Unique roles for histone H3K9me states in RNAi and heritable silencing of transcription

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Heterochromatic DNA domains have important roles in the regulation of gene expression and maintenance of genome stability by silencing repetitive DNA elements and transposons. From fission yeast to mammals, heterochromatin assembly at DNA repeats involves the activity of small noncoding RNAs (sRNAs) associated with the RNA interference (RNAi) pathway1–9. Typically, sRNAs, originating from long noncoding RNAs, guide Argonaute-containing effector complexes to complementary nascent RNAs to initiate histone H3 lysine 9 di- and trimethylation (H3K9me2 and H3K9me3, respectively) and the formation of heterochromatin10–17. H3K9me is in turn required for the recruitment of RNAi to chromatin to promote the amplification of sRNA11,15,18. Yet, how heterochromatin formation, which silences transcription, can proceed by a co-transcriptional mechanism that also promotes sRNA generation remains paradoxical. Here, using Clr4, the fission yeast Schizosaccharomyces pombe homologue of mammalian SUV39H H3K9 methyltransferases, we design active-site mutations that block H3K9me3, but allow H3K9me2 catalysis. We show that H3K9me2 defines a functionally distinct heterochromatin state that is sufficient for RNAi-dependent co-transcriptional gene silencing at pericentromeric DNA repeats. Unlike H3K9me3 domains, which are transcriptionally silent, H3K9me2 domains are transcriptionally active, contain modifications associated with euchromatic transcription, and couple RNAi-mediated transcript degradation to the establishment of H3K9me3 domains. The two H3K9me states recruit reader proteins with different efficiencies, explaining their different downstream silencing functions. Furthermore, the transition from H3K9me2 to H3K9me3 is required for RNAi-independent epigenetic inheritance of H3K9me1. Our findings demonstrate that H3K9me2 and H3K9me3 define functionally distinct chromatin states and uncover a mechanism for the formation of transcriptionally permissive heterochromatin that is compatible with its broadly conserved role in sRNA-mediated genome defence.

To determine whether the different methylation states of H3K9 make distinct contributions to heterochromatin formation, we designed mutations in the active site of Clr4 that inhibit H3K9me3 catalysis. SET domain methyltransferases contain a ‘switch position’ that governs the degree of methylation (Fig. 1a, highlighted green)19. Phenylalanine in the switch position, such as in Neurospora crassa DIM-5 and mammalian SUV39H1/2, allows the catalysis of di- and trimethylated lysine, while tyrosine predominantly allows lysine mono- and dimethylation, such as in Arabidopsis thaliana SUVH4/KYP (Fig. 1a)19. At the switch position, the Clr4 SET domain contains phenylalanine (F449), equivalent to F294 in DIM-5 and F363 in SUV39H1 (Fig. 1a, b; highlighted green). Moreover, the amino acid at the position equivalent to the S. pombe I418 was previously proposed to affect methylation states, with bulkier residues such as proline in G9a correlating with dimethylation (Fig. 1a, b; highlighted blue)18,20.

We constructed strains expressing endogenous Clr4 protein with either a F449Y or I418P mutation (Extended Data Fig. 1a). Clr4(F449Y) was incorporated into the CLRC methyltransferase complex and interacted with Swi6 with efficiency similar to wild-type Clr4 (Extended Data Fig. 1b, c). Immunoblotting and quantitative mass spectrometry of an enriched histone fraction showed that H3K9me3 levels were dramatically decreased in clr4F449Y cells, while H3K9me2 levels were increased (Extended Data Fig. 1d, e). Furthermore, chromatin immunoprecipitation followed by high throughput sequencing (ChIP–seq) or quantitative PCR (ChIP–qPCR) showed that while pericentromeric H3K9me2 levels were increased in clr4 mutants relative to clr4+ cells (Fig. 1c; Extended Data Fig. 2a, b), H3K9me3 was lost in clr4F449Y cells and reduced in clr4I418P cells (Fig. 1d; Extended Data Fig. 2c). The increase in H3K9me2 levels in the clr4 mutants is probably a result of available H3K9me2 that would otherwise be trimethylated in clr4+ cells. Moreover, the close correspondence between H3K9me2 peaks in clr4+ and clr4F449Y cells indicated that H3K9me3 was not required for the formation of extended H3K9me3 domains at pericentromeric DNA repeats (Fig. 1c, d; Extended Data Fig. 2c). The increase in H3K9me2 levels in the clr4 mutants is probably a result of available H3K9me2 that would otherwise be trimethylated in clr4+ cells. Moreover, the close correspondence between H3K9me2 peaks in clr4+ and clr4F449Y cells indicated that H3K9me3 was not required for the formation of extended H3K9me3 domains at pericentromeric DNA repeats (Fig. 1c, d; Extended Data Fig. 2c).

In agreement with the growth silencing assay, clr4+ cells displayed increased growth on plates lacking uracil and grew poorly on FOA plates (Fig. 1e, left) indicating a loss of clr4+F449Y silencing. clr4I418P had a milder silencing defect, consistent with partial loss of H3K9me3 in this mutant (Fig. 1d, e) in agreement with the growth silencing assay, ort1R::ura4+ transcriptions were derepressed in clr4+F449Y and to a lesser extent in clr4I418P cells (Fig. 1e, right). Furthermore, we observed increased RNA polymerase II (Pol II) occupancy at the endogenous pericentromeric dg and dh repeats, with clr4F449Y cells having similar levels of Pol II occupancy to that of clr4+ cells (Fig. 1f; Extended Data Fig. 4b). Surprisingly, despite the observed loss of transcriptional gene silencing (TGS) in clr4F449Y cells, dg and dh transcript levels were 10- to 15-fold lower in clr4F449Y cells than in Δclr4 cells (Fig. 1g). Together with the absence of detectable H3K9me3 in clr4F449Y cells (Fig. 1d), this result suggests that H3K9me2 may be sufficient for promoting RNAi-dependent co-transcriptional gene silencing (CTGS) of the dg and dh transcripts.

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Values are shown as fold increase in RNA levels in mutant over wild-type cells. Arrowheads indicate primer locations for ChIP–qPCR analysis (Extended Data Fig. 4a for insert location). 

Together with previous observations that siRNA accumulation is not completely H3K9me-dependent at H3K9me2 ChIP–seq RPM 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 0

In contrast to A. thaliana, these mutations in the indicated methyltransferases (bottom). chromodomains (CD) and SET domains, and the mutations used in this study (top). Sequence alignment for the SET domain region containing these mutations in the indicated methyltransferases (bottom). A. thaliana, Arabidopsis thaliana; H. sapiens, Homo sapiens. 

Crystal structure of N. crassa DIM-5 catalytic pocket in complex with a histone H3 N-terminal peptide (yellow), showing side chain of lysine 9 in the catalytic pocket. 

N. crassa DIM-5 catalytic pocket in complex with a histone H3 N-terminal peptide (yellow), showing side chain of lysine 9 in the catalytic pocket. 

Figure 1 | Clr4 SET domain mutations that block H3K9me3 result in defective TGS. a. Diagram illustrating location of the Clr4 chromodomains (CD) and SET domains, and the mutations used in this study (top). Sequence alignment for the SET domain region containing these mutations in the indicated methyltransferases (bottom). A. thaliana, Arabidopsis thaliana; H. sapiens, Homo sapiens. 

Crystal structure of N. crassa DIM-5 catalytic pocket in complex with a histone H3 N-terminal peptide (yellow), showing side chain of lysine 9 in the catalytic pocket. 

N. crassa DIM-5 catalytic pocket in complex with a histone H3 N-terminal peptide (yellow), showing side chain of lysine 9 in the catalytic pocket. 

Figure 2 | Transcription-permissive H3K9me3 helps recruit RNAi and precedes H3K9me3 establishment. a. Model for recruitment of the RNAi machinery (RITS, RDR2 and Dcr1) and Clr4-containing CLRC to a nascent pericentromeric transcript. b. Northern blot of dg and dh siRNAs in cells with the indicated genotypes. Ratios are determined by fold increase in siRNA levels in mutants over wild-type cells. snoR69 was used as an internal control. For gel source data, see Supplementary Fig. 1. Image represents three (dg siRNA) or two (dh siRNA) individual experiments. c. Chp1 ChIP–seq reads mapped to the pericentromeric repeat regions on the right arm of chromosome 1. Right, sum of normalized reads mapped to pericentromeric regions. Reads were randomly assigned to the dg and dh repeats of each chromosome and are presented as reads per million (RPM, y axis). 

d. Same as c but showing H3K9me3 ChIP–seq reads. e. Left, otr1R::ura4+ transgene silencing assay (see Extended Data Fig. 4a for insert location). N/S, non-selective medium; – Ura, minus uracil medium; + FOA, 5-FOA-containing medium. Image represents three individual experiments. Right, RT–qPCR analysis of otr1R::ura4+ transcript. Error bars, s.d.; n = 3 biological replicates.

Consistent with functional RNAi-dependent CTGS (Fig. 2a) in both clr4F449Y and clr4I418P cells, dg siRNA levels were similar or higher than in clr4+ cells and dh siRNA levels were only reduced by about twofold, in contrast to Δclr4 cells, which contain trace amounts of dg and dh siRNAs (Fig. 2b). Furthermore, the pericentromeric recruitment of the RITS complex subunits Chp1 was increased in both clr4F449Y and clr4I418P cells, presumably owing to increased H3K9me2 (Fig. 2c, Extended Data Fig. 5a–c), while Ago1 localized to the dg and dh repeats in clr4F449Y with near wild-type efficiency (Extended Data Fig. 5d).

Together with previous observations that siRNA accumulation is completely H3K9me-dependent at dh and partially H3K9me-dependent at dg (ref. 15), these results demonstrate that H3K9me2 is sufficient for dg siRNA amplification (Fig. 2a), with H3K9me3-dependent steps making partial contribution to siRNA processing at the dh repeats. We therefore conclude that the partial silencing of endogenous pericentromeric transcripts in cells lacking H3K9me3 (Fig. 1g) is a result of H3K9me2- and RNAi-dependent co-transcriptional degradation of RNAs transcribed from pericentromeric repeats.

Since increased Pol II occupancy was associated with high levels of H3K9me2 (Fig. 1c, f; Extended Data Fig. 4b), we tested whether H3K9me2 domains contain histone modifications that are linked to transcription22. Chp1 ChIP–seq and Chp1 qPCR showed increased levels of transcription-associated H3K4me3, H3K36me3, H3K14 acetylation (H3K14ac), and H4K16ac in clr4F449Y relative to clr4+ cells (Fig. 2d, e; Extended Data Fig. 5e–h). Additionally, quantitative mass spectrometry analysis of nuclear histones purified from clr4+ cells showed a nearly 1-to-1 ratio of H3K9me2 and H3K9me3, with about 30% of the H3K9me2 peptide, but 0% of the H3K9me3 peptide, containing K14ac (Extended Data Fig. 6). We also detected high levels of H3K9me1 in both clr4+ and Δclr4 cells (Extended Data Fig. 6b), suggesting that fission yeast harbour another enzyme (or multiple enzymes) that catalyses H3K9me1. These results demonstrate that H3K9me2 domains are associated with transcription–coupled histone modifications commonly found in euchromatic domains and raise the possibility that transcriptionally permissive H3K9me2 domains may precede H3K9me3 and TGS during heterochromatin establishment.
To test this hypothesis, we examined whether H3K9me3 precedes H3K9me2 during de novo RNAi-dependent heterochromatin establishment in wild-type cells. We treated S. pombe cells with trichostatin A (TSA), a histone deacetylase inhibitor previously shown to partially disrupt heterochromatin22, and harvested them at several time points after TSA removal (Fig. 2f). Successful re-establishment of heterochromatin was demonstrated by gradual and partial restoration of silencing at the otr1R::ura4+ transgene over a period of several hours (Extended Data Fig. 7a). In agreement with ChIP–seq results (Extended Data Fig. 7b, c), Chp1–IP assays revealed faster recovery kinetics for H3K9me2 relative to H3K9me3 (Fig. 2g). Such temporal separation between H3K9me2 and H3K9me3 may allow the establishment of large domains of transcription- and RNAi-dependent H3K9me3 throughout several kilobases of pericentromeric chromatin before silencing of transcription, which, if it occurred too early, would interfere with siRNA-mediated spreading.

Efficient silencing requires the recruitment of HP1, Swi6 and Chp2, which bind to H3K9me and promote TGS at least in part by recruiting the Clr3 histone deacetylase complex22,23. We next explored if H3K9me2 states affect the recruitment of those downstream factors that, unlike Chp1, are not required for H3K9me3 establishment at pericentromeric repeats (Extended Data Fig. 8a, b). In clr4F449Y cells, Swi6 association with pericentromeric dhl repeats was nearly abolished, while its association with dgl repeats was reduced by about threefold (Fig. 3a; Extended Data Fig. 9a). We also observed weak localization of GFP–Swi6 to a single perinucleolar point in clr4F449Y cells, consistent with its weak association with pericentromeric H3K9me2 (Extended Data Fig. 9b). Chp2 recruitment was also impaired, but to a lesser extent (Fig. 3b; Extended Data Fig. 9c). Moreover, the reduced Swi6 and Chp2 recruitment to pericentromeric DNA repeats in clr4F449Y cells was accompanied by a reduction in Clr3 recruitment (Fig. 3c; Extended Data Fig. 9d). Therefore, defective TGS, associated with the loss of H3K9me3, results at least in part from a defect in HP1 and Clr3 recruitment. To further investigate whether the differential recruitment of chromodomain proteins was a result of their differing affinities for H3K9me2 and H3K9me3, we performed quantitative mass spectrometry using isobaric mass tags to examine the ability of unmodified H3 (H3K9me0), H3K9me2, and H3K9me3 tail peptides to associate with chromodomain proteins in S. pombe extracts (Fig. 3d, e). This assay evaluates binding to histone tails in the context of each protein’s association with its native binding partners. Chp1 and Chp2 bound to H3K9me2 and H3K9me3 peptides with similar efficiency, while Swi6 and Clr4 associated with H3K9me3 peptides more efficiently than with H3K9me2 peptides (Fig. 3f). Together with previous findings26, these results suggest that efficient recruitment of Chp1/RNAi and reduced recruitment of Swi6 to pericentromeric DNA repeats in clr4F449Y cells is a result of their different affinities for H3K9me3 relative to H3K9me3, and provide an explanation for efficient H3K9me2-dependent RNAi versus H3K9me3-dependent TGS.

We next examined the mechanism that governs the transition from H3K9me2- to H3K9me3-dependent TGS. Clr4 localizes to pericentromeric DNA repeats by interaction with the RNAi machinery as well as through the interaction of its chromodomain with H3K9me3 (reviewed in ref. 3). In cells with stably expressed Clr4 carrying a W31G mutation in its chromodomain (Extended Data Fig. 1a), we observed a drastic loss of H3K9me3, but not H3K9me2, throughout pericentromeric DNA repeats, demonstrating that the interaction of Clr4 with the RNAi machinery was sufficient for its recruitment and H3K9me2 catalysis (Fig. 3g, h; Extended Data Fig. 9e, f). ChIP experiments also revealed that in contrast to greatly reduced association of Clr4(W31G) with chromatin, Clr4(F449Y) associated with pericentromeric DNA repeats at levels close to that of wild-type Clr4, demonstrating that Clr4 can bind to H3K9me2 via its chromodomain (Fig. 3i; Extended Data Fig. 9g). Together, these results suggest that following RNAi-mediated recruitment of Clr4 to establish H3K9me2, the association of the chromodomain of Clr4 with H3K9me2 is required for transition to H3K9me3 and TGS. We propose that this association promotes H3K9 trimethylation by either increasing the residence time of Clr4 on chromatin or by inducing a conformational change that favours trimethylation.

Having determined the role of H3K9me3 states in RNAi-dependent heterochromatin establishment, we sought to examine their possible role...
H3K9me3 is required for epigenetic inheritance. a. Experimental strategy for testing requirements for epigenetic inheritance. b. Silencing assays of 10xtetO-ade6 on low-adencine medium lacking tetracycline (−tet) or containing tetracycline (+tet) to assess establishment and maintenance, respectively, in Δclr4 cells, which either lack endogenous clr4 (Δclr4), or contain clr4, clr4W31G or clr4I418P alleles. Image represents three individual experiments. c. H3K9me2 ChIP–seq reads mapped to the 10xtetO-ade6+ region. Both 10xtetO-ade6− (green) and mugI35− (black) located 5 kb upstream of 10xtetO-ade6 were used for ChIP–qPCR analysis (see Extended Data Fig. 10). d. Same as c, but after 24 h of growth in +tet medium. e, H3K9me2 ChIP–seq reads mapped to pericentromeric repeats on the right of chromosome 1. The sum of normalized reads is indicated on the right. Data are presented as reads per million (RPM, y axis). The bottom two tracks have a tenfold expanded y axis scale to highlight the complete loss of H3K9me2 in the clr4I418P,Δago1 double-mutant cells. f. Schematic summary of the unique roles of H3K9 methylation states. Top, H3K9me2 mediates co-transcriptional degradation of nascent transcripts (RNAi–CTGS) and H3K9me2 silencing. Bottom, the formation of H3K9me3 domains, which requires the chromodomain (CD) of Clr4, results in efficient recruitment of HP1 and transcriptional gene silencing (RNAi–TGS). H3K9me3, but not H3K9me2, can be epigenetically inherited. See Supplementary Information for additional discussion.

In epigenetic inheritance, we first used a system that allows reversible and RNAi-independent heterochromatin establishment. In this system, a Clr4 protein lacking its chromodomain is fused to the bacterial TetR protein (TetR–Clr4-I) and binds to an array of tet operator (10xtetO) sites located upstream of an ade6 reporter gene (Fig. 4a), resulting in ade6 silencing and formation of red colonies on medium with limiting adenine (Fig. 4b, left)27,28. In cells containing a wild-type endogenous copy of clr4, and lacking epe1+, which promotes H3K9 demethylation, this ectopically established silencing is epigenetically inherited after tetracycline-induced release of TetR–Clr4-I (Fig. 4b, right, second row)27,28. Notably, replacement of clr4 with clr4I418P, or clr4W31G abolished the epigenetic inheritance of ade6 silencing (Fig. 4b, right). Similarly, consistent with previous results using Clr4 lacking its chromodomain27,28, replacement of clr4 with clr4W31G abolished heterochromatin maintenance (Fig. 4b). TetR–Clr4-I established broad domains of H3K9me2 in clr4I418P, clr4W31G, and clr4 cells lacking epe1+ (Fig. 4c; Extended Data Fig. 10a, b), which were maintained after releasing TetR–Clr4-I in clr4+ cells, but declined to background levels in clr4I418P and clr4W31G cells, similar to the levels observed in Δclr4 cells (Fig. 4d; Extended Data Fig. 10a, b). These results indicate that H3K9me3 is critical for epigenetic inheritance of silencing at an ectopic locus. We then asked whether H3K9me3 was required for the epigenetic maintenance of H39kme at endogenous pericentromeric DNA repeats. RNAi mutant cells contain residual H3K9me at pericentromeric DNA repeats that is epigenetically maintained27. We observed a complete loss of H3K9me2 when the clr4I418P mutant was combined with Δago1 (Fig. 4e, bottom rows; Extended Data Fig. 10c), indicating that H3K9me3 was required for RNAi-independent epigenetic inheritance of H3K9me at endogenous pericentromeric repeats.

In summary, we discovered that RNAi and CTGS are associated with a transcriptionally permissive type of heterochromatin that is defined by H3K9me2 (Fig. 4f). Transition from H3K9me2 to H3K9me3, which requires the association of the chromodomain of the Clr4 methyltransferase with H3K9me2, is required for efficient recruitment of HP1 and subsequent TGS (Fig. 4f). We propose that the slower kinetics of de novo H3K9me3 establishment relative to H3K9me2 establishment allow the siRNA- and transcription-dependent spreading of H3K9me before the onset of H3K9me3-dependent TGS. Our findings also indicate that H3K9me3 is required for the epigenetic inheritance of domains of H3K9me following their establishment by either endogenous RNAi or artificial recruitment of Clr4 to an ectopic site. This requirement for H3K9me3 is probably due to the higher affinity of the Clr4 chromodomain for H3K9me3 relative to H3K9me213,28, which would ensure stable binding of Clr4 to parentaly inherited histones followed by methylation of newly deposited histones. Histone H3K9me states therefore determine not only the mechanism of silencing within heterochromatin but also its potential epigenetic heritability. The uncoupling of TGS from H3K9me is likely to be critical for H3K9me-dependent sRNA generation in other organisms, and may be regulated by H3K9me states, as described here, or other factors that would allow transcription within heterochromatin16–18. Finally, the conservation of H3K9 methylation and its role in chromatin regulation, together with the diversity of methyltransferases that preferentially catalyse H3K9me1, H3K9me2 or H3K9me3, suggest that mechanisms similar to what we have described in fission yeast are likely to be used in other organisms. Our findings regarding H3K9me3 states also further highlight the remarkable utility of lysine methylation states and their power in signalling distinct downstream chromatin-dependent events.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online version.

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METHODS

Strain construction. Strains used in this study are listed in Supplementary Table 1. All Flag-tagged genes were expressed under the control of their endogenous promoters and terminators. SPY3244, SPY3245, SPY3248 and SPY6326 were constructed by integrating full-length wild-type or mutant cDNA open reading frames and integrating endogenous cdi4 promoter and terminator sequences about 450 bp upstream of the trp1 gene in the SPY3086 strain27.

Growth assays. Cells were grown in YES to log phase, 1 × 107 cells were pelleted, suspended in 5 ml of water and then serially diluted tenfold. Three microdrops of each dilution were spotted on the appropriate growth medium. FOA was used at 1 mg ml−1 and tetracycline at 2.5 μg ml−1. Plates were incubated at 32°C for 3–4 days. YE plates were used for assaying ade6 reporter strains and were additionally incubated at 4°C overnight (to enhance the colour red) before imaging.

H3K9me erasure by TSA and recovery. Cells were inoculated in 50 ml YEA containing 25 μg ml−1 TSA and grown at 36°C for 15 h. Cells were then pelleted and washed to remove TSA, suspended in YEA at 32°C to re-establish heterochromatin, and harvested at 0, 3, 5 and 7 h for ChIP analysis.

Chromatin immunoprecipitation (ChIP). ChIP was performed as previously described30. For conditions for fixation and immunoprecipitation (IP) are briefly described here. 50-ml cultures of cells were grown overnight (or 24 h when the mirVana miRNA Isolation Kit (Ambion), loaded on 17.5% polyacrylamide/7 M urea gel, transferred to positively charged nylon membrane (Roche 1417240), and probed with a mixture of 32P-labelled DNA probes corresponding to dg or dh siRNA sequences described previously33.

Quantitative mass spectrometry assay for association of silencing complexes with histone H3 peptides. Exponentially growing wild-type cells expressing 3 × Flag–Clr4 under its endogenous promoter (SPY4636) (2.25 × 107 cells) were harvested by centrifugation, transferred to a 50 ml tube, washed twice in TBS (50 mM Tris (pH 7.6), 150 mM NaCl), and frozen at −80°C (approximately 20 g of cells). All subsequent steps were performed at 4°C. The frozen cells were resuspended in 1 volume of ice-cold lysis buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 1 mM EDTA (pH 8.0), 0.5 mM DTT, 10% glycerol, 0.25% Triton X-100) and alkylation with 10 mM iodoacetamide (room temperature, 30 min in the dark). Excess iodoacetamide was quenched with 15 mM dithiothreitol (room temperature, 15 min in the dark). TCA precipitation followed by one acetone and one methanol wash was performed before protease digestion. Samples were resuspended in 200 mM HEPES, pH 8.5 and digested at room temperature for 1.5 h with LysC protease at a 100:1 protein-to-protease ratio after which trypsin was added at a 100:1 protein-to-protease ratio for 6 h at 37°C. Acetonitrile was added to a final concentration of approximately 30% (v/v), after which TMT reagents were added. Following incubation at room temperature for 1 h, the reaction was quenched with hydroxylamine to a final concentration of 0.3% (v/v) for 15 min. The TMT-labelled samples were pooled at a 1:1 ratio for all samples, vacuum centrifuged to near dryness and fractionated using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Pierce 84868). Twelve fractions were collected using: 5%, 10%, 12.5%, 15%, 17.5%, 20%, 22.5%, 25%, 35%, 50%, 60% and 80% acetonitrile. From here, every sixth sample was combined, yielding a total of 6 samples. Samples were subsequently acidified with 1% formic acid and vacuum centrifuged to near dryness. Each fraction was desalted via StageTip, lyophilized and subjected to disulfide bond reduction.

Exponentially growing wild-type cells expressing Flag–Clr4 under its endogenous promoter (SPY4636) (2.25 × 107 cells). All Flag-tagged genes were expressed under the control of their endogenous promoters and terminators. SPY3244, SPY3245, SPY3248 and SPY6326 were constructed by integrating full-length wild-type or mutant cDNA open reading frames and integrating endogenous cdi4 promoter and terminator sequences about 450 bp upstream of the trp1 gene in the SPY3086 strain27.

Growth assays. Cells were grown in YES to log phase, 1 × 107 cells were pelleted, suspended in 5 ml of water and then serially diluted tenfold. Three microdrops of each dilution were spotted on the appropriate growth medium. FOA was used at 1 mg ml−1 and tetracycline at 2.5 μg ml−1. Plates were incubated at 32°C for 3–4 days. YE plates were used for assaying ade6 reporter strains and were additionally incubated at 4°C overnight (to enhance the colour red) before imaging.

H3K9me erasure by TSA and recovery. Cells were inoculated in 50 ml YEA containing 25 μg ml−1 TSA and grown at 36°C for 15 h. Cells were then pelleted and washed to remove TSA, suspended in YEA at 32°C to re-establish heterochromatin, and harvested at 0, 3, 5 and 7 h for ChIP analysis.

Chromatin immunoprecipitation (ChIP). ChIP was performed as previously described30. For conditions for fixation and immunoprecipitation (IP) are briefly described here. 50-ml cultures of cells were grown overnight (or 24 h when the mirVana miRNA Isolation Kit (Ambion), loaded on 17.5% polyacrylamide/7 M urea gel, transferred to positively charged nylon membrane (Roche 1417240), and probed with a mixture of 32P-labelled DNA probes corresponding to dg or dh siRNA sequences described previously33.
tolerance for total protein level analysis. The product ion tolerance was set to 0.9 Da. These wide mass tolerance windows were chosen to maximize sensitivity in conjunction with Sequest searches and linear discriminant analysis. TMT tags on lysine residues and peptide N termini (+229.163 Da) and carbamidomethyl-
atio n of cysteine residues (+57.021 Da) were set as static modifications, while oxidation of methionine residues (+15.995 Da) was set as a variable modification. Peptide–peptide matches (PSMs) were adjusted to a 1% false discovery rate (FDR). PSM filtering was performed using a linear discriminant analysis, as described previously. For the antibodies, a TMT reporter ion signal-to-noise ratio that is less than 100, or no MS3 spectra with more than eight TMT reporter ion channels missing, MS3 spectra were rejected. For analysis of histone post-translational modifications using total fission yeast histones, histones were prepared by modifications of a previously described protocol. One litre of culture (SPY137 or SPY815) grown to an OD 600 of 0.9–1.0 was centrifuged at 3,200g for 5 min at 4°C. The supernatant was centrifuged again at 3,200g for 5 min at 4°C. Nuclei were pelleted by centrifuging at 3,200g for 5 min at 4°C. SW40 Ti rotor. The pellet was resuspended in buffer C (0.34 M sucrose, 20 mM Tris–HCl, pH 7.4, 50 mM KCl, 5 mM MgCl2, and complete EDTA-free protease inhibitor cocktail (Roche)), layered on a 2 ml sucrose cushion at the bottom of the tube, and centrifuged at 30,000g for 30 min at 4°C. Nuclei were resuspended in buffer D (10 mM Tris, pH 8.0, 0.5% NP-40, 75 mM NaCl, and complete EDTA-free protease inhibitor cocktail (Roche)) and pelleted by centrifuging at 3,200g for 4°C for 5 min at a total of three times. Nuclei were resuspended in buffer E (10 mM Tris–HCl, pH 8.0, 400 mM NaCl, complete EDTA-free protease inhibitor cocktail (Roche)) and dialyzed against buffer F (500 mM NaCl, complete EDTA-free protease inhibitor cocktail (Roche)). Yeast powder was resuspended in 1 volume of ice-cold lysis buffer containing 300 mM NaCl (20 mM HEPES (pH 7.5), 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA (pH 8.0), 0.5 mM DTT, 10% glycerol, 0.25% Triton X-100, 1 mM phenyl-methylsulfonyl fluoride (PMSF), complete protease inhibitor cocktail (Roche)), sonicated 6 s ON, 1 min OFF for a total of 2 min and clarified at 4°C by two consecutive rounds of centrifugation at 16,000g for 25 min and 10 min, respectively. The cleared lysates were diluted to a protein concentration of 25 mg ml\(^{-1}\) with ice-cold lysis buffer containing 500 mM NaCl and incubated with Protein G Dynabeads (Life Technologies) cross-linked with amino-epoxy (Sigma F1804) antibody for 4 h at 4°C. The beads were collected on magnetic stands, washed four times with 7 ml ice-cold lysis buffer containing 500 mM NaCl, followed by two washes with ice-cold lysis buffer containing 100 mM NaCl (20 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA (pH 8.0), 0.5 mM DTT, 10% glycerol, 0.25% Triton X-100, 1 mM phenyl-methylsulfonyl fluoride (PMSF), complete protease inhibitor cocktail (Roche)). Beads were resuspended in 500 μl of ice-cold lysis buffer containing 100 mM NaCl and incubated with 5,000 units of Benzonase (Novagen 71205-3) for 20 min at 25°C with mild shaking. Beads were washed twice with ice-cold lysis buffer containing 100 mM NaCl, 500 μl aliquoted with 1 ml of 500 mM NaCl at 37°C for 20 min. The eluted proteins were dried overnight using vacuum centrifugation. Yeast proteins were subjected to disulfide bond reduction with 5 mM tris(2-carboxyethyl)phosphine (room temperature, 30 min) and alkylation with 10 mM iodoacetamide (room temperature, 30 min in the dark). Ectoiso-
dase was quenched with 15 mM dithiothreitol (room temperature, 15 min in the dark). TCA precipitation followed by one acetone and one methanol wash was performed before protease digestion. Samples were resuspended in 100 mM HEPES, pH 8.5 and digested at room temperature for 13 h with LysC protease at a 1:100 protein-to-protease ratio. The sample was desalted via a Zeba Spin Desalting Column (Millipore, 7K MWCO), reconstituted in 500 μl of 50 mM HEPES, and dialyzed against 500 mM NaCl, 5 mM MgCl2, 1 mM EDTA (pH 8.0), and complete protease inhibitor cocktail (Roche). Beads were resuspended in 100 μl of ice-cold lysis buffer containing 100 mM NaCl and incubated against 500 μl of LysC (Thermo Scientific). Data were acquired using data-independent acquisition (DIA) as previously described. Beads were resuspended in 1 ml of ice-cold lysis buffer containing 100 mM NaCl, 500 μl aliquoted with 1 ml of 500 mM NaCl at 37°C for 20 min. The eluted proteins were dried overnight using vacuum centrifugation. Protein concentrations were determined using a NanoPhotometer (Thermo Scientific) coupled with a NanoDrop 8000c spectrophotometer (Thermo Scientific). Yeast proteins were subjected to disulfide bond reduction with 5 mM tris(2-carboxyethyl)phosphine (room temperature, 30 min) and alkylation with 10 mM iodoacetamide (room temperature, 30 min in the dark). TCA precipitation followed by one acetone and one methanol wash was performed before protease digestion. Samples were resuspended in 100 mM HEPES, pH 8.5 and digested at room temperature for 13 h with LysC protease at a 1:100 protein-to-protease ratio. The sample was desalted via a Zeba Spin Desalting Column (Millipore, 7K MWCO), reconstituted in 500 μl of 50 mM HEPES, and dialyzed against 500 mM NaCl, 5 mM MgCl2, 1 mM EDTA (pH 8.0), and complete protease inhibitor cocktail (Roche). Proteins were separated on a 10 μm inner diameter microcapillary column packed with approximately 25 cm of Acaccore C18 resin (2.6 μm, 150 Å, Thermo Fisher Scientific). For each analysis, we loaded approximately 1 μl of sample. The column was washed with a 1 h gradient of 0 to 25% acetonitrile in 0.125% formic acid with a flow rate of approximately 300 nL min\(^{-1}\). The scan sequence began with an Orbitrap MS1 spectrum with the following parameters: 10,000 resolution, 1.0 ECD isolation target, 1.0 ECD, 1.0 ECD, and 1.0 ECD, 1,000 Th automatic gain control (AGC) target 1 x 106, maximum injection time 250 ms, and centroid spectrum data type. We selected the top 20 precursors for MS2 analysis which consisted of HCD high-energy collision dissociation with the following parameters: resolution 17,500, AGC 1 x 106, maximum injection time 60 ms, isolation window 2 Th, normalized collision energy (NCE) 30, and centroid spectrum data type. The underfill ratio
was set at 1%, which corresponds to a 1.1 × 10⁴ intensity threshold. In addition, unassigned and singly charged species were excluded from MS2 analysis and dynamic exclusion was set to automatic. Data analysis was performed as described in the section 'Quantitative mass spectrometry assay for association of silencing complexes with histone H3 peptides' except that samples were not TMT labelled and the product ion tolerance was set to 0.03 Da.

GST–Swi6 pulldown assay. Swi6 protein coding sequence was PCR amplified and inserted between BamH1 and XhoI restriction sites of pGEX-6p-1 expression vector (GE Healthcare). This produced a Swi6 fusion protein with an N-terminal GST tag separated by a Precision Protease cleavage site. GST and GST–Swi6 were expressed in BL21 Codon Plus (Agilent Technologies), grown in 1 l of LB media supplemented with 100 μg ml⁻¹ ampicillin at 37 °C to an OD₆₀₀ of 0.6, and induced with 0.5 mM of IPTG for 4 h. Cells were harvested and suspended in buffer A (50 mM Tris–HCl and 0.5 M NaCl, pH 8.0) containing 1 mg ml⁻¹ lysozyme, and nuclease at 4 °C for 1 h. The mixture was then sonicated and centrifuged at 32,000 g for 20 min and the supernatant was loaded onto a Glutathione Sepharose 4B Column (GE Healthcare) equilibrated with buffer A. The column was washed with buffer A and the protein was eluted with buffer B containing 10 mM reduced glutathione. Eluted fractions were dialysed overnight into buffer A and the reduced glutathione and then subjected to size exclusion chromatography using a Highload 16/60 Sephadex 200 pg column (GE Healthcare) equilibrated with buffer A. Fractions containing pure protein were pooled and immobilized on glutathione magnetic agarose beads (Pierce).

Ten microlitres of magnetic beads with immobilized GST–Swi6 or GST were incubated with 230 μl of whole-cell lysate (approximately 16 mg ml⁻¹), prepared as follows: 200 ml of cells (SPY137, SPY4636, and SPY4642) were grown in YEA to OD₆₀₀ of 1.6. Cell pellets were suspended in lysis buffer (20 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 0.5 mM DTT, 1 mM PMSF, and complete EDTA-free protease inhibitor cocktail (Roche)), and beads at 5,000 r.p.m. for 5 × 45 s with MagNa Lyser (Roche). Samples were incubated for 3 h at 4 °C. Beads were washed 3 times with lysis buffer and suspended in SDS sample buffer. 0.3% of input and 50% of bound proteins were run on 4–12% gradient SDS–PAGE gel, transferred to nitrocellulose membrane, and blotted with mouse anti-Flag conjugated to HRP (Sigma A8592) at 1:5,000 dilution.

Data reporting. No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment. All quantitative experiments are presented as means ± s.d. of three independent biological experiments.

Data availability. The raw and processed ChIP-seq data are publicly available at the NCBI Gene Expression Omnibus under accession number GSE83495.

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Extended Data Figure 1 | Analysis of mutant Clr4 proteins and their effect on histone H3K9me. a, Western blot of N-terminal 3 × Flag-tagged Clr4 showing that SET mutations (F449Y or I418P) or a chromodomain mutation (W31G) do not affect Clr4 protein stability (top). The same blot stained with Ponceau dye is shown as a loading control (bottom). Image represents two individual experiments. b, Flag purification of wild-type Clr4 and Clr4(F449Y) showing both proteins are incorporated into the CLRC methyltransferase complex. c, Pull-down assays showing that wild-type Clr4 and Clr4(F449Y) interact with recombinant GST–Swi6 with similar efficiency. d, Coomassie staining (top) and western blot (bottom) of histones enriched for H3K9me using Swi6 affinity pull-down from wild-type and clr4F449Y cells showing that Clr4(F449Y) primarily catalyses H3K9me2. Image represents three individual experiments. e, Quantitative mass spectrometry of histones showing the redistribution of H3K9 methylation states in clr4+ and clr4F449Y cells. The histones were isolated using Swi6 affinity pull-down to increase detection sensitivity. See Extended Data Fig. 6 for quantitative mass spectrometry of H3 tail modifications in total wild-type histones. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 2 | ChIP analysis of H3K9me levels in wild-type clr4 and clr4-mutant cells. a, Map of the pericentromeric DNA region to the right of centromere 1. Arrowheads indicate the location of primers used for ChIP–qPCR in b and c. b, c, ChIP–qPCR analysis of H3K9me2 (b) and H3K9me3 (c) levels at the dg and dh repeats in cells with the indicated genotypes (clr4dead indicates clr4H410L, C412A). Error bars, s.d.; n = 3 biological replicates. d, Expanded view of H3K9me2 ChIP–seq reads mapped to the pericentromeric repeat regions on the right arm of chromosome 1 in Δclr4, clr4+, clr4F449Y and clr4I418P cells. The location of centromere 1 (cen1), innermost repeats (imr1R), outermost dg and dh repeats, and inverted repeat centromere (irc) sequences are indicated. Chromosome 1 coordinates are indicated above the tracks. Reads were randomly assigned to the dg and dh repeats of each chromosome. e, Same as d but showing H3K9me3 ChIP–seq reads.
Extended Data Figure 3 | Clr4 mutants have reduced H3K9me spreading at mating type and telomeric regions. a–g, ChIP–seq data showing changes in H3K9me2 and H3K9me3 levels outside of RNAi-dependent nucleation regions (indicated by solid black bars below tracks) at mating type (mat) and telomeric DNA regions (tel1L, tel1R, tel2L, tel2R, tel3L and tel3R) in Δclr4, clr4Δ, clr4F449Y and clr4I418P cells. tel3L and tel3R represent reads from the rDNA repeats. H3K9me2 reads were randomly assigned to repeated sequences. The reads at cenH are therefore shared with those at the pericentromeric dg and dh repeats (with which cenH shares 98% sequence identity); H3K9me2 reads that map uniquely to the mating type locus are shown (a, right panel). When only unique reads are mapped, a large fraction of total reads corresponding to repeated sequences at centromeres, telomeres, and the mat locus, are removed. This changes the normalized peak heights, which are affected by fewer total mapped reads. Data are presented as reads per million (y axis).
Extended Data Figure 4 | ChIP–qPCR analysis showing increased Pol II levels at pericentromeric DNA repeats of clr4-mutant cells. a, Map showing the location of the heterochromatin reporter ura4+ inserted to the right of cenI. b, ChIP–qPCR data showing changes in the association of RNA Pol II with the dg and dh pericentromeric DNA repeats in clr4 mutant cells. qPCR primer locations are indicated by arrowheads in a. Error bars, s.d.; n = 3 biological replicates.
Extended Data Figure 5 | Clr4 mutant cells have increased levels of Chp1 and activating histone marks at pericentromeric DNA repeats.

a, ChIP–qPCR showing increased Chp1 recruitment to pericentromeric DNA repeats. Error bars, s.d.; n = 3 biological replicates.

b, ChIP–seq data showing increased Chp1 reads mapping to pericentromeric regions of chromosome 2 in clr4<sup>F449Y</sup> and clr4<sup>I418P</sup> mutants compared to wild-type (wt) cells.

c, Same as b, but showing pericentromeric regions of chromosome 3.

d, ChIP–qPCR analysis of Flag–Ago1 recruitment to pericentromeric DNA repeats in wild-type clr4 and clr4<sup>F449Y</sup> cells. Error bars, s.d.; n = 3 biological replicates.

e, f, ChIP–qPCR analysis of H3K4me3 (e) and H3K36me3 (f) levels at pericentromeric DNA repeats in wild-type clr4 and clr4-mutant cells. dg2 primer location is indicated by empty arrowhead. Error bars, s.d.; n = 3 biological replicates.

g, ChIP–seq data showing increased H3K14ac mapped reads at pericentromeric regions of chromosome 1 in clr4<sup>F449Y</sup> cells.

h, ChIP–seq data showing increased H4K16ac mapped reads at pericentromeric regions of chromosome 1 in clr4<sup>F449Y</sup> cells. Data are presented as reads per million (y axis).
Extended Data Figure 6 | Quantitative mass spectrometry of histone H3 tail modifications. a, Steps for the isolation of chromatin-bound histones and their analysis by liquid chromatography and tandem mass spectrometry (LC–MS/MS). b, Results of quantitative mass spectrometry analysis of modifications associated with the indicated H3 tail tryptic peptide in clr4+ and Δclr4 cells.

| H3 9-17: KSTGGKAPR | Normalized % | clr4+ | Δclr4 |
|--------------------|--------------|-------|-------|
| unmodified         | 83.36        | 74.60 |
| K9me1              | 6.40         | 15.06 |
| K9me2              | 0.66         | 0     |
| K9me3              | 1.02         | 0     |
| K9ac               | 3.94         | 4.68  |
| K14ac              | 2.38         | 2.84  |
| K9me1, K14ac       | 1.86         | 2.63  |
| K9me2, K14ac       | 0.30         | 0.06  |
| K9me3, K14ac       | 0            | 0     |
| K9ac, K14ac        | 0.09         | 0.14  |
Extended Data Figure 7 | H3K9me2 precedes H3K9me3 during heterochromatin establishment. a, Tenfold serial dilution of cells plated on non-selective (N/S) and FOA-containing (+ FOA) medium to evaluate re-establishment of otr1R::ura4+ silencing at 0, 3, 5, 7 h after TSA removal. Untreated and Δclr4 cells serve as positive and negative control for otr1R::ura4+ silencing, respectively. Image represents three individual experiments.  

b, H3K9me2 ChIP–seq reads mapped to pericentromeric repeats to the right of cen1 in untreated and TSA-treated cells at the indicated time points following TSA removal. The highlighted region (darker blue) displayed the greatest loss of H3K9 methylation resulting from TSA treatment. Read ratio (indicated on the right) was obtained by normalizing the sum of reads mapping to the highlighted region for TSA-treated compared to untreated cells. 

c, Same as b, but showing H3K9me3 ChIP–seq reads. See Fig. 2g for ChIP–qPCR analysis.
Extended Data Figure 8 | Chp2 and Swi6 are not required for the formation of H3K9me2 or me3 domains at pericentromeric DNA repeats. a, b, ChIP–seq data showing that unlike Chp1, Chp2 and Swi6 are not required for RNAi-mediated H3K9 methylation, as indicated by similar levels of H3K9me2 (a) and H3K9me3 (b) mapped reads at pericentromeric regions of chromosome 1 in wild-type (wt), Δchp2, and Δswi6 cells. Δclr4 serves as a control for specificity of the anti-H3K9me antibodies. Data are presented as reads per million (y axis).
Extended Data Figure 9 | H3K9me states regulate the recruitment of HP1 proteins and Clr4. 

**a.** Swi6 ChIP-qPCR analysis at dg and dh. Error bars, s.d.; n = 3 biological replicates. **b.** Live cell imaging using confocal microscopy showing the localization of GFP–Swi6 and Cut11–mCherry in clr4+, clr4F449Y, and Δclr4 cells. In wild-type clr4+ cells, GFP–Swi6 foci representing centromeres, telomeres, and the mating type locus are predominantly localized at the nuclear periphery (marked by Cut11–mCherry nuclear pore component). These peripheral GFP–Swi6 foci are lost in Δclr4 cells, but in clr4F449Y cells, in which H3K9me2 is restricted primarily to pericentromeric repeats, one fluorescent focus corresponding to centromeres (indicated by white arrows), which cluster at the nuclear periphery independently of H3K9me, is maintained. The peripheral GFP–Swi6 focus in clr4F449Y cells is weaker than that in clr4+ cells, which is probably a results of the lower affinity of Swi6 for H3K9me2 relative to H3K9me3. Correspondingly, diffuse GFP–Swi6 signals are observed throughout the nucleoplasm in clr4F449Y cells. Three representative cells for each genotype are presented. 18 of 18 clr4+, 15 of 16 clr4F449Y, and 0 of 10 Δclr4 cells had perinuclear localization of GFP–Swi6. 

**c–g.** Flag–Chp2 (c), Flag–Clr3 (d), H3K9me3 (e), H3K9me2 (f), and Flag–Clr4 (g) ChIP-qPCR analysis at dg and dh in clr4 wild-type and mutant cells. Error bars, s.d.; n = 3 biological replicates.
Extended Data Figure 10 | H3K9me3 is required for epigenetic inheritance of H3K9 methylation. a, b, ChIP–qPCR analysis showing loss of H3K9me2 at the 10x tetO-ade6+ reporter gene (a) and the adjacent mug135+ gene (b) upon the release of tethered TetR–Clr4-I by the addition of tetracycline (−tet versus +tet). c, ChIP–qPCR analysis showing loss of H3K9me2 at pericentromeric dg and dh DNA repeats in ∆ago1;clr4 Δ183P double-mutant cells. Arrowheads indicate the location of primers used for ChIP–qPCR. Error bars, s.d.; n = 3 biological replicates.