Role of Histone H3 Lysine 9 Methylation in Epigenetic Control of Heterochromatin Assembly

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progeny tested at generation 10. To construct the wild-type coisogenic strain, we backcrossed Windsor into *ri r e* for 10 generations by selecting e^+ daughters to yield *ri r* InR^+ e^+ / *ri r* InR^+ e^+.
8. Membranes were prepared from adult heads and immunoprecipitated with an INR-specific polyclonal antibody. Kinase activity was measured as described (*32*).
9. S. Sciacchitano *et al.*, unpublished data.
10. Life tables were constructed at 25°C by the extinct cohort method. Within a 24-hour eclosion period, 100 adults of mixed sex were introduced into 1-liter demography cages. Every 2 days, dead individuals were aspirated from cages and counted, and fly-medium vials were changed. Three to four replicate cages were concurrently assayed for each genotype.
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16. Corpora allata incubation and radiochemical assay for JH biosynthesis followed the protocol of (*33*). In each datum, three pairs of CA were incubated. Radioactive JH homologs were visualized by thin-layer chromatography separation and scanning.
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20. Triglyceride was measured from sonicated flies with Stanbio Triglyceride Liquicolor. Activity of Cu/Zn-superoxide dismutase was measured from sonicated flies by a protocol modified for 96-well plates from (*34*). Both assays were scaled by total sample protein. Metabolic rate was estimated from volume oxygen consumed as measured by gas analysis (Applied Electrochemistry) for a cohort of known number and mass over 4 hours.
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24. Cohorts of adult females, distributed among three replicate cages with 130 to 194 flies per cohort, were aged while untreated, exposed to ethanol, or exposed to methoprene delivered in ethanol. Every 4 days, 10 μl of solution was applied to a wick suspended in a standard demography cage, which was sealed except for a 2-cm cotton plug.
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REPORTS

Role of Histone H3 Lysine 9 Methylation in Epigenetic Control of Heterochromatin Assembly

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The assembly of higher order chromatin structures has been linked to the covalent modifications of histone tails. We provide in vivo evidence that lysine 9 of histone H3 (H3 Lys^9) is preferentially methylated by the Clr4 protein at heterochromatin-associated regions in fission yeast. Both the conserved chromo- and SET domains of Clr4 are required for H3 Lys^9 methylation in vivo. Localization of Swi6, a homolog of *Drosophila* HP1, to heterochomatic regions is dependent on H3 Lys^9 methylation. Moreover, an H3-specific deacetylase Clr3 and a β-propeller domain protein Rik1 are required for H3 Lys^9 methylation by Clr4 and Swi6 localization. These data define a conserved pathway wherein sequential histone modifications establish a "histone code" essential for the epigenetic inheritance of heterochromatin assembly.

The organization of the higher order chromatin structure has been linked to the posttranslational modifications of histone tails, including acetylation, phosphorylation, and methylation (*1*). It has been suggested that distinct combinations of covalent histone modifications, also referred to as the "histone code," provide a "mark" on the histone tails to recruit downstream chromatin-modifying proteins (*2*, *3*). This is best illustrated by recent studies indicating that the conserved bromodomain of several transcriptional coactivators bind specifically to acetylated lysine residues on histone tails (*4*, *5*). The mechanisms responsible for the establishment and maintenance of multiple covalent modifications within the same or different histone tail are not fully understood.

Modifications of histone tails have also been linked to heterochromatin assembly. Histones H3 and H4 are largely hypoacetylated in heterochromatic chromosomal regions in organisms as diverse as yeast, flies, and mammals (*6*–*8*). In fission yeast, hypoacetylation of histones is associated with the silent mating-type region and centromeres (*9*, *10*), chromosomal domains that share many parallels with heterochromatic regions in higher eukaryotes (*11*). Centromeric regions comprising a central core of unique sequences surrounded by inner (*imr*) and outer (*otr*) repeats are assembled into silenced chromatin structures (*12*). Similarly, a large ~15-kb chromosomal domain at the mating-type (*mat2/3*) region, including the *mat2* and *mat3* loci and an interval between them, known as the *K*-region, is maintained in a silent epigenetic state (*13*, *14*). Among the trans-acting factors that affect silencing at these regions, Clr3 and Clr6 belong to family of histone deacetylases (HDACs) (*15*). Swi6 and Clr4 proteins contain a chromodomain, an evolutionarily conserved motif initially identified in HP1 and *Polycomb* proteins (*16*–*18*). Recently, both Clr4 and its mammalian counterpart, SUV39H1, have been shown to have intrinsic histone H3-specific methyltransferase (HMTase) activity in vitro (*19*). However, it is not known whether histones are the physiological targets of these methyltransferases in vivo.

Consistent with previous findings, recombinant Clr4 (rClr4) was found to contain HMTase activity exclusively for histone H3 (Fig. 1B). To identify the specific residue of H3 methylated by rClr4, we used synthetic peptides derived from the NH_2-terminus of H3 as substrates in an in vitro HMTase assay (*20*, *21*). Clr4 preferentially methylated the H3 1-20 unmodified peptide but failed to methylate the H3 19-35 unmodified peptide (Fig. 1C), indicating that the target residue of Clr4 HMTase resides in the first 20 amino acids of H3. To determine this target residue, we developed a synthetic H3 1-20 peptide set that contained covalent modifications on different amino acids. With these peptides as substrates, only acetyl or methyl modifications on Lys^9 effectively blocked rClr4 HMTase activity, indicating that Clr4, like its mammalian homolog SUV39H1 (*19*), selectively methylates Lys^9 of H3. Furthermore, similar to SUV39H1, rClr4 HMTase activity

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was inhibited by phosphorylation of serine 10 (Fig. 1C). These results demonstrate that enzymatic features of the Su(var)3-9 protein family are evolutionarily conserved from fission yeast to humans.

A recent study demonstrated that the conserved SET domain and two flanking cysteine-rich regions were required for SUV39H1 HMTase activity in vitro (*19*). To determine whether the conserved domains, the chromo, SET, and cysteine-rich regions, were also critical for Clr4 HMTase activity, we tested mutant Clr4 proteins for HMTase activity. Although mutations in the chromodomain [$Trp^{31} \rightarrow$ Gly (W31G) and $Trp^{41} \rightarrow$ Gly (W41G)] had little effect on Clr4 HMTase activity, mutations in the SET domain [$Gly^{328} \rightarrow$ Ser (G378S)] and both cysteine-rich regions [$Arg^{320} \rightarrow$ His (R320H) and $Gly^{486} \rightarrow$ Asp (G486D)] greatly reduced Clr4 HMTase activity, indicating that these three regions are critical for Clr4 HMTase activity in vitro (Fig. 1, A and B).

To test the hypothetical correlation between H3 Lys^9 methylation and silencing, we developed an H3 Lys^9–methyl specific antibody. In an enzyme-linked immunosorbent assay, the H3 Lys^9–methyl antibody specifically recognized the H3 1-20 Lys^9–methyl peptide in a wide range of antibody dilution (*22*). Moreover, the H3 Lys^9–methyl antibody did not detect recombinant histone H3 (rH3) alone compared with the HeLa core histone positive control but did detect rH3 selectively methylated by rClr4 (Fig. 2A), further demonstrating the specificity of this antibody. Using this antibody in chromatin immunoprecipitation (ChIP) experiments (*10*) (Fig. 2B), we found that the H3 Lys^9–methyl modification is specifically localized at the silenced chromosomal regions. H3 Lys^9 methylation and Swi6 were preferentially enriched at a marker gene (*Kint2::ura4*$^+$) inserted within the silenced *mat2/3* chromosomal domain, compared with control *ura4DS/E* locus at the endogenous location (Fig. 2C). Similarly, H3 Lys^9 methylation was also preferentially enriched at the *ura4*$^+$ marker inserted within the highly repressed innermost repeat (*imr1R::ura4*$^+$) and the outer repeat (*otr1R::ura4*$^+$), but not at the weakly repressed central core (*cnt1::ura4*$^+$) of *cen1* (Fig. 2D). In addition, H3 Lys^9 methylation coincided with the presence of Swi6 at these regions (Fig. 2D) (*23*). These findings suggest that H3 Lys^9–methyl modification and Swi6 are preferentially localized to silent chromosomal regions and that Swi6 localization is functionally dependent on H3 Lys^9 methylation.

We next sought to determine the biological effect of Clr4 on H3 Lys^9 methylation and Swi6 localization at *mat2/3* region and centromeres. Compared with the relatively high levels of Swi6 and H3 Lys^9 methylation at both *Kint2::ura4*$^+$ and *otr1R::ura4*$^+$ in wild-type cells, Swi6 and H3 Lys^9 methylation were absent in a *clr4*Δ strain at both loci (Fig. 3, A and B). This result suggests that H3 Lys^9

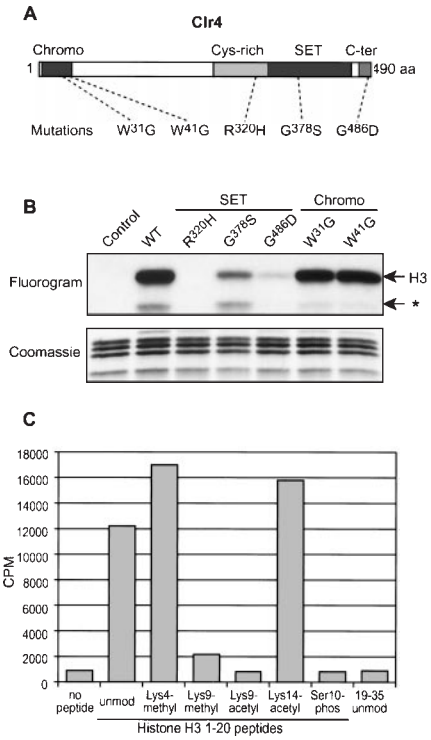

Fig. 1. Clr4 selectively methylates lysine 9 of histone H3. (**A**) Schematic representation of the Clr4 protein. Conserved domains and locations of the five mutations are shown. aa, amino acid. (**B**) Effect of mutation in Clr4 on its HMTase activity in vitro. rClr4 wild-type and mutant proteins were analyzed in an in vitro HMTase assay with chicken core histones as the substrate and *S*-adenosyl-[methyl-^{3}H]-methionine as the methyl donor. Samples were separated by 15% SDS-PAGE and visualized by Coomassie staining (bottom). Fluorography indicates the relative HMTase activity of each of the rClr4 proteins (top). The asterisk (*) indicates a histone H3 proteolytic breakdown product. (**C**) rClr4 was used in an in vitro HMTase assay with the following covalently modified peptides from the first 20 amino acids of the NH_2-terminal tail of H3 as substrates: unmodified, dimethylated at lysine 4, dimethylated at lysine 9, acetylated at lysine 9, acetylated at lysine 14, or phosphorylated at serine 10. An unmodified H3 19-35 was also assayed.

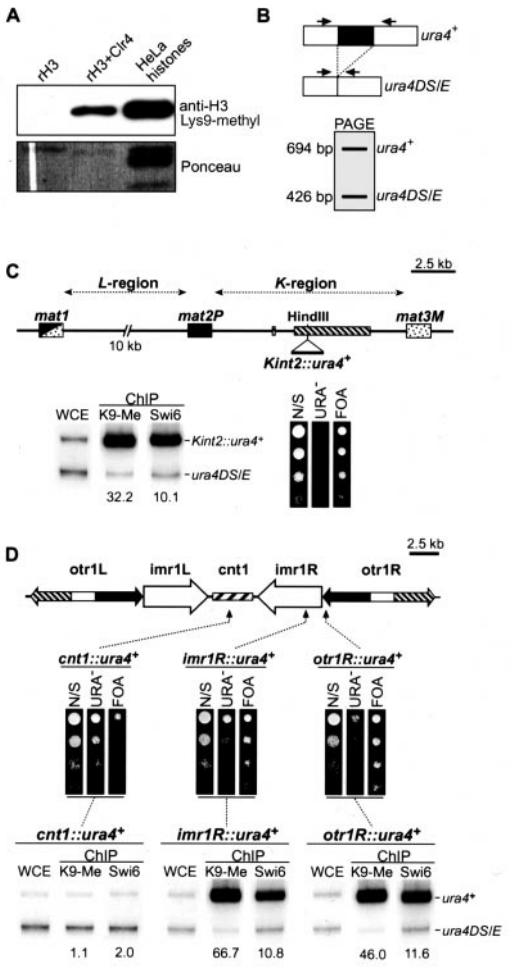

Fig. 2. Presence of H3 Lys^9 methylation at the *mat* and *cen1*. (**A**) Specificity of the H3 Lys^9–methyl antibody. Recombinant histone H3 (rH3) was incubated alone or in the presence of rClr4 (rH3+Clr4) in an HMTase assay. The samples, including the HeLa core histone positive control, were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and visualized by Ponceau staining (bottom). Western blot analysis with the H3 Lys^9–methyl antibody is shown (top). (**B**) DNA isolated from immunoprecipitated chromatin (ChIP) or whole-cell extracts (WCE) was quantitatively analyzed with a competitive polymerase chain reaction (PCR) strategy, whereby one set of primers amplifies a 694–base pair (bp) and a 426-bp size products from *ura4*$^+$ at silenced loci and the control *ura4DS/E* minigene at the endogenous location, respectively. PCR fragments were resolved on polyacrylamide gel and then quantified with phosphoimager. (**C**) A physical map of the mating-type locus indicating the *Kint2::ura4*$^+$ insertion site (top). The cross-hatched box indicates a part of the *K*-region homologous to centromeric repeats. Results from ChIP analysis with the H3 Lys^9–methyl (K9-Me) or Swi6 antibody are shown. The ratios of *ura4*$^+$ and control *ura4DS/E* signals present in WCE were used to calculate relative precipitated fold enrichment shown underneath each lane. Serial dilution plating assay was used to measure *ura4*$^+$ expression (middle). (**D**) Schematic representation of *cen1* and the corresponding *ura4*$^+$ insertion sites (top). Levels of H3 Lys^9 methylation and Swi6 at the three insertion sites were determined by ChIP analyses (bottom). Expression of *ura4*$^+$ markers was assayed with serial dilution plating assay.

is the physiological target of Clr4 HMTase activity and that Clr4 appears to be the exclusive in vivo H3 Lys^9–specific HMTase at *mat* and *cen* loci. In comparison with our in vitro result showing that only the SET domain is required for Clr4 HMTase activity (Fig. 1B), we found that both the chromo- and SET domains are required for H3 Lys^9 methylation and Swi6 localization in vivo (Fig. 3, C and D). Taken together, these results indicate that the chromodomain is presumably required for targeting Clr4 to the *mat2/3* region and centromeres, whereas the SET domain and associated cysteine-rich regions of Clr4 constitute the catalytic site.

The Swi6 levels at *mat* and *cen* in different *clr4* mutant backgrounds were directly correlated with H3 Lys^9 methylation levels (Fig. 3, C and D), further suggesting that Swi6 localization at silent chromosomal domains is functionally dependent on H3 Lys^9 methylation. The importance of our in vivo analyses was further highlighted by observations that some mutations in Clr4 that decrease its HMTase activity in vitro do not substantially decrease H3 Lys^9 methylation and Swi6 localization in vivo. In addition, mutations in the SET domain and the NH_2-terminal cysteine-rich regions of Clr4 (G378S and R320H) greatly reduce H3 Lys^9 methylation and Swi6 localization at the *mat* locus; however, these mutations have moderate or negligible effects at *cen1* (Fig. 3, C and D). These mutations also have weak effects on centromeric silencing compared with mating-type silencing. Our results are consistent with the notion that enzymatic defects displayed by recombinant monomeric proteins in vitro can be "rescued" by functioning in the context of a multisubunit complex in vivo. Moreover, the functional organization of the *mat2/3* region and centromeres may differ, and an additional factor(s) may help promote Clr4 activity at centromeres.

Mutations in the *clr3* HDAC, which specifically deacetylates H3 Lys^{14} (*24*), affects silencing at *mat* and *cen* (*12*, *15*). ChIP analysis demonstrated that a *clr3-735* mutant partially defective in H3 Lys^{14} HDAC activity (*24*) displayed a moderate decrease in H3 Lys^9 methylation and Swi6 localization at *otr1::ura4*$^+$ (Fig. 4A), coincident with the apparent reduction in its HDAC activity. This result suggests that H3 Lys^{14} acetylation inhibits Clr4 HMTase in vivo (*22*). To further investigate the functional interaction between Clr3 and Clr4, we created a double-mutant strain containing the *clr3-735* and *clr4R320H* mutations, a *clr4* mutation that had the least effect on H3 Lys^9 methylation at *otr1R::ura4*$^+$ (Fig. 3D). ChIP analysis of the double mutant demonstrated that H3 Lys^9 methylation and Swi6 localization were nearly abolished when compared with the single mutants (Fig. 4A). These findings indicate that Clr3 acts synergistically with Clr4 to effectively localize Swi6 to heterochromatic domains. In other words, deacetylation of H3 Lys^{14} by Clr3 is required for H3 Lys^9 methylation by Clr4 and for Swi6 localization either indirectly, by altering Clr4 activity, or directly or both. These data also support the theory that residues neighboring Lys^9, and potentially their modification states, play an important role in establishment of the appropriate H3 Lys^9–methyl mark.

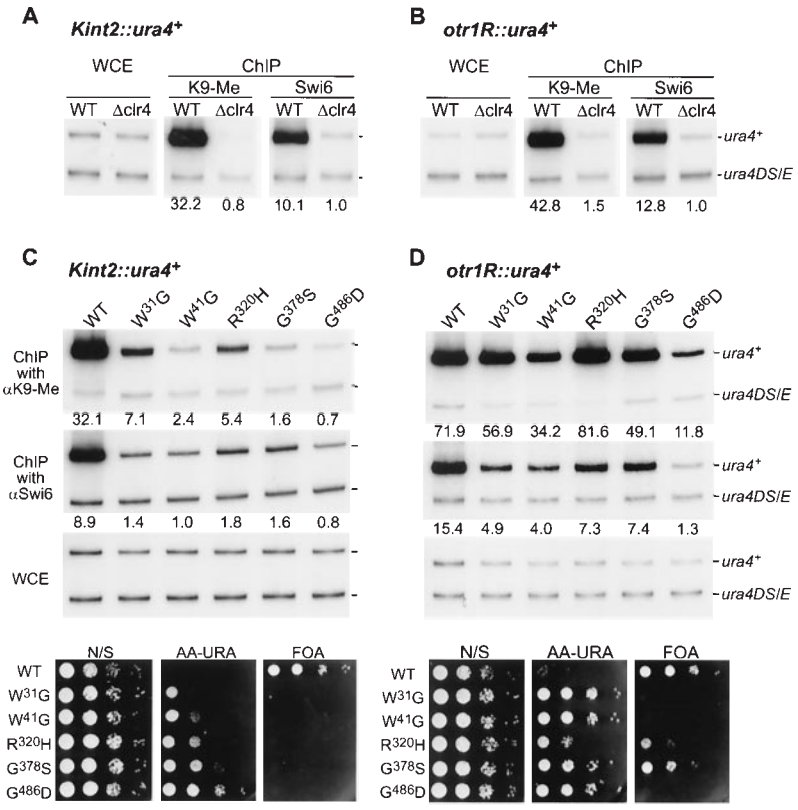

Fig. 3. The effect of *clr4* mutations on H3 Lys^9 methylation and Swi6 localization at *mat* and *cen1*. (**A** and **B**) Deletion of *clr4* abolishes H3 Lys^9 methylation and Swi6 localization at *mat* and *cen1*. ChIP analyses were used to compare the levels of H3 Lys^9–methyl or Swi6 in wild-type (WT) or Δ*clr4* strains. (**C** and **D**) Results from ChIP analyses to determine effect of mutant alleles on H3 Lys^9 methylation and Swi6 localization (top) and silencing phenotypes (bottom) are shown. Mutations in *clr4* have varying effects on H3 Lys^9 methylation, Swi6 localization, and silencing at *Kint2::ura4*$^+$ and *otr1R::ura4*$^+$.

Previous studies have shown that *rik1*$^+$ affects silencing as well as Swi6 localization at silent loci (*12*, *25*). Computational analyses revealed that Rik1 contains β-propeller domains typically found within WD-40 repeat proteins and are theorized to participate in protein:protein interactions (*26*). A mutation in *rik1* completely abolished H3 Lys^9 methylation and Swi6 localization at both *mat* and *cen* compared with wild type (Fig. 4B). WD-40 proteins are involved in many aspects of chromatin remodeling and histone metabolism, such as chromatin assembly and acetylation or deacetylation of histones (*27*). Therefore, the β-propeller domains of Rik1 may form a complex with Clr4 to recruit its HMTase activity to heterochromatic regions and may play a role in coupling other trans-acting factors, such as Swi6 and histone deacetylases.

The possible role of Swi6 on Clr4-dependent methylation of H3 Lys^9 was also tested. Strains carrying *swi6-115* (W269R) mutation that severely reduced Swi6 protein levels were used. As expected, Swi6 localization at both *mat* and *cen* was abolished as demonstrated by ChIP analysis (Fig. 4C). The *swi6-115* mutation did not cause any detectable change in H3 Lys^9 methylation when compared with the wild-type strain. These data indicate that Swi6 is dispensable for Clr4 function and suggest that Swi6 acts downstream of Clr4 H3 Lys^9 methylation.

Collectively, the above results allow us to define a temporal sequence of events leading to establishment of the silenced chromatin state with regard to the covalent modifications of the H3 NH_2-terminal tail (Fig. 5). We propose that HDACs and HMTases act cooperatively to establish a "histone code" that is then recognized by Swi6. More specifically, we propose that the HDACs, Clr6 and/or Hda1 (*11*), deacetylate H3 Lys^9, whereas Clr3 deacetylates H3 Lys^{14} before H3 Lys^9 methylation by the Clr4/Rik1 HMTase complex. Swi6 binding to the H3 Lys^9–methyl modification would then result in self-propagating

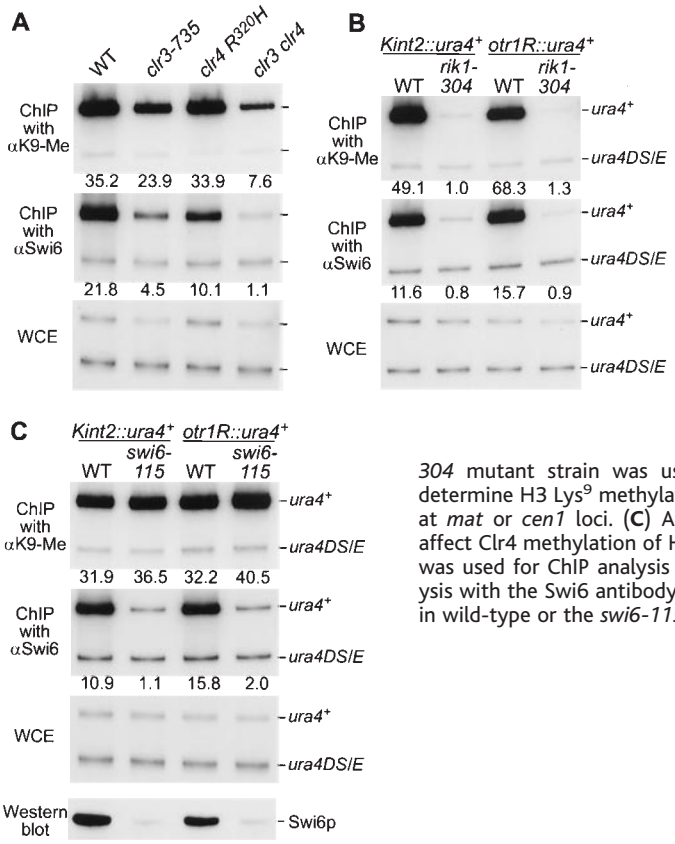

Fig. 4. Functional Clr3 and Rik1, but not Swi6, are required for H3 Lys^9 methylation by Clr4. (**A**) Clr3 and Clr4 act synergistically to silence *cen1*. The *clr3-735*, *clr4* $R^{320}H$, and *clr3clr4* double-mutant strains were used for ChIP analyses to determine H3 Lys^9 methylation and Swi6 localization at *otr1R::ura4*$^+$. (**B**) A *rik1* mutation abolishes H3 Lys^9 methylation and Swi6 localization at *mat* and *cen1*. A *rik1-304* mutant strain was used for ChIP analysis to determine H3 Lys^9 methylation and Swi6 localization at *mat* or *cen1* loci. (**C**) A *swi6* mutation does not affect Clr4 methylation of H3 Lys^9. A *swi6-115* strain was used for ChIP analysis (top). Western blot analysis with the Swi6 antibody shows Swi6 protein level in wild-type or the *swi6-115* mutant strain (bottom).

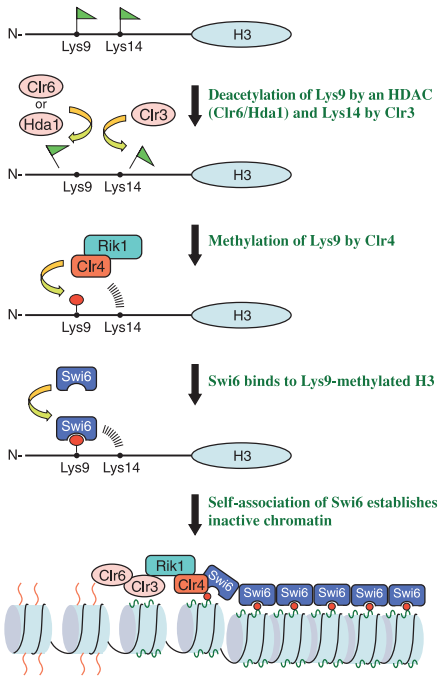

Fig. 5. A stepwise model for heterochromatin assembly leading to epigenetic silencing in *Schizoaccharomyces pombe*. Green flags and red lollipops represent acetyl and methyl modifications, respectively. A nucleosome core particle is composed of DNA (black line) wrapped around a histone octamer (blue). Orange or green protrusions represent the NH_2-terminal tails of histones with or without acetyl modifications, respectively.

heterochromatin assembly (*10*). Because the heterochromatin-binding domain of Swi6 was mapped to its chromodomain (*28*), it is most likely that this protein motif has evolved to recognize the H3 Lys^9–methyl modification.

It was recently shown that Swi6 remains associated with the *mat2/3* region throughout the cell cycle where it acts as an important determinant of the epigenetic cellular memory, promoting inheritance of the silenced state (*10*). Because the mouse homolog of Swi6, M31, associates with Su(var)3-9 (*29*), a similar interaction between Clr4 and Swi6 is predicted. The close association of Clr4 enzymatic HMTase activity, followed by recruitment and binding of Swi6 to Lys^9 methyl "marks" in H3 through its chromodomain, suggests a pathway of epigenetic inheritance. The extent to which the chromodomain of Clr4 recognizes H3 Lys^9–methyl marks is unknown, but it would provide the enzyme a means to bind chromatin as it performs subsequent methylation events. On the basis of the conservation of Clr4/SUV39H1 and Swi6/HP1 proteins and the presence of H3 Lys^9–methyl modification in higher eukaryotes (*30*) (Fig. 2A), we predict that a similar mechanism may be responsible for higher order chromatin assembly in organisms ranging from fission yeast to humans. Considering the parallels between transcriptional repression by *Polycomb* group proteins in flies and mammals and silencing in fission yeast, it is likely that histone methylation coupled with histone deacetylation may help localize *Polycomb* in pathways that lead to the regulation of homeotic gene expression (*22*).

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20. Five milligrams of HeLa or chicken core histones was incubated with 0.55 mCi of *S*-adenosyl-L-[methyl-3H]methionine (3H-AdoMet; 72 Ci/mmol; 1 mM final) and 2 mg of recombinant Clr4 wild-type or mutant proteins in 25 ml of HMTase buffer [50 mM tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol in 10% glycerol] for 1 hour at 30°C. SDS loading buffer was added to half of each sample and boiled followed by separation on a 15% SDS–polyacrylamide gel electrophoresis (PAGE) gel. The resulting histone bands were visualized by Coomassie staining and fluorography. For the peptide analysis, 5 mg of each peptide derived from the NH_2-terminus of human histone H3 containing a COOH-terminal cysteine was used. Half of the sample was spotted on Whatman P-81 filter paper and washed four times for 10 min in 50 mM $NaHCO_2$ (pH 9.0), followed by liquid scintillation counting..
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