets are 70-mer probes specially designed within 750 bases from the 3′ end of the open reading frame. The fluorescence-labeled probes were hybridized to a chip (Phalanx Biotech) for 16 h at 60°C. After performing the washing steps, the DNA chips were scanned using a ScanArray Lite (PerkinElmer Life Sciences, Billerica, MA, USA). Image analysis was performed with GenePix Pro v 6.0 (Molecular Devices). The raw data were then filtered for signal quality (three standard deviations above background) and spot quality (minimum diameter). The data were subjected to Lowess normalization with GeneTraffic v 3.2 (Iobion). The data were then exported for input into Cyber-T to assign Bayes *P*-values to determine whether the mutant was significantly different from the wild-type (WT) for each open reading frame (ORF). Changes in relative expression were identified as significant by ranking the Bayesian *P* values and applying a false discovery rate algorithm to account for multiple testing. The false discovery rate threshold was set at 5%. If, for any mutant, an ORF was determined to be significantly different from the WT, this ORF was included in the cluster analysis. Cluster analysis was performed with Cluster v 2.12 and visualized with Treeview v 1.6. Clusters were analyzed for enrichment of gene classes with FunSpec.

Micrococcal nuclease (MNase) digestion

Micrococcal nuclease (MNase) digestion was performed for nucleosome position analysis. Chromatin pellets were resuspended in NPS buffer (0.5 mM Spermidine, 0.075% IGEPAL, 50 mM NaCl, 10 mM Tris–Cl (pH = 7.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol) and digested with an appropriate concentration of MNase at 37°C. Enzyme activity was stopped by the addition of EDTA to 10 mM. Soluble, digested chromatin was collected after centrifugation at 4°C. After reverse-crosslinking by proteinase K digestion at 65°C overnight, nucleosomal DNA was purified with Phenol chloroform extraction. DNA between 100 and 200 bp was purified by gel extraction and finally used as template for quantitative-PCR. All the primers used are listed in Supplementary Table S2.

Chromosome conformation capture (3C)

The chromosome conformation capture (3C) analyzes of yeast strains were performed exactly as described previously (50). Yeast strains were grown to an OD_{600} of 0.4–0.8 and fixed with 3% formaldehyde at RT for 20 min. Fixation was stopped by the addition of glycine to a final concentration of 0.3 M for 5 min and the cells were then collected and washed twice with ice-cold Tris-buffered saline (TBS) (100 mM Tris, pH 7.5, 0.9% NaCl). The restriction enzyme used for digestion was Sau3A and the digestion buffers used were those recommended by the manufacturer of the enzyme. All the primers used are listed in Supplementary Table S2.

RESULTS

H2B ubiquitylation genetically interacts with the Asf1 histone chaperone

We previously demonstrated that H2Bub and Asf1 function in concert to regulate nucleosome dynamics and promote cell survival during hydroxyurea (HU)-induced replication stress (45). To further investigate the functional interaction between H2Bub and histone chaperones, we first

examined the survival of different histone chaperone mutants combined with H2Bub-deficient mutant (*htb-K123R*) when exposed to various stressors. We found that deletion of *ASF1* sensitizes H2Bub-lacking cells to HU. *ASF1* deletion produced a minor sensitization to ultraviolet radiation, but did not sensitize cells to a DNA alkylating agent, methyl methanesulfonate (Figure 1A, left panel). These results suggest that H2Bub and Asf1 have specific and overlapping functions in DNA replication stress, induced by HU. In addition to their roles in replication, H2Bub and Asf1 have been implicated in regulating nucleosome assembly during transcriptional elongation (20,42). We consistently observed that the *htb-K123R* and *asf1*Δ double mutants exhibited a strong growth defect in 6-Azauracil (6-AU) , a compound which reduces the nucleotide pool for RNA Polymerase II elongation (Figure 1A, right panel). In contrast, deletion of either *CAC1* or *HIR1* chaperone genes from the *htb-K123R* strain did not further reduce cell growth on 6-AU medium (Figure 1A). Thus, these results suggest a collaborative role for H2Bub and Asf1 in transcriptional regulation.

H2Bub and Asf1 are important for gene regulation and telomere-specific silencing

Both H2Bub and Asf1 have been implicated in the regulation of transcription repression (17,46,51) and we thus investigated whether these two factors have combined effects on gene expression. To address this question in an unbiased manner, we used cDNA microarrays to compare mRNA levels of *htb-K123R*, *asf1*Δ and *asf1*Δ *htb-K123R* strains to those of WT. Depletion of H2Bub or Asf1 affected the gene expression profile (both upregulated and downregulated genes are shown) and a combinatorial effect was observed in double mutant cells (Figure 1B). We found that the expression of 536 genes in *asf1*Δ and 334 genes in *htb-K123R* was increased by at least 1.75-fold, and a modest combinatorial effect was observed for double mutant cells (729 genes) (Figure 1B). On the other hand, 331 genes in *asf1*Δ, 302 genes in *htb-K123R* and 738 genes in double mutant cells were decreased by at least 1.75-fold. These affected genes (both upregulated and downregulated) were further functionally annotated and classified under various Gene Ontology (GO) terms (*Saccharomyces Genome Database*). The upregulated genes were enriched in genes that were classified under *cell cycle*, *sporulation* and *cell wall organization* GO terms (Supplementary Table S3). The downregulated genes were functionally grouped among *response to pheromone* and *conjugation with cellular fusion* GO terms (Supplementary Table S4). We further categorized upregulated or downregulated genes according to their distance from telomeres (Figure 1C). Focusing on 275 genes located within 20 kb of a telomere, we found that that ∼20% (60/275) in *htb-K123R* cells and ∼30% (95/275) in *asf1*Δ were upregulated or derepressed (Figure 1C). However, more than 40% of those same genes in sub-telomeric regions of *asf1*Δ *htb-K123R* cells were significantly de-repressed (114/275) (Figure 1C). Therefore, the results suggest a modest combinatorial effect of H2Bub and Asf1 in telomere-specific gene silencing.

Ablation of both H2Bub and Asf1 eliminates the cellular response to sex pheromone

Next, we sought to examine the role of H2Bub and Asf1 at a second silenced chromatin region, the *HM* locus (6). We found that both *asf1*Δ and *htb-K123R* mutant cells (*MAT a* type) responded to sex pheromone α-factor in a dose-dependent manner (Figure 2A). Furthermore, deletion of either the *CAC1* or *HIR1* histone chaperone did not abolish the cellular response to α-factor in *htb-K123R* cells (Supplementary Figure S1). However, *asf1*Δ *htb-K123R* double mutants exhibited a poor response to sex pheromone (Figure 2A). Consistent with this finding, depletion of H2B-specific E3 ligase Bre1 in *asf1Δ* cells leads to the loss of response to α-factor (Supplementary Figure S2A). However, the deletion of E2 ubiquitin-conjugating enzyme Rad6 in *asf1Δ* cells did not produce a similar defect. Rad6 is not only the E2 enzyme for Bre1 but is also involved in post-replication repair through Rad18 and ubiquitin-mediated N-end rule protein degradation through Ubr1 (52–55). Thus, the pleiotrophic effects of *rad6Δ* may lead to a phenotype that is divergent from that of *bre1Δ*.

H2Bub is the signal that allows for H3K4 methylation by Set1 and H3K79 methylation by Dot1 (47). To gain further insight into whether the effect of H2Bub on pheromone response was mediated by downstream methylation events, we deleted Set1 or Dot1 in *asf1*Δ cells and found that both of the double mutant cells still responded to α-factor as well as *asf1Δ*. (Supplementary Figure S3). However, the growth of triple mutant *dot1*Δ *set1*Δ *asf1*Δ was equivalent to that in *asf1*Δ *htb-K123R* in the presence of α-factor (Supplementary Figure S3). Thus, the results suggest that the synergistic effects of H2Bub and Asf1 are likely linked to the downstream H3 methylation.

To further confirm the synergistic effects of H2Bub and Asf1 in pheromone response, we treated cells with α-factor to induce G1 arrest of *MAT a* cells, and then measured the cellular DNA content by flow cytometry. As expected, α-factor treatment led to G1 arrest in WT, *htb-K123R* and the majority of *asf1Δ MAT a* cells within 3 h. However, a large proportion of double mutant cells remained at different stages of the cell cycle, even after 5 h incubation (Figure 2B). Therefore, flow cytometry analysis confirmed the inability of the double mutants to respond to sex pheromone.

Silencing of *HML* loci requires both H2Bub and Asf1

The inability of the *asf1*Δ *htb-K123R* double mutant to respond to sex pheromone may also affect the repression of mating genes. Thus, we compared gene expression changes between different mutants and WT cells through a non-biased clustering analysis. Under treatment with α-factor, a group of ∼40 repressed genes in *asf1*Δ *htb-K123R* mutants clustered together at the top of the heat map (Figure 2C). This group was highly enriched with genes related to *sexual reproduction* ($P = 5.34 \times 10^{-13}$) and *response to pheromone* ($P = 5.34 \times 10^{-13}$) (Figure 2C). Enrichment of genes in these categories suggests that both a- and α-related expression are present in this mutant, thereby preventing them from responding to sex pheromone. In further support of this hypothesis, we found that the expression level of *HMLα2* was increased 17.6-fold in *asf1*Δ *htb-K123R* (*MAT*

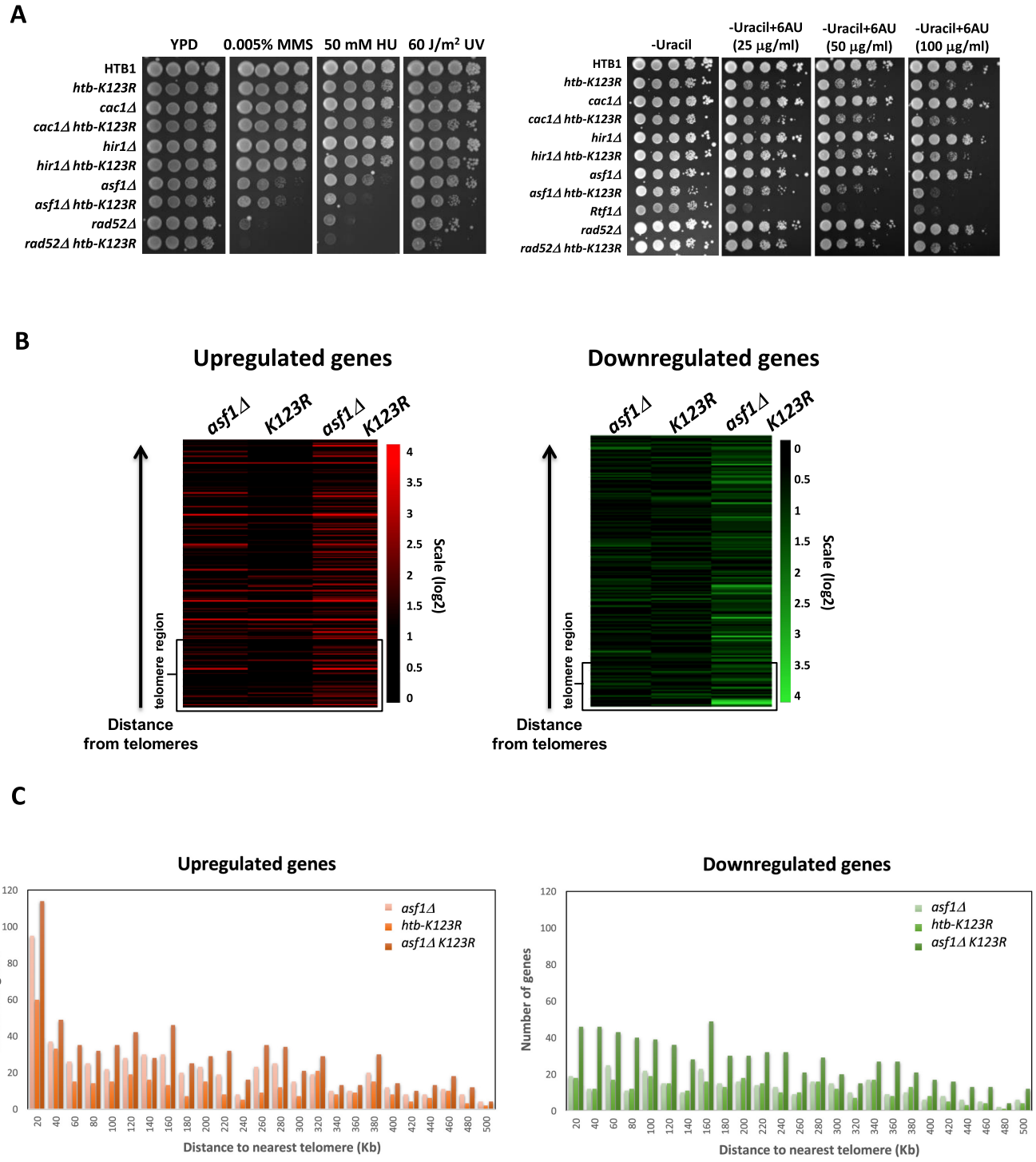
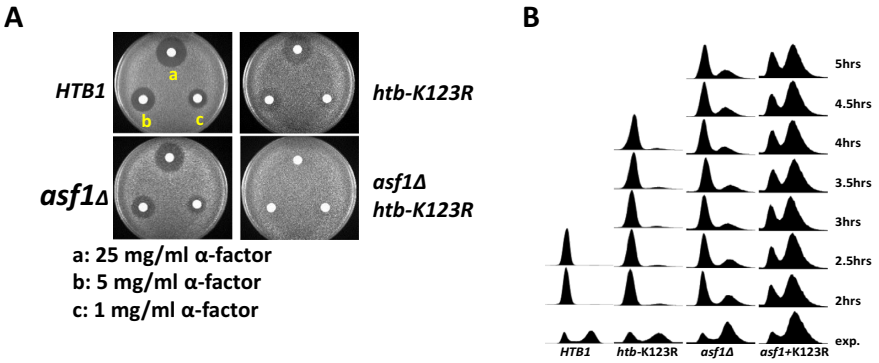


Figure 1. Cells lacking both anti-silencing function 1 (Asf1) and H2Bub exhibit defects in stress-resistance and gene silencing. (**A**) Specific genetic interactions between H2Bub and the Asf1 histone chaperone. Cell growth was observed for 10-fold serial dilutions of yeast cells spotted onto non-selective YPD plates, or plates containing one of the following chemicals at 30°C for 3–5 days: methyl methanesulfonate (MMS; causes DNA damage), hydroxyurea (HU; source of replication stress), UV irradiation (leads to DNA damage) or 6-Azauracil (6-AU; affects transcriptional elongation). The *rad52Δ* strain served as a control for MMS, HU and UV treatment, while *rtf1Δ* served as a control for 6-AU treatment. (**B**) Positional cluster analysis shows gene expression changes in *asf1Δ*, *htb-K123R* and *asf1Δ htb-K123R* mutant cells. Only genes whose expression was increased or decreased by at least 1.75-fold in at least one of these strains are shown (775 upregulated genes and 826 downregulated genes). Genes located within 20 kb of their respective telomeres are marked on the left as the telomere region. (**C**) H2Bub and Asf1 are required for gene silencing within the proximity of telomeres. Transcripts from each strain were isolated and analyzed by Phalanx Yeast OneArray®. The numbers of genes affected in the mutants compared to wild-type (WT) were plotted against their position from telomeres in kilobase pairs (kb). The numbers of upregulated and downregulated genes are shown as red and green bars, respectively.



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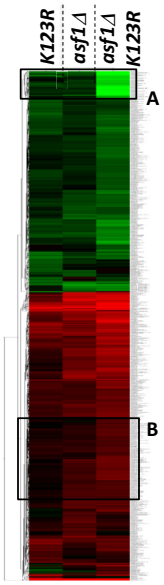

A

GO Term	*p* value	Genes
sexual reproduction	5.34e-13	PCL2, SST2, ASG7, CIK1, FAR1, FIG1, PRM8, HYM1, FIG2, PRM10, PRM3, PRM4, AGA1, PRM6, GPA1, KAR4, KAR5, FUS1, FUS2, PRM1, PRM2, AFR1, FUS3
response to pheromone	4.83e-10	SST2, FAR1, FIG1, PRM8, FIG2, PRM10, PRM3, PRM4, AGA1, GPA1, PRM6, KAR4, FUS1, FUS2, PRM1, AFR1, FUS3

B

GO Term	*p* value	Genes
cell cycle process	< 0.0001	VIK1, STU1, APC1, SPO13, KIP2, CHS2, SIC1, RAX2, SHE1, SPC29, SPS100, MSH6, DSN1, CSM1, CDC5, MIF2, RDH54, CDC20, EMI1, YCS4, TUB1, ESP1, MPC54, BUD27, ACE2, SLK19, PDS5
DNA repair	< 0.01	MSH6, REV1, PIF1, PSY3, HHF1, RDH54, HHT1, HTA1, POL1, DPB2, HTB1, RLF2, PDS5
response to DNA damage stimulus	< 0.05	MSH6, REV1, PIF1, PSY3, HHF1, RDH54, HHT1, HTA1, POL1, DPB2, HTB1, RLF2, PDS5
DNA replication	0.072	MSH6, REV1, EST2, CDC5, CSM1, POL1, DPB2

Figure 2. H2Bub and Asf1 are important for cellular response to sex pheromone. (**A**) Loss of mating type silencing in *asf1Δ htb-K123R* mutant cells (Halo assay). The indicated strains (mating type a) were treated with various doses of α-factor (1, 5 and 25 mg/ml) and the inhibition of cell growth indicates that mating type silencing was well maintained. (**B**) Cells lacking both Asf1 and H2Bub cannot respond to alpha factor. Isogenic WT (HTB1), *htb-K123R*, *asf1Δ* and *asf1Δ htb-K123R* cells were arrested at G1 phase with α-factor at 30°C for 2–5 h; exp: cells at exponential stage; h: the time in hours under α-factor treatment. Cells at each time point were stained with SYBR Green and the DNA content was determined by flow cytometry. (**C**) Transcriptional profiles of *htb-K123R*, *asf1Δ* and *asf1Δ htb-K123R* mutants under α-factor treatment. Cells were treated with α-factor for 3 h and transcripts were isolated and analyzed by Phalanx Yeast OneArray®. Red and green represent upregulated and downregulated genes, respectively. Genes that were not significantly affected appear black in the heat map. Genes that exhibited a 1.75-fold change or greater between the WT and at least one of the mutants were selected and used in gene clustering analyzes. Table A: downregulated genes were clustered into two predominant GO terms, *sexual reproduction* and *response to pheromone*. Table B: upregulated genes were clustered into several GO terms.

a) as compared to WT, but this gene was only marginally expressed in the two single mutants (Supplementary Table S5).

Activation of the *HML* gene may result from a defect in *HM* silencing (10). To test this hypothesis, we removed the entire *HML* locus (to mimic the repression of *HML*) from the double mutants; such removal was found to restore the cellular response to sex pheromone (Figure 3A). We also confirmed that deletion of the *HML* locus restored expression of repressed mating genes (including *PRM2*, *PRM3*, *PRM6*, *FIG1* and *FUS2*) in the *asf1Δ htb-K123R* mutant (Figure 3B). These results suggest that H2Bub and Asf1 may mediate transcriptional repression at the *HML* locus.

H2Bub and Asf1 collaboratively promote the establishment of silencing

Chromatin factors are involved in repressing the silenced mating loci and thus we hypothesized that H2Bub and Asf1 may be involved in heterochromatin formation. To test this hypothesis, we utilized a reporter gene encoding a fast-folding, high-turnover GFP protein at the *HML* locus (*hml::GFP*), which allowed real-time monitoring of the

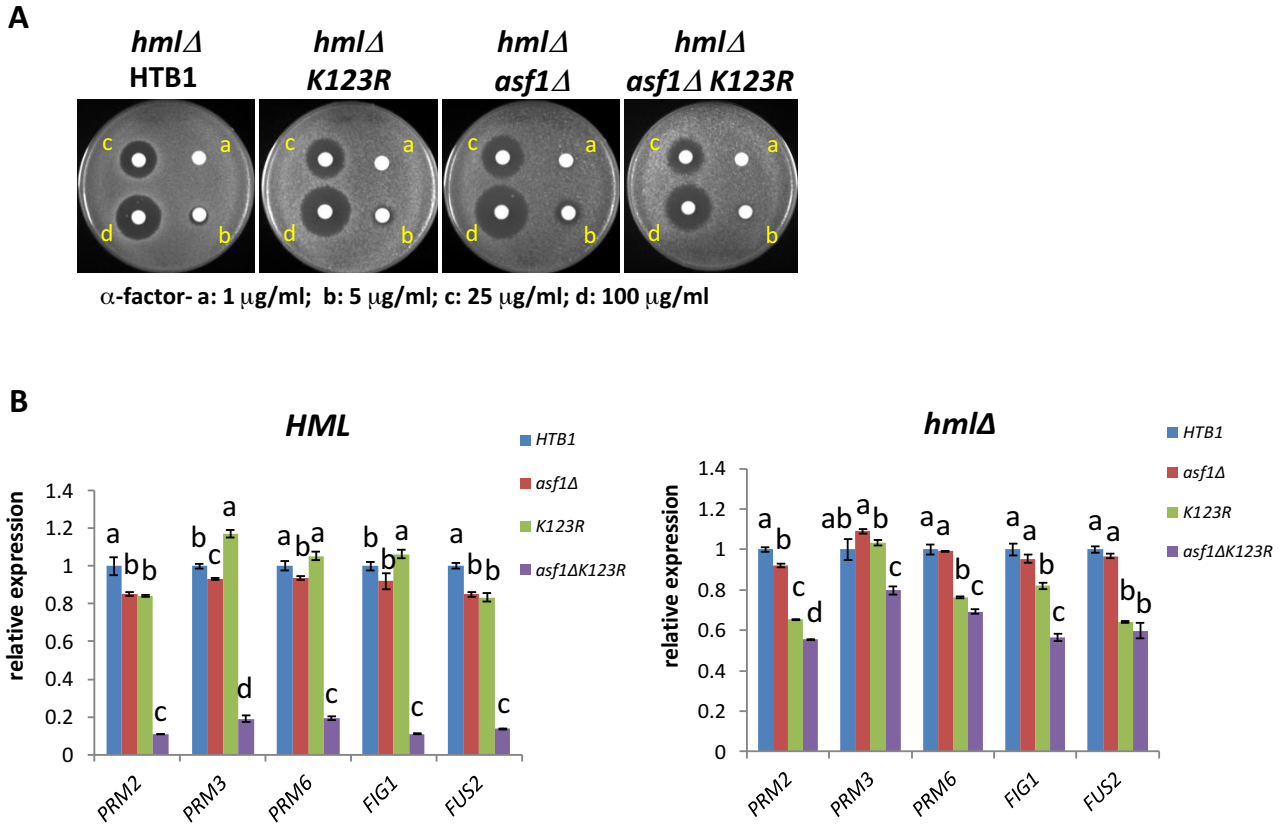

Figure 3. Asf1 and H2Bub are required for the silencing of *HML* loci. (**A**) Deletion of the *HML* cassette restores the response to pheromone in *asf1 htb-K123R* double mutant cells. The halo assay was performed using WT, *htb-K123R*, *asf1Δ* and *asf1Δ htb-K123R* cells, all with *HML* deletion. (**B**) Deletion of the *HML* cassette restores gene expression of *MAT a* in response to pheromone in *asf1 htb-K123R* double mutant cells. The expression levels of several sexual reproduction genes (*PRM2*, *PRM3*, *PRM6*, *FIG1*, *FUS2*) were measured in strains with a WT (*HML*) or *hmlΔ* background by reverse transcriptase-polymerase chain reaction and normalized to *ACT1*. The value for the WT was set as 1 for each group and all values are shown as the mean ± SEM ($n = 3$). Means with different letters are significantly different ($P < 0.05$).

kinetics of chromatin silencing. The *hml::GFP* construct is subject to SIR protein-mediated repression and can be de-repressed by nicotinamide (NAM)-inhibition of Sir2 (Figure 4A) (48). Comparing the expression of *hml::GFP* between WT and the mutant cells revealed that Asf1 and H2Bub play individual roles in the repression of *hml::GFP* (Figure 4B). Deletion of both showed a combinatorial effect on *HML* silencing (Figure 4B), which is consistent with our preceding results (Figures 2 and 3). Interestingly, we also noted that the GFP fluorescence intensity in various mutant NAM-treated cells were not the same as WT NAM-treated cells (Figure 4B and Supplementary Figure S4). This was not due to a non-specific effect of NAM, since NAM treatment did not enhance the fluorescence intensity in *sir2Δ* cells (Supplementary Figure S4). Surprisingly, the GFP fluorescence in *rad6Δ*, *bre1Δ* or *htb-K123R* cells exceeded that in *sir2Δ* and *sir2Δ asf1Δ* cells when treated with NAM (Supplementary Figure S4). One possible explanation is that the Rad6-Bre1-H2Bub cascade may be involved in the turnover of GFP protein. If so, we would expect an accumulation of GFP protein in cells lacking H2Bub after de-repression by NAM. An alternative possibility is that the URA3 promoter, which drives GFP expression, is highly activated in these mutants, leading to a high level of GFP expression.

We next measured the kinetics of *HML* silencing by removing nicotinamide from the medium and subsequently monitoring the reduction of *hml::GFP* expression. While *hml::GFP* expression began to decrease immediately after release from nicotinamide in WT and single mutant cells, expression in the double mutant remained high for about 2 h (Figure 4C). By 6 h after release, *hml::GFP* expression in all strains had reached a steady state. However, the GFP fluorescence intensity remained highest in the double mutant, indicating strong resistance to gene silencing (Figure 4C). Taken together, these results indicated that the histone mark, H2Bub and the histone chaperone, Asf1, are important for the establishment of *HML* silencing.

H2Bub and Asf1 fine tune nucleosome dynamics and SIR protein occupancies at *HM* loci

The establishment and progression of silenced chromatin requires appropriate nucleosome assembly. H2Bub and Asf1 have been shown to participate in gene transcription and DNA replication through regulating nucleosome dynamics (20–23,44,45). Thus, we examined whether these

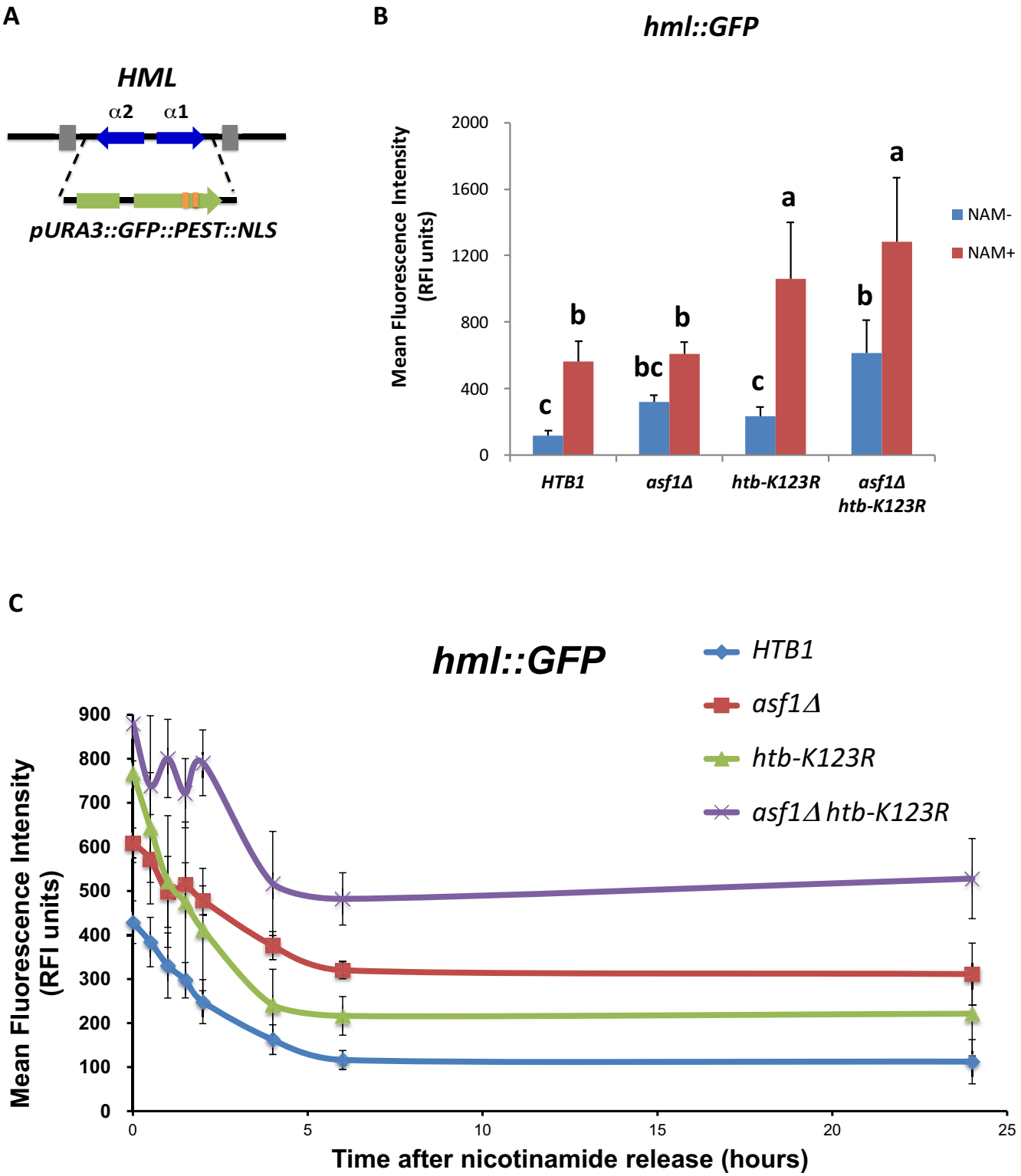

Figure 4. Asf1 and H2Bub cooperate to regulate the establishment of *HML* repression. (**A**) Schematic of the *HML* replacement by GFP encoding gene. A gene encoding a nuclear-localized, destabilized version of GFP expressed from the *URA3* promoter was integrated into the *HML* locus, replacing the α1 and α2 genes. (**B**) Fluorescence intensity of cells containing the *hml::GFP* reporter. Fluorescence intensity was measured by flow cytometry; profiles are shown for both nicotinamide (NAM, a Sir2 inhibitor)-treated (red column, NAM+) and untreated (blue column, NAM−) cells. Data are shown as the mean ± SEM ($n = 3$). Means with different letters are significantly different ($P < 0.05$). (**C**) The kinetics of silencing establishment are disrupted in the *asf1* and *htb-K123R* double mutant. Isogenic cultures of the indicated strains were grown in 5 mM NAM to de-repress *hml::GFP::PEST::NLS*. NAM was removed by washing prior to measuring the establishment of silencing. The mean fluorescence intensity in relative fluorescence units was plotted against time for the four cultures; intensity is shown as the mean ± SEM ($n = 3$).

two factors exert a combined effect on nucleosome formation at silenced mating loci. We first tested the chromatin accessibility in WT and mutant strains. Intriguingly we found that chromatin extracted from *asf1*Δ mutant was more vulnerable to MNase digestion than that from *asf1*Δ *htb-K123R* (Figure 5A), while chromatin from *htb-K123R* was more resistant to MNase (Figure 5A). To further assess the regional digestion pattern of MNase, we conducted a primer-walking experiment with a series of 17 overlapping primers walking through the E silencer and 22 primers walking through the *α1–α2* ORF of *HML* (Figure 5B). DNA encompassed within a mono-nucleosome is protected from digestion and can be detected by PCR. Consistent with the results from MNase digestion experiments, nucleosome occupancy was reduced dramatically in *asf1*Δ cells at *HML* locus and diminished to a similar level in *htb-K123R* and double mutant cells (Figure 5B). These results suggest complicated interactions between H2Bub and Asf1 in regulating nucleosome stability at *HML* loci and also imply that nucleosome formation is not the only factor through which H2Bub and Asf1 may promote *HML* silencing.

Physical spreading of SIR proteins plays a key role in the formation of silenced chromatin (9,12) and heterochromatin stabilization relies on persistent interactions between the SIR complex and nucleosomes (32,56). Thus, we next asked whether these two chromatin factors may affect the recruitment of SIR proteins at *HML*. Chromatin occupancy of Sir2 and Sir3 at *HML* loci were monitored by chromatin immunoprecipitation (ChIP). Interestingly, we found that Sir2 was diminished in *ASF1*-deletion mutants (*asf1*Δ and *asf1*Δ *htb-K123R*) while Sir2 binding remained relatively high in *htb-K123R* mutants, sometimes even exceeding WT levels (Figure 5C). In contrast, Sir3 binding was enriched at *HML* loci in WT, but decreased in *asf1*Δ and *htb-K123R* mutants, with the lowest binding observed in the double mutant cells (Figure 5C). Taken together, these results indicated that H2Bub and Asf1 contribute to *HML* silencing through a collaborative action in regulating Sir2 and Sir3 recruitment and/or binding.

H2Bub and Asf1 are required for the formation of the higher-order structure at silent mating loci

It was previously proposed that *HML* and *HMR* form a silenced superstructure containing tightly packed nucleosomes, with the promoter buried in a secondary loop that prevents RNA polymerase access *in vivo* and antibody access *in vitro* (16). The long-range interaction between *HML* and *HMR* is known to depend on silencing proteins Sir2, Sir3 and Sir4 (7). Thus, based on our preceding observations (Figures 4 and 5), we speculated that this higher-order structure is not formed in the double mutant. If this hypothesis is correct, *HML* would be expected to interact with surrounding chromatin areas in the absence of H2Bub and Asf1. We tested this prediction by performing 3C assays (50). We found that in WT cells, the E silencer did not interact with chromatin in the *HML* region (primer A with primer D in Figure 6A), but did exhibit low levels of interaction with regions outside of the *HML* region (primers A–C, A–E and A–F in Figure 6A). In contrast, the E silencer did interact with the *HML* region in double mutant cells (primers A–D) and the interactions with the surrounding areas were enhanced (primers A–C and A–F in Figure 6A).

Next, we were curious whether the differences in higher order structuring may affect chromatin accessibility in the *HML* region. We tested this by adjusting the concentration of MNase, such that equal digestion of DNA from WT and double mutant cells was observed, thereby preserving nucleosome positioning in the genomes of both strains (Figure 6B). ChIP was then performed on cell lysates to detect the accessibility of core histone subunits (H3) at the *HML* loci in different strains. We found that histone H3 molecules around the *α1–α2* region within the *HML* were more amenable to ChIP than those at the E silencer (primers E1–7) (primer positions indicated in Figure 6C). In addition, the epitopes of Histone H3 were more accessible to the anti-H3 antibody in double mutant cells (Δ H3 IP between α1/α2 and E element, $P < 0.01$). Taken together, these results suggest that the formation of the higher-order superstructure at *HML* is also affected in the absence of H2Bub and Asf1, as evidenced by increased regional interactions *in vivo*.

DISCUSSION

Heterochromatin contributes to eukaryotic chromosome segregation fidelity and genome stability by serving as a heritable form of gene repression. Furthermore, formation of heterochromatin at yeast silent mating loci (*HML* and *HMR*) is required for robust mating ability. In this study, we have identified a specific genetic interaction between two highly conserved chromatin factors: ubiquitylated histone H2B and the histone chaperone, Asf1. We found that depletion of H2Bub or Asf1 alone does not affect the mating phenotype. However, defect in mating phenotype can be found in *asf1*Δ *htb-K123R* mutants (Figure 2A). This finding seems to be quite different from that in telomeric silencing, which suggests a de-repression in telomeric silencing by *asf1*-deletion and to a lesser extent in *htb-K123R* (Figure 1) (29,46,47). We demonstrated that these two chromatin factors coordinately affect nucleosome assembly and SIR protein recruitment that contribute to heterochromatin repression at yeast silent mating loci, *HML* (Figure 3 and Supplementary Table S5). We monitored heterochromatin establishment in real time using a GFP reporter system [invented by Osborne *et al.* (48)], through which we observed that transcriptional repression at *HML* shows attenuated initiation and never becomes fully established in the absence of both H2Bub and Asf1 (Figure 4A–C). We proceeded to show that the recruitment of general repressors, Sir2 and Sir3, to *HML* E/I silencers and subsequent spreading into α1/α2 genes is abolished in the double mutant (Figure 4D). Finally, we found that nucleosome occupancy is diminished and higher-order heterochromatin organization formation is incomplete in the absence of H2Bub and Asf1 (Figures 5 and 6). Taken together, our results identify a previously unappreciated role of H2Bub and Asf1 in the regulation of heterochromatin formation and maintenance at silenced mating loci.

Pioneering work from two independent studies led to the conclusion that cell cycle progression between early S and

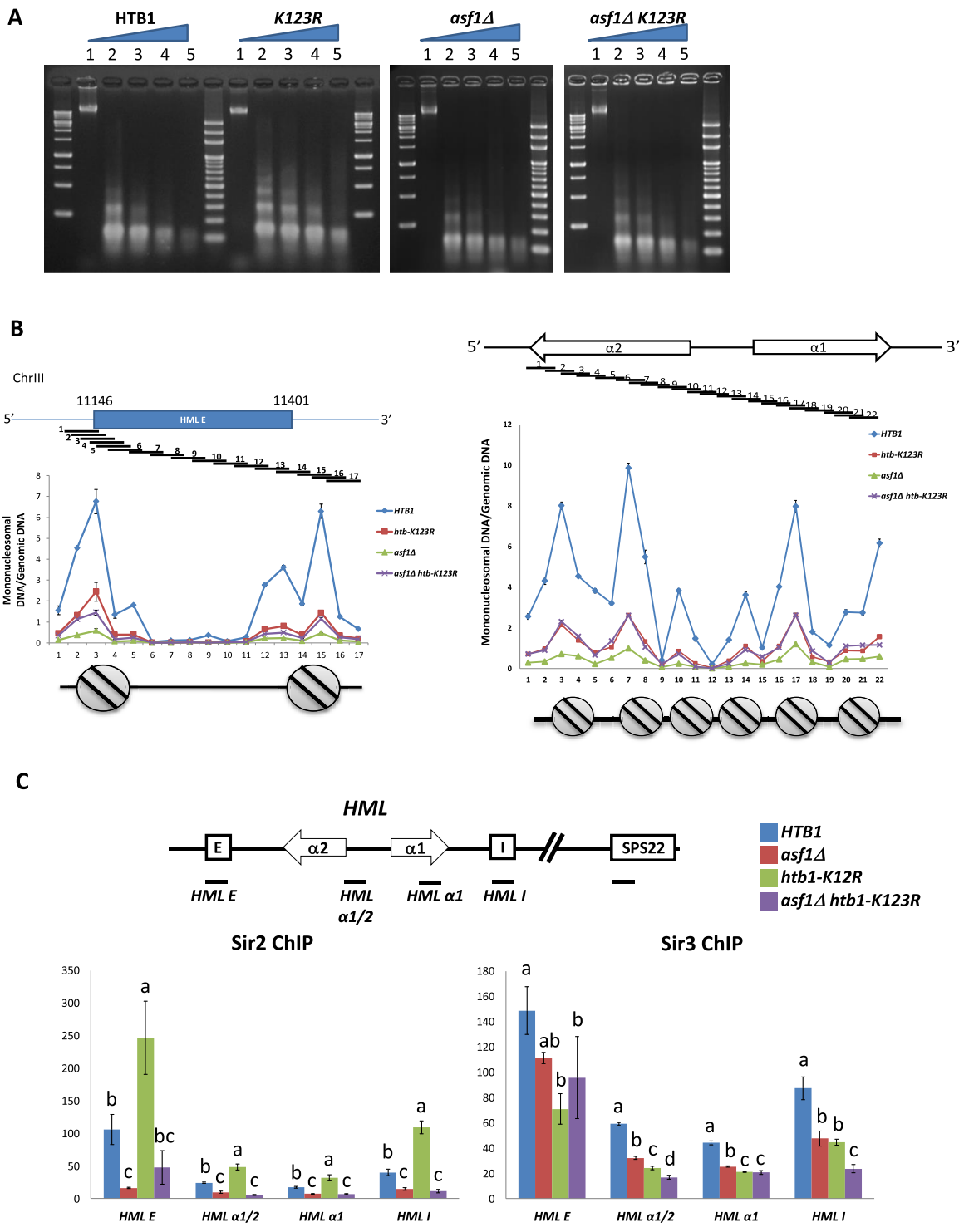

Figure 5. Asf1 and H2Bub fine-tunes nucleosome occupancy and Sir protein recruitments at silenced mating loci. (**A**) Agarose gel image of chromatin DNA following micrococcal nuclease (MNase) digestion. Chromatin extracted from each strain was treated with different doses of MNase (0.01, 0.25, 0.5 and 0.1 U MNase/OD unit of cells) for 15 min at 37°C. MNase-undigested (0.5 OD unit) and -digested chromatin DNA (2.5 OD unit) were loaded onto a 2% agarose gel. (**B**) Nucleosome occupancy at the *HML* locus in different strains. Schematic of the primer pairs against the indicated positions of the *HML* locus. Nucleosomal DNA enrichment (normalized to signals derived from undigested genomic DNA) is shown at the indicated positions along the *HML* locus in each strains after treated with MNase (0.5 U MNase/OD unit of cells) for 15 min at 37°C. Data are shown as the mean ± SEM ($n = 3$). (**C**) The recruitment of silent information regulatory (SIR) proteins (Sir2 and Sir3) is decreased in *asf1Δ* and *htb-K123R* mutant cells. Top: schematic of the relative locations of the primer pairs used. Primers against *SPS22* were used for normalization. Bottom: chromatin immunoprecipitation (ChIP) was performed using anti-Sir2 antibody (Ab) or anti-myc Ab (myc-tagged Sir3) to detect the relative occupancy of Sir2 or Sir3, respectively, on chromatin in isogenic WT (HTB1), *asf1*Δ, *htb-K123R* and *asf1*Δ *htb-K123R* cells. Data are shown as the mean ± SEM ($n = 3$). Means with different letters are significantly different ($P < 0.05$).

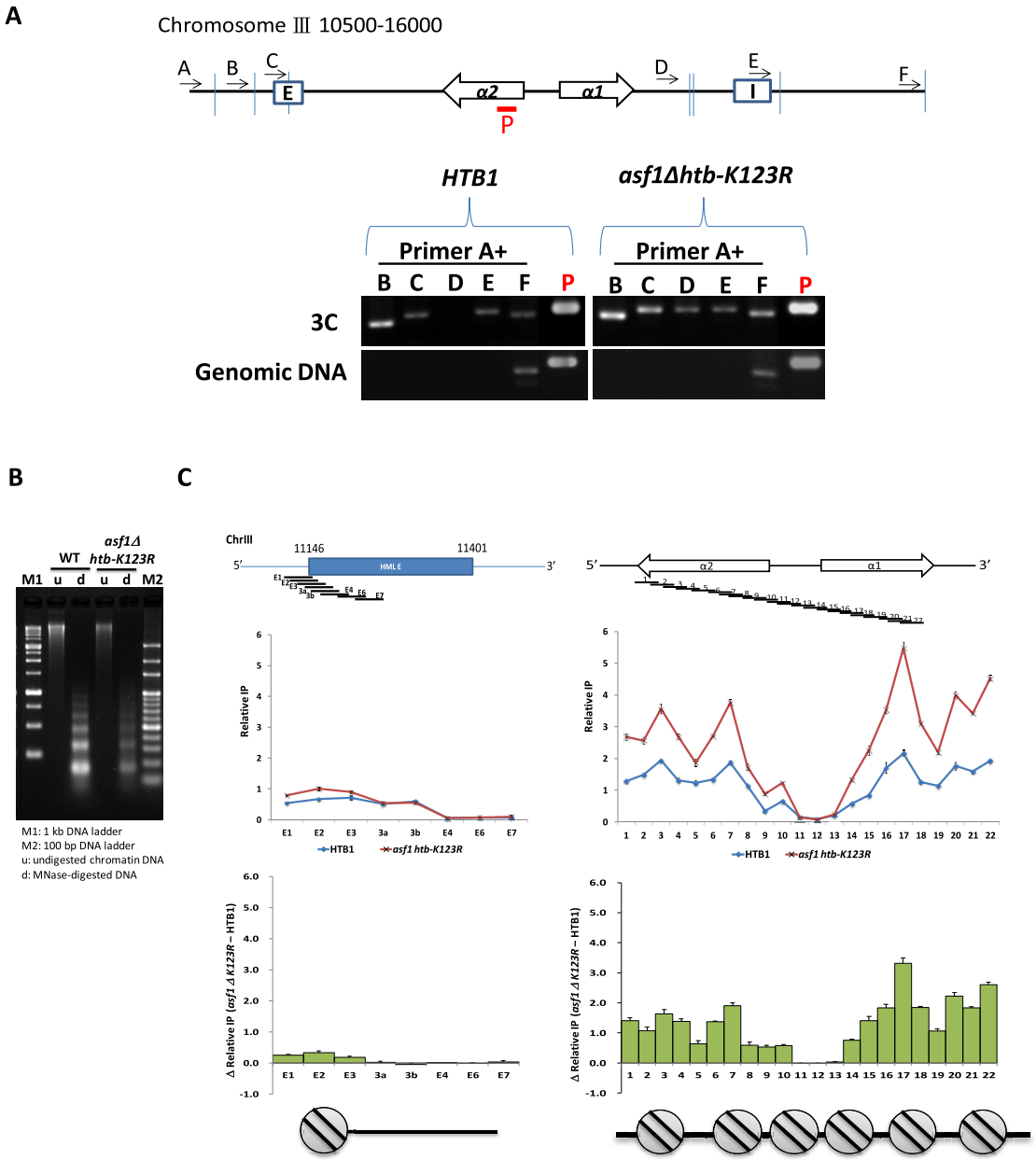

Figure 6. H2Bub and Asf1 are critical for higher-order heterochromatin organization at silenced mating loci. (**A**) Chromosome conformation capture (3C) assay reveals a more flexible chromatin structure in *asf1Δ* and *htb-K123R* double mutants as compared to WT. Schematic of the primers used in 3C assay. Log phase cells were fixed with 3% formaldehyde at room temperature for 20 min and then digested with the Sau3A restriction enzyme. The linear range for the quantitative PCR reactions was determined by titrating the cross-linked and control templates after intra-molecular ligation and cross-link reversal. The products were separated on agarose gels; primers at site P were used as a positive control. (**B**) Agarose gel image of WT (*HTB1*) and *asf1Δ htb-K123R* chromatin DNA prior to chromatin immunoprecipitation. Chromatin was cross-linked for 15 min and treated with 0.5 or 0.25 U MNase/OD (WT and *asf1Δ htb-K123R* cells, respectively) for 10 min at 37°C. Sheared chromatin DNA fragments were then visualized on a 2% agarose gel. (**C**) Nucleosome accessibility at the *HML* locus is increased in double mutants. ChIP was performed using an antibody against the H3 C-terminus to detect the relative occupancy of histone H3 across the *HML* locus in WT (*HTB1*) and *asf1Δ htb-K123R* cells. Primers against telomere (VI-R) were used for normalization. Data are shown as the mean ± SEM ($n = 3$). The differences between nucleosome DNA of WT and *asf1Δ htb-K123R* cells at each position are shown as Δ relative IP.

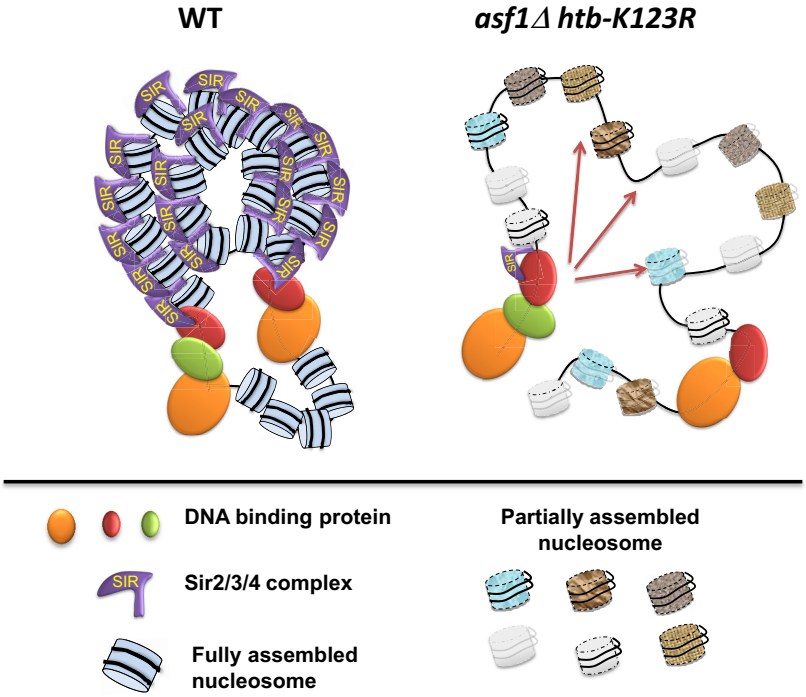

Figure 7. A proposed model. Anti-silencing function 1 (Asf1) and histone H2B ubiquitylation (H2Bub) collaboratively maintain transcriptional silencing at the *HML* locus by fine-tuning nucleosome assembly and Sir protein recruitment, both of which are critical for heterochromatin maintenance. The recruitment of general repressors Sir2 and Sir3 to *HML* E/I silencers and subsequent spread into α1/α2 genes is significantly reduced in double mutants. In addition, the loss of nucleosomes at *HML*, which in turn affects higher-order heterochromatin organization, is pronounced in the absence of H2Bub and Asf1.

M phase, but not DNA replication per se, is necessary for the establishment of silencing (57,58). These studies thus indicated a requirement for the assembly of repressive chromatin after the cell cycle, however, the precise mechanisms that regulate assembly have not been fully described. Both Asf1 and H2Bub have been shown to play roles in nucleosome formation and/or silencing. Asf1 interacts with Hir1 and together these two proteins promote *HM* loci silencing in a process that involves proliferating cell nuclear antigen (25). The *Drosophila* homolog of yeast Asf1 was previously co-purified with acetylated histone H3 and H4, and this homolog was found to stimulate the nucleosome assembly activity of CAF-1 *in vitro* (17). Yeast CAF-1 has been proposed to mediate *HM* loci silencing by contributing to the early stages of formation and subsequent maintenance of heterochromatin (26,59). Moreover, Asf1-Rtt109-mediated H3K56 acetylation, a modification that is associated with S phase, promotes efficient replication-coupled nucleosome assembly (20,21,60). Bre1, the E2 ubiquitin ligase required for the ubiquitylation of histone H2B, is recruited to replication forks (44) where it may promote H2Bub-mediated nucleosome formation. On the other hand, H2Bub also mediates nucleosome reassembly during gene transcription (41–43). Therefore, Bre1 may be expected to cooperate with Asf1 at the level of nucleosome assembly, during heterochromatin formation. To our surprise, the observations in this study suggest that Asf1 promotes nucleosome assembly at *HML* (Figure 5A and B), while H2Bub facilitates the decompaction of silenced chromatin (Figure 5A and B), which is similar to its role in facilitating chromatin relaxation during DNA repair (61–63). Thus, lack of nucleosomes can only partially explain this defective phenotype in double mutants (Figure 2A).

The establishment of repressed chromatin requires appropriate nucleosome assembly (19,25,26), the appearance of specific histone marks (8,32–35,64) and the physical binding and spreading of SIR proteins (9,12). Our study argues that Bre1-H2Bub and Asf1 may not only coordinate in controlling nucleosome assembly, but also fine-tune histone marks and SIR protein (Sir2 and Sir3) recruitment at the *HML* locus (Figure 5). Sir2 deacetylates histone tails of adjacent nucleosomes and this is important for spreading of silencing (31,65). The depletion of *ASF1* leads to a reduction of nucleosomes at *HML* loci and also diminished histone acetylation (H3K56 acetylation), which correspond to impaired recruitment of Sir2 (Figure 5B and C, *asf1*Δ and *asf1*Δ *htb-K123R*). In contrast, the loss of H2Bub (*htb-K123R* mutants) creates a more condensed chromatin environment which may favor Sir protein binding and enhances the stabilization of Sir2 at *HML* (Figure 5B and C, *htb-K123R*). Sir3 binds to the N-tail of histones (66) and may be hindered by H3K4me and H3K79me. Thus, loss of H2Bub may decrease the downstream methylation signals, which act as boundary histone marks to concentrate Sir proteins at their action sites (35–37). Furthermore, the loss of H2Bub-mediated demarcation would lead to promiscuous binding of Sir3 and the reduction of Sir3 occupancy specifically in heterochromatin (*htb-K123R* and *asf1*Δ *htb-K123R* in Figure 5C). Continuous presence of Sir proteins is required for chromatin silencing (12,67–69). Thus, in cells lacking both

Asf1 and H2Bub, both Sir2 and Sir3 recruitment are reduced at *HM* loci (Figure 5C), leading to a de-repression of *HML* gene (Figure 3). The diminished binding of Sir2 and Sir3 at the E/I silencer in double mutants further disrupts the silenced superstructure of *HML* (Figures 5C and 6). In summary, the two chromatin factors, Asf1 and H2Bub, appear to play important, distinct roles in regulating the stoichiometric assembly of Sir2 and Sir3 proteins at silenced mating loci.

Thus, for the first time, we identify that the histone marker, H2Bub, cooperates with Asf1 histone chaperone to establish and stabilize facultative heterochromatin. We propose a model (Figure 7) in which H2Bub and Asf1 are required to fine-tune nucleosome positioning and the recruitment of SIR complex at silenced mating loci. Our results also strongly support the existence of specialized chromatin organization at the silenced mating type loci (16), and imply that H2Bub and Asf1 plays important roles in maintaining higher-order chromatin structures at *HML* loci.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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