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Positive Charge of Arginine Residues on Histone H4 Tail Is Required for Maintenance of Mating Type in *Saccharomyces cerevisiae*

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Transcriptional gene silencing is regulated by the chromatin structure, which is by various factors including histones. *Saccharomyces cerevisiae* contains transcriptionally silenced regions such as telomeric regions and hidden mating (HM) loci. The positively-charged amino acids on the histone H4 tail were reported to be critical for the telomeric silencing in yeast, by interacting with Dot1, a specific methyltransferase for the 79th lysine on histone H3. However, Dot1 did not affect gene silencing within HM loci, but whether the positively-charged amino acids on the H4 tail affect HM silencing has not been defined. To elucidate the function of the H4 tail on HM silencing, we created several *MAT*a-type yeast strains bearing the substitution of arginine with alanine or lysine on the histone H4 tail and checked the sensitivity of *MAT*a-type yeast to alpha pheromone. The arginine point mutants substituted by alanine (R17A, R19A, and R23A) did not show sensitivity to alpha pheromone, but only two arginine mutants substituted by lysine (R17K and R19K) restored the sensitivity to alpha pheromone-like wild type. These data suggested that the basic property of arginine at 17th and 19th positions in the histone H4 tail is critical for maintaining HM silencing, but that of the 23rd arginine is not. Our data implicated that the positive charge of two arginine residues on the histone H4 tail is required for HM silencing in a manner independent of Dot1.

Keywords: *Saccharomyces cerevisiae*, arginine residues, histone H4, HM silencing

In eukaryotic cells, gene expression is regulated by the structure of chromatin. The chromatin is composed of a nucleosome, which is a histone octamer wrapped by 147 bp of DNA, and its structure is regulated by various factors including dynamic modifications of histones [1, 2]. The histone modifications such as acetylation, methylation, and phosphorylation are implemented by several histone-modifying enzymes, and affect the interaction between histone proteins and DNA molecules, and subsequently change the chromatin structure [3].

The chromatin is generally divided into euchromatin and heterochromatin depending on the DNA packaged level. Unlike euchromatin, in which DNA is packaged at a relatively low level, heterochromatin is packaged at a very high level of DNA, making it difficult to access proteins necessary for the transcription, thereby suppressing gene expression. Regulation of gene expression within heterochromatin and euchromatin is critical to maintaining gene stability [4].

Saccharomyces cerevisiae, budding yeast, is known to have no heterochromatic region. However, the telomeric regions and hidden mating (HM) loci in *S. cerevisiae* could be considered to be heterochromatic regions in that DNA is packaged at a high level and the expression of genes within these regions is suppressed. There are two mating types, *MAT*a and *MAT*α, of haploid *S. cerevisiae* and two different haploid *S. cerevisiae* can create a diploid by mating together. Both haploid yeast strains contain both genes for

the mating type. However, the mating type is determined according to the gene in the *MAT* locus regardless of both genes within HM loci, which are always silenced. Therefore, the maintenance of gene silencing within HM loci is critical to determine the yeast mating type. When *MAT*a haploid *S. cerevisiae* meets a *MAT*α haploid cell, the *MAT*a haploid cell arrests its growth for the preparation of mating. That means that the growth of *MAT*a haploid yeast strains is inhibited by the alpha factor which *MAT*α haploid yeast strains secrete [5, 6].

Unlike eukaryotes including mammals and fission yeast *Schizosaccharomyces pombe*, *S. cerevisiae* mediates gene silencing through the Silent Information Regulator (SIR) complex. In the regions of HM loci, telomere, and rDNA repeats, which are known to be the transcriptionally silenced regions in *S. cerevisiae*, gene expression is suppressed by the SIR complex, although there are some differences in the associated proteins [7]. In addition, H3K9 methylation is prevalent in the heterochromatin region of most eukaryotes including *S. pombe*, but this common histone modification does not appear in *S. cerevisiae* [8].

Many studies have been performed to elucidate gene silencing in *S. cerevisiae*. Previous studies have reported that Dot1, a methyltransferase specific for H3K79, regulates telomeric silencing and HM silencing [9, 10] and another research has shown that Dot1 controls telomeric silencing but not HM silencing [11]. Briggs group found that the basic patch of histone H4 tail regulates telomeric gene silencing through interaction with Dot1 [12]. In this study, to identify whether these factors are also important for the regulation of HM silencing and have the same function as in telomere, we performed some experiments.

In a previous study performing the mating assay with mutant strains deleting some regions of N-terminal tail in histone H4, a significant decrease of mating ability was observed in the strains with removed N-terminal tails on histone H4. These results suggest that the N-terminal tail of H4 is required for suppressing gene expression in HM locus [13]. The 16th lysine residue in histone H4 was considered to be required for maintaining gene silencing in an HM locus because the substitution of the lysine-16 within the N-terminal tail of histone H4 (H4K16) to alanine caused de-repression of silenced genes within HM loci [14]. Based on these studies, we further confirmed the association of the N-terminal tail of histone H4 with maintaining the gene silencing in HM loci using the properties of haploid *S. cerevisiae*. First, we performed disc assay using two histone H4 mutant strains (H4Δ*1-23* and H4Δ*4-23*) obtained by removing 1~23 or 4~23 residues from the tail of histone H4 (Fig. 1A). After plating 100 μl of cells diluted into 10^6 cells/ml on the YPD plates, three sterilized paper discs were placed on three points on the plate. 5 μl alpha factor of three different concentrations (1, 0.1, and 0.01 μg/μl, respectively) were spotted onto the paper discs, and the plates were incubated at 30°C for 3 days. H4K16A mutant strain was used as a negative control, and wild type was used as a positive control. The acetylated H4K16 is a substrate of Sir2, a histone deacetylase, and the removal of an acetyl group from acetylated H4K16 by Sir2 is critical in heterochromatin spreading and maintenance of gene silencing [15, 16]. However, the H4K16A mutant strains have lost their acetylated site due to the switch from lysine to alanine. While wild-type yeast strain has clear areas around the discs containing alpha factor, H4Δ*1-23* and H4Δ*4-23* do not show clear zones around the discs containing alpha factor like H4K16A mutant strain, which does not maintain HM silencing (Fig. 1A). To identify residues in histone H4 tail affecting HM silencing, we observed loss of HM silencing in the strains containing variously substituted residues of histone H4. We focused on arginine residues including Arg-17, Arg-19, and Arg-23, which may be competent for histone modifications. Arg-17 and Arg-19 of histone H4 tail were reported to influence telomeric silencing as parts of the basic patch on the histone H4 tail [12]. As a result, it was confirmed that HM silencing was not maintained in mutants containing substituted Arg-17, Arg-19, or Arg-23 with alanine of histone H4 (Figs. 1B and 1C). Based on our results, we confirmed that two arginine residues (Arg-17 and Arg-19) located in the histone H4 tail are critical to maintaining HM silencing as well as telomeric silencing. In addition, our data showed that Arg-23 on the histone H4 tail is required for the maintenance of HM silencing (Figs. 1B and 1C).

A previous study verified that the change of positive charge in basic patch residues of histone H4 tail resulted in the loss of telomeric silencing [12]. To determine whether these changes in charge also affect HM silencing, we substituted arginine of H4R17, H4R19, and H4R23 into lysine. As a result, in contrast to H4R17A or H4R19A substitution, substitution of H4R17 or R19 with lysine leads to recovery of HM silencing in both disc assay and serial dilution assay. Surprisingly, H4R23K mutant didn't recover HM silencing in both disc assay and serial-dilution assay (Figs. 2A and 2B). Also, the substituted mutant of Arg-23 with Alanine (R23A mutant) formed a partly clear zone around the paper disc containing alpha factor compared with R17A or R19A mutants (Fig. 1B). This data suggested that Arg-23 had an important function for the maintenance

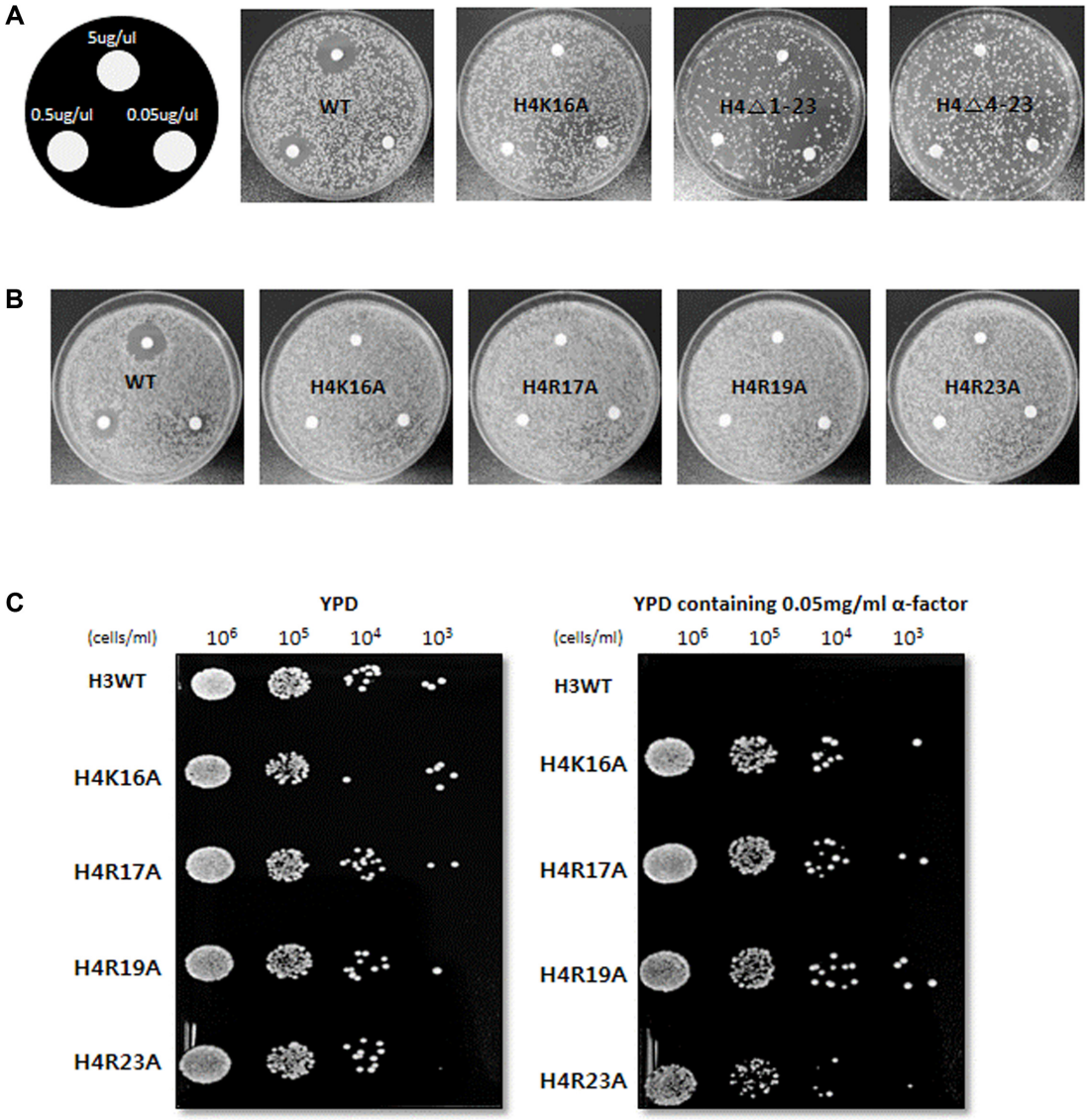

Fig. 1. The arginine residues of the histone H4 tail are important for the maintenance of HM silencing.
(**A**) Deletion of N-terminal tail of histone H4 leads to loss of HM silencing. Disc assay was performed with mutant strains deleting 1-23 or 4-23 residues of histone H4 tail. After plating 100 μl of cells with a concentration of 10^6 cells/ml on the YPD plates, three sterilized paper discs were placed on three points on the plate. 5 μl alpha factors of three different concentrations, 1, 0.1, and 0.01 μg/μl, were spotted onto the paper discs and the plates were incubated in the 30°C incubator for 3 days. *MAT*a-type cells stop cell cycles to prepare for mating and form round-shaped clear zones depending on the concentration of alpha factor. If a mutation causes loss of HM silencing, however, *MAT*a-type cells can grow irrespective of alpha factor. While wild-type cells, used as a positive control, maintain HM silencing and form a clear zone around the disc containing alpha factor, H4K16A mutant cells, used as a negative control, grow irrespective of alpha factor. In mutant strains deleting 1-23 or 4-23 residues of histone H4 tail, clear zone disappeared and it implicates loss of HM silencing. (**B**) Substitutions of arginine residues on histone H4 tail with alanine cause defects in HM silencing. As described in Fig. 1A, disc assay was performed with mutant strains substituting Lys-16, Arg-17, Arg-19, or Arg-23 of N-terminal tail in histone H4 with alanine. Contrary to WT showing clear zone around the disc containing alpha factor representing HM silencing, clear zone disappeared in K16A, R17A, R19A, and R23A substitution mutants and it implicates loss of HM silencing. The concentration of alpha factor is same in (**A**). (**C**) HM silencing defects resulting from substitutions of positively-charged arginine residues of N-terminal tail of histone H4 were reproducibly observed in serial dilution assay. Serial dilution assay was performed with strains containing substitutions of H4R17, H4R19, and H4R23 with alanine to confirm HM silencing defects observed in Fig. 1B. 5 ul of serially diluted (10^6–10^3 cells/ml) each strain was spotted onto YPD plate containing 0.05 mg/ml α-factor and were incubated in 30°C for 3 days. Each substituted mutant strain forms colonies on the plate containing alpha factor.

of HM silencing by the different mechanism from that of Arg-17 and Arg-19.

Taken together, we found that well-maintained positive charge in several residues is crucial for HM silencing maintenance, like telomeric silencing, and other unknown factors in addition to a charge are also required. For the maintenance of HM silencing, SIR complex should be well-recruited in HM loci [7]. Because Sir2 is a subunit of SIR complex, Sir2-ChIP followed by subsequent quantitative-PCR targeting *HML* in ChromosomeIII was performed in these point-mutated strains to confirm the distribution of Sir2 of SIR complex (Fig. 3A). Although H4R17A and H4R19A strains showing defective HM silencing had a low level of Sir2-binding, H4R17K and H4R19K strains showing HM silencing recovery had a significant binding level of Sir2 comparable to wild type (Fig. 3B). We confirmed

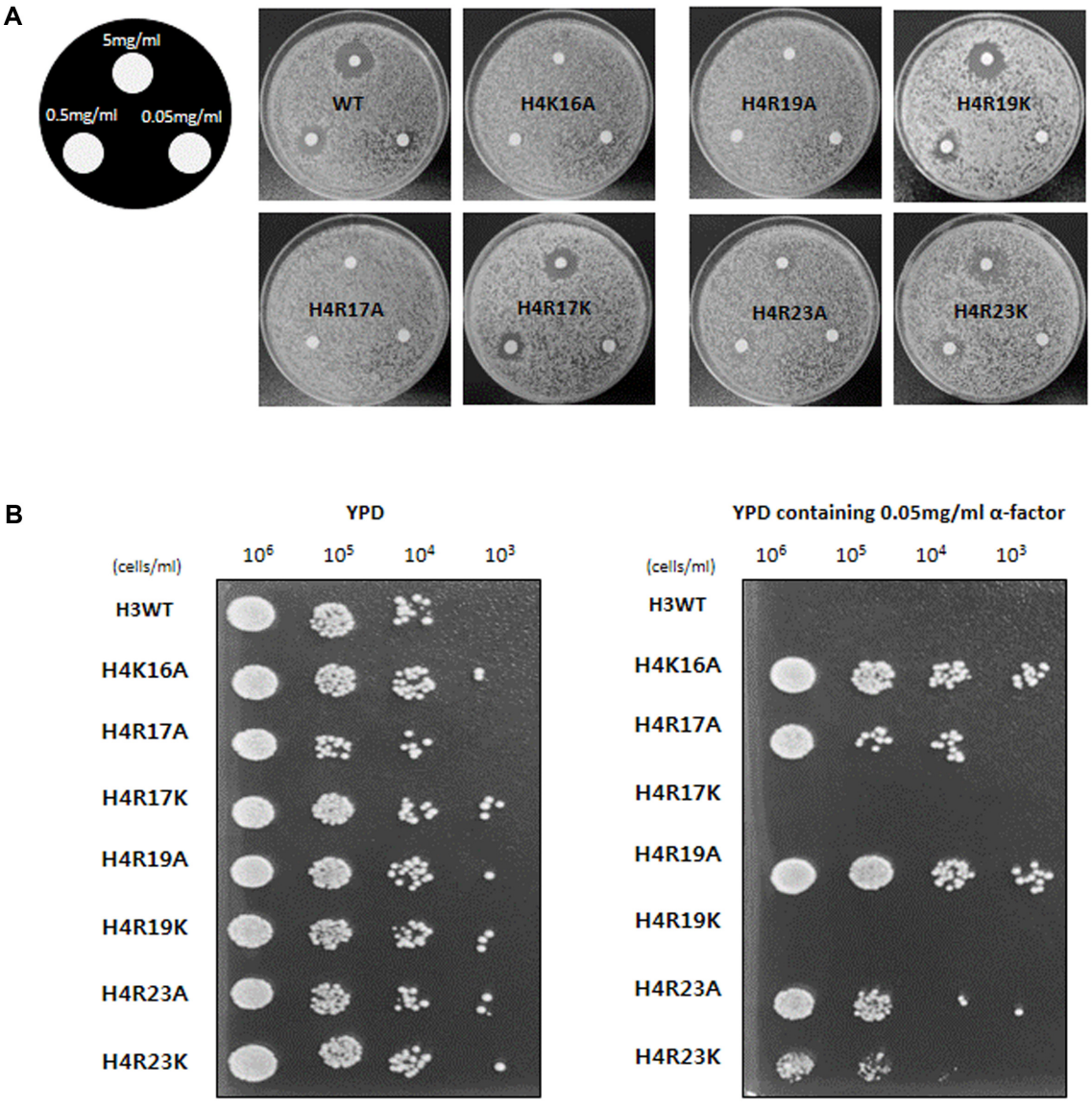

Fig. 2. The charge of some arginine residues on histone H4 is critical for the maintenance of HM silencing.
(**A**) Conservation of charge through substitutions of H4R17, H4R19 with lysine in place of alanine enables the maintenance of HM silencing. As described in Fig. 1A, we implemented disc assay to identify whether substitutions of H4R17, H4R19, and H4R23 with lysine, positively-charged amino acid, can complement HM silencing defects observed in alanine substitutions. (**B**) Serial dilution assay was performed as described in Figure1C to identify if conservation of charge through substitutions of H4K16, H4R17, H4R19, and H4R23 with lysine in place of alanine enables maintenance of HM silencing reproducibly, like disk assay shown in Fig. 2A.

through ChIP-q-PCR data that the maintenance of a positive charge in arginine residues of the histone H4 tail is crucial for maintaining HM silencing. On the basis of the previous study concerning Dot1, which is not required for HM silencing, we compared the Sir2-binding level of Dot1-deleted strain with that of wild-type strain and confirmed that the Sir2's abundance within *HML* region has no detectable changes (Fig. 3C). Therefore, we concluded that the basic patch of histone H4 tail regulates telomeric silencing through interaction with Dot1, but Dot1 is not critical for HM silencing.

Gene silencing in telomere, one of the silenced regions in budding yeast, is regulated by both SIR complex and the interaction between basic patch residues in histone H4 and histone H3K79 methyltransferase Dot1. To identify if these factors also have crucial functions for HM silencing maintenance, we carried out several experiments. The basic patch in histone H4 was critical for telomeric silencing, and some arginine residues competent for histone modifications were critical for HM silencing as well as telomeric

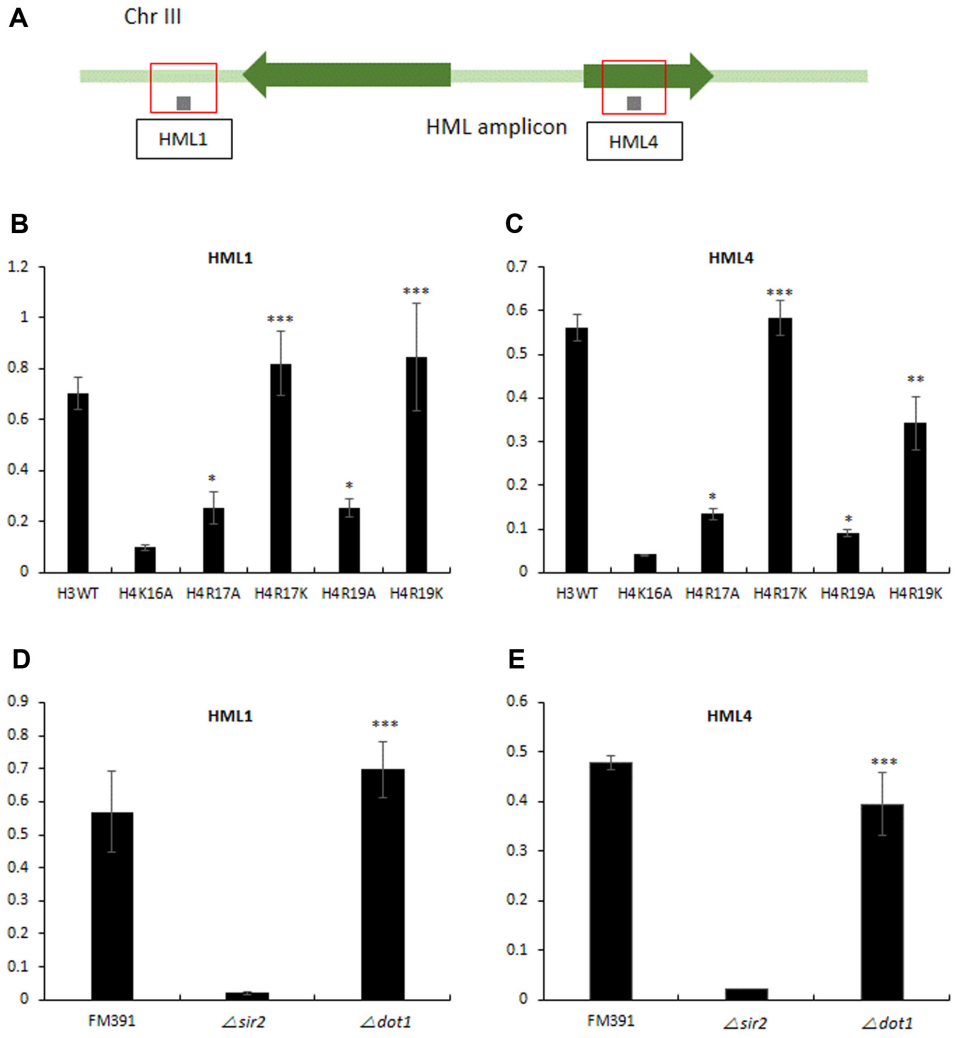

Fig. 3. The recruitment of Sir2 onto HM loci depends on positive charge in the N-terminal tail of histone H4 with Dot1-independent manner.

(**A**) Schematic representation of the chromatin immunoprecipitation (ChIP) PCR probes targeting HML1 and HML4 of HML region localized in chromosomeIII. HML1 and HML4 are pointed out by the lined box. (**B** and **C**) ChIP analysis of HML1 and HML4 using anti-Sir2 antibody was carried out with mutant strains substituting arginine of N-terminal tail in histone H4 with alanine or positively-charged lysine. Quantification of data from three independent experiments normalized to input using the same chromatin. (**D** and **E**) ChIP analysis of HML1 and HML4 using anti-Sir2 antibody was carried out with Dot1-deleted strain. Quantification of data from three independent experiments normalized to input using the same chromatin (*$p < 0.001$, **$p = 0.05$, ***$p > 0.2$).

silencing. Through substitutions of three arginine residues including R17, R19 and R23 of histone H4 tail into alanine or lysine and subsequent disc assay and serial dilution assay in the plate containing alpha-factor, we found out that what maintains arginine residues in histone H4 tail with its original charge is important for HM silencing maintenance.

However, not only H4R23A but H4R23K also lost its capability to repress HM loci. It may suggest other intrinsic functions of H4R23, such as the putative target of various histone modifications or structurally crucial intrinsic property. The arginine residue of histone has been reported to be involved in the regulation of chromatin structure and its subsequent transcription [17]. The loss of the asymmetrical dimethylation of H4R3 by PRMT1 within insulator region caused the decreased barrier function of chromatin [18, 19]. However, the symmetric methylation by PRMT5 is required for the recruitment of DNMT3A, DNA methyltransferase, within the promoter region and the subsequent repression of gene expression [20, 21]. These studies suggested that the function of arginine methylation is dependent on its methyltransferase and methylation pattern [22, 23]. So, the strategy of how H4R23 contributes to HM silencing remains to be determined.

Contrary to telomeric silencing, which is affected by the interaction between Dot1 H3K79 methyltransferase and the basic patch of N-terminal in histone H4, loss of Dot1 didn't influence the recruitment of Sir2 into several HM loci. Therefore, it may be suggested that Dot1 is dispensable for the recruitment of SIR complex into several HM loci. However, by serial dilution assay, we found that Dot1 contributes to HM silencing to some extent in a different manner from recognized mechanisms (Fig. S1). It suggests a divergence in silencing mechanisms between telomere and HM loci in budding yeast. Other intrinsic functions of the basic patch in histone H4, except for the role of a docking site for Dot1, appearing in telomeric silencing, may get involved specifically for HM silencing. Also, our data implicate Dot1's role in HM silencing maintenance, independently of conventional mechanisms, as well as its appearing in telomeric silencing, and regarding Dot1's interaction with H4 tail, followed by enhancement of Sir complex recruitment.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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