Spreading and epigenetic inheritance of heterochromatin require a critical density of histone H3 lysine 9 tri-methylation

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Heterochromatin assembly requires methylation of histone H3 lysine 9 (H3K9me) and serves as a paradigm for understanding the importance of histone modifications in epigenetic genome control. Heterochromatin is nucleated at specific genomic sites and spreads across extended chromosomal domains to promote gene silencing. Moreover, heterochromatic structures can be epigenetically inherited in a self-templating manner, which is critical for stable gene repression. The spreading and inheritance of heterochromatin are believed to be dependent on preexisting H3K9 tri-methylation (H3K9me3), which is recognized by the histone methyltransferase Clr4/Suv39h via its chromodomain, to promote further deposition of H3K9me. However, the process involving the coupling of the “read” and “write” capabilities of histone methyltransferases is poorly understood. From an unbiased genetic screen, we characterize a dominant-negative mutation in histone H3 (H3G13D) that impairs the propagation of endogenous and ectopic heterochromatin domains in the fission yeast genome. H3G13D blocks methylation of H3K9 by the Clr4/Suv39h methyltransferase and acts in a dosage-dependent manner to interfere with the spreading and maintenance of heterochromatin. Our analyses show that the incorporation of unmethylated histone H3G13D into chromatin decreases H3K9me3 density and thereby compromises the read-write capability of Clr4/Suv39h. Consistently, enhancing the affinity of Clr4/Suv39h for methylated H3K9 is sufficient to overcome the defects in heterochromatin assembly caused by H3G13D. Our work directly implicates methylated histones in the transmission of epigenetic memory and shows that a critical density threshold of H3K9me3 is required to promote epigenetic inheritance of heterochromatin through the read-write mechanism.

heterochromatin | epigenetic | histone methylation | gene silencing

The assembly of distinct chromatin domains within eukaryotic genomes facilitates diverse chromosomal processes, including the maintenance of genome stability, and the regulation of gene expression, DNA replication and recombination. Histones and their post-translational modifications are components of epigenetic mechanisms that partition the genome into “open” euchromatin or “closed” heterochromatin domains (1–3). Euchromatin domains, marked by histone acetylation and histone H3 lysine 4 methylation (H3K4me), are generally more accessible to transcription machinery, whereas repressive heterochromatin domains are hypoacetylated and contain the histone H3 lysine 9 methylation (H3K9me) mark (4, 5). Histone H3 proteins bearing di- and tri-methylation marks (H3K9me2/3) are recognized by heterochromatin protein 1 (HP1) family proteins (6–8), which together serve as a recruitment scaffold for multiple effectors (2). Considering that histone modifications define gene expression patterns specific to different cell types, determining their role in the assembly and propagation of distinct chromatin domains is critical for understanding normal development and disease states.

The fission yeast Schizosaccharomyces pombe is an excellent model system for studying heterochromatin assembly. Small facultative heterochromatin islands target developmentally and environmentally regulated genes, and large constitutive heterochromatin domains coat pericentromeric, subtelomeric, and the silent mating-type (mat) regions (9–12). Heterochromatin assembly is a multistep process that includes nucleation and spreading. The de novo nucleation of heterochromatin occurs at specific sites, such as repeat elements within constitutive heterochromatin domains, from where heterochromatin factors spread to surrounding sequences (13, 14). RNAi machinery (13, 15), as well as factors involved in nuclear RNA processing and noncanonical RNA polymerase II termination (12, 16–19), nucleate heterochromatin by targeting the multisubunit Clr4 methyltransferase complex (ClrC) (20) that is responsible for mono-, di-, and tri-methylation of histone H3K9 (H3K9me1/2/3) (6, 21). Clr4 has a unique dual activity allowing it to bind to methylated H3K9 via its chromodomain (“read”) and catalyze H3K9 methylation (“write”), a feature that is essential for heterochromatin to spread (22, 23). The H3K9me2/3 marks also provide a binding site for other chromodomain proteins, including the HP1 family members Chp2 and Swi6 (24), which in turn target effectors such as the Snf2-histone deacetylase (HDAC) repressor complex (SHREC) involved in transcriptional gene silencing (25, 26).

The principle that preexisting heterochromatin can be epigenetically inherited in a self-templating manner during both mitosis and meiosis was uncovered through studying heterochromatin...
assembly at the silent mat region in S. pombe (13, 27, 28). Further insights into epigenetic transmission of chromatin structure soon followed. The first major finding established the importance of Clr4 read-write activity in the epigenetic inheritance of heterochromatin (22). This work showed that parental H3K9 methylated nucleosomes and their associated factors are a "molecular bookmark" and provide an epigenetic template for loading Clr4 (via the Clr4 chromodomain) to promote clonal propagation of heterochromatin. Another key finding was that constitutive heterochromatin domains show markedly lower turnover of histones compared to euchromatin (29). The HDAC Clr3 and the homolog of mammalian over preserves epigenetic memory for the inheritance of heterochromatin (29, 30). Moreover, the nuclear peripheral subdomain provides an ideal microenvironment for loading factors that facilitate epigenetic inheritance (31, 32). Despite significant progress, evidence directly linking methylated histones to epigenetic memory and a detailed molecular mechanism underlying heterochromatin inheritance have yet to emerge.

Here, we report that a mutant isolated from our previous genetic screen affects heterochromatin propagation (30). The mutation maps to one of three genes encoding histone H3 and converts glycine residue 13 to aspartic acid in human H3 amino-terminal tail (H3G13D). H3G13D, which cannot be methylated by Clr4, acts in a dominant-negative manner to impair the spreading and epigenetic inheritance of heterochromatin. H3G13D is distinct from the previously described H3K9M dominant mutation that enhances the association between the catalytic SET (SUVA9-3, Enhancer-of-zeste and Trithorax) domain of the methyltransferase and the H3 tail, thereby trapping Clr4 and blocking H3K9me (33). Rather, our work shows that H3G13D reduces the density of the H3K9me3 mark, which is recognized by Clr4 to further promote deposition of H3K9me. We find that enhancing the affinity of Clr4 for H3K9me2 nucleosomes is sufficient to overcome the heterochromatin defects caused by H3G13D. These results demonstrate that epigenetic propagation of heterochromatin relies on a critical density threshold of H3K9me3 to promote Clr4 read-write activity. Our findings may also elucidate how mutations in histone tails, including those linked to cancer (34–39), perturb the chromatin landscape in cells.

Results

EMS32 Harbors a Mutation in hht2 that Affects Heterochromatin Maintenance. The heterochromatin domain comprising the silent mating-type loci mat2P and mat3M and the interval between them (referred to as the K-region) serves as a paradigm for understanding the assembly and maintenance of repressive chromatin. Heterochromatin nucleated at the centromere-homologous cenH element spreads across the ~20 kb region surrounded by IR-L and IR-R inverted repeat elements (5, 13) (Fig. L4). Cells in which the nucleation center cenH is replaced with ura4+ (Ka:ura4+) establish heterochromatin stochastically. Once assembled, the heterochromatic state is stably propagated in cis via a mechanism involving suppression of histone turnover and Clr4 read-write activity (13, 22, 30, 32). The Ka:ura4+ reporter allows the identification of factors whose requirement in heterochromatin propagation is otherwise masked by de novo nucleation mechanisms (22). To map the EMS32 mutation, we backcrossed EMS32 to a non-mutagenized parental strain and performed tetrad analyses in which silencing of the ura4+ reporter was monitored to isolate wild-type (WT) and EMS32 segregates. Whole-genome sequencing and nucleotide variant analysis of three WT and three EMS32 segregates revealed a G-to-A nucleotide substitution unique to EMS32 mutants that mapped to hht2. In S. pombe, the histone H3 protein is encoded by three alleles, hht1+, hht2+, and hht3+ (40). Conventional DNA sequencing confirmed the G-to-A nucleotide conversion in hht2 (Fig. 2A), which changes glycine to aspartic acid at position 13 (G13D) in the amino-terminal tail of histone H3.

H3G13D Has a Dominant-Negative Effect on Heterochromatin. To further investigate H3G13D, we used a highly sensitive reporter to detect heterochromatin changes at the silent mat region. Deletion of a local silencer REII, which acts redundantly with heterochromatin, silences mat2P, whereas detection of heterochromatin assembly defects (31, 32, 42). In nonswitching mat1-M cells lacking the REII element, impaired heterochromatic silencing of mat2P leads to "haploid meiosis," an aberrant sporulation phenotype resulting from coexpression of M and P mating-type information in haploid cells. Haploid meiosis is detected by exposing yeast colonies to iodine vapor. Colonies undergoing haploid-meiosis stain dark brown when exposed to iodine vapor, whereas WT colonies appear yellow. The ura4+ reporter inserted near mat2P (mat2P::ura4+) provides an additional readout for heterochromatic silencing. Consistent with defects in heterochromatin maintenance, REIIΔ hht2G13D cells showed de-repression of mat2P and the ura4+ reporter (Fig. 2B). H3G13D did not impact the expression of histone H3 or its incorporation into chromatin (SI Appendix, Fig. S2A–C). Importantly, deletion of hht2 in the mutant strain restored silencing and heterochromatin-specific H3K9me2/3, indicating that hht2G13D is a dominant-negative mutation (Fig. 2B and SI Appendix, Fig. S2A). Notably, unlike the sequence of mat2P and the ura4+ reporter in cells lacking Clr4, hht2G13D exhibited a variated phenotype, with single colonies showing a mixture of light and dark staining, and a smaller proportion of cells undergoing haploid meiosis (Fig. 2C). Together, these results indicate that hht2G13D is a dominant-negative mutation associated with heterochromatin and gene-silencing defects.

H3G13D Impairs Heterochromatin Spreading and Propagation. The variated phenotype of hht2G13D cells is reminiscent of other mutants with defects in heterochromatin propagation, including fli3A and amo1A (30, 32, 42). To test if similar defects could explain the phenotype of REIIΔ hht2G13D cells, we mapped H3K9me2 and H3K9me3 at the silent mat region. Importantly, hht2G13D cells showed a considerable reduction in H3K9me3, notably at regions distal to the cenH nucleation center (Fig. 2D). At cenH, H3K9me3 was only marginally reduced and correlated with an increase in H3K9me2 (Fig. 2D), consistent with the results described above (Fig. 1). ChIP-qPCR (Chromatin immunoprecipitation-quantitative polymerase chain reaction) analysis confirmed these results (Fig. 2E).

We then asked if hht2G13D affects the localization of the ClrC methyltransferase complex, which spreads from cenH to surrounding sequences via the Clr4 read-write mechanism (22). Indeed, cells carrying hht2G13D showed a considerable decrease in the epitope-tagged ClrC component Ral2 in the regions surrounding cenH (Fig. 2 D and E). This result is consistent with H3G13D.
interfering with the spreading and/or maintenance of heterochromatin by hindering Clr4 read-write activity.

We next tested whether \( hht2^{G13D} \) affects the propagation of heterochromatin at an ectopic site. We used an inducible system wherein cells express Clr4 fused to a TetR\textsuperscript{off} DNA-binding domain (TetR-Clr4) and harbor an \( ade6^+ \) reporter located downstream of six tetracycline operators (\( 6\text{xtetO-ade6}^+ \)). Following the release of TetR-Clr4 in cells lacking the anti-silencing factor Epe1 (43), heterochromatin at the ectopic site can be maintained for multiple generations via a mechanism involving the read-write activity of endogenous Clr4 (32, 44, 45). Cells expressing H3G13D showed impairment of the epigenetic maintenance of heterochromatin at the ectopic site (Fig. 2F). This is consistent with identification of \( hht2^{G13D} \) as a mutant that affects heterochromatin silencing at an ectopic site (46). Thus, H3G13D impairs heterochromatin maintenance at both endogenous and ectopic sites.

**Mutation of Shelterin Components Mitigates the Effect of H3G13D on Heterochromatin.** To better understand the mechanism by which H3G13D affects heterochromatin formation, we employed an unbiased genetic screen that relies on the REII\textsuperscript{Δ} mat2P::ura4+ reporter system to identify suppressors of the H3G13D silencing defect. Mutagenesis was followed by screening for colonies that showed suppression of the haploid meiosis phenotype and the ability to grow on counter selective medium containing 5-fluoroorotic acid (FOA). Several suppressor candidates were isolated (SI Appendix, Fig. S3A), backcrossed, and subjected to tetrad analyses. Suppressor mutations were identified by whole-genome sequencing and nucleotide variant analysis. Interestingly, suppressor mutations mapped to genes encoding the Shelterin components Taz1, Ccq1, and Rap1 (SI Appendix, Fig. S3A and B). Consistently, \( taz1\Delta, ccq1\Delta, \) or \( rap1\Delta \) could also suppress the silencing defects in H3G13D cells (Fig. 3A). The suppression was linked to partial restoration of heterochromatin as indicated by the increase in H3K9me3 levels at the silent mat region in \( taz1\Delta hht2^{G13D} \) compared with \( hht2^{G13D} \) alone (Fig. 3B).

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**Fig. 1.** Identification of a mutation that affects the maintenance of heterochromatin. (A) Schematic of the \( S.\ pombe \) mating-type (mat) locus (Top). Genetic screen using \( K\Delta:ura4^+ \) cells to identify factors that affect the maintenance of heterochromatin (Bottom). (B) Expression analysis of \( K\Delta:ura4^+ \) (Left) and \( Kint2::ura4^+ \) (Right) in the indicated strain backgrounds. (C) ChIP-qPCR (mean ± SEM, \( n = 2 \)) of \( K\Delta:ura4^+ \) (Left) and \( Kint2::ura4^+ \) (Right) strains. The fold enrichment of H3K9me3 and H3K9me2 at mat2P is shown for WT and the EMS32 mutant background. (D) ChIP-chip analysis of H3K9me3 and H3K9me2 enrichment at heterochromatic loci in the indicated \( K\Delta:ura4^+ \) strains. See also SI Appendix, Fig. S1.
Shelterin forms a complex with ClrC and is involved in heterochromatin assembly at telomeres and other loci (47–50). When Shelterin-mediated heterochromatin assembly is compromised, silencing factors are released and redistributed to other heterochromatic loci (51). Therefore, loss of Shelterin components might suppress heterochromatin defects in H3G13D cells by increasing the available pool of silencing factors, such as Clr4, at the mat locus.

Indeed, our attempts to identify EMS32 by complementation using a genomic DNA library yielded several clr4+ containing clones as suppressors of the silencing defect. Consistently, insertion of 3 to 4 copies of clr4+ into the genome partially suppressed the silencing and heterochromatin defects in hht2G13D (Fig. 3 C and D). Together, these results show that increasing the available pool of Clr4 is sufficient to partially overcome the inhibitory effect of H3G13D on heterochromatin assembly at the silent mat region.

H3G13D Impairs H3 Lysine 9 Methylation by Clr4. We next introduced the G13D mutation into a strain carrying a single copy of the histone H3 gene, hht2+ (52). The single copy hht2G13D cells exhibited de-repression of the ade6+ reporter inserted at the
outer pericentromeric repeat (otr1R::ade6+), similar to cells in which H3K9 was mutated to arginine or alanine (Fig. 4A) (52). Unlike cells in which only one of the three copies of histone H3 was mutated (Fig. 1D), single-copy hht2G13D cells showed a complete loss of H3K9me2/3 at pericentromeric repeats (Fig. 4B). Consistent with the loss of H3K9 methylation in vivo, Clr4 was unable to methylate the H3G13D substrate in vitro (Fig. 4C and SI Appendix, Fig. S4). These results suggest that hht2G13D affects heterochromatin assembly by preventing the methylation of H3K9 by Clr4, thereby reducing the requisite level of H3K9 methylated nucleosomes for Clr4 read-write activity.

Relative Abundance of H3G13D Potentiates H3K9me Spreading and Gene-Silencing Defects. Since Clr4 binds to and catalyzes methylation of H3K9 to propagate heterochromatin (22, 23), the un-methylatable H3G13D might “poison” the system by decreasing the
density of H3K9me3 critical for Clr4 read-write activity. To test this, we increased the relative abundance of H3G13D by placing epitope-tagged WT H3 or H3G13D under the control of the thiamine repressible nmt1 promoter and monitored silencing at the mat region in REIIA background cells. H3 expression increased upon reduction of the thiamine concentration. Remarkably, an increase in H3G13D, but not WT H3, corresponded with an increase in the haploid meiosis phenotype (Fig. 5 A and B and SI Appendix, Fig. S5A), indicating defective heterochromatic silencing. The silencing defect correlated with a decrease in H3K9me3 levels (Fig. 5 C and D). In particular, an increase in H3G13D, but not WT protein levels, was linked to a concomitant reduction in the spreading of H3K9me3 to regions surrounding the centH nuclearation center at the silent mat region (Fig. 5C and SI Appendix, Fig. S5B). These results suggest that H3G13D acts in a dosage-dependent manner to interfere with heterochromatin spreading.

Enhanced Affinity of Clr4 for H3K9me Suppresses Heterochromatin-Spreading Defects. H3G13D likely affects heterochromatin spreading by compromising the read-write activity of Clr4 (22). However, H3G13D could trap Clr4 by stably binding to its catalytic SET domain, similar to H3K9M (33). To determine if interference with read-write activity is the primary defect, we attempted to suppress the heterochromatin defects in H3G13D cells by enhancing Clr4 binding to methylated H3K9. Clr4 sequentially catalyzes mono-, di-, and trimethylation of H3K9; however, the conversion of H3K9me2 to H3K9me3 occurs relatively inefficiently and is the most sensitive to compromised Clr4 read-write activity (23). Consistently, hht2G13D cells showed a disproportionate reduction in H3K9me3 levels and a considerable enrichment of H3K9me2 (Figs. 1 and 2A). To enhance Clr4 binding in hht2G13D cells, we replaced the Clr4 chromodomain (CD) with the Chp1 CD (Fig. 6A), which binds H3K9me2 with high affinity (23, 53). The expression of the Clr4Chp1CD chimeric protein under the control of native clr4-gene promoter had no effect on cell viability or REIIA mat2P::ura4+ reporter gene silencing. Remarkably, Clr4Chp1CD suppressed the silencing defect at the mat locus in hht2G13D cells (Fig. 6B). This suppression corresponded with an increase in the levels of H3K9me3 and Raf2 across the silent mat interval (Fig. 6C) and subtelomeric heterochromatic domains (SI Appendix, Fig. S6A). These results indicate that H3G13D disrupts heterochromatin spreading by interfering with the read-write activity of Clr4 (Fig. 7). Interestingly, Clr4Chp1CD expression also led to a considerable increase in H3K9me2 at heterochromatin islands (SI Appendix, Fig. S6B), indicating that factors influencing the binding of Clr4 to methylated nucleosomes control the stability of facultative heterochromatin domains.

We then tested if Clr4Chp1CD could restore heterochromatin silencing in other mutants with reduced H3K9me3 levels, including cells lacking the FACT (Facilitates Chromatin Transcription) subunit Pob3, the SMARCAD1 homolog Fft3, or Amo1 involved in nuclear peripheral tethering of heterochromatin. Remarkably, Clr4Chp1CD suppressed the silencing defect in all three mutants (SI Appendix, Fig. S7A). We then expressed Clr4Chp1CD in hht2K9M mutant cells (33). The introduction of the hht2K9M mutation into REIIA mat2P::ura4+ cells resulted in the loss of heterochromatin silencing at the silent mat region (SI Appendix, Fig. S7 A and B). Unlike cells expressing H3G13D, the silencing defect in cells expressing H3K9M could not be rescued by Clr4Chp1CD (SI Appendix, Fig. S7 A and B). Thus, the mechanism by which H3K9M influences heterochromatin is likely distinct from that involving H3G13D. Ultimately, the characterization of this unique mutant hht2K9M allele has revealed that a critical threshold of preexisting H3K9me3 is required for propagation of heterochromatin by the read-write activity of Clr4.
an effective local concentration of Clr4 to promote the read-write activity that supports spreading and maintenance of heterochromatin (Fig. 7).

We characterized a mutation that directly implicates H3K9me in the transmission of epigenetic memory. The dominant-negative hht2G13D mutation affects heterochromatin propagation and results in a variegated phenotype. H3G13D is distinct from other histone mutants, including H3K9M that, in addition to blocking H3K9me, traps catalytic Clr4 to inhibit heterochromatin assembly (33). H3G13D cannot be methylated by Clr4 and disrupts the epigenetic loop mechanism involving Clr4 read-write activity. H3G13D directly blocks H3K9 methylation by Clr4, although it could impact ubiquitination of H3K14 that stimulates Clr4 activity (54). Regardless, H3G13D reduces the level of H3K9me3, which is required for the epigenetic-templated recruitment and stimulation of Clr4 enzymatic activity to promote heterochromatin propagation (22, 23). Consistently, H3G13D hinders the spread of CrtC and H3K9me from the nucleation site to surrounding regions in a dosage-dependent manner. Moreover, increasing the effective local concentration of Clr4 suppresses heterochromatin defects in hht2G13D cells. A key finding, however, is that the Clr4-Chp1CD chimeric protein, which can recognize H3K9me2 with high affinity, restores propagation of heterochromatin in hht2G13D cells. In addition to underscoring the requirement for recognition of methylated H3K9 by Clr4 for heterochromatin propagation, these results reveal that a critical density of H3K9me3 supports epigenetic stability of repressive chromatin domains through the read-write mechanism.

The finding that a critical threshold of H3K9me3 is essential for faithful propagation of heterochromatin may shed light on how factors such as HP1, HDACs, and the anti-silencing factor Epe1 affect heterochromatin assembly. The oligomerization of HP1 (55) localized across heterochromatin domains might enhance the density of H3K9me3 nucleosomes and their associated Clr4 to promote the spreading and inheritance of heterochromatin. Similarly, deacetylation of histones by HDACs, such as Clr3, may promote nucleosome–nucleosome interactions and suppress histone turnover to enhance H3K9me3 density (29, 30, 56). Along these lines, Epe1 stimulates histone exchange (29) and therefore might lower H3K9me3 density to destabilize heterochromatin (43–45, 57). On other hand, perinuclear tethering of heterochromatin and phase separation might increase the local concentration of modification-specific histone-binding proteins, including Clr4, to stabilize heterochromatin. In any case, maintaining a critical threshold of H3K9me3 is required for transgenerational inheritance of heterochromatin and stable gene silencing. Indeed, modified parental histones, which are deposited at their original location on daughter strands during replication (58, 59), are believed to recruit histone-modifying enzymes such as Clr4 to modify newly assembled nucleosomes (60).

Our findings may also illuminate mechanisms that maintain repressive chromatin domains in other systems. In particular, they may explain the recent observation in mouse embryonic stem cells that the linker histone H1 facilitates spreading of H3K9 methylation (61). In addition to the direct interaction between H1 and histone methyltransferases (61), stabilization of nucleosomes by
linker histones might preserve H3K9me3, which in turn facilitates anchoring of Suv39h1/2 via the chromodomain to promote spreading of heterochromatin. Similarly, dense chromatin was shown to facilitate PRC2-mediated H3K27 methylation (62). Although the mechanisms are likely to be more complex, histone K-to-M mutations frequently linked to cancer might compromise repressive chromatin assembly by affecting the local population of modified histones required to support the read-write activity of modifying enzymes (35, 36, 63, 64). Future studies will be needed to determine if a critical density of H3K9me3, which also facilitates DNA methylation (65), is a conserved requirement for the assembly and propagation of heterochromatin domains in other systems.

**Materials and Methods**

**Strains, Media, and Plasmid Construction.** *S. pombe* yeast strains used in this study are listed in SI Appendix, Table S1. Deletion and epitope-tagged strains were generated by standard PCR-based methodology and transformation or genetic crosses followed by tetrad dissection (SI Appendix). Standard techniques were used to culture *S. pombe*. All experiments were performed in yeast extract rich medium supplemented with adenine (YEAA) at 32 °C unless indicated otherwise. For serial dilution assays, tenfold dilutions of a mid log-phase culture were plated on PMG (Pombe Minimal Glutamate medium), PMG-ura, or PMG-ade6+ plates (incubated at 30 °C) were stained with iodine vapor to detect haploid meiosis. The relative affinities for H3K9me2 and H3K9me3 are represented by arrow heads. ChIP-chip analysis of H3K9me3 and Raf2-myc enrichment at the silent mat locus in the indicated REII mat2P::ura4+ strains, plotted using a sliding window average of three probes. Raf2-myc data shown for WT and hht2G13D are also presented in Fig. 2D (Left). ChIP-qPCR (mean ± SEM; n = 2) analysis of H3K9me3 and Raf2-myc fold enrichment at mat2P and cenH in the indicated REII mat2P::ura4+ strains (Right). See also SI Appendix, Figs. 56 and 57.

**ChIP.** ChIP experiments were performed as described previously (9) with some modifications (SI Appendix). Immunoprecipitated and input DNA were analyzed by performing qPCR or DNA microarray as described in SI Appendix.

**Microscopy and Image Analysis.** To quantify haploid meiosis, cells were collected from PMG plates grown at 30 °C for 2 to 3 d and imaged on a glass slide using a DeltaVision Elite microscope (GE Healthcare) with a 100× 1.4NA Plan Super Apochromat oil lens. Percent haploid meiosis was calculated by manually counting cells from images opened in Fiji.

**Methyltransferase Assay.** To examine the enzymatic activity of Clr4 on H3 peptide substrates (SI Appendix), histone methyltransferase assays were performed with equal microgram quantities of recombinant Clr4 and H3N-GST in methyltransferase buffer (50 mM Tris [pH 8.0], 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF]) supplemented with [3H]labeled-SAM for 1 h at 30 °C. The samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie to visualize the proteins. Gels were then treated with Amplify (GE Healthcare), dried, and subjected to fluorography to detect [3H]-labeled substrates.

See SI Appendix for additional details and descriptions of genetic screening, Western blotting, protein purification, and mass spectrometry analyses.

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**Fig. 6.** Enhancing the affinity of Clr4 for H3K9me2 can suppress the heterochromatin spreading defect in cells expressing H3G13D mutant protein. (A) Schematic representation of chromodomain-containing Clr4 and Chp1 proteins as well as chimeric Clr4Chp1CD. Their relative affinities for H3K9me2 and H3K9me3 are represented by arrow heads. (B) Expression analysis of mat2P::ura4+ in the indicated strain backgrounds. Additionally, plates were stained with iodine vapor to detect haploid meiosis. (C) ChIP-chip analysis of H3K9me3 and Raf2-myc enrichment at the silent mat locus in the indicated REII mat2P::ura4+ strains, plotted using a sliding window average of three probes. Raf2-myc data shown for WT and hht2G13D are also presented in Fig. 2D (Left). ChIP-qPCR (mean ± SEM; n = 2) analysis of H3K9me3 and Raf2-myc fold enrichment at mat2P and cenH in the indicated REII mat2P::ura4+ strains (Right). See also SI Appendix, Figs. 56 and 57.
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Data Availability. Microarray data are deposited in the Gene Expression Omnibus with accession number GSE162701.

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